UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL – UFRGS
CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

TESE DE DOUTORADO

ESTUDOS *IN VITRO* E *IN SILICO* DOS MECANISMOS MOLECULARES DA SENESCÊNCIA CELULAR EM GLIOBLASTOMAS

JOSÉ EDUARDO VARGAS

Orientador: Dr. Guido Lenz

Co-orientador: Dr. Diego Bonatto

Porto Alegre

-2013-

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL – UFRGS
CENTRO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

TESE DE DOUTORADO

ESTUDOS *IN VITRO* E *IN SILICO* DOS MECANISMOS MOLECULARES DA SENESCÊNCIA CELULAR EM GLIOBLASTOMAS

Tese de doutorado apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de doutor em Biologia Celular e Molecular.

JOSÉ EDUARDO VARGAS

Orientador: Dr. Guido Lenz

Co-orientador: Dr. Diego Bonatto

Porto Alegre

- 2013-

Dedico esta tese especialmente a três mulheres maravilhosas com as quais a vida me presenteou. Alicia, minha mãe, Verônica, minha irmã e Fabiana, minha namorada, por me ensinar e ter me ensinado através do amor.

AGRADECIMENTOS

Ao meu orientador, Dr. Guido Lenz, pela liberdade de deixar-me andar guiado pela minha curiosidade e por criar possíveis soluções para enfrentar as dificuldades que se apresentaram; pela presença e adição de ideias para a estruturação e amadurecimento desta tese; pelas criticas construtivas que viraram valiosos ensinamentos que vou aproveitar durante minha vida acadêmica e pessoal. Por oferecer maior compreensão e apoio nas etapas finais de desenvolvimento desta tese.

Ao meu co-orientador Dr. Diego Bonatto, por me mostrar o mundo até então desconhecido para mim como a biologia de sistemas, da qual fiquei maravilhado; pelas palavras de conforto convincentes quando necessárias; pela paciência, presença e ensinamentos que contribuiram para o meu desenvolvimento como pesquisador tanto na biologia sintética como experimental.

À CAPES pela minha bolsa, FAPERGS, FINEP e CNPq pelo suporte financeiro.

Aos professores Henrique Ferreira e Andrés Cañedo, minha comissão de acompanhamento, por aportar ideias e sugestões.

Ao Dr. Martín Bonamino e o Dr. Andrés Cañedo por acreditar e ajudar a finalizar o trabalho presente na seção ANEXO desta tese.

À banca, pela disponibilidade e paciência.

Aos funcionários do PPGBCM, especialmente à Silvia e ao Luciano por estarem sempre presentes para resolver algum problema burocrático.

Aos amigos, em especial o Eduardo, o Andrew e o Tiago por contribuir em boa parte a fazer mais prazeroso o trabalho no laboratório. Compartilhando inúmeros chimarrões, cervejas, risadas e também trocando conselhos em momentos não tão felizes. A Patilu por estar presente sempre oferecendo sua amizade desde que estou no Brasil. Também agradeço a eles a compreensão de vários fins de semana que fiquei trabalhando. A Lauren pelos ensinamentos e idéias no inicio deste doutorado. Aos demais colegas do laboratório 115, Ale T, Ale P, Emilly, Pítia, Fran, Mardja, Marcos, Darlan e Camila pelo companheirismo, ajuda e bons momentos de descontração vividos nestes anos que foram fundamentais para permitir o desenvolvimento desta tese.

À minha iniciação científica Tais, pela ajuda e paciência.

Aos amigos argentinos aqui no Brasil, Diego, grande pessoa; Carolina, Guillermo, Roberto, Martin, Natalia, Néstor, Liliana, Leonardo, Luis, Manuel, Mónica, Facundo e Viviana. Ao Julián pelas ótimas conversas e apoio, mates, tererês e pelos jogos de basquete. Às amigas e boas pessoas da Colômbia, Grethel, Lyda e Victória. À minha colega de Peru, Elvira pelos seus conselhos.

Aos amigos do Laborátorio de Cardiologia Molecular e Celular, Lucinara, Melissa e Andreia.

Aos colegas do laboratório 210 em especial a Cristiano, Larissa e Diana pela troca de material de laboratório e ajuda quando precisei.

Aos colegas do laboratório 219 pelas conversas, trocas científicas e momentos agradáveis.

À minha namorada, Fabiana, pessoa incrível e carinhosa, pelo seu imprescindível apoio, grande amizade, paciência e compreensão nos momentos de estresse e nos numerosos fins de semana que foram dedicados a esta tese. Também agradeço a ajuda incodicional prestada na hora de corrigir meu português. A sua familia, pessoas maravilhosas. Lígia pelo apoio, carinho, compreensão e amizade; Tusto pela parceria, churrascos maravilhosos e amizade; Ao Flávio pelo apoio, ótimas conversas e amizade. E a todos os outros integrantes destas famílias que se esforçaram com meu portunhol e me adotaram.

À minha familia, pelo auxílio em momentos cruciais e pela compreensão necessária para com um doutorado. Por estarem presentes e me ressaltarem o que é verdadeiramente importante na vida e me apoiarem plenamente nos meus acertos ou erros. Minha mãe, pela ética e moral invaloráveis, pelos conselhos valiosos, torcida e carinhos infinitos. Meu pai pelo seu grande apoio e compreensão. Minha tia e minha avó pela torcida e carinho.

Minha irmã, pelo auxílio incondicional, sabedoria, carinho e compreensão infinita ainda estando separada por um oceano. A meu irmão pelo carinho e por simplesmente dizer o correto no momento indicado.

A Deus, por dar-me entedimento para descobrir como aprender com os erros cometidos. Por me oferecer escolhas e a partir destas me permitir amadurecer.

A todos os pesquisadores que contribuíram para os conhecimentos utilizados nesta tese;

A todos que contribuíram de alguma forma para esse trabalho e não foram aqui citados, meus sinceros agradecimentos.

“Uma vida não questionada não merece ser vivida.”

Apologia de Sócrates

Platão (428-348 ac)

SUMÁRIO

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ABL1 - Oncogene derivado de leukemia murina (do inglês, *Abelson murine leukemia viral oncogene homolog 1*);

ADP - Difosfato de adenosina (do inglês, *adenosine diphosphate*);

AKT1 - Proteína homóloga celular ao oncogene viral v-AKT. Também conhecida como PKB - Proteína cinase B;

AMP - Monofosfato de adenosina (do inglês, *adenosine monophosphate*)

AMPK - Quinasa ativa por monofosfato de adenina (do inglês, *adenosine monophosphate protein kinase*);

ATM - Proteína mutada da ataxia telangiectasia (do inglês, *ataxia telangiectasia mutated*);

ATP - Trifosfato de adenosina (do inglês, *adenosine triphosphate*)

ATR - Proteína relacionada à ATM e Rad3 (do inglês, *ataxia telangiectasia and RAD3 related*);

BAX - Proteína Bcl2 associada ao X (do inglês, *Bcl2-associated X Protein)*;

BCL2 - Proteína 2 de linfoma de células B *(*do inglês, *B-cell Lymphoma Protein 2);*

BRCA1 - Câncer de mama 1 (do inglês, *breast cancer 1*);

BUBRI - Também conhecido como Bub1, proteína não inibida por benzimidazoles 1 (do inglês, *budding uninhibited by benzimidazoles 1*);

CDK - Quinase dependente de ciclina (do inglês, *cyclin-dependent kinase*);

CDKi - Inibidor de quinase dependente de ciclina (do inglês, *cyclin-dependent kinase inhibitor*);

CENP-E - Proteína do centrômero E (do inglês, *centromere protein E*);

CENP-P - Proteína do centrômero P (do inglês, *centromere protein P*);

CHK1 - Quinase de ponto de checagem 1 (do inglês, *checkpoint Kinase 1)*;

CHK2 - Quinase de ponto de checagem 2 (do inglês, *checkpoint Kinase 2*);

CS - Senescência celular (do inglês, *cellular senescense*);

DBS - Dano na dupla cadeia de DNA (do inglês, *DNA double-strand break*)

DNA - Ácido desóxido rebonucleico (do inglês, *deoxyribonucleic acid*);

DDR - Resposta de dano ao DNA (do inglês, *DNA damage response*);

DNA-PK - Quinase dependente de DNA (do inglês, *DNA-dependent protein kinase*);

ERK - Quinase regulada extracelularmente (do inglês, *extracellularly regulated kinase);*

ERO - Especies reactivas de oxigeno;

FADH - Dinucleotido de flavina reducido (do inglês, *flavin adenine dinucleotide reduced*);

G1 - Etapa 1 do ciclo celular (do inglès, *Gap 1*);

G2 - Etapa 2 do ciclo celular (do inglês, *Gap 2*);

GBM - Glioblastoma multiforme;

GSH - Gluotationa reductasa (do inglês, *glutathione reductase*)

HAT - Enzima acetiltransferasas (do inglês, *histone* acetyltransferase enzymes);

HDAC - Desacetilases de histonas (do inglês, *histone deacetylases*);

HDACi - Inibidor de desacetilases de histonas (do inglês, *histone deacetylases inhibitor*);

hTERT - Subunidade catalitica da telomerasa;

IGFP-7 - Proteina associado ao fator de crescimento de insulina 7 (do inglês, *insulin-like growth factor binding protein- 7*)

KO - do inglês, *knockout*;

M - Mitose;

MAD2 - Proteina deficiente de parada da mitose 2 (do inglês, *mitotic arrest deficient-like 2*);

MEK - Quinase ativada por mitogenos (do inglês, *mitogen active- protein kinase*)

MIC - Mutações gênicas indutoras de câncer;

MRE11 - Proteína homologa da recombinação meiótica 11 (do inglês, *meiotic recombination 11*);

MRN - Complexo sensor de dano ao DNA (do inglês, *MRE11–RAD50–NBS1 DNA damage sensor complex*);

mTOR - Proteína alvo de rapimicina (do inglês, *mammalian target of rapamycin*);

MYC - Oncogene viral de mielocitomatose (do inglês, *mielocytomatosis viral oncogene*);

NADH - Dinucleotido de adenina nicotinamida (do inglês, *nicotinamide adenine dinucleotide*);

NBS1 - Proteína derivada do sindrome de Moebius 1 (do inglês, *moebius syndrome 1*);

OIS - Oncogenes indutores de senescência (do inglês, *oncogene induce senescence*);

PTEN - Proteína homóloga fosfatase/tensina deletada do cromossomo 10 (do inglês, *Phosphatase and Tensin Homologue deleted on chromosome 10*);

POG - Privação de oxigênio e glicose;

RAD3 - Proteína de reparo derivada de rabdomisarcoma 3 (do inglês, *rhabdomyosarcoma 3*);

RAF - Proteína da familia de RAS (do inglês, *RAS family protein*)

RAS - Oncogene derivado de sacorma viral (do inglês, *rat sarcoma viral oncogen homolog*);

RASSF1A - Proteína associada a RAS (do ingles, *RAS association (RALGDS/AF-6) domain family member 1 A*);

RB - Proteina de retinoblastoma (do inglês, *retinoblastoma*);

RIPH - Redes de interação de proteínas humanas;

RS - Senescência replicativa (do inglês, *replicative senescence);*

SIRT - Sirtuína (do inglês, *sirtuin 1*);

SNC - Sistema nervoso central;

SP1 - Proteina especifica 1 (do inglês, *specificity protein 1*);

SP3 - Proteina especifica 3 (do inglês, *specificity protein 3*);

SPARC - Proteína ácida secretada com motivos ricos de cisteína (do inglês, *secreted protein, acidic, cysteine-rich- osteonectin*);

STAT - Transdutor de sinal ativador da transcrição (do inglês, *signal Transducer and Activator of Transcription*);

TERT - Do inglês, *telomerase reverse transcriptase*;

TMZ - Temozolamida;

UCP2 - Proteína desacopladora 2 (do inglês, *uncoupling protein 2*);

ZBP-89 - Proteína associada á forma DNA Z (do inglês, *Z-DNA binding protein 89*)

γH2AX - Familia da protena H2A, membro X (do inglês, *H2A histone family, member X*).

RESUMO

Os gliomas representam a maioria dos tumores do sistema nervoso central, sendo o glioblastoma o mais maligno entre eles. O tratamento destes tumores ainda é ineficiente e a sobrevida média dos pacientes é de aproximadamente um ano após o diagnóstico. A remoção cirúrgica, quando possível, acompanhada de radioterapia e quimioterapia é o tratamento padrão. A tendência é que a quimioterapia assuma um papel mais importante, uma vez que possibilita a ativação de mecanismos endógenos antitumorais, como senescência celular. Este mecanismo possui potencial de induzir a perda irreversível da capacidade da divisão de células tumorais. O resveratrol e a quercetina, dois polifenóis naturais, induzem senescência em diferentes linhagens tumorais, incluindo os glioblastomas. Ambos têm efeitos pleiotrópicos e induzem a ativação de vários alvos moleculares, sendo *SIRT1,* um desses alvos. SIRT1 é uma histona desacetilase (HDAC) associada ao desenvolvimento de vários tipos de tumores. Por este motivo, foi avaliada a indução de senescência em células de glioblastomas pelo resveratrol e quercetina combinados com butirato de sódio, um inibidor de HDAC classe I e II. Os resultados mostram um efeito combinado do butirato de sódio e estes polifenóis para induzir a senescência nas linhagens celulares U87 e C6, mas não em astrócitos normais de ratos. Os co-tratamentos induzem perda de capacidade proliferativa e aumento do número de células positivas para β-galactosidase, um marcador de senescência. Estes resultados foram acompanhados de parada no ciclo celular na fase G2 na linhagem U87, mas nenhum efeito foi observado sobre o ciclo de células C6. A análise por *Western Blot* sugere o aumento do supressor tumoral p21 após co-tratamentos. Além disso, o co-tratamento com quercetina e butirato de sódio aumenta a produção de espécies reativas de oxigênio, mas não os níveis de gama-H2AX (um marcador de dano). Estes dados sugerem que o butirato de sódio em combinação com os polifenóis tem potencial terapêutico para a supressão de glioblastomas. Por outro lado, um dos problemas em biologia e medicina é entender como células pré-cancerosas e cancerosas entram em senescência ou conseguem evitá-la. A chave para a compreensão da indução da senescência e do seu *bypass* é a ativação da enzima telomerase (TERT), presente em 85 % dos tumores. Na segunda parte desta tese, ferramentas de biologia de sistemas foram utilizadas para projetar uma rede câncer e

senescência humana com o objetivo de estudar a relação entre senescência e câncer (incluindo glioblastoma). Os resultados mostram uma interação direta entre TERT e oncogenes como MYC, E2F1, AKT1 e ABL1, que podem induzir a senescência. Além disso, uma associação metabólica forte entre estes oncogenes e sua capacidade de induzir senescência foi mostrada através de análise por ontologia gênica. Estes oncogenes também atuam como reguladores da transcrição e/ou pós-transcrição da subunidade catalítica de TERT. Resultados obtidos a partir da análise de centralidade da rede juntamente com dados de microarranjos derivados de tumores foram utilizados para conceber um modelo dinâmico da regulação de TERT em câncer. Com base nestes resultados, foi gerado um modelo hipotético no qual a expressão de TERT pode ser transitória ou induzida por espécies reativas de oxigênio, aumentando a sobrevivência de diferentes tipos de tumores, como o glioblastoma. Este estudo fornece novas evidências para a relação entre senescência e câncer. Além disso, esta hipótese, se verificada experimentalmente, pode permitir a formulação de novas abordagens quimioterapêuticas para o tratamento de gliobastomas

ABSTRACT

Gliomas are the majority of central nervous system tumours, being glioblastoma the most malign among them. The treatment for these tumors is still inefficient and survival average of patients is approximately one year after diagnosis. Surgical removal, if possible, followed by radiotherapy and chemotherapy is the standard treatment. The trend is that chemotherapy assumes a greater role, since it allows the activation of antitumour mechanisms, such as cellular senescence. This mechanism has potential to induce irreversible loss of division ability in tumor cells. Resveratrol and quercetin, two natural polyphenols, are able to induce senescence in different cancer models, including glioblastoma. Resveratrol and quercetine are molecules with pleiotropic effects and leads to *SIRT1* activation. SIRT1 is a histone deacetylase (HDAC) which has been linked positively to cancer development. Therefore, we analyzed the ability of sodium butyrate, a HDAC class I and II inhibitor combined with resveratrol or quercetin to induce senescence in glioblastoma cell lines. Results show a combined effect of sodium butyrate and these polyphenols to induce senescence in U87 and C6 cell lines, but not in normal rat astrocytes. Co-treatments induce a loss of proliferative capacity and increase the number of positive cells for β-galactosidase, a senescence marker. These results were accompanied with cell cycle arrest in G2 checkpoint in U87, but no effect on cell cycle phase's distribution in C6 was observed. Western blott analysis suggests that these arrests are result of tumour suppressor p21 increase. Moreover, co-treatments increased reactive oxygen species, but not gamma-H2AX levels (a damage marker). The data underline that tumor cells can be driven towards cellular senescence by sodium butyrate combined with polyphenols, which may further arise as a possibility for tumor suppression. On the other hand, one of the challenging problems in biology and medicine is to understand how pre-cancerous or cancer cells enter in senescence or can avoid it. The key to understanding senescence induction and its bypass is the telomerase (TERT), which is present in 85 % of tumors. In a second part of this thesis, system biology tools were used to design a human cancer-senescence network with the aim to study the relationship between senescence and cancer (included glioblastoma). The results show a direct interaction between TERT and oncogenes such as MYC, E2F1, AKT1 and ABL1, which can induce senescence. Moreover, a strong metabolic

association between these oncogenes and their ability to induce senescence was shown by gene ontology analysis. These oncogenes also act as transcriptional and/or post-transcriptional regulators of TERT catalytic subunit. Results obtained from centrality analysis and microarray data derived from tumors were used to design a dynamic model for TERT regulation in cancer. Based on these results, a hypothetical model was generated in which TERT expression may be transient or induced by reactive oxygen species, increasing the survival of different tumor types, such as glioblastoma. This study provides new evidence for the relationship between senescence and cancer. Furthermore, this hypothesis, if experimental verified, can enable the development of new chemotherapy approaches to glioblastoma treatment.

and ABL1, which can induce senescence. Moreover, a strong metabolic association between these oncogenes and their ability to induce senescence was shown by gene ontology analysis. These oncogenes also act as transcriptional and/or post-transcriptional regulators of TERT catalytic subunit. Results obtained from centrality analysis and microarray data derived from tumors were used to design a dynamic model for TERT regulation in cancer. Based on these results, a hypothetical model was generated in which TERT expression may be transient or induced by reactive oxygen species, increasing the survival of different tumor types, such as glioblastoma. This study provides new evidence for the relationship between senescence and cancer. Furthermore, this hypothesis, if experimental verified, can enable the development of new chemotherapy approaches.

ESTRUTURA DA TESE

Esta tese está organizada em seções dispostas da seguinte maneira: Introdução; Desenvolvimento - artigo científico publicado e artigos a serem submetidos; Discussão; Conclusões; Perspectivas; Referências bibliográficas e Anexo.

A Introdução apresenta o embasamento teórico que nos levou a formular a proposta do trabalho e á formulação dos objetivos gerais e específicos desta tese.

Os materiais, métodos, resultados e as referências específicas de cada artigo encontram-se no corpo de cada trabalho, os quais estão apresentados na forma de artigos científicos denominados capítulos 1, 2 e 3 da seção de Desenvolvimento.

A seção Discussão contém uma interpretação geral e uma análise global dos resultados obtidos nos diferentes trabalhos, bem como a interpretação das hipóteses que surgiram neste trabalho.

A seção Conclusões aborda as principais conclusões obtidas nesta tese.

A seção Perspectivas suscita as novas idéias propostas para testar as novas hipóteses que as conclusões geraram.

A seção Referências Bibliográficas cita as referências utilizadas na seção introdução e discussão.

Na seção Anexo encontra-se, um manuscrito publicado, não relacionado aos temas abordados nesta tese, mas produzido em parte durante o tempo de desenvolvimento do doutorado. No anexo também é incluido o *Currículum Vitae* baseado no modelo padrão lattes/CNPq do autor deste trabalho como as fontes de financiamento que permitiram a execução dos artigos aqui apresentados.

1. INTRODUÇÃO

1.1. Gliomas

Os gliomas são as neoplasias mais comuns do sistema nervoso central (SNC). Estes tumores podem ser classificados de acordo com sua morfologia e características clínicas em glioblastomas (também conhecidos como glioblastomas multiformes - GBM), astrocitomas, oligodendrogliomas e ependimomas [1].

Outra classificação envolve o grau de malignidade que varia em uma escala de I a IV [2]. Tumores de grau I são biologicamente benignos e podem ser curados cirurgicamente, quando for considerado operável no momento do diagnóstico. Já os tumores de grau II, são neoplasias de baixo grau que podem se desenvolver por longo curso clínico, mas não são curáveis cirurgicamente. Os tumores de grau III são malignos e podem levar a óbito em poucos anos e os tumores de grau IV são altamente malignos e letais dentro de 9 a 15 meses [1]. Cerca de 70% dos gliomas grau II podem se transformar em grau III e IV dentro de 5 a 10 anos do diagnóstico inicial, [3].

Dentre os gliomas, os tumores mais comuns são os astrocitomas (cerca de 3 a cada 10 tumores cerebrais). Os astrocitomas de baixo grau (pilocitico – grau I, difuso – grau II) possuem um crescimento lento; os de grau intermediário (anaplásico – grau III) crescem em um ritmo moderado a acelerado; os de grau elevado (GBM – grau IV) possuem um rápido crescimento [4].

Os dados sobre a incidência de GBM são escassos na literatura, sendo na maioria das vezes descritos para a população norte-americana, onde estes tumores são bastante freqüentes [5, 6]. Neste país, são diagnosticados aproximadamente 22.500 casos de tumores no SNC em adultos, sendo 14.000 destes casos gliomas por ano [6, 7], o que dá uma incidência de 5 por 100.000 por ano [6].

No Brasil foram registradas 3.590 mortes por esse tipo de câncer em 2008 (CBTRUS, 2011). De acordo com a Consulta Pública 30/2010 no sítio eletrônico do Governo Federal do Brasil, os tumores cerebrais malignos representam apenas 2% dos cânceres que ocorrem no país, mas são os que apresentam as maiores taxas de mortalidade em adultos [7]. Para os tumores mais agressivos, os GBM, o tratamento padrão consiste em cirurgia seguido de quimioterapia. Uma das principais dificuldades destes tumores é que mesmo retirando a massa tumoral podem ocorrer recidivas devido ao desprendimento de algumas células tumorais, o que ocasiona a invasão do tecido normal adjacente. Assim, a cirurgia deve ser seguida de radioterapia com quimioterapia adjuvante com temozolomida (TMZ) [1].

A quimoterapia continua tendo um papel central nos tratamentos de gliomas malignos. Duas meta-análises sugerem que a quimioterapia adjuvante permite um aumento modesto da sobrevida em pacientes (10% em 1 ano) [7-9]. Desde que a combinação de radioterapia e TMZ foi proposta, em torno do ano de 2005, a sobrevida dos pacientes aumentou apenas uma média de dois meses, indicando que os tratamentos atuais vêm melhorando pouco ao longo dos anos, com o agravante do número crescente de pacientes [6, 10]. A combinação com radioterapia estende esta margem de sobrevida de 12 para 15 meses. Entretanto, mesmo utilizando estas abordagens, 74% dos pacientes morrem em menos de dois anos após o diagnostico. Pouco mais dos 3% dos pacientes com GBM permanecem vivos após cinco anos de diagnóstico [6, 11]. A malignidade destes tumores está diretamente relacionada a anormalidades genéticas do glioblastoma (ativação patológica ou supressão de vias de transdução de sinais intracelulares), o que aumenta sua agressividade [12].

1.1.1. Ciclo celular e gliomas

1.1.1.1. Conceito e regulação do ciclo celular

O ciclo celular consiste em uma série de quatro períodos principais regulados pela expressão, ativação e interação protéica que tem como objetivo produzir uma célula filha com a mesma informação genética que a célula que deu-lhe origem. Em eucariotos, o ciclo celular pode ser divido em dois períodos definidos : a interfase, durante a qual a célula cresce ao acumular nutrientes necessários para a mitose e divide o seu material genético; e a mitose (fase M) durante a qual ocorre a segregação do material genético previamente duplicado para os pólos celulares, seguido da citocinese (divisão celular), o que resultaem duas células-filhas idênticas [13, 14].

A interfase e a mitose apresentam diferentes “subfases” cada uma com características diferenciais. A interfase, é dividida em 3 fases principais, a G1 (*Gap* 1), S (de síntese do DNA) e G2 (*Gap* 2), além de um estágio denominado G0 ou “G zero”. Neste estágio, a célula se mantém metabolicamente ativa, mas interrompe seu crescimento permanecendo em um estado de quiescência até receber estímulos externos que permitam a continuidade de seu ciclo [13].

Uma das principais características do ciclo celular é ser unidirecional, ou seja, para que aconteça uma fase é preciso passar pela fase anterior [15]. Assim, durante a fase G1, a célula aumenta seu tamanho e sintetiza proteínas necessárias para a síntese de DNA, a qual acontece na fase S. A passagem da fase G1 para a fase S requer a

passagem por uma etapa de checagem ou *chekpoint* de G1 mediado por uma série de proteínas que avaliam o tamanho e ambiente celular [13]. Durante a fase S ocorre o processo de síntese do DNA, nesta fase o material genético é duplicado. No final da fase S, foi descrito outra etapa de checagem, mas relacionado ao processo de replicação do DNA. Durante a fase G2, ocorre um período de alta síntese protéica e de organelas. A quantidade de material genético da fase S é verificada junto às condições necessárias para entrar na fase M, constituindo uma etapa de checagem adicional. Finalmente, a fase M produz células por divisão celular com a mesma quantidade de informação genética que da célula que lhe deu origem [13].

Em nível molecular, as classes de proteínas envolvidas diretamente no controle do ciclo celular são as ciclinas, quinases dependentes de ciclinas (CDK – *Cyclin-Dependent Kinases*) e inibidores de quinases (*CDKi- Cyclin-dependent kinases inhibitors*). A principal função das CDK é fosforilar vários substratos envolvidos na progressão do ciclo celular. O substrato das CDK mais bem caracterizado (principalmente CDK2, 4 e 6, envolvidas nas fases G_1 e S) é a proteína RB (proteína do retinoblastoma), responsável pela interação física com o fator de transcrição E2F no citoplasma, portanto, deixando este no estado inativo. A fosforilação da RB pelas CDK libera E2F, que pode se translocar para o núcleo e ativar a expressão de vários genes-alvo que estão relacionados com a progressão do ciclo [13]. Os principais reguladores do ciclo celular estão representados na figura 1.

Durante a fase G1, ocorre o acúmulo de ciclina D1, que se associa com as CDK 4/6. A formação e aumento do complexo ciclina D-CDK4/6 sinaliza positivamente para a passagem pelo ponto de controle de G1 e pela fosforilação da proteína RB. Isto induz a desestabilização do complexo RB/E2F que culmina com a liberação de E2F. Esta proteína permite o início da transcrição de proteínas envolvidas nas fases seguintes do ciclo celular, especialmente as ciclinas E e A, CDK1 e c-MYC. Ao mesmo tempo, a ciclina D1 é ubiquitinada para degradação no proteossomo [14, 16]. Entre os inibidores de CDK 4/6, destacam-se p14, p15, p16 e p18, os quais estão envolvidos na parada no ciclo celular [17]. Outras fosforilações acontecem na proteína RB, assim, a fosforilação no resíduo S780 pelo complexo ciclina E-CDK2 permite a passagem de G1 para a fase S. As funções de CDK2 são contrabalanceadas pela atividade de CDKi da família Cip/Kip, que incluem p21 [18], p27 [18], e p57 [18]. Neste aspecto, é importante que a proteína c-Myc, traduzida na fase tardia de G1, iniba a atividade destes inibidores (p21 e p27). Além destes, existem outros inibidores de p21 e p27. Um exemplo é o AKT, o

qual, após estímulado com fatores de crescimento que levam à proliferação celular, bloqueia a atividade destes p21 e p27 [14, 19, 20].

Na fase S, ciclinas E são fosforiladas pela sua própria CDK, o que causa sua degradação [21]. Ao mesmo tempo, a ciclina A-CDK2 se liga a E2F e fosforila o seu parceiro transcricional DP-1, evitando a ligação de E2F ao DNA e impedindo sua atividade transcricional [22, 23]. A primeira etapa de verificação da replicação do DNA é ativada quando há elevado dano ao DNA ou falhas na maquinaria de replicação. Este ponto envolve fundamentalmente a proteína CDK2, além de proteínas relacionadas ao reparo do DNA como CHK1/2, ATM, ATR, p53 e p21, entre outras. Assim, p21 induz a inibição da CDK2 e CDK1, o que leva à parada do ciclo na fase G_2 [22].

Por outro lado, na fase G_2 o tamanho celular e a presença dos componentes celulares, incluindo organelas e lipídeos, suficientes para a produção de duas células filhas, define a etapa de checagem de G2 a M. No entanto, não está totalmente claro quais são os mecanismos que ligam o tamanho celular à progressão no ciclo. Uma das vias de sinalização mais importantes para regular tamanho celular é a via da mTOR (*mammalian Target of Rapamycin*), que responde à insulina, aminoácidos e níveis intracelulares de ATP [24].

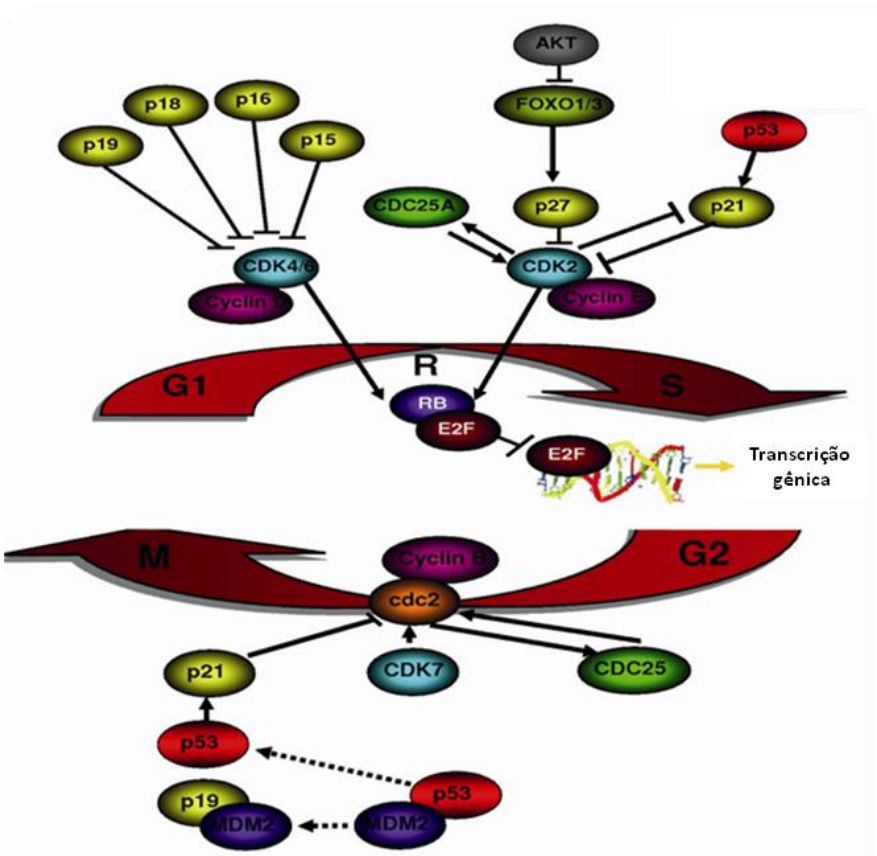


Figura 1. Representação das principais proteínas envolvidas na regulação do ciclo celular em cada uma das fases.

1.1.1.2. Alterações em proteínas chaves do ciclo celular em gliomas

Nos gliomas, de maneira similar a outros tumores, são detectadas alterações no ciclo celular. Nestes tumores cerebrais é descrita a perda da função normal de pRb, o que pode resultar da expressão alterada dos genes de p16 e CDK4, bem como a perda da própria proteína através de alterações no seu próprio gene. A via da p16/RB parece ser uma via importante tanto em GBM primário quanto em secundário. A deleção homozigota de p16 foi mais frequente em GBM primário do que em secundário [25, 26], mas não houve diferença significativa na frequência total das alterações de p16 (como deleção ou metilação do promotor) [25, 26]. A amplificação do gene CDK4 foi vista em 18 % e a deleção homozigota ou mutação no gene RB foi vista em 11 % das amostras [26, 27].

Mutações em outros supressores tumorais também são descritas. Assim, mutações no gene da p53 também ocorrem em GBM primários [26, 27]. Mutações ou deleções homozigotas do gene da p53 foram encontradas em 35 % dos casos em um

estudo de coorte consistindo predominantemente de GBM primários. A amplificação de MDM2 (proteína que regula a estabilidade de p53) está presente em 14 % dos GBM [26, 27] e exclusivamente em GBM primários sem a mutação no gene de p53 [25]. Também se produzem alterações em outros supressores tumorais como p14 [26]. As principais modificações gênicas em GBM estão representadas na figura 2.

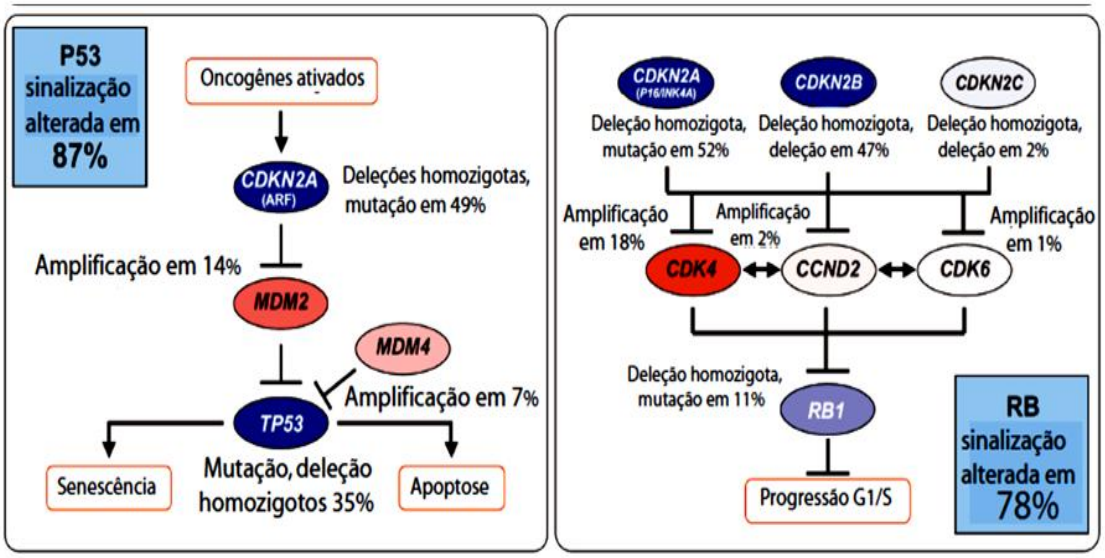


Figura 2. Vias de sinalização alteradas em GBM, p53 e pRB. Em vermelho indica modificações gênicas, sendo os mais frequentes indicados pelo vermelho mais intenso. Do contrário, a cor azul indica a alterações inativadoras, sendo mais frequentes em tons mais escuros. Para cada componente alterado consta a natureza da alteração e percentagens de tumores afetados. Nas caixas em azul se encontram as percentagens finais de alterações em gliomas com no mínimo um componente conhecido da correspondente via. Adaptado de Cancer Genome Atlas pela doutora Patricia Luciana da Costa López (2008) [27, 28].

1.1.2. Senescência celular

1.1.2.1. Conceito e principais formas de senescência

A senescência celular (CS) é definida como uma perda irreversível da capacidade proliferativa, mesmo em condições ótimas para o crescimento ou sob estímulo de mitógenos [29]. Esta parada na proliferação é acompanhada por mudanças morfológicas características deste estado. Células em CS apresentam aspecto achatado, multinucleado e citoplasma granulado [30]. O metabolismo basal nestas células é diminuído, mas funcional [31]. De maneira similar, o metabolismo lisossômico é afetado, observando-se aumento da massa lisossômica e modificação das atividades de suas enzimas. A enzima β-galactosidase ácida, presente nestas organelas, aumenta sua atividade sendo esta propriedade utilizada como um marcador clássico de CS (Figura 1). Porém, outros marcadores específicos têm sido descritos para identificação de células

neste estado, como a lipofuscina [32] e mais recentemente a detecção de focos de heterocromatina resultante da alteração da cromatina – incluindo a detecção de metilação de histonas e a fosforilação de γH2AX [33, 34].

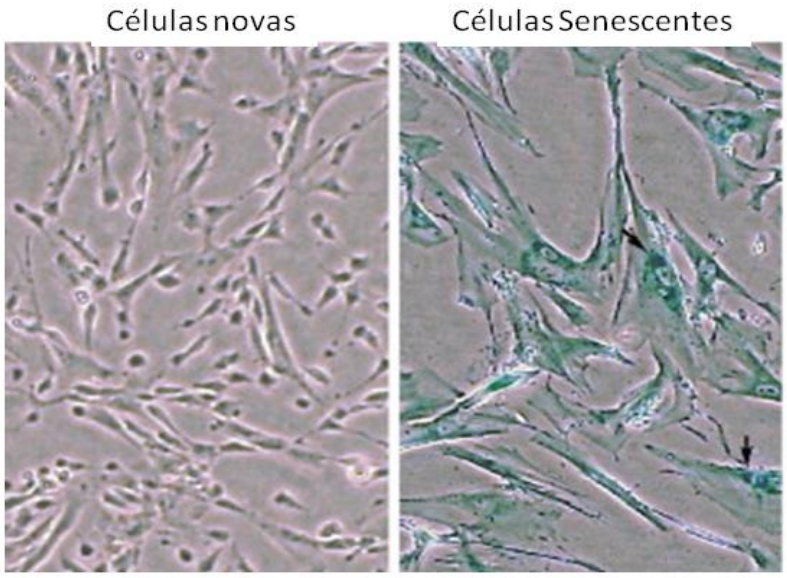


Figura 3. Comparação morfológica entre fibroblastos (TIG7) com poucas divisões (novos) e senescentes (envelhecidos). A coloração azul corresponde á atividade enzimática da β-galactosidase ácida. Estas fotomicrografias foram adaptadas de Arivzahagan, *et al* 2004 [35].

Por outro lado, diversos estudos sugerem que CS pode atuar como mecanismo endógeno que impede o crescimento descontrolado de células que potencialmente poderiam se tornar tumorigênicas [36, 37]. Assim, a indução de CS nestas células, poderia ser utilizada como uma possível terapia para esta doença [38].

Há dois tipos principais de CS: (1) Senescência replicativa (*replicative senescence* -RS) e (2) senescência induzida por estresse (*stress-induced senescence* – SIS). Nesta última se destaca a senescência induzida por oncogenes (*oncogene induce senescence* - OIS) como primeira barreira antitumoral [36].

1.1.2.2. Senescência replicativa

Em 1965, Hayflick e Moorhead observaram que células somáticas normais (fibroblastos) têm o potencial limitado de replicação em cultura [29]. Assim, nestas células a velocidade das suas divisões diminui, juntamente com mudanças morfológicas e bloqueio definitivo da capacidade proliferativa. O número de divisões até as células alcançarem este estado é conhecido como o limite de Hayflick, sendo esta forma de CS denominada como RS. No nível molecular, RS é resultante de problemas na cinética de replicação do DNA, uma vez que a DNA polimerase não consegue replicar o segmento terminal da fita retardatária de cromossomos lineares. No final das fitas que serão

replicadas de modo descontínuo não haverá fita molde de DNA para permitir a ligação com um novo *primer* depois que o último fragmento de Okasaki tiver o *primer* de RNA removido. Assim, esta região final (correspondente ao último *primer*) não tem como ser replicada, ficando exposta à ação de DNAses. Como este processo acontece em cada divisão celular, ocorre a perda de DNA localizado nos extremos dos cromossomos [39]. Os segmentos de DNA terminais são conhecidos como telômeros (do grego, Telo = fim, meros = medida). O encurtamento dos telômeros por falhas na replicação do DNA levou à hipótese de que o seu comprimento regula o número de replicações da célula *in vitro* e *in vivo* [40]. Assim, o encurtamento dos telômeros atua como um "relógio molecular" que sinalizaria a eventual RS, observada em células humanas em cultura.

Os telômeros estão constituídos de repetições de uma sequência de 6 nucleotídeos (sequência TTAGGG), com a função de preservar a integridade dos genomas [41]. Os telómeros têm uma estrutura em alça essencial à sua atividade, caracterizada por uma alça T e uma alça D. A chamada alça T é a maior de todos, e a alça D, é uma abertura da cadeia telomérica de DNA acompanhada da ligação por complementaridade da extremidade 3' [41-43]. A proteína TRF2 desempenha um papel importante no processo de formação dos telômeros, uma vez que ela assegura a ligação entre a extremidade mais distante e a "base" ou início da alça [44]. Toda esta estrutura traduz-se em uma enorme estabilidade para a terminação telomérica e impede a degradação do telômero por ação de DNAses [45] (Figura 4).

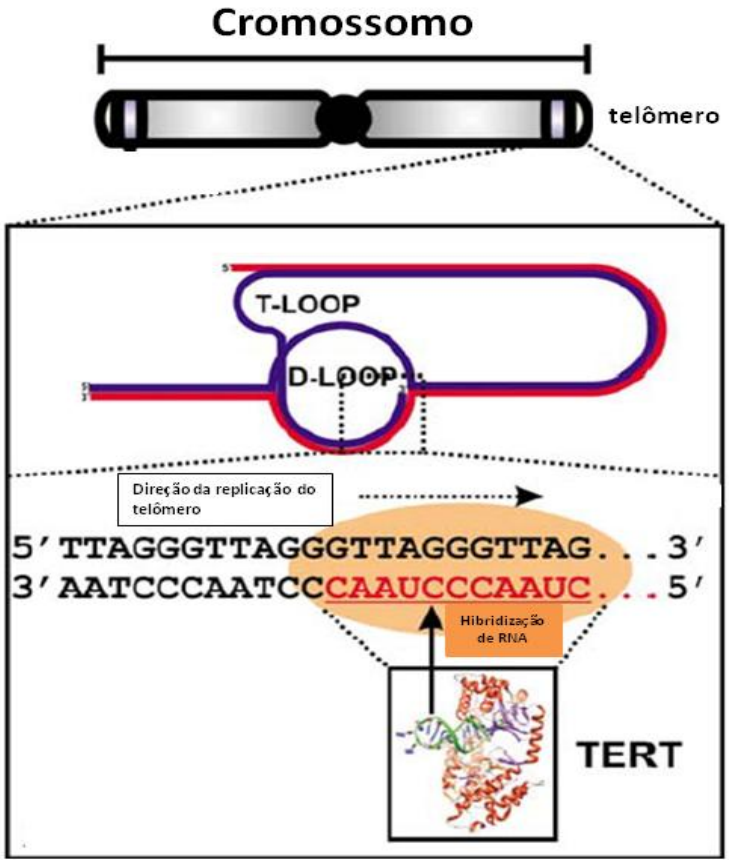


Figura 4. Estrutura das regiões dos telômeros e esquema da atividade da enzima telomerase (TERT). Está figura foi adaptada de Vargas, *et al* 2012 [36].

Quando o encurtamento progressivo do telômero atinge um tamanho mínimo necessário para a formação do loop, este telômero atingiu o ponto crítico, a partir do qual não é possivel que replicação aconteça. Este tamanho crítico dos telômeros pode danificar o DNA genômico, principalmente através da formação de pontes inter-cromossômicas, ativando vias de reparação do DNA. Nas regiões teloméricas são observadas o recrutamento de um grupo de proteínas, entre as quais se encontram ATM, ATR, RAD3 entre outras [46], proteína relacionada a ATM e RAD3 (ATR - ataxia telangiectasia and Rad3 related) e DNA-PK [47]. Sabe-se que o complexo MRE11-RAD50-Nbs1 (MRN) atua como um sensor para ATM, recrutando-a para o local da quebra na molécula de DNA [48]. A ATM é uma proteína homóloga a integrantes da família da PI3K [49] e, após sua ativação, ela fosforila a histona H2AX, que parece recrutar fatores de reparo, como RAD51 e BRCA1 (câncer de mama 1 - breast cancer 1) [50, 51] (figura 5). A ATM também causa a estabilização de p53 e induz a ativação da CHK2, uma proteína quinase relacionada aos pontos de checagem na transição G1/S, permitindo sua fosforilação e resultando na parada da progressão do ciclo celular por

ativação de p21 (figura 5). No entanto, independente do sistema de reparo, quando uma região telomérica encurta além de seu ponto critico, pode-se induzir a fusão de extremos cromossômicos para conseguir uma estabilidade estrutural relativa ou ativação de mecanismos necessários para estender estas regiões novamente [52-54].

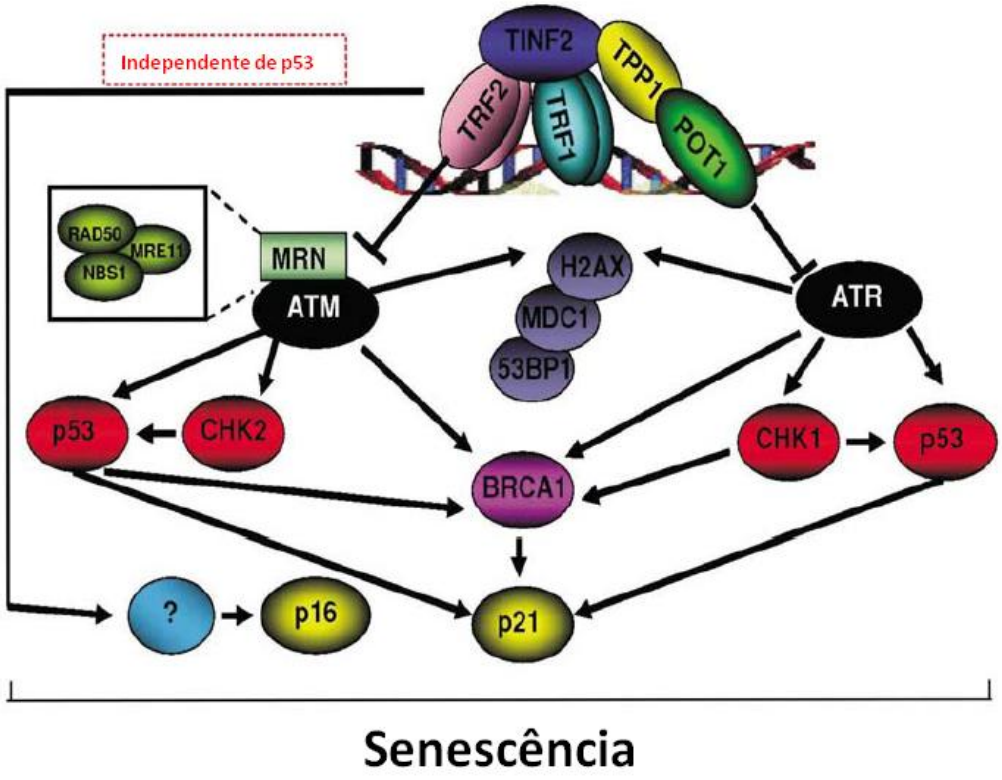


Figura 5. Ativação de sinais de dano ao DNA nas regiões dos telômeros que podem ativar RS. Está figura foi adaptada de Vargas, *et al* 2012 [36].

A telomerase (TERT) é uma transcriptase reversa constituída de uma sequência curta de RNA que serve de molde para a síntese do telômero [55], como mostrado na figura 4. O mecanismo pela qual esta enzima é ativa, ainda permanece desconhecido. A expressão de TERT ocorre nas células de linhagem germinativa, nas células-tronco e nas células neoplásicas, havendo nestas células uma regeneração dos telômeros e prevenção da senescência replicativa. No entanto, a maioria das células humanas somáticas normais apresenta pouca ou nenhuma atividade de TERT. Quando os telômeros chegam a um comprimento mínimo, específico para cada célula, ocorre a sinalização que determina a senescência celular [36].

Cerca de 85% das células de tumores malignos expressam TERT [56], o que apoia a hipótese de que a alta atividade da TERT é importante para a tumorigênese e / ou manutenção do tumor. Diversos estudos confirmam a correlação da reativação de TERT com a progressão maligna de vários tipos tumorais [57, 58]. Por exemplo, estudos através de um modelo de indução de linfoma de Burkitt em camundongo expressando o oncogene Eμ-myc, observaram que ao realizar o *Knockout* (KO) do gene da TERT, a formação de tumor era significativamente reduzida [59, 60]. Este mecanismo não foi bloqueado pela superexpressão de *Bcl2*, o que sugere o mecanismo de senescência e não de apoptose. Além disso, os animais KO apresentaram forte coloração de senescência nos gânglios linfáticos em comparação aos gânglios dos animais controles. Assim, o bloqueio da expressão ou atividade da TERT pode ser utilizado como uma abordagem antitumoral [36].

Outra maneira de analisar RS é do ponto de vista metabólico. A teoria dos radicais livres, proposta em 1956 por Denham Harman, estabelece que o envelhecimento celular derivado de RS advém dos efeitos deletérios nas organelas celulares, causados pelas espécies reativas de oxigênio (ERO) [61]. As espécies reativas de oxigênio, como o oxigênio singlete (O_2) e os radicais superóxido (O^{2-}) e hidroxila (OH), são geradas fisiologicamente nos organismos aeróbios [61]. Aproximadamente 90 % das espécies reativas de oxigênio são produzidas por mitocôndrias em decorrência da fosforilação oxidativa. Os elétrons derivados do NADH (nicotinamida-adenina-dinucleotídeo) ou de FADH (flavina-adenina-dinucleotídeo) podem reagir diretamente com o oxigênio ou com outros receptores de elétrons em vários pontos da cadeia transportadora, gerando ERO [62]. A associação entre TERT e mitoncôndrias também foi estudada. Estudos recentes sugerem que hTERT (subunidade catalítica de TERT) é exportada do núcleo à matriz mitocondrial para proteger o DNA [63]. No entanto, o mecanismo molecular pelo qual hTERT é exportada para cumprir uma função diferente do que a extensão dos telômeros, ou seja, uma função "não clássica" ainda não foi descrito.

1.1.2.3. Senescência induzida por oncogenes

Nos estágios iniciais de transição para transformação tumoral ocorre uma série de mutações que produzem células mais agressivas, as quais são seleccionadas positivamente pelo tecido e/ou pelo microambiente do tumor [64]. A transição neoplásica é caracterizada pelo aumento da expressão de oncogenes que levam a câncer

[65]. Estes podem ser ativados por rearranjos cromossômicos, como consequência da clastogênese, ou por alterações genéticas estruturais, tais como a fusão , a justaposição de elementos potenciadores [65, 66] ou a amplificação do gene [65].

No entanto, há quase três décadas foi observado que as células normais são resistentes à transformação oncogênica, indicando que as células não tumorais provavelmente possuem mecanismos que impedem a atividade de oncogenes [65], promovendo a entrada em senescência. Esta forma de senescência é conhecida como senescência induzida por oncogenes (OIS). Este processo foi verificado para oncogenes como *Ras, Raf, E2F, Stat5* e *Akt,* que podem induzir CS em condições normais de cultura celular. Um modelo interessante para OIS são os sinais da pele. Os sinais estão caracterizados pela presença de melanócitos ligeiramente alterados, com tamanhos e formas variados [67, 68]. Nos sinais existe uma alta frequência de mutações em *Braf*. Este gene codifica uma proteína serina/treonina-quinase que faz parte da via de sinalização RAS/RAF/MEK/ERK. Esta via regula o crescimento, sobrevivência e diferenciação celulares e pode ser ativada por diversos receptores de membrana, incluindo receptores de tirosina quinases ou receptores acoplados à proteína G [69]. O estímulo destes receptores leva à ativação da GTPase RAS, que por sua vez ativa uma das quinases da família RAF entre os quais a B-RAF. As quinases RAF continuam, depois da cascata, ativando MEK1/2 que por sua vez ativam ERK1/2. As proteínas ERK ativadas podem fosforilar as suas proteínas alvo no citoplasma ou fazer translocação para o núcleo, onde os seus alvos são fatores de transcrição que regulam a expressão de genes envolvidos na proliferação, diferenciação e a sobrevivência celular [69].

Existem diversas mutações oncogênicas no gene *Braf*. A mais frequente é a B-RAF que consiste na substituição do aminoácido valina no sitio 600 por um ácido glutâmico, situada na alça de ativação do domínio catalítico da quinase. Trata-se de uma alteração de troca de sentido (*missense*) que provoca uma ativação da quinase independentemente de Ras. Isso leva à ativação da via de sinalização a jusante, estando presente, sobretudo em cânceres da pele, da tiróide e do cólon [70-72]. Apesar da ativação de B-RAF promover a proliferação celular, CS é induzida nestas células [71]. A ativação oncogênica de B-RAF é regulada através de uma retroalimentação negativa via ERK1/2 que induz a liberação de protéinas difusíveis como IGFBP-7, a qual atua como inhibidor de B-RAF induzindo senescência aguda. Esta senescência é dependente da ativação do CDKi p16 [36]. Esta via de sinalização é apresentada na figura 6.

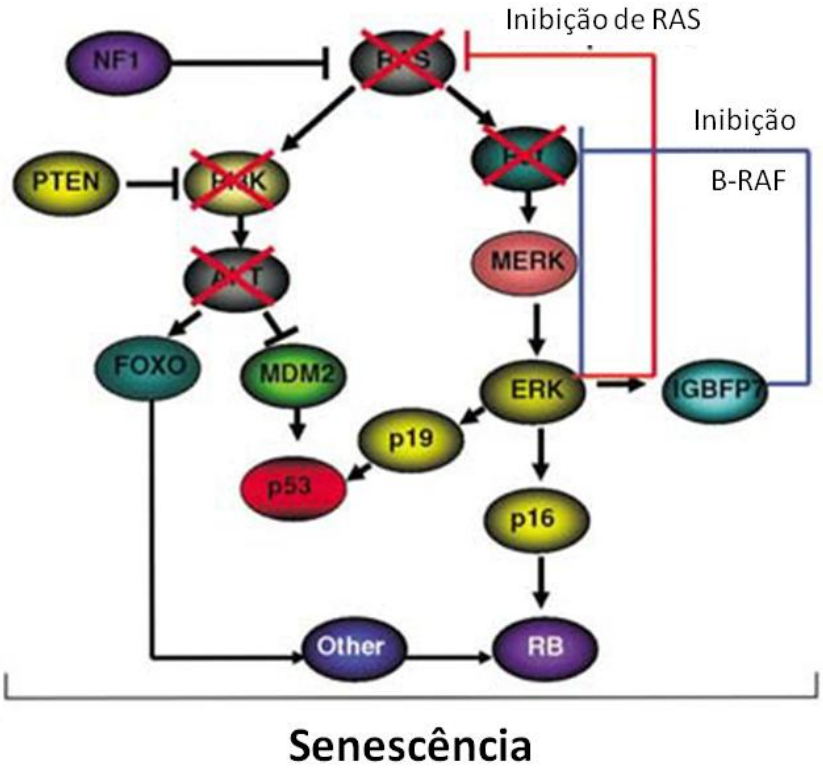


Figura 6. Mecanimos de inibição da via B-RAF em nevus (sinal da pele). Está figura foi adaptada de Vargas, *et al* 2012 [36].

Existem outros mecanismos para induzir OIS. Assim OIS pode ocorrer através de estresse na replicação do DNA que ativa sinais de dano, que por sua vez ativam a p53, induzindo a senescência [36, 73]. Os oncogenes ativam sinais de proliferação, o que produz um desbalanço na fisiologia celular e na replicação do DNA. O estresse na replicação do DNA produz uma replicação de DNA ineficiente que faz com que as forquilhas de replicação do DNA progridam lentamente ou parem. No entanto, existem OIS que atuam por uma via independente de resposta ao dano do DNA (*DNA damage response*-DDR) como foi mostrado AKT1. AKT1 pode induzir senescência em ausência de sinais da dano ao ativar mTOR em células com PTEN deletadas [74].

1.2. Derivados naturais como antitumorais

A utilização de plantas medicinais no Brasil foi a base da farmacologia até meados do século XX, quando houve uma forte expansão da indústria farmacêutica no mundo. Nesta época, o Brasil era um país essencialmente rural, com amplo uso da flora medicinal. Com o início da industrialização, o conhecimento tradicional começou a ser

posto em segundo plano. O acesso a medicamentos sintéticos e a falta de comprovação da ação farmacológica das plantas tornou o conhecimento da flora medicinal sinônimo de atraso tecnológico. Essa tendência seguiu o que já acontecera em outros países em processo de urbanização [75]. Entretanto, no século XXI as plantas medicinais têm ocupado um lugar relevante com o intuito de melhorar a qualidade de vida na terceira idade e prevenir doenças [76-79]

Nesta tese, dois componentes derivados de plantas e presentes na dieta humana (resveratrol e quercetina) e outro derivado do metabolismo intestinal (butirato de sódio) foram estudados em relação ao seu potencial antitumoral. Nas últimas décadas, estes compostos têm se destacado pelo seu potencial anticarcinogênico, sem afetar em forma nociva o tecido sadio. Por este motivo, os mecanismos endógenos pelos quais eles induzem ações antitumorais foram explorados mais profundamente.

1.2.1. Resveratrol

O Resveratrol (3,4',5-trihidroxiestilbeno) é um polifenol pertencente ao conjunto de compostos denominados fitoalexinas [80]. A estrutura de sua molécula consiste em dois anéis fenólicos unidos ligações covalentes duplas, que permite orientações *cis* e *trans* (figura 1). Estas duas formas isômericas: *trans*- e *cis*-resveratrol (3,4',5-trihidroxi-*trans*-estilbeno e 3,4',5-trihidroxi-*cis*-estilbeno) e outras diversas formas análogas, dentre elas os isômeros *trans*- e *cis*-piceido (*trans*-resveratrol *3-O-β-glucoside* e *cis*-resveratrol *3-O-β-glucoside*) são majoritariamente presentes na natureza. O isômero *trans*-resveratrol é comercializado no estado sólido e estável. No entanto, quando esta isoforma é exposta a radiação ultravioleta (UV), ela se torna um racemato das formas *cis*- e *trans*-resveratrol [81].

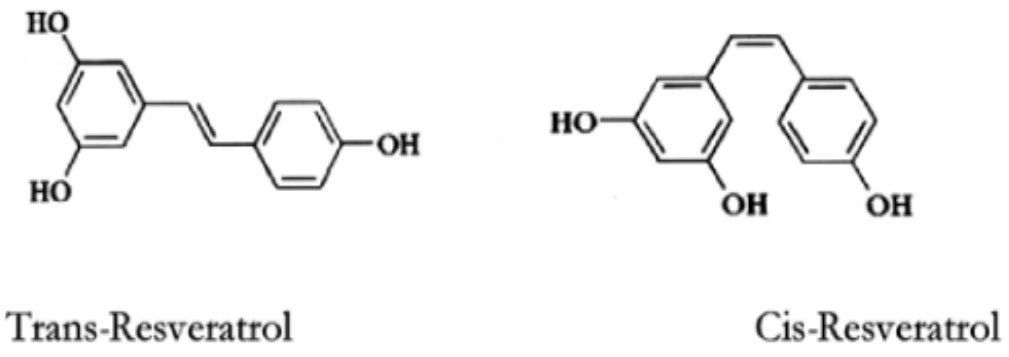


Figura 7. Estrura química de *trans*- e *cis*-resveratrol

1.2.1.1. Fontes de resveratrol

O resveratrol é sintetizado naturalmente por uma ampla variedade de plantas em resposta à exposição à radiação UV ou pelo estresse mecânico produzido pela ação de patógenos, agentes químicos e físicos [82]. O primeiro estudo relatando os efeitos benéficos do consumo de vinho foi divulgado em 1979 mostrando uma diminuição da taxa de mortalidade por doenças cardíacas isquêmicas [83] . Em 1992 surgiu o primeiro trabalho associando este efeito ao resveratrol [84].

No mesmo ano, Renaud e Lorgeril destacaram que alta ingestão de gorduras saturadas estava correlacionada positivamente com a taxa de mortalidade por doenças cardíacas e coronarianas [26]. Contudo, na França se configurava uma situação paradoxal, já que havia baixas taxas destas doenças, ainda que a dieta fosse rica em gorduras saturadas. Este paradoxo foi atribuído ao vinho, e assim recebeu o nome de Paradoxo francês [26]. O agente protetor presente no vinho era o resveratrol, posteriormente confirmado experimentalmente [85-87].

Nas espécies de videiras *Vitis vinifera* e *Vitis labrusca* foi encontrada a maior capacidade de síntese de resveratrol [82, 88] sendo as variedades Pinot Noir, Merlot e Cabernet Sauvignon as que possuem a maior quantidade deste polifenol [81, 89]. Nas análises realizadas em diferentes amostras de uva, o resveratrol apresentou uma concentração na película do fruto de 27,5 μg/g, sendo que no bagaço da uva, cascas e sementes após a extração do sumo, apresentou uma concentração de apenas 6,0 μg/g [82, 90]. Em todos estes casos, foram encontrados compostos: *trans*-resveratrol, *trans*- e *cis*-piceido, não sendo constatada a presença do isômero *cis*-resveratrol [89].

Nos vinhos tintos, foram encontradas concentrações de resveratrol entre 0,82 e 5,75 mg/L [91]. No entanto nos vinhos brancos os níveis são de 1,8 mg/L [88, 89]. Por outro lado, nos vinhos rosés, resultantes da mistura de vinho tinto e branco ou de um contato menor com a casca da uva no mostro, a quantidade de resveratrol atinge valores intermediários de aproximadamente 2,15 mg/L [88, 89]. Os demais produtos industrializados derivados da uva como os sucos, geléias e as gelatinas também apresentam resveratrol em concentrações inferiores a 0,15 mg/L [81]. Os sucos de uva de origem brasileira são considerados uma boa fonte do composto, pois apresentaram concentrações relativamente elevadas (0,19 a 0,90 mg/L), como descrito em [92].

Alem das videiras, existem outras fontes de resveratrol. Aproximadamente 72 plantas distribuídas em 12 familias foram descritas como capazes de produzi-lo [93].

Dentre estas plantas se destacam: o eucalipto (*Eucaliptus wandoo*, Myrtaceae) e o amendoim (*Arachis hypogea*, Fabaceae).

1.2.1.2. Absorção, metabolismo e biodisponibilidade do resveratrol

O resveratrol pode ser administrado por vias oral e intravenosa. Em seres humanos, constatou-se que aproximadamente 70% do composto são absorvidos pelo organismo, independente da via de administração [94]. Este dado foi obtido pela análise da biodistribuição do resveratrol marcado com carbono 14 em indivíduos saudáveis, que receberam resveratrol marcado por via oral (25 mg). A radioatividade dos 30 % não absorvidos foi observada na urina, bile e fezes. A concentração máxima observada no plasma após 1 h de administração por via oral foi de aproximadamente 22 nM, com um segundo pico após 6 h. Por via intravenosa, a concentração de resveratrol no plasma foi de 36 - 45 nM e não houve um segundo pico. Após administração intravenosa, o resveratrol foi distribuído por todo organismo, e provavelmente absorvido pelos tecidos antes de chegar à circulação entero-hepática. A meia vida da radioatividade total encontrada no plasma foi de 10 h, para ambas as vias de administração [94]

O transporte do resveratrol dentro dos tecidos é realizado pela difusão trans-epitelial (transporte passivo) e pelo processo mediado por uma proteína carregadora [95]. Ambos os transportes são facilitados pela alta lipossolubilidade deste composto e elevada afinidade pela albumina, proteína que facilita o seu transporte [95]. A absorção do resveratrol se dá basicamente no intestino. No fígado e duodeno ele é modificado para trans-resveratrol-3-O-glicuronídeo, trans-resveratrol-4´-O-glicuronídeo, e trans-resveratrol-3-O-sulfatoma e ainda nestas formas produz uma ampla variedade de efeitos pleiotrópicos [96]. Devido a sua solubilidade, o resveratrol consegue atravessar a barreira hematoencefálica (BHE) atingindo o SNC. Em ratos tratados por via oral com 20 mg/Kg de resveratrol, a concentração encontrada no cérebro foi de 0.11 nmol/g após 10 minutos [26, 97].

1.2.1.3. Senescência celular e resveratrol: alvos moleculares

Uma ampla quantidade de estudos sugere que resveratrol cumpre um papel duplo, induzindo senescência em células tumorais de diferentes linhagens, e protegendo células normais do processo de envelhecimento. Em 1997, foi publicado o primeiro estudo demonstrando o efeito antitumoral do resveratrol, no qual se observou uma

redução de 98 % no crescimento de câncer de pele em camundongos após aplicação tópica [93, 98]. Outros estudos confirmam este achado demonstrando que células tumorais possuem um potencial reduzido de invasão de vasos sanguineos após administração de resveratrol [99, 100]. Este efeito pode se dar devido ao fato de que a transformação celular é bloqueada em presença de resveratrol [101, 102]. Uma proteína chave neste processo de transformação celular, a telomerase (descrita na seção*1.1.2.2*), possui a expressão reduzida pela adminstração do resveratrol em experimentos *in vitro* utilizando linhagens tumorais [103-105].

As vias metábolicas também são afetadas e o fenótipo senescente é detectado após tratamento com resveratrol. Assim, estudos *in vitro* mostram inibição na ribonucleotido redutase [106, 107], ciclo-oxigenases [108, 109], e diferentes cinases [110-112]. Porém efeitos benéficos foram descritos em tecidos saudáveis. Desta forma, diversos estudos têm proposto a utilização do resveratrol como fármaco para tratamento de patologias, tais como diabetes, hipertensão, aterosclerose, osteoartrite e doenças neurais [113-118]. O efeito protetor do resveratrol foi associado à diminuição de processos inflamatórios e defesa contra espécies reativas de oxigênio (ERO), fatores importantes para o desenvolvimento de várias destas doenças [119-121].

Para estudar mais profundamente os efeitos do resveratrol, diferentes abordagens experimentais *in vitro*, foram desenvolvidas. Em cultura primária de neurônios, o resveratrol estimula a biogênese mitocondrial pela ativação da via da proteína quinase ativada por AMP (AMPK) [122] e induz o aumento de sua funcionalidade através de proteínas chamadas sirtuinas [123]. Estas proteínas possuem capacidade de desacetilação de histonas NAD-dependentes, o que modula a transcrição de vários genes envolvidos no metabolismo básico (AKT e proteínas desacopladoras UCP2). As sirtuinas estão relacionadas ao aumento da longevidade, com efeitos similares à restrição calórica. Neste sentido, o efeito de antienvelhecimento mediado por resveratrol ativa sirtuinas em *Caernohabiditis elegans* e *Drosophila melanogaster* estendendo a vida destas duas espécies [14, 124, 125]. Por outro lado, alguns estudos ja demonstraram resultados controversos, aos citados, quanto a ativação de (sirtuina 1) SIRT1 por resveratrol [126-129]. Aonde resveratrol não aumentou a longevidade de algumas cepas de leveduras. Além disso, foi demonstrado que o resveratrol somente ativa SIRT1 *in vitro*, quando testado em substrato acetilado ligado a um nucleo fluorescente [126]. Dois estudos recentes enfatizaram a problematica de ativação de SIRT1 por resveratrol. O primeiro, utilizando dois conhecidos alvos acetilados de SIRT1, PGC1α e p53, demonstrou que o aumento na atividade catalitica de SIRT1,

mediada por resveratrol é presente somente na presenca do peptideo marcado com fluoroforo (*Fluor-de-lys*). O estudo ainda sugere que o composto pode modular, indiretamente, a atividade de SIRT1 [130, 131].

Em camundongos que receberam baixas doses de resveratrol, concluiu-se que o composto mimetizou parcialmente a restrição calórica e esta foi independente de SIRT1 [132], sugerindo que o mecanismo pode ser diferente *in vivo*, e apresentar maior complexidade por estar relacionado com a dose administrada. No entanto, recentemente, foi confirmado que o resveratrol aumenta a expectativa de vida com manutenção da homeostase celular em mamíferos afetando á atividade SIRT1 [133]. Ele atua inibindo cAMP fosfodiesterases, as quais induzem a liberação de cálcio do retículo endoplasmático, resultando na ativação de SIRT1, como mostrado na figura 8.

A ativação da SIRT1 tem duplo papel, reprimindo ou induzindo o crescimento tumoral [134]. No entanto, ao nosso conhecimento nenhum artigo relaciona este fato com a possivél regulação farmacológica de SIRT1 para aumentar o efeito antitumoral de resveratrol em GBM.

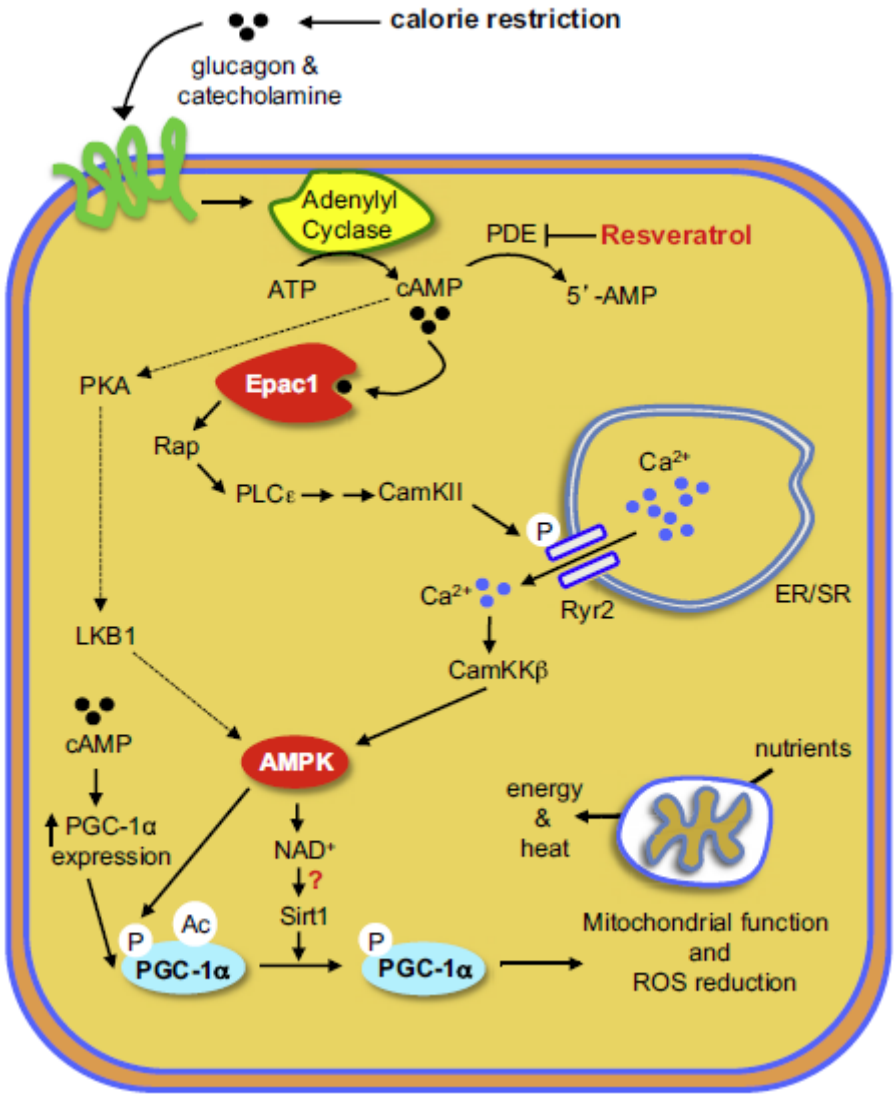


Figura 8. Modelo sugerido sobre o mecanismo pelo qual o resveratrol simula os efeitos da restrição calórica, adaptado de Park [133].

1.2.1.4. Gliomas e resveratrol: efeitos pleiotrópicos

Como foi descrito na seção anterior, o resveratrol tem um amplo espectro de ação, podendo atingir diversos alvos moleculares. Cabe também ressaltar que o efeito do resveratrol varia de acordo com o tipo celular e com o estado metabólico da célula. Em gliomas, o efeito citotóxico do resveratrol vem sendo caracterizado desde 2004, quando Tseng e colaboradores demonstraram que o resveratrol inibe seu crescimento (RT2) em camundongos, induzindo apoptose das células tumorais aumentando a sobrevida e inibindo a angiogênese tumoral [26, 135]. Outro estudo semelhante foi realizado utilizando implante subcutâneo de células de neuroblastoma em camundongos que foram tratados com resveratrol. Neste estudo, foi demonstrado um aumento da apoptose e diminuição do crescimento das células cancerosas [136]. Na literatura

cientifica, também são encontrados vários enfoques *in vitro*, utilizando o resveratrol em linhagens de gliomas U251, U87-MG e C6 que confirmam seu efeito antitumoral por indução de morte e senescência [137-140]. Estudos do nosso grupo e de outros, demonstraram *in vitro* e *in vivo*, que os efeitos do resveratrol são dependentes da dose e da concentração nas linhagens de gliomas U87-MG, GL261 e C6. Por exemplo em C6, a concentração de resveratrol 50 μM induz apoptose em 48 h, mas a concentração de 10 μM provoca senescência após 12 dias de tratamento [140].

1.2.2. Quercetina

A quercetina pertence ao grupo dos flavonóides, nome dado aos pigmentos derivados da benzo-g-pirona encontrado em plantas. Estes compostos possuem uma estrutura em comum de três anéis (A, C e B) com um esqueleto carbônico C6 – C3 – C6 (Figura 01). O primeiro anel "A"é aromático, o segundo anel "C' é um anel heterocíclico associado a um oxigênio e ligado por meio de uma ligação C-C ao terceiro anel aromático "B", revisado em [141]. Esta estrutura básica permite a possibilidade de substituições nos anéis benzênicos A e B, tais como hidroxilação, glicosilação, metilação, sulfatação e glicuronidação, que podem ser resultantes de processos metabólicos [141] . A estrutura da quercetina está representada na figura 9.

A estabilidade da quercetina é limitada para a forma aglicona (sem açucar associado) observada nas seguintes condições: no plasma, em acetonitrila e em água à temperatura ambiente [141, 142]. Neste estudo, foi observarada a instabilidade da quercetina em diferentes valores de pH (pH 2,7, 7 e 10) e temperatura (4ºC e -20ºC), a qual foi atribuída à instabilidade da estrutura do anel central que pode resultar na fragmentação do mesmo. No entanto, em condições ácidas, a quercetina mostrou-se mais estável [141].

Nas plantas, os flavonóides respondem à luz, protegendo-as contra os danos ocasionados pela radiação UV e controlando os níveis de auxinas, (reguladores do crescimento e diferenciação) [143]. Outras funções descritas são atividade antibacteriana e antifúngica e participação na pigmentação de frutas [144, 145].

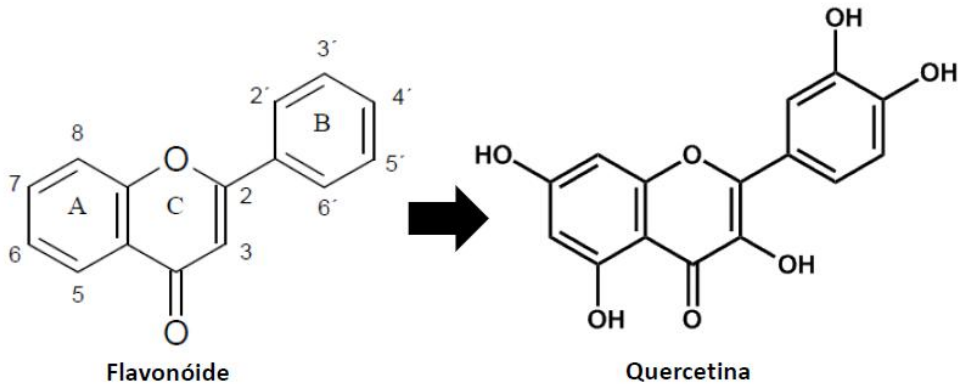


Figura 9. Estrutura química da quercetina. Na esquerda é representado o esqueleto carbonado dos flavonóides; na direita a estrutura da quercetina, grupos hidroxila são incorporados nos anéis heterocíclicos da estutura de flavonóides.

1.2.2.1. Fontes da quercetina

Dois estudos avaliaram mais de 50 amostras, entre diferentes variedades de frutas, hortaliças, verduras e alguns derivados destas, consumidas no Hawaii, e verificaram que as concentrações de quercetina nestes alimentos foram bastante variadas [146, 147]. Encontraram elevadas concentrações de quercetina em cebola (284-486 mg/kg), couve (100 mg/kg), vagem (32-45 mg/kg), brócolis (30 mg/kg), repolho (14 mg/kg) e tomate (8mg/kg). Considerando frutas, a concentração média de quercetina encontrada foi de 15 mg/kg, com a maçã tendo os maiores níveis, entre 21 e 72 mg/kg. Em bebidas (vinho Blanco e café) a quantidade encontrada foi de aproximadamente 1 mg/L. O chá preto é a bebida que apresenta maior concentração de quercetina, em torno de 10-25 mg/L.

1.2.2.2. Absorção, metabolismo e biodisponibilidade da quercetina

Na dieta, a quercetina pode ser encontrada como β-glicosídeo, ou seja, com um açúcar ligado na posição 3 do anel C [141]. No entanto, a quercetina pode ser encontrada agliconada. A natureza da glicosilação é conhecida por influenciar sua eficiência de absorção [148]. Assim, um estudo com voluntários humanos ileostomizados mostrou que 52 % da quercetina glicosídica é absorvida no intestino delgado, enquanto apenas 24 % da forma aglicona é absorvida [149]. No entanto, outros estudos sugerem que a natureza hidrofílica dos grupos glicosídeos tem sua absorção alterada pelo intestino delgado, acontecendo uma clivagem da ligação β-glicosídica no intestino grosso, quando a microflora presente entra em ação. Porém, outros estudos afirmam que glicosilação e glicuronidação ocorrem durante a passagem através do epitélio [141, 150]

No cólon, a absorção da quercetina glicosídica acontece após a hidrólise pela microflora residente. Entretanto se a quercetina for absorvida intacta, a deglicosilação pode ser efetuada pela β-glicosidase citosólica presente em células intestinais [141]. Após a absorção no intestino, a quercetina pode ser conjugada [141]. A etapa subsequente de sua metabolização é a conjugação que pode acontecer nos rins e no fígado. Assim, a quercetina pode se conjugar com o ácido glicurônico ou com o ácido sulfúrico aumentando sua solubilidade e sua eliminação [141]. Outra modificação que pode ocorrer na quercetina é a metilação no fígado pela ação da O-metiltransferase [141]. A extensiva O-metilação da quercetina, em adição a outras reações de conjugação podem justificar a falta de toxicidade pela adição de grupos glicuronídeos e sulfatídeos [141].

Em relação à biodistribuição da quercetina, de Boer e colaboradores (2005) encontraram uma ampla distribuição tecidual desta após 11 semanas de suplementação na dieta de ratos [26, 151]. Após administração de 500 mg/Kg, a concentração de quercetina no cérebro foi de 0,06 nmoles/g e, quando a suplementação foi realizada por 3 dias observou-se uma concentração de 0,7 nmoles/g, sugerindo que este composto consegue atravessar a BHE e alcançar concentrações detectáveis no cérebro [26, 152].

1.2.2.3. Quercetina e senescência: alvos moleculares

Mutações em p53 estão entre as anormalidades genéticas mais comuns em cânceres humanos [153]. Em linhagens celulares de câncer de mama, foi observado que a quercetina (248 μM) regula negativamente a expressão de proteína p53 mutada para níveis quase indetectáveis [154, 155]. Concentrações mais baixas resultam em uma menor redução, indicando um mecanismo dose dependente [155]. Além disso, há inibição do acumulo de p53 pelo que induz uma parada na fase G2-M do ciclo celular [155].

Por outro lado, a etapa de controle de G1 também é controlado pelo gene p53. A quercetina induz senescência em células T de leucemia humana [156]. Numa concentração de 70 μM, 64 % das células estavam em G0 /G1 comparado com 50 % em culturas controle. Esta parada em G1 também foi observada em células de cânceres gástricos tratados com quercetina [156]. Os principais alvos que interagem com a quercetine são mostrados na figura 10.

As tirosina-quinases são uma família de proteínas localizadas na, ou próximo à membrana celular. Estas proteínas estão envolvidas na transdução de sinais de fatores

de crescimento ao núcleo. Elas também estão envolvidas na oncogênese, assim seus inibidores têm sido utilizados como agentes antitumorais [157]. Em pacientes com câncer avançado, a administração intravenosa de quercetina (dosagens 60-1700 mg/L conduziu à inibição de tirosina-quinases de linfócitos em menos de uma hora, em 9 dos 11 casos [158]. Esta inibição foi mantida 16 horas pós-administração do polifenol [158]. Assim, a quercetina foi o primeiro composto inibidor da tirosina quinase testados em humanos e com fase clínica em desenvolvimento [158].

Em modelos animais a quercetina leva á indução de apoptose [159], aumentando a relação Bax (proteína BCL2 associada ao X - *Bcl2 associated X protein*)/Bcl-2 [160], aumentando a liberação de citocromo-c da mitocôndria, aumentando a ativação de caspase-3 e caspase-9 [161].

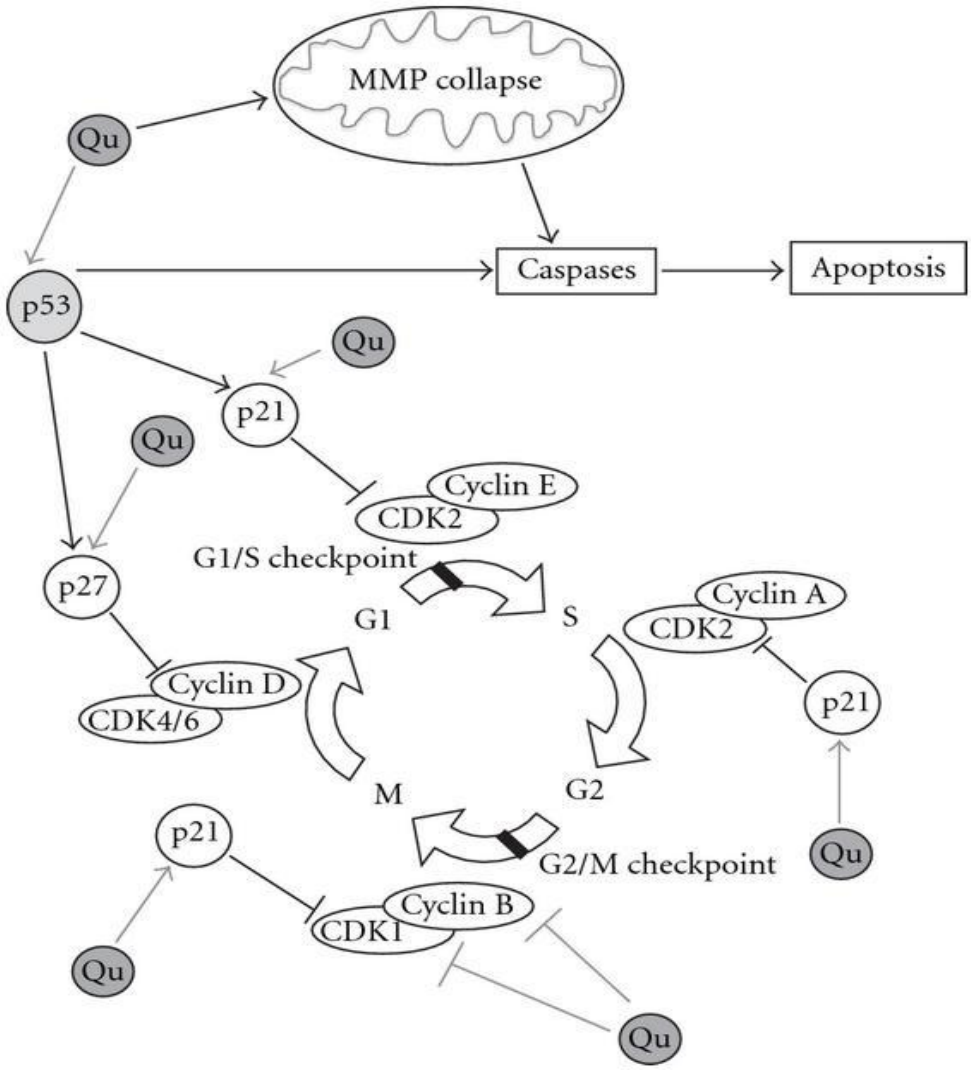


Figura 10. Alvos moleculares do ciclo celular que são afetados pela quercertina. A quercetina é representada com a sigla Qu dentro das esferas cinzas. Figura adaptada de Gibellini [162].

1.2.2.4. Gliomas e quercetina: efeitos pleiotrópicos

Diversos estudos utilizando linhagens de glioma analisaram o efeito antitumoral da quercetina *in vitro* [26, 163-165]. Braganhol e colaboradores (2006) demostraram que a quercetina induz morte por apoptose e necrose na linhagem de glioma humano U138 [165]. No entanto, este composto protege culturas organotípicas de hipocampo de rato da morte celular induzida por privação de oxigênio e glicose. Resultados similares foram obtidos nas linhagens U87-MG, U251 e A72 quando tratadas com quercetina [166]. Este flavonóide sensibiliza as células à apoptose pela diminuição da expressão de survivina, uma proteína anti-apoptótica [166]. Esta mesma via de sinalização junto com AKT e ERK parecem ser os principais mecanismos afetados por quercetina em glioblastomas, como sugerido por Kim e colaboradores (2008) [163].

1.2.3. Modificações epigenéticas induzidas por fármacos: uma estratégia antitumoral

A unidade básica da cromatina é o nucleossomo constituido, aproximadamente, por 146 pares de bases do DNA enoveladas a um octâmero de histonas. Essas proteínas básicas foram consideradas como componentes meramente estruturais, mas nos últimos 10 anos foram reconhecidas pelo importante papel que desempenham na manutenção do equilíbrio dinâmico da cromatina [167].

Modificações pós-traducionais acontecem em forma preferencial nos extremos aminoterminais das histonas, sendo acetilação, fosforilação, metilação, ubiquitinação, as mais estudadas [168]. Estas modificações induzem mudanças na estrutura dos cromossomos e alteração na expressão de seus genes, através de dois mecanismos distintos. No primeiro, todas as modificações alteram a carga eletrostática das histonas e isto pode modificar as propriedades estruturais das histonas e ligantes do DNA [169]. No segundo, estas modificações pós-traducionais podem gerar, estabilizar, romper ou ocluir domínios de interação na cromatina para proteínas regulatórias, como fatores de transcrição, proteínas envolvidas na condensação da cromatina e reparo do DNA [170, 171].

Duas modificações regulam diretamente a expressão gênica: acetilação e metilação. A acetilação facilita a expressão de genes (genes ativos), enquanto a metilação pode estar associada tanto a genes ativos como a silenciosos [169]. Assim, a metilação nas lisinas 4, 36 e 79 da histona H3 (H3), por exemplo, está associada a genes ativos, enquanto nas

lisinas 9 e 27 está associada a genes silenciosos [172]. Porém, entre estas duas modificações, mudanças no padrão de acetilação do DNA são preferencialmente mais estudadas pelo seu envolvimento direto em diferentes estados patológicos. Assim, o desequilíbrio da acetilação e desacetilação das histonas em regiões promotoras contribui para a desregulação da expressão gênica e tem sido associado à carcinogênese e à progressão do câncer [173]. No entanto, mutações na histona H3.3 foram recentemente associadas a GBM [174-176]. A análise do exossoma de 48 amostras de GBM pediátricos revelou mutações somáticas na via da remodelação da cromatina H3.3-ATRX (-thalassaemia/mental retardation syndrome X-linked) -DAXX (death-domain associated protein), responsável do recrutamento da H3.3 nas heterocromatinas pericentricas e nos telômeros [174]. Pela primeira vez, mutações em histonas foram associadas a doenças específicas. Isto reforça a idéia da complexidade do câncer e a difilcudade de encontrar terapias adequadas para esta doença, na qual tanto mutações como mudanças epignéticas sobre histonas atuam em forma conjunta para aumentar a malignidade tumoral.

Duas famílias de enzimas: as histonas acetiltransferases (HAT) e as histonas desacetilases (HDAC) regulam os níveis globais da acetilação do DNA. As HAT transferem grupos acetil para resíduos de lisina aminoterminal nas histonas, resultando na expansão local da cromatina e no aumento da acessibilidade de proteínas regulatórias do DNA [177]. Já as HDAC catalisam a remoção de grupos acetil, levando à condensação da cromatina e repressão transcricional [178]. A modulação farmacológica das HDAC tem sido utilizada para o tratamento de diferentes tipos de patologias, dentre elas o câncer [179]. Existem vários compostos com atividade inibidora de HDAC descritos e estes basicamente são classificados em quatro grupos estruturais: os inibidores de HDAC (HDACi) do tipo ácidos hidroxâmicos, entre eles Trichostatin A, SAHA, PXD101, LBH589, 4SC-201; os ácidos graxos de cadeia curta, como o butirato de sódio, Privanex e o ácido valpróico. Esses três grupos agem inibindo HDAC de classes 1 e 2 [180]. O quarto grupo estrutural é composto por benzamidas, MS-275 e MGCD-0103, que inibem além da classe 1 e 2, também a classe 3 [180]. Estudos com inibidores de HDAC têm se mostrado eficazes em diminuir a taxa de proliferação e aumentar a apoptose em diversos tipos tumorais, estando, muitos deles, sendo testatos em ensaios clínicos fase I, II e III [180].(tabela 1).

Tabela 1**. HDACi utilizados como antitumorais**. Adaptado de khan e Thaneg, 2012 [180].

Grupo químico	Composto	Concentração de uso	Fase clínica
Hidroximato	SAHA (vorinostat)	nM	II, III
	PXD101 (belinostat)	nM	I, II
	LBH589 (panobinostat)	nM	II, III
	ITF2357 (givinostat)	nM	I, II
	PCI-24781 (CRA-024781)	nM	I
	JNJ-26481585	µM	I
	4SC-201 (enitinostat)	µM	I, II
	Trichostatin A (TSA)	nM	I, III
Benzamida	MS-275 (entinostat)	µM	II
	MGCD0103 (mocetinostat)	µM	II
Tetrapeptídeos cíclicos	FK2228 (romidepsina)	nM	I, II
Ácidos Alifáticos	Acido valproico	mM	I, II, III
	Butirato de sódio	mM	II
	Pivanex (NA-9)	mM	I, II

1.2.3.1. Butirato de sódio

Cada composto tem um mecanismo de ação próprio, mas a grande maioria dos inibidores de HDAC interfere no sítio catalítico da enzima, o que induz o bloqueio do acúmulo de sustrato de histonas acetiladas [181]. O butirato de sódio é um ácido graxo de cadeia curta com fórmula molecular, $C_4H_7O_2Na$ (figura 11), sendo um HDACi de origem natural. Este HDACi é originado a partir da fermentação de fibras alimentares pelas bactérias do cólon. Este ácido graxo participa na manutenção da saúde das células enterocíticas [182].

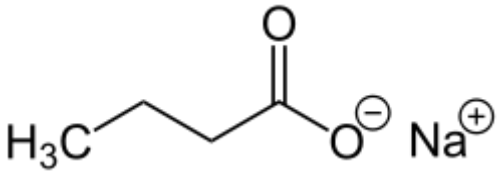


Figura 11. Estrutura molecular do butirato de sódio.

1.2.3.2. Absorção, metabolismo e biodisponibilidade do butirato de sódio

O butirato de sódio é o primeiro HDACi derivado de mamíferos descrito, encontrado como derivado principal da fermentação de amidos resistentes O butirato de sódio é consumido quase completamente pela mucosa colônica, sendo este produzido no cólon numa razão de aproximadamente 15 %, junto com acetato (65%) e propionato (20%) [183]. No entanto, as concentrações podem variar de acordo com a porção do intestino onde ocorre a fermentação da fibra ou com o tipo de fibras [184]. Os ácidos graxos de cadeia curta, como este, apresentam múltiplas funções que promovem a saúde no intestino grosso [182, 184, 185]. O aumento de sua produção é um dos fatores mais importantes para que ele seja o substrato (energético) preferencial das células colônicas [182, 185]. Diversos estudos mostram o impacto do butirato de sódio no intestino grosso para potencial tratamento de colite ulcerativa, ou de outros distúrbios intestinais [186]. Porém, a quantidade de necessária para produzir efeitos benéficos no cólon, deve ser similar à fisiológica (12-24 mM) [185, 186].

Ácidos orgânicos indissociados, como ácido butirico, podem atravessar a membrana e ser absorvidos rapidamente pelo intestino delgado, por isso dificilmente chegarão ao intestino grosso [187]. No entanto, o butirato de sódio microencapsulado consegue chegar à parte posterior do trato gastrointestinal aumentando a estabilidade deste composto [188].

1.2.3.3. Butirato de sódio e senescência: alvos moleculares

O butirato de sódio foi descrito como um inibidor não competitivo de HDAC, ou seja, ele não se associa a seu sítio ativo. O mecanismo molecular pelo qual o NaB atua inibindo HDAC ainda permanece desconhecido, no entanto esta atividade já foi descrita para este composto que induz parada na proliferação celular em diferentes linhagens tumorais [189-191]. Esta diminuição da capacidade de divisão celular está associada à estimulação ou repressão de genes específicos [192-196]. Existem no genoma eucarioto de mamíferos sequências de DNA dentro de promotores, as quais regulam a expressão de genes que são ativos ou reprimidos em presença de butirato de sódio. Estas regiões são denominadas *elementos de resposta a butirato* [192-196]. Estes elementos de resposta podem ser divididos em dois grupos distintos, de acordo com sua estrutura ou interação com estas sequências. Assim, um grupo de genes são induzidos ou reprimidos por uma sequência comum dentro dos elementos de resposta, o que sugere que existam fatores de transcrição conservados que se ligam nestes sítios [192]. Além disso, outro grupo inclui a indução da expressão de p21 e outros genes, mediada pela união das

proteínas SP1 e SP3 aos *elementos de resposta a butirato* [197]. SP1 e SP3 foram associadas com actividade de HDAC em células de câncer da mama humano [197]. Ambas as proteínas imunoprecipitaram junto com HDAC 1 e 2, o que sugere que elas recrutam HDAC induzindo a desacatilação destas regiões.

O promotor de p21 tem seis sítios de ligação a SP1 (ou elementos de resposta a butirato) [197]. Além disso, o SP1 está associado com a proteína dedo de zinco 89 (ZBP-89). Esta proteína recruta p300, que é uma HAT [197]. Assim, ZBP-89 recruta uma HAT ao promotor de p21, e ao mesmo tempo, SP3 recruta HDAC 1 e 2 para o mesmo promotor, estabelecendo um dinamismo na acelitação das histonas (figura 12). A inibição da actividade de HDAC com o buitirato de sódio permite que a atividade de HAT de p300 aumente, induzindo o aumento os níveis de acetilação das histonas no promotor [197], como mostrado na figura 12. A hiperacetilação das histonas provoca a abertura da cromatina e induz a expressão de p21 [197]. Este mecanismo sugere que esta indução de p21 leva à parada no ciclo celular. Vários exemplos em câncer são descritos na literutura sugerindo que p21 é o principal indutor de senescência celular [197]. No entanto, a ativação de p27 também tem sido descrita como indutora de senescência [198-201], embora os mecanismos moleculares pela qual esta é ativada, ainda não são conhecidos.

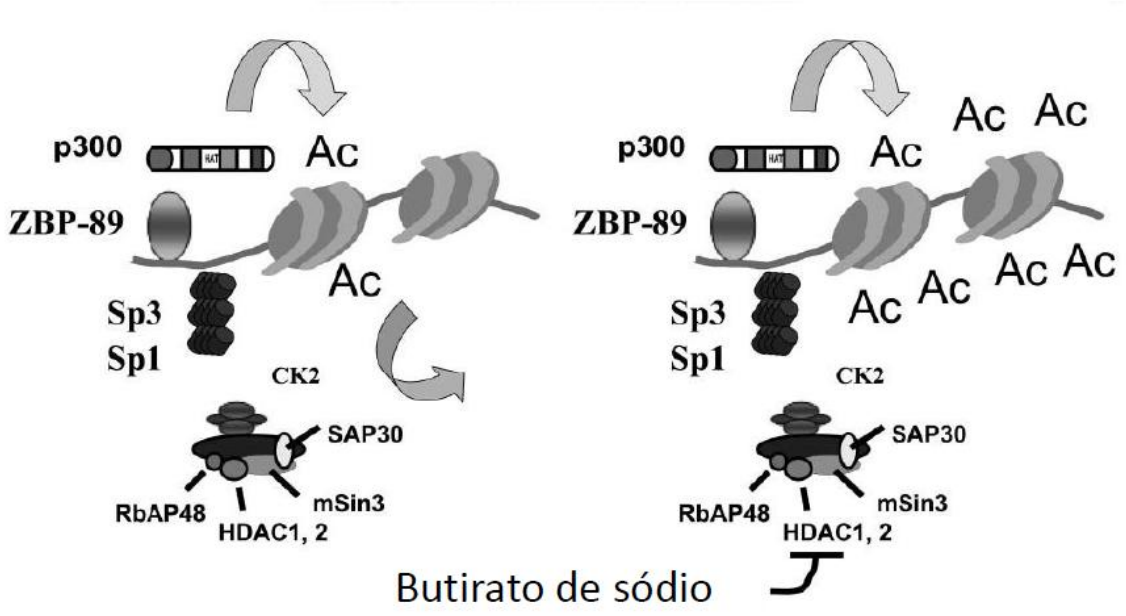


Figura 12. Mudanças epigenéticas na região promotora de p21 sob efeito de butirato de sódio. Ac representa grupos acetila (adaptado de Davie, 2003 [197])

1.2.3.4. Gliomas e butirato de sódio: efeitos pleiotrópicos

Poucos estudos utilizando linhagens de glioma analisaram o efeito antitumoral do butirato de sódio *in vitro* [202-204]. Dois estudos demonstraram que o butirato de sódio induz morte por apoptose na linhagem de glioma humano U87-MG sem afetar células normais (cultura primária de astrócitos) [204]. No entanto, este não é o único mecanismo ativado, a senescência celular é induzida nas linhagens de rato C6 e T98G humana [202, 203].

Como descrito acima, a remodelação da cromatina induz alteração no padrão da expressão gênica das células tratadas. Entretanto, os mecanismos que induzem senescência ou morte celular em glioblastoma ainda devem ser estudados mais profundamente.

1.2.4. Resveratrol, Quercetina e Butirato de sódio: efeitos em glioblastomas

Estudos epidemiológicos têm associado um alto consumo de frutas e verduras a um menor risco de desenvolver câncer, doenças cardíacas e doenças neurodegenerativas ao longo da vida. Assim, estudos avaliando o efeito da combinação de dois ou três compostos (quercetina, resveratrol, genisteína, catequinas, etc) começaram a ser realizados, mostrando um efeito sinérgico ou aditivo destes compostos [140, 205-207] em modelos tumorais, o que corrobora a hipótese de que o efeito benéfico dos vegetais não reside em apenas um composto isolado, mas sim na combinação destes.

O resveratrol interage com numerosos alvos moleculares afetando múltiplas cascatas de sinalização [208]. No entanto, o mecanismo mais descrito é a ativação em forma indireta da SIRT1 após administração deste polifenol, como descrito na seção *1.2.1.3*. O resveratrol retarda o envelhecimento celular em mamíferos [133], o que mimetiza a condição de restrição calórica. Estudos demonstraram que os níveis de SIRT1 estão aumentados em roedores e tecidos humanos em resposta a restrição calórica [209-211], este aumento acontece devido às mudanças metabólicas e à tolerância ao estresse, como resultado da dieta. No entanto, a SIRT1, quando desregulada, está relacionada ao desenvolvimento de câncer. Vários relatos mostram que esta desacetilase é superexpressa em vários modelos de câncer, como por exemplo, em câncer de pulmão humano [212], de próstata [213] e leucemia [214]. Além disso, os níveis de acetilação em K16-H4 e K9-H3, substratos de SIRT1, são alterados em diferentes tumores [215, 216]. Assim, estudos mostram que a perda de monoacetilado K16-H4 é um evento comum e precoce no desenvolvimento do câncer [217]. De maneira similar, a atividade alterada da SIRT1 em câncer também pode interferir em

proteínas-chave que regulam importantes funções celulares. Por exemplo, a superexpressão de SIRT1 inibe a função de p53 [218] e sua inibição em células de leucemia produz a ativação de p53 [219].

Ku70, outra proteína de resposta ao estresse e reparação do DNA é regulada por SIRT1. Esta proteína é desacetilada permitindo a sobrevivência em longo prazo de células cancerosas danificadas [209]. Neste sentido, o tratamento com inibidores da SIRT1 por HDACi tem efeito antitumoral pela indução da expressão de genes supressores tumorais e aumento nos níveis de acetilação de K16-H4 e K9-H3 em linhagens celulares de câncer do cólon [216]. Por outro lado, a quercetina também pode atuar como inibidor de HDAC como demonstrado na linhagem de leucemia humana HL-60 e em um modelo tumoral de câncer de hamster (HBP) [220-222]. Assim, a eficácia na indução de senescência pela combinação de resveratrol e quercetina pode ser devido a esta inibição [140]. No entanto, para testar de forma mais objetiva se o resveratrol poderia ter um efeito pró-tumoral pela ativação de SIRT1, investigamos a combinação de HDACi com resveratrol. Na mesma linha, se o efeito pró-senescência da quercetina for somente através da inibição da HDAC, um HDACi não teria efeito aditivo quando quercetina estiver presente. Neste sentido, a inibição farmacológica de HDAC combinada com o tratamento com o resveratrol ou a quercetina poderia apresentar novas evidências sobre a atividade antitumoral destes compostos.

1.3. Câncer: Um fenômeno complexo

O genoma humano, assim como o de outras células eucariotoas, é composto por milhares de genes codificadores para proteínas, onde estas comumente interagem entre si, estabelecendo um sistema bioquimicamente complexo que responde pela homeostase celular [223]. Uma característica deste sistema complexo é a sua não linearidade, onde mudanças simples em uma parte do sistema podem gerar efeitos que se propagam por todo este. Considerando a complexidade dos sistemas biológicos e a sua não linearidade, torna-se necessário o desenvolvimento de ferramentas para entendimento de tais sistemas, a fim de descobrir princípios comuns que regem a sua organização e funcionamento.

Neste contexto, o estudos dos mecanismos moleculares que levam às inúmeras patologias, como o câncer, é um grande desafio do ponto de vista biológico.Assim, considerando os processos tumorais, duas características principais resultam no

aumento da complexidade desta doença. Primeiramente, o câncer é uma patologia altamente heterogênea em relação ao tipo de célula e origem do tecido e, por fim, é uma doença que envolve a desregulação de múltiplas vias de sinalização que regem processos celulares fundamentais, tais como a morte, proliferação, diferenciação e migração celular.

Portanto, compreender esta desregulação multivariada (epigenética ou clastogênico) exige a interpretação global de diferentes plataformas experimentais de grande escala, tais como o sequenciamento do genoma, a análise de transcriptomas, proteomas e metabolomas de células tumorais e os tecidos circundantes.

A fim de gerar um conjunto de ferramentas capazes de interpretar dados de larga escala desenvolveu-se uma área da Bioinformática denominada de Biologia de Sistemas.

1.3.1. Biologia de sistemas

A Biologia de Sistemas consiste de um conjunto de análises matemáticas que visam complementar a visão tradicional de pesquisa biológica. Os "sistemas" ou "níveis de complexidade" da Biologia de Sistemas compreendem a interação entre duas ou mais proteínas necessárias para a realização de determinada função na célula. Este princípio pode ser aplicado às interações observadas entre componentes celulares de um tecido ou até interações observadas entre indivíduos em um contexto ecológico [224]. Por estes motivos, a Biologia de Sistemas integra ferramentas computacionais para analisar interações funcionais entre moléculas, como proteínas ou genes, a partir da análise de bancos de dados [224, 225].

Um sistema que pode ser estudado por meio da biologia de sistemas é a senescência celular e suas diversas formas (RS e OIS), que podem ser inferidas a partir de mapas de rotas bioquímicas [226]. Estas rotas podem ser deduzidas por redes de interações entre metabólitos e macromoléculas, entre as quais muitas são proteínas ou compostos químicos sintetizados pela própria célula ou células vizinhas [224, 227]. Portanto, os experimentos de larga escala (*high throughput*) são um passo importante para a compreensão deste mecanismo antitumoral e o câncer.

1.3.1.1. Conceitos básicos de biologia de sistemas

O crescimento dos bancos de dados de informações biológicas e sua interpretação constituem um grande desafio computacional. Este problema pode ser resolvido através de estratégias matemáticas e de informática que compreendem a integração de um amplo número de dados em grafos ou também chamadas de redes de interação [228].

As redes de interação são estruturas complexas, formadas por elementos unitários (nós) ligados por conectores conformando uma ampla variedade de conexões [224, 228]; a estrutura de um grafo é representada na figura 13. Na Biologia de Sistemas, os nós podem representar genes, proteínas, pequenas moléculas, drogas, ou qualquer outra entidade capaz de interagir em um sistema. Os conectores representam a natureza da interação e podem ser diretos ou indiretos, representando interações físicas, ativações, inibições, regulações ou qualquer outra relação entre os nós. Assim, uma célula pode ser descrita como uma rede de moléculas de diferentes naturezas designadas como "nós", conectadas através de reações químicas denominadas "conectores" [224, 229, 230].

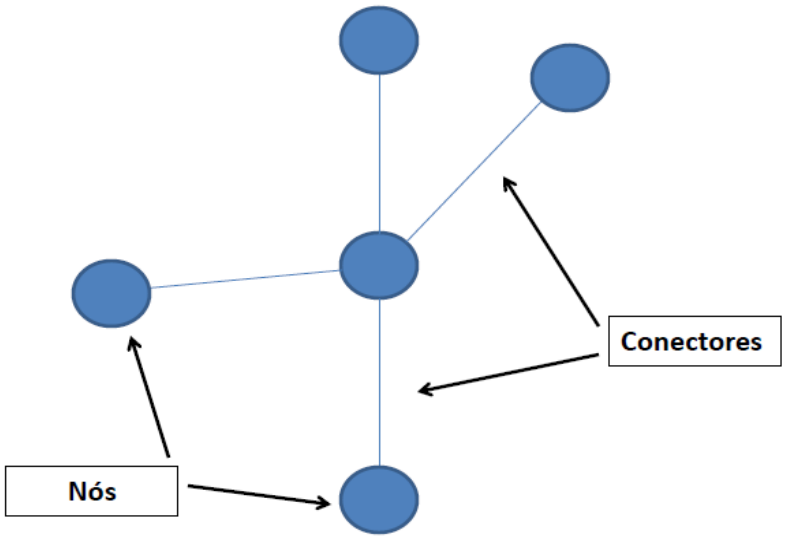


Figura 13. Exemplo de uma rede de interação e seus componentes. Os círculos representam nós (proteínas) e as conexões entre estes conectores que referem a interações químicas ou físicas entre proteínas.

1.3.1.2. Modularidade biológica

Inúmeras observações em Biologia de Sistemas indicaram que inúmeras sub-redes distintas a partir de uma rede maior. Estas sub-redes podem ser definidas como módulos [231]. De uma forma bastante simplificada, um módulo ou agrupamento em uma rede é um grupo de nós altamente interconectados entre si, com baixo valor de diâmetro [228]. Aparentemente, grupos de genes, proteínas ou metabólitos são capazes de formar módulos específicos que constituem processos biológicos fundamentais para que uma célula consiga exercer uma determinada função. Os processos biológicos estão agrupados no conceito de ontologias gênicas. Estas ontologias gênicas são determinadas a partir do cruzamento de diferentes bases de dados experimentais e dados da literatura científica [224, 232]. Assim, diferentes funções podem ser atribuídas a uma rede ou sub-rede no contexto celular [224, 228].

Existem vários exemplos de modularidade biológica (figura 14). De fato, a maioria das moléculas em uma célula faz parte de um complexo intracelular com atividade modular, como os complexos ribossômicos [231], necessários para o processo de tradução [233] ou o avanço do ciclo celular [234]. Desta maneira, para que seja possível estudar a natureza modular de uma rede, bem como se os módulos estão relacionados a um determinado mecanismo celular, são necessárias ferramentas e métodos de medida fornecidos pela Teoria dos Grafos.

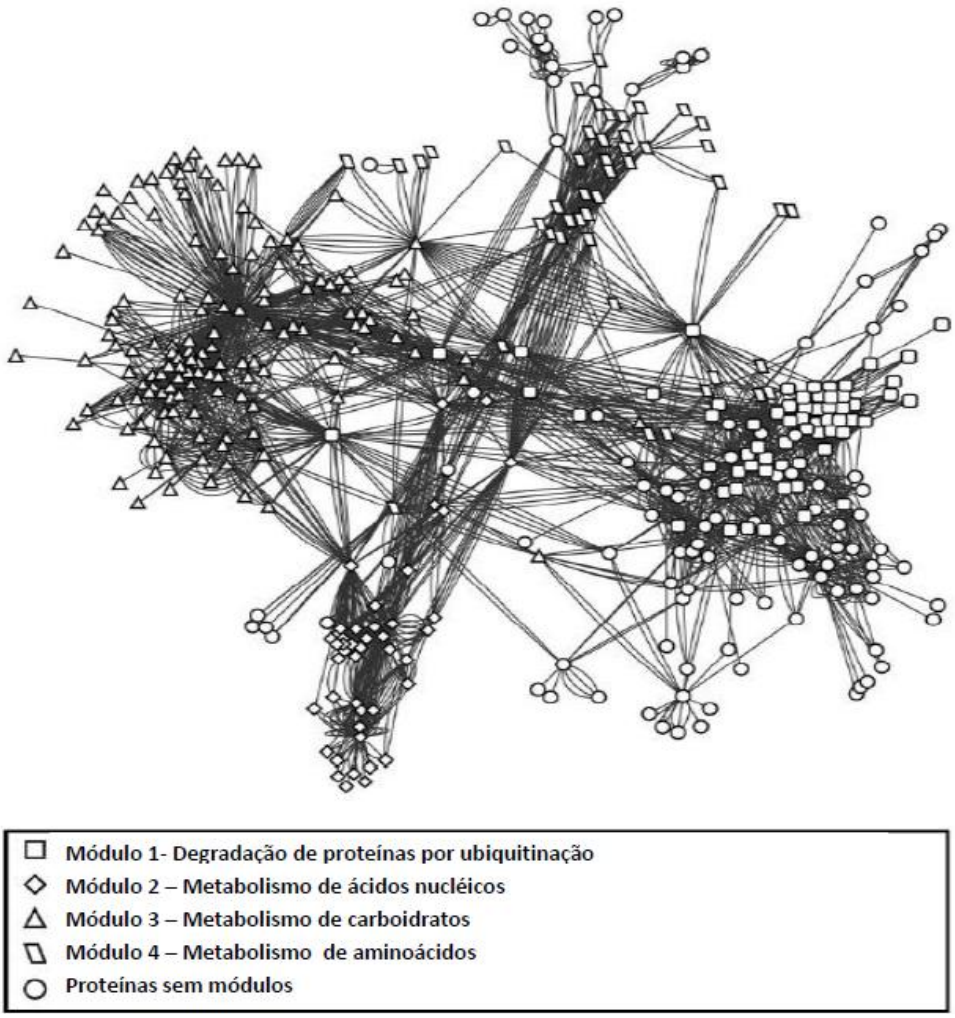


Figura 14. Exemplo de uma rede de interação mostrando modularidade em leveduras. Cada grupo de nós pertencentes a um determinado módulo está indicado por formatos geométricos específicos junto com a sua ontogenia respectiva (adaptado de Barea *et al*, 2011 [235]).

1.3.1.3. Estrutura de redes: princípios de centralidades

A análise da estrutura global de redes não se limita à presença de motivos ou módulos, mas compreende também a determinação de centralidades dentro da estrutura da rede [224, 236].

As centralidades constituem um conjunto de ferramentas computacionais que permitem identificar nós que possuem uma posição relevante na rede [237]. Assim, foram desenvolvidos alguns critérios, tais como centralidade e intermedialidade (*betweenness*) [224, 237], que definem a importância do nó na rede.

As centralidades têm sido estudadas em sistemas biológicos para identificar proteínas-chaves para um organismo ou que possuem uma posição importante em um determinado processo biológico [237]. Entre as medidas de centralidade, o grau (*degree*) representa o quanto o nó está conectado a outros nós adjacentes. Em outras

palavras, o grau do nó representa a "popularidade" de um determinado nó em uma rede. Nós com alto grau de conectividade são denominados de *hubs*, sendo proteínas cujas alterações prejudicam a homeostase do organismo (Figura 2). O termo de intermedialidade representa quanto um nó específico está entre todos os outros nós na rede ou entre determinados processos.

De forma geral, o grau e a intermedialidade demonstram a influência de um nó em uma rede na propagação de uma informação por toda a rede. Desta forma, define-se o conceito de gargalo (*bottleneck*) como uma medida de centralidade que define todos os nós com altos valores de intermedialidade, configurando-se como pontos centrais que controlam a propagação de informação para outros nós integrantes da rede. Os gargalos também indicam todos os nós que estão entre agrupamentos altamente interconectados, onde a remoção destes pode dividir a rede inteiramente [224, 237], um exemplo destes paramétros é mostrado na figura 15. Além disso, todos estes conceitos podem ser aplicados para a análise de redes de interação tumorais.

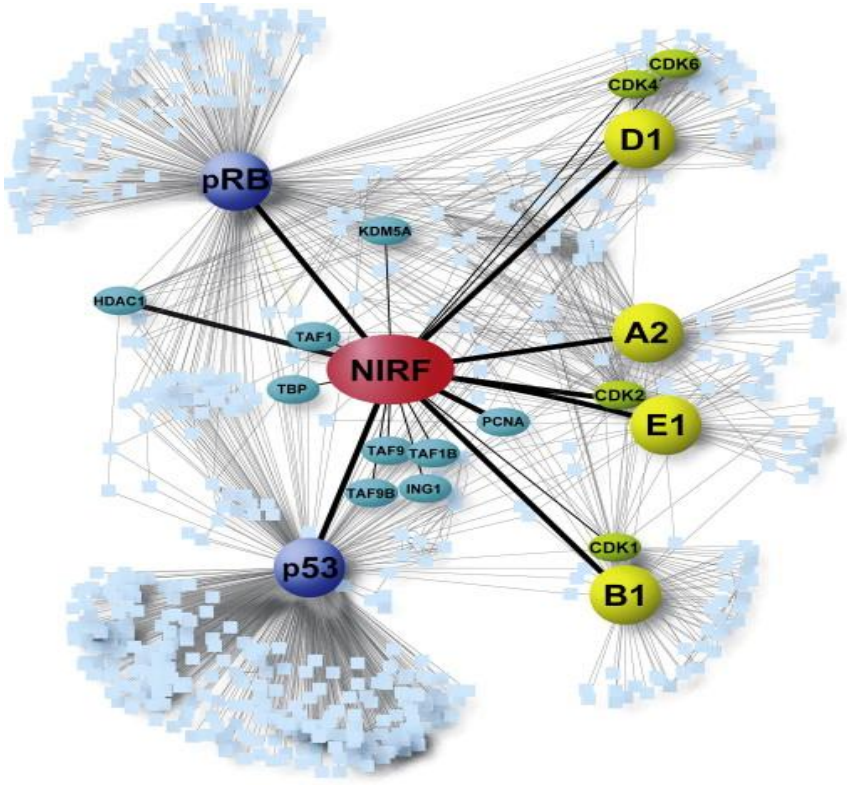


Figura 15. Rede de interações entre uma ubiquitina quinase (NIRF; nó vermelho), proteinas que regulam o ciclo celular (RB e p53 em azul; CDK e ciclinas em amarelo) e outras que modificam diretamente a cromatina (HDAC1, TAFs, TBP, ING1 e PCNA em azul claro). pRB e p53 representam dois *hubs* principais, conectados através de NIRF1. Mori, *et al* 2012 [238].

1.3.2. Biologia de sistemas aplicada ao estudo do câncer

Dentro de uma rede biológica de células normais, existe uma relação temporal dinâmica e precisa na formação dos complexos protéicos [239]. Por exemplo, de de Lichtenberg [239] realizou a análise do ciclo celular através de uma rede de interação proteíca baseada em dados proteômicos de levedura e mostrou que dois princípios básicos regem o ciclo celular: (1) o ciclo celular está contituído por genes expressos em forma periódica e outros constitutivamente; (2) essa expressão garante que os complexos proteícos possam ser constituidos nos momentos adequados, o que proporciona o controle da proliferação celular (figura 16). Isto mostra que os genes não apenas trabalham juntos, mas também são regidos por determinadas regras subjacentes.

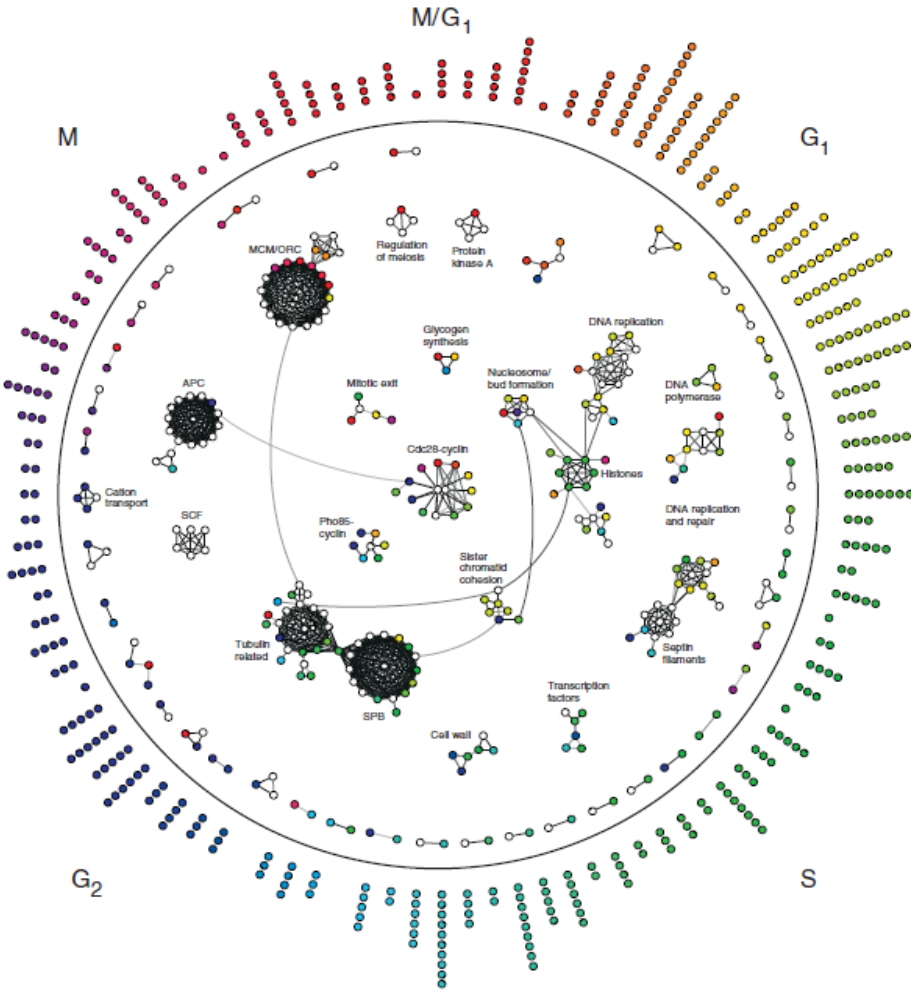


Figura 16. Rede temporal de interações entre proteínas que regulam o ciclo celular de leveduras. Nós circulares são as proteínas envolvidas no ciclo celular que podem formar complexos ou ter outro tipo de interação não física. Nós brancos representam proteínas estáticas, enquanto os nós coloridos inferem o pico de expressão das proteínas. Proteínas sem interação estão fora do círculo e igualmente posicionadas de acordo a seu pico de expressão. de Lichtenberg, *et al* 2005 [239].

De maneira similar, os genes trabalham em conjunto para desempenhar suas funções no câncer. Porém, cânceres são causados geralmente pela mutação de vários

genes que levam à desregulação de múltiplas vias de sinalização [240, 241]. Atualmente, a identificação de biomarcadores que podem caracterizar cânceres continua sendo um desafio.

Os métodos tradicionais detectam genes diferencialmente expressos entre amostras de câncer e normais, porém, existe a possibilidade de erros devido ao tamanho amostral reduzido ou ao conceito de que os genes funcionem independentemente. Portanto, vias desreguladas podem servir como biomarcadores melhores em comparação a um único gene [242]. Da mesma forma, redes de proteínas tumorais apresentam algumas características intrínsecas que as diferencia das redes de células normais, as quais estão expostas a seguir.

1.3.2.1. Mutações gênicas indutoras de câncer influenciam as redes de interação de proteínas humanas

A teoria dos grafos é aplicada tanto como para determinar seus efeitos em redes de interação de proteínas humanas (RIPH) a partir de padrões mutacionais no genoma. As mutações gênicas indutoras de câncer (MIC) promovem a proliferação celular e o escape (*bypass)* da senescência celular [243, 244]. Análises genômicas permitiram observar que as MIC acontecem de forma menos frequente em genes duplicados e mais frequente em *singletons* ou genes únicos (16,3% e 83,7%, respectivamente), independente de suas funções moleculares [245]. Porém, existem mutações que podem afetar diretamente a homeostase celular. Por exemplo, um oncogene mutado ou expresso em níveis elevados ajuda a transformar uma célula normal em célula cancerosa [246]. O mesmo pode acontecer quando um gene supressor de tumor é mutado, o que leva à redução ou a perda da sua função [247].

1.3.2.3. Mutações gênicas indutoras de câncer produzem proteínas e redes altamente interconectadas.

A análise de redes de proteínas humanas sugere que proteínas derivadas de genes duplicados interagem com um maior numero de outras proteínas que as proteínas derivadas de *singletons*, porém os coeficientes de modularidade são mais baixos, ou seja, possuem uma interconectividade menor dentro da RIPH [248, 249]. O câncer produz alterações destes padrões dentro das RIPH. Assim, Rambaldi e colaboradores (2008) [245], mapeando um conjunto de MIC por meio da análise de genomas tumorais por sequenciamento, observaram que proteínas mutadas apresentam maior numero de

nós interagindo e maior coeficiente de modularidade do que proteínas normais. Interessantemente, resultados semelhantes foram mostrados por Jonsson e Bates [250], os quais utilizaram uma base de dados de interação de proteínas diferente para caracterizar MIC [250]. Neste estudo, um método computacional baseado no princípio de interações de ortólogos foi aplicado para construir uma rede de interação humana de proteínas [250]. Com esta estratégia, os autores concluíram que proteínas resultantes de MIC constituem a base de interação em RIPH. Através destes resultados, Rambaldi (2008) propôs que MIC são intrinsecamente frágeis e propensas a induzir alterações na estrutura de RIPH, ou seja, produzem *hubs* altamente interligados que podem produzir efeitos simultâneos em vários processos (*bottleneck*). Assim, os oncogenes ativam vias de sinalização que estimulam o desenvolvimento tumoral (vias de proliferação), ou os genes supressores tumorais metilados ou mutados provocam alterações na regulação de RIPH de células normais. Ambos os casos induzem câncer [251].

1.3.2.4. A sinalização celular do câncer está determinada pela estrutura de suas redes

A alta conectividade das proteínas derivadas de MIC e a elevada modularidade influenciam diretamente a sinalização celular. Para entender como estas características afetam as vias de sinalização é preciso considerar dois princípios: *"positive feed-forward regulatory loop"* e *"bi-fan regulatory loop"*. No primeiro, por exemplo, se considerarmos três proteínas: A, B e C; A regula B e B regula C; mutações que afetam A (oncogene) podem alterar a via de sinalização, amplificado assim seu sinal intracelular e induzindo câncer [252], figura 17. Seguindo o mesmo raciocínio, no caso de *"bi-fan regulatory loop"*, podemos considerar quatro proteínas: A, B, C e D; A regula C e D, e B regula C e D concomitantemente. Assim, mutações que afetem A não necessariamente induzirão a amplificação do sinal. Considerando estes princípios, a regulação mais frequente nos módulos de RIPH de cãncer é a *"positive feed-forward regulatory loop"* [253], figura 17.

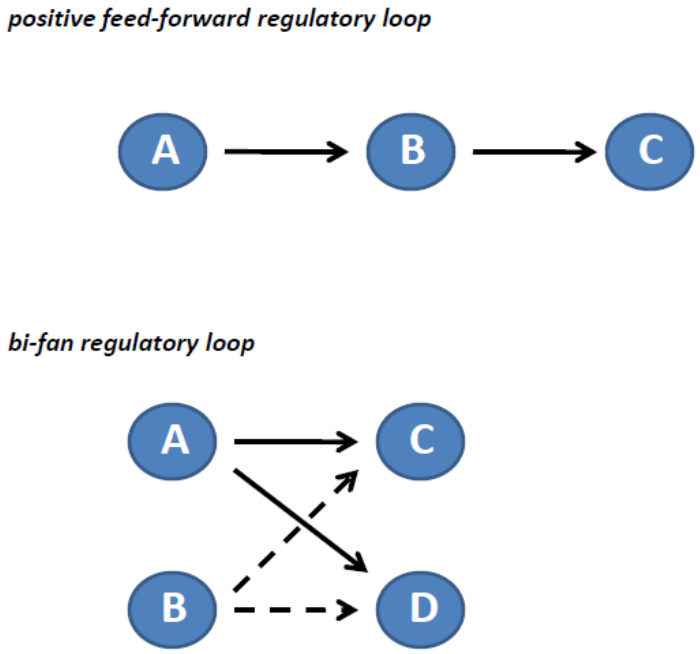


Figura 17. Representação de dois possíveis mecanismos de regulação da sinalização celular. Nós circulares são representadas proteínas (A,B,C e D) envolvidas numa cascata de sinalização hipotética. As setas refletem a direção como o alvo de regulação.

Nas redes de sinalização, os fluxos de informação poderiam convergir produzindo um número redundante e limitado de respostas [254]. Estes pontos de convergência podem ser proteínas (nós) que ao serem alteradas induzem a perda da homeostase celular provocando câncer [253]. Assim, a alta conectividade de proteínas produzida por MIC pode induzir o aumento de nós convergentes, os quais em RIPH de células normais pertenceriam a vias de sinalização diferentes [253].

O conhecimento gerado a partir do estudo de RIPH de câncer ajudará na geração de hipóteses experimentalmente testáveis e descoberta dos mecanismos subjacentes da tumorigênese. Porém, a aplicação de biologia de sistemas para o estudo dos mecanismos endógenos antitumorais e sua sinalização está em fase inicial [255-258]. Até o momento, a senescência celular foi analisada no contexto de envelhecimento fisiológico [259]. Atualmente, segundo nosso conhecimento não existem estudos focando a análise da senescência celular, seu *bypass* ou sua indução através da análise de RIPH de GBM ou outros tipos de cânceres.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar *in vitro* e *in silico* os mecanismos moleculares associados à indução ou escape da senescência celular em glioblastomas.

2.2. Objetivos Específicos

- Produzir uma revisão crítica e integrada sobre senescência como um mecanismo endógeno antitumoral, sua indução e os mecanismos utilizados por diferentes tipos tumorais (incluído glioblastomas) para evitá-la (Capítulo 1).
- Avaliar o efeito sobre a proliferação celular do butirato de sódio combinado com resveratrol ou quercetina em uma linhagem de glioma humano e em uma linhagem de glioma de rato (Capítulo 2).
- Investigar se estes compostos causam a indução de senescência celular associada à inibição de SIRT1 por butirato de sódio em presença de resveratrol. Também analisar vias alternativas relacionadas à senescência como o estresse oxidativo e dano ao DNA (Capítulo 2).
- Gerar redes pequenas de interação de proteínas que sejam representativas de duas formas de senescência, senescência replicativa e induzida por oncogenes (Capitulo 3);
- Analisar a modularidade da telomerase em glioblastomas através da utilização de Biologia de Sistemas (Capítulo 3).
- Definir a relevância biológica da telomerase nessas redes de acordo, com sua popularidade e intermedialidade (Capítulo 3);
- Utilizar dados de microarranjos derivados de glioblastomas e outros tipos tumorais para elaborar uma hipótese sobre o mecanismo de ativação da telomerase e de seu efeito sobre a sobrevida tumoral (Capítulo 3).

3. DESENVOLVIMENTO

3.1. CAPÍTULO I - Senescence; an endogenous anticancer mechanism

José Vargas, Bruno César Feltes, Joice de Faria Poloni, Guido Lenz, Diego Bonatto

Periódico: **Frontiers in Bioscience**

Estado: **Publicado**

Referência: **J. Vargas, B.C. Feltes, F. Poloni J de, G. Lenz, D. Bonatto, Senescence; an endogenous anticancer mechanism Front Biosci 17 (2012) 2616-2643.**

[Frontiers in Bioscience 17, 2616-2643, June 1, 2012]

Senescence; an endogenous anticancer mechanism

Jose Vargas[1], Bruno Cesar Feltes[2], Joice de Faria Poloni[2], Guido Lenz[1], Diego Bonatto[3]

[1]Laboratory of Cellular Signaling and Plasticity, Department of Biophysics and Center of Biotechnology, Federal University of Rio Grande do Sul, Av. Bento Goncalves, 9500, Porto Alegre, Brazil, [2]Institute of Biotechnology, University of Caxias do Sul, Rua Francisco Getulio Vargas 1130, Caxias do Sul, Brazil, [3] Laboratory of Molecular Radiobiology, Department of Molecular Biology and Biotechnology, Federal University of Rio Grande do Sul, Av. Bento Goncalves, 9500, Porto Alegre, Brazil

TABLE OF CONTENTS

1. ABSTRACT

Pre-malignant tumor cells enter a state of irreversible cell cycle arrest termed senescence (cellular senescence; CS). CS is a part of the aging program and involves multiple signaling cascades and transduction mechanisms. In general, senescence can be divided into replicative senescence and premature senescence. Replicative senescence (replicative CS) has been described for all metabolically active cells that undergo a spontaneous decline in growth rate. Notably, ectopic expression of telomerase holoenzyme (hTert) can prevent replicative CS. In cancer cells, premature senescence induced by oncogenes, named oncogene-induced senescence (oncogene induced CS; OIS), play an important role in preventing the development of cancer. Oncogene induced CS can be promoted by the loss of tumor suppressor genes, such as PTEN. Additionally, other interesting mechanisms, like selective microRNA expression, epigenetic modifications, or even stress conditions, are also able to activate the senescence program. Here, we will critically review the literature on the role of senescence in preventing the development of cancer and discuss the potential of senescence modulation for generating new molecular tools that could be explored as anticancer treatments.

2. INTRODUCTION

Cellular senescence (CS), which was first described in 1961 by Leonard Hayflick and Paul Moorhead, is a complex phenomenon characterized by irreversible growth arrest. This growth arrest is accompanied by changes in cellular structure, chromatin organization, and gene expression (*1*). Morphologically, senescent cells in culture grow in size, become flattened and show increased cytoplasmic granularity (*2*). In addition, a large increase in lysosome mass is observed upon the induction of senescence; the measurement of lysosomal β-galactosidase activity is a classical biomarker of senescence (*3*), as it could be observed in proliferating C6 rat glioblastoma cell treated with sodium butyrate, a drug that induces cellular senescence (Figure 1A). Chromatin remodeling, induced by proteins that bind to DNA, is another hallmark of senescence. One DNA-binding protein known to be involved in senescence is the heterochromatin protein 1 (HP1) family member HP1γ, which has been established as a marker for senescence-associated heterochromatin foci (SAHF) in human and murine cells (*4,5*).

Table 1. Types of senescence

Type of senescence[a]	Abbreviation	Mechanism	Reference
Replicative senescence	RS	Senescence dependent on telomere length and blocked by hTert expression.	(6 to 15)
Premature senescence	PS	Any senescence that is not replicative; includes SIPS, OIS and PICS.	(16 to 29)
Stress-induced premature senescence	SIPS	Senescence induced by several kinds of stresses, such as DNA damage, oxidative stress and culture conditions. Includes OIS and some aspects of PICS.	(16 to 29)
Oncogene-induced senescence	OIS	Senescence induced by oncogenes such as Ras^{G12V}, cMYC or $BRaf^{E600V}$; involves signaling pathways such as p38MAPK and the DNA damage response.	(16 to 23)
PTEN loss-induced cellular senescence	PICS	Shares several features with OIS but can also be induced through mechanisms that do not involve DDR or p53 signaling.	(24 to 29)

[a]"Types" do not refer to specific, independent mechanisms but rather to different terminologies used in the field of senescence.

Senescence itself is biochemically diverse, and the environmental/molecular signals that induce senescence are heterogeneous. Moreover, senescence can be divided into two major categories: replicative senescence (replicative CS; RS), which is normally induced after cells undergo a large number of divisions, and premature senescence (premature CS; PS), which results mainly from DNA damage and/or oncogenic signals. Several premature senescence sub-types have been defined, including stress-induced premature senescence (stress-induced premature CS; SIPS), oncogene-induced senescence (oncogene induced CS; OIS) and PTEN loss-induced cellular senescence (PTEN loss-induced CS; PICS) (Table 1).

Replicative senescence (replicative CS; RS) is characterized by an irreversible loss of replicative capacity and is associated with telomere dysfunction, as observed in the so-called Hayflick cell division limit (*6*). The Hayflick limit refers to the total number of divisions that a normal cell can undergo before arrest due to critical telomere shortening (*7*). As will be discussed later, telomeres and proteins that are necessary to maintain telomeric structure (e.g., telomerase) are fundamental components of the senescence pathway, and virtually all basic and applied research related to tumor development and senescence considers telomeres and their associated elements to be major targets for drug development.

Recently, many studies have shown that replicative CS is involved in tumor suppression activity and aging, with replicative CS acting as a barrier to cellular immortalization (*8*). In this context, it has been observed that many cell populations require replicative CS to maintain the balance between the rates of cell division and cell death and thus achieve tissue homeostasis (*9*). However, cell populations can tend toward an increase in cell division (tumorigenesis) or cell death (tissue aging) through the combined effects of three major factors: (i) cell lifespan, (ii) number of cell divisions (*10,11*), and (iii) stress caused by external factors (*30*). All of these factors are responsible, to different degrees, for an increase in molecular damage and mutations that cause genetic instability and could lead to tumor development or aging (*10*). It is important to note that the fate of each cell (tumor or aging) is strongly influenced by the cellular environment.

The ectopic expression of some oncogenes (e.g., Ras^{G12V}, $B\text{-}Raf^{V600E}$ and c-MYC) in fibroblast cells induces senescence in a telomere-independent fashion and concomitantly activates the DNA damage response (DDR), which can thus be considered stress-induced premature CS (*16*). This form of senescence, called "oncogene-induced senescence" (oncogene induced CS), is displayed by a variety of cell types (*17*). Oncogene induced CS is rapidly activated when oncogenic stress is present, resulting in the death of neoplastic cells *in vitro* (*18*). However, the role of oncogene induced CS *in vivo* is not clear, although data gathered by some authors have shown that oncogene induced CS could also serve as an *in vivo* antitumoral mechanism. For example, the expression of mutant K-ras oncogene family members (e.g., K-rasG12V and N-rasG12D) can induce senescence in the mammary gland (19) or the bladder (20). Another oncogene, B-raf (a downstream effector of K-ras), induces senescence in melanocytes (*16, 21 23*).

The loss of PTEN causes an increase in PIP3, which in turn leads to the activation of Akt and other proteins. This process, termed PTEN loss-induced cellular senescence (PTEN-loss induced CS; PICS), has some interesting differences from the oncogene induced CS induced by Ras overexpression. While oncogene induced CS requires the hyper-replication response, which involves the DDR, PTEN-loss induced CS occurs even in the presence of S-phase blockers or ATM inhibitors, suggesting a fundamental difference between PTEN-loss induced CS and oncogene induced CS or replicative CS (*1*).

The link between oncogene induced CS and replicative CS is mainly determined by cell cycle-regulatory mechanisms (e.g., post-transcriptional and post-translational modifications) that are intimately involved in tumor progression (Figure 1B). In this sense, transcriptional factors (e.g., p53; Figure 1B) can act as key elements in oncogene induced CS and replicative CS by blocking the activity of different cyclins, CDCs, and CDKs proteins (Figure 1B), leading to a permanent cell cycle arrest, a hallmark of cellular senescence. It should be noted that different cyclin-CDC-CDK complexes found within the cell are cell cycle-dependent, but all lead to gene transcription induction by means of retinoblastoma (RB)-E2F complex (Figure 1B). However, the suppression of cyclin-CDC-CDK complexes by different transcriptional factors supports the notion that senescence can be activated independently of cell cycle's phase (Figure 1B). Notably, and due to the complexity of protein complexes involved, the identity of the molecular pathway that links oncogene induced CS and replicative CS is not known, despite intensive efforts in the last several years to understand the molecular basis of senescence. However, some molecular elements that are active during senescence have been described. For example, the activity of cyclin-dependent kinases (CDKs), proteins whose functions are strongly

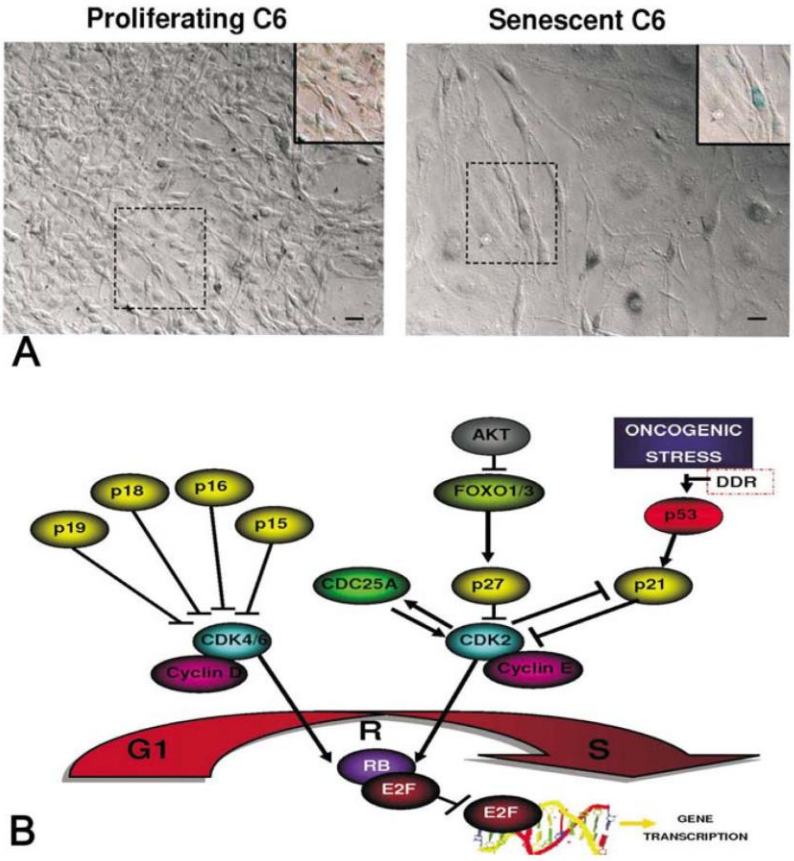


Figure 1. Phenotypic characteristics (A) and molecular mechanisms (B) associated with senescence. Both proliferating C6 rat glioblastoma cells and cells treated with sodium butyrate (A) display the hallmarks of senescence, which include the expression of β-galactosidase (senescence-associated β-galactosidase, SA-β-Gal; dotted box) and altered morphology (solid box). Cells were treated with sodium butyrate for 2 days in culture, followed by 10 days in drug-free medium (senescent C6 cells) and compared to control cells not exposed to sodium butyrate during the same time in culture (proliferating C6 cells). Cells were then stained with X-gal and visualized by optical microscopy (scale bar = 10 μm). Interestingly, the molecular pathways that lead to senescence are closely associated with the control of the different phases of cell cycle (B). In fact, many proteins that drive the cell toward senescence, such as p15, p16, p18, p19, p21, p27, and p53 (B) block the activity and gene expression of cyclins, cell division cycle (CDCs), and cyclin-dependent kinases (CDKs), resulting in cell cycle arrest and senescence. In addition, oncogene-induced senescence (OIS) leads to the activation of a DNA damage response (DDR) pathway (B) that in turn results in p53-dependent cell senescence.

associated with tumorigenic processes, is essential for cell cycle control and consequently senescence. CDKs and, therefore, cell cycle progression are controlled by specific phosphorylation and dephosphorylation mechanisms as well as by their interactions with specific proteins, including CDK inhibitors (*31,32*). Two families of CDK inhibitors have been described: the CDKN1 family [CDKN1A (p21), CDKN1B (p27) and CDKN1C (p57)] and the CDKN2 family [INK4A (p16), INK4B (p15), INK4C (p18), and INK4D (p19)] (for review, see *33*). Among these, p21, p27, and p16INK4A are the most directly involved in senescence induction.

Retinoblastoma protein (Rb) is a major CDK target that, upon phosphorylation by CDKs, releases the transcription factor E2F, which in turn regulates several genes involved in the progression from G1 to S phase (*34,35*). The tumor suppressor protein p53 plays an important role in senescence induction by several oncogenes and stresses, but it is less important in replicative CS (*36*) (Figure 1B). Detailed descriptions of the roles of Rb and p53 in the different forms of senescence will be provided in the following sections.

However, it is noteworthy from a therapeutic point of view that cancer cells lacking functional Rb, p53, and other tumor suppressors retain the capacity for senescence induction, at least *in vitro*. This indicates that other proteins/mechanisms associated with senescence are

still functional. For example, the treatment of the osteosarcoma cell line SAOS-2 and the prostate cancer cell line DU145 with doxorubicin, an anthracycline-derived antitumor drug that promotes DNA damage by inducing crosslinks and oxidative stress (*37,38*), induced senescence in more than 50% of cells *in vitro* (*37,38*), even though these cell lines do not express p53 or Rb. In fact, recent data suggest that DNA damage could be a common causative agent that underlies several different forms of cellular senescence (Figure 1B), including not only telomere dysfunction but also oncogene induced CS (*39-41*).

Here, we will review the recent progress in elucidating the biology of cellular senescence. We will consider data from both basic and applied biomedical areas and discuss how this information is being used to develop new anticancer therapies and to understand the mechanisms of cancer progression.

3. JANUS'S FACES: SENESCENCE AND TELOMERIC (IN)STABILITY IN TUMORS

In Roman mythology, Janus is the two-faced god of beginnings and endings, transitions, gates, doors, doorways, and time. Janus symbolizes the progression from past to future, of one condition to another. Facing the passage of time and sensing the cumulative damages induced by metabolism and environment, all cells are prone to senescence and, ultimately, aging.

Telomeres, the structures present at the chromosome ends (*42*), have multiple roles in the maintenance of genomic integrity (*42*). Telomere shortening, or "erosion", in mammalian cells has been strongly associated with senescence (*32*) and protection against cancer. However, telomeres display a characteristic Janus face: instead of becoming senescent, cells can develop chromosomal instabilities due to telomere erosion and induce the development of tumors (*42, 43*).

Senescence and telomeres share a complex relationship, which has been covered in great detail in several previous reviews (*44, 45*). This review will focus on the potential applications of telomere shortening in cancer treatment and diagnostics.

3.1. Telomere shortening: cancer biomarker and/or antitumor target?

In eukaryotes, specifically vertebrates, telomeres are composed of short, hexameric, guanosine-rich repeat sequences (TTAGGG) that are located at chromosome ends and form a 3' single-stranded overhang. This overhang forms a structure that folds back into itself such that no free single-stranded DNA remains (Figure 2A) (*46-48*). The main function of telomeres is to protect chromosome ends against degradation (telomere erosion) and potential end fusion due to the gradual shortening of DNA upon each round of replication in mitotically active cells (*49,50*). Telomere erosion is variable from tissue to tissue and the degree of erosion depends on the age of the donor organism (*44,51-53*). Human telomeres are 5-15 kb in length (*47*) and lose approximately 2-4 kb of length over the lifespan of a cell (*54*).

Some cells can evade telomere erosion by expressing telomerase reverse transcriptase (TERT; Figure 2A), the major enzyme responsible for telomere extension during DNA replication. In a fibroblast model, hTERT-transduced cells had very long telomeres with an extended life span because replicative CS induction was blocked (*12*).

Interestingly, 85% of malignant tumor cells exhibit increased TERT expression (*13*), leading to the maintenance of telomeres and enabling continuous cell division (*14,15*) and supporting the hypothesis that high telomerase activity is important for tumorigenesis and/or tumor maintenance. Advanced-stage tumors display longer telomeres (*55*), suggesting that complete telomere elongation may be necessary to sustain a large number of cell divisions with telomere shortening in tumorigenesis (*56-57*). Another study of 86 patients with primary gastric adenocarcinoma confirmed the correlation of TERT reactivation with malignant progression compared with early gastrocarcinogenesis (*58*).

Direct *in vivo* evidence for the potential antitumor effect of telomere erosion was presented by Feldser and Cosme-Blanco (*59, 60*) and discussed by Sedivy (*61*). Knockout of the telomerase gene in mice expressing the Eμ-myc oncogene, an established model of Burkitt's lymphoma, significantly reduced tumor formation. This mechanism was not blocked by overexpression of Bcl2, suggesting that senescence, rather than apoptosis, is involved in the protective effect of the telomerase knockout. Additionally, KO animals presented strong senescence staining in lymph nodes compared to WT animals. The protective effect of telomerase KO required p53 (*59*). In parallel to these experiments, the use of a knock-in mouse model bearing the p53 allele $p53^{Arg172Pro}$, which is able to mediate the induction of senescence but not apoptosis, showed that spontaneous tumorigenesis is potently repressed by TERC KO. Surprisingly, chemically induced skin tumorigenesis is not blocked by Terc KO, indicating that telomerase-dependent senescence plays different roles depending on the tumorigenic stimulus (*60*).

Despite these data indicating the anti-tumorigenic role of short telomeres and senescence induction, short telomeres induce gross chromosomal rearrangements (GCRs) (*61*), a consequence of the formation of anaphase bridges and chromosome breakage, particularly if senescence is blocked. Repetition of the breakage-fusion-bridge cycle leads to aneuploidy and further chromosome fusions, loss of heterozygosity, and/or gene amplification (*62*). Although this hypothesis remains to be tested directly from an epidemiological perspective (*63*), one recent study has shown that both cancer incidence and cancer mortality are associated with telomere shortening. In this work, 92 out of 787 participants (11.7%) developed multiple cancer types, and a statistically significant inverse relationship between telomere length and cancer incidence and mortality was observed (*63*). This information is consistent

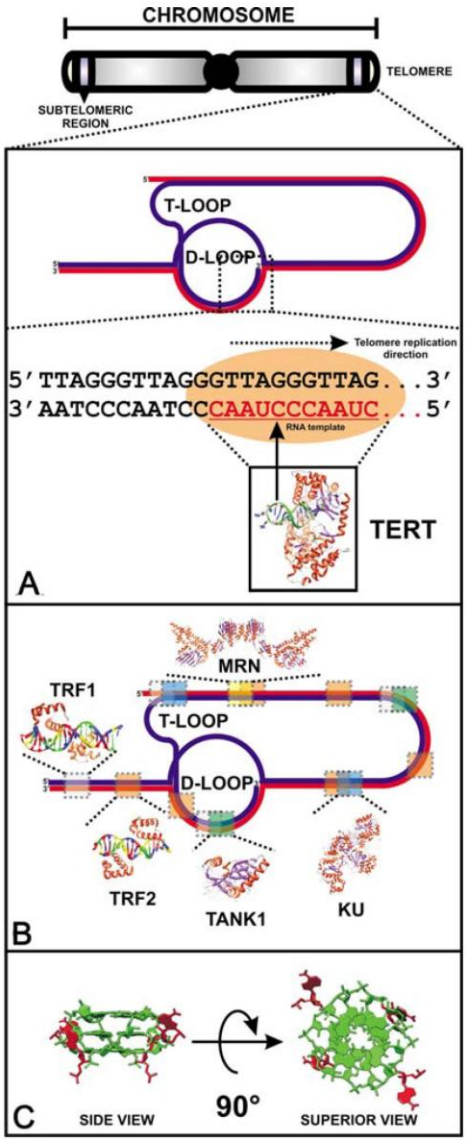


Figure 2. A schematic view of telomere structure (depicting T-loop and D-loop structures) and short hexameric repeat sequences (TTAGGG) (A). The synthesis and maintenance of these repeat sequences are mainly carried out by the telomerase reverse transcriptase [TERT; Protein Data Bank (PDB) number 3KYL), which contains in its three-dimensional structure (inset box) a short RNA molecule that is complementary to the telomere repeat sequence and allows its extension by DNA replication (A). The major protein complexes that participate in the maintenance of telomere structures (T-loop and D-loop; B), including guanosine (G)-quadruplex elements (C), are present in the extremities of the eukaryotic chromosome (yellow marks). Considering the data about telomere dynamics accumulated to date, it is clear that several proteins are necessary to conserve the secondary and tertiary structures of telomeres, including TRF1 and TRF2 (PDB numbers 1W0T and 1W0U, respectively), TANK1 (PDB number 2RF5), KU (PDB number 1JEQ), and the complex MRE11-RAD50-NBS1 (MRN; PDB number 3QG5), whose three-dimensional structures are depicted in (A). Other proteins (e.g., POT1, TIN2, RAP1, and TPP1) that are required for telomere structure maintenance are not shown in (B). Interestingly, many of these proteins combine to form different complexes (colored dotted boxes) that are responsible for particular aspects of telomere biology, such as capping induction or maintenance of G-quadruplexes (PDB number 2KBP). The three-dimensional structure of G-quadruplex DNA is shown from two different angles (C).

with previous evaluations of the correlation between shortened telomeres and different types of cancer (*64-67*). Therefore, the role of telomere erosion in both tumor suppression and tumor induction must be considered. The model that seems to emerge from these studies suggests that short telomeres are anti-tumorigenic during the early phases of tumor formation but can be pro-tumorigenic in late, established tumors in which senescence induction mechanisms are already lost, and the aneuploidy produced increases the tumor's heterogeneity.

Considering the available data, innovative therapeutic strategies that employ telomere/TERT-based drugs with broad anticancer activities are abundant in the specialized literature. However, the unexpected side-effects of these drugs are dependent on the model chosen to study senescence and tumor treatment. This imposes a challenge for the development of new treatments, which will be the subject of this review. It is also important to emphasize that senescent cells can maintain long telomeric 3'-overhangs (*68,69*), suggesting that senescence can be induced in ways that are independent of telomere length or telomerase activity, as will be discussed below.

3.2. Telomere structure as a target for a potential therapeutic intervention

The leading cause of telomere erosion is the DNA replication process itself. During semiconservative replication, the DNA holoenzyme complex synthesizes a lagging strand made up of Okazaki fragments, with each fragment requiring a new RNA primer that is then extended by DNA polymerase. At the end of the chromosome, however, there is not enough DNA to serve as a template for another RNA primer, and the DNA replication machinery is unable to fill the gap between the final priming event and the end of the chromosome. Thus, the lagging strand is shorter than the original strand that was used as a template, producing a 3'-overhang (*7*). In 1999, electron microscopy studies suggested that the 3'-overhang can loop back and integrate into the duplex repeat tract, forming a lasso-like structure called the "T-loop" (Figure 2B). This hypothesis was confirmed by an analysis of telomeres in different organisms, which revealed that the 3'-single-stranded overhang can invade the double-stranded telomeric tracts, displacing the homologous strand of the same telomere (*70*).

In fibroblasts, the telomeric 3'-overhang and the telomeres work in a coordinated way to maintain an intact telomere structure and, consequently, chromosomal integrity and genome stability. When telomeres become too short to maintain the T-loop structure, tumorigenesis can be induced (*71*). Supporting this hypothesis, immortalized fibroblasts they presented short telomere and telomeric 3'-overhangs showed a high degree of dicentric chromosome induction, an indicator of end-to-end chromosome fusion (*71*). Therefore, a minimal length of telomeric 3'-overhang may be necessary to maintain a proper telomere end-capping structure and ensure genomic stability in normal cells (*72*).

One probable mechanism for the maintenance of telomeric 3'-overhangs is related to the presence of a secondary DNA structure termed G-quadruplex (Figure 2C), which is found in telomeres (*73-75*). The consecutive formation of G-quadruplex structures in single-stranded telomeric overhangs protects double-stranded DNA ends from being recognized as DSBs and protects against nuclease hydrolysis. Moreover, G-quadruplex structures are necessary for the effective packing of telomeric DNA into the protective capping state. However, the G-quadruplex and T-loop structures may provide conformational flexibility for chromosome ends in response to different environmental conditions.

Considering the importance of G-quadruplex and T-loop DNA in protecting telomeres against degradation (*73*), these DNA structures may be useful for the development of drugs to induce senescence in tumors, which will be reviewed further in this manuscript.

In addition to forming specialized DNA secondary structures, telomeres are bound by a multiprotein complex, known as shelterin or the telosome, that forms the end-capping structure. The shelterin complex includes tankyrase, telomeric repeat-binding factor 1 (TRF1), telomeric repeat-binding factor 2 (TRF2), TRF1-interacting protein 2 (TIN2), repressor-activator protein 1 (RAP1), protection of telomeres 1 (POT1), and TPP1 (formerly named PTOP/PIP1/TINT1) (*74*) (Figure 2B). Various experiments have been designed to investigate the functions of the telosome in the context of senescence.

The disruption of genes involved in telomeres/telosome function can lead to the induction of either apoptosis or senescence. For example, fibroblasts respond to POT1 depletion with a strong induction of senescence, which is independent of telomerase (*75*). The role of POT1 in protecting telomeres has been also evaluated in breast cancer cells exposed to anti-POT1 small-interfering RNAs (siRNAs) (*75,76*). POT1 knockdown leads to telomere dysfunction, activating apoptosis by positively modulating p53 and Bax expression and downregulating the expression of some anti-apoptotic genes, like Bcl-2.

TRF2 inhibition by a dominant-negative form of TRF2 (TRF2ΔBΔM) in mouse hepatocytes resulted in telomere dysfunction and the generation of GCRs, which induced p53-independent apoptosis and p53-dependent senescence. Interestingly, the overexpression of a different TRF2 dominant-negative mutant caused apoptosis in tumor cells but senescence in normal fibroblasts (*77*).

3.3. Telomerase, a key in cancer development: therapeutic perspectives

Considering the close association of TERT, telomere structure, and telosome length with senescence, it has been suggested that the development of drugs that alter the functionality of these molecules/DNA structures could lead to the development of anticancer strategies aimed at inducing telomere erosion and, therefore, senescence.

Currently, four major molecular tools are used to induce senescence in multiple tumor models: (i)

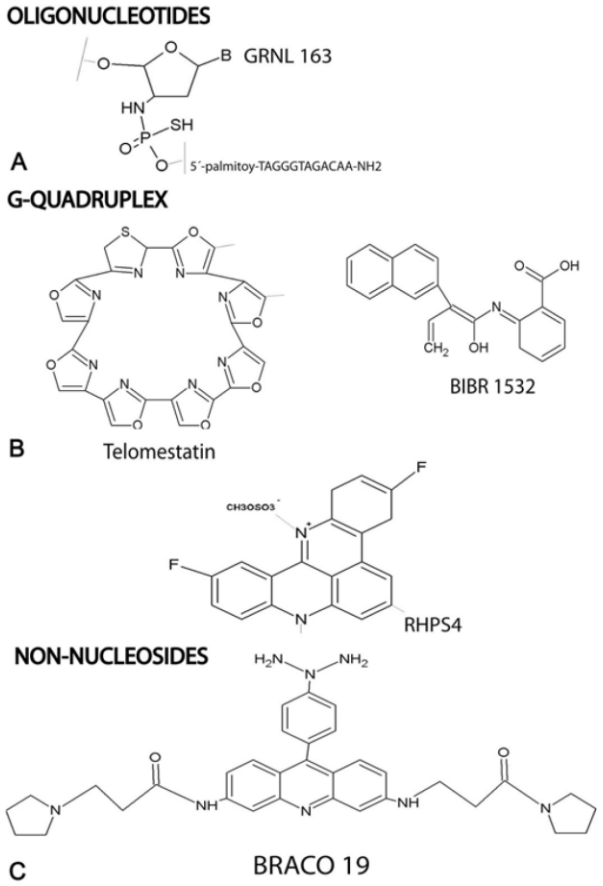


Figure 3. Molecular representations of representative compounds from different families of senescence-inducing drugs (A to C) used as telomere secondary structure stabilizers and TERT inhibitors. Please refer to the main text of the manuscript for additional details.

oligonucleotides (Figure 3A), (ii) G-quadruplex interacting agents (Figure 3B), (iii) non-nucleoside compounds (Figure 3C), and (iv) RNA interference (RNAi). These molecular tools will be discussed in detail below.

3.3.1. Oligonucleotides: a quasi-ideal molecular tool for senescence induction in tumor cells

The most potent and most frequently studied oligonucleotide used for TERT inhibition is GRN163L (Imetelstat). This molecule is a 13-mer oligonucleotide derived by palmitoylation of the parent molecule thiophosphoramidate GRN163 (Figure 3A) and is under clinical development by the Geron Corporation (Delaware, USA) (*78*). The DNA sequence of GRN163 is complementary to the mRNA sequence of hTERT, leading to the degradation of hTERT mRNA and thus limiting cell growth in multiple types of tumors (*79, 80*).

Initially, efficacy studies of GRN163 and GRN163L were conducted in mouse xenograft models representing a range of different human tumor types including lung (*81-84*), breast (*84-87*), liver (*88*), brain (*84, 89*) and hematological cancers, such as multiple myeloma and lymphoma (*90, 91*). The data collected from these animal models indicated that GRN163 and GRN163L have a strong TERT-inhibitory effect (*84*), sometimes blocking the induction of cancer metastases in the animals (*84, 86*).

Interestingly, the pharmacological inhibition of hTERT by GRN163 and/or GRN163L and the induction of senescence in tumors types are largely dependent upon telomere length and cell type. For example, little effect on growth was seen in U266 multiple myeloma cells (which exhibit long telomeres) (*87*) after 56 days of *in vitro* culture in the presence of GRN163, whereas growth inhibition was apparent in MM.1S multiple myeloma cells (which exhibit short telomeres) after only 28 days (*80, 90*). Other examples of the inverse correlation between the efficacy of GRN163 and telomere length in different tumor and biologic models, including in cancer stem cells (CSCs),

support the idea that long telomeres inhibit the activation of the senescence pathway. CSCs are naturally resistant to drug treatment (*91*), and TERT appears to regulate the clonogenic expansion of CSCs from multiple myelomas (*91*). The treatment of CSCs with GRN163L reduced cell proliferation *in vitro* five-fold after three weeks and 100-fold after five weeks, compared to controls. The administration of GRN163 *in vivo* resulted in significant survival of the animals compared to controls (*91*). Additionally, a recent preclinical assay suggests that GRN163L can cross the blood–brain barrier and inhibit telomerase in human glioblastoma cells, including glioblastoma CSCs, preventing cancer recurrence (reviewed in *92*). GRN163L is currently undergoing phase I and II clinical trials as a treatment for breast and lung cancers, myeloma, and chronic leukemia, all of which are driven in part by CSCs. An upcoming trial will test GRN136L in combination with paclitaxel and bevacizumab in breast cancer (*92*).

Despite the strong potential of oligonucleotides to induce senescence in different tumor types, this therapeutic approach has some major issues that impair its use *in vivo*. The lack of stability and bioavailability of oligonucleotides is an unsolved problem, although alternative approaches that use chemically modified oligonucleotides (e.g., peptide nucleic acids, or PNAs) are being developed. PNAs are modified oligonucleotides that contain a non-ionic backbone in which the deoxyribose linkages have been replaced by *n*-(2-amino-ethyl) glycine units, making PNAs resistant to degradation by endo- and exonucleases. PNAs bind to complementary nucleic acids with very high affinity (*93*), and these molecules are being evaluated as hTERT antagonists (*94, 95*). The cellular uptake of PNAs is very poor, requiring electroporation or the formation of DNA-PNA complexes that can be efficiently transfected by cationic lipids (*96,97*).

3.3.2. G-quadruplex structure: an innovative approach to induce senescence in tumor cells

Recently, the discovery that telomere-disrupting agents can also inhibit TERT activity led to intensive research into G-quadruplex-stabilizing compounds. These telomere-disrupting agents interact with the TTAGGG repeats of telomeres and stabilize G-quadruplexes, inhibitingtelomere elongation and also hindering the ability of the telomeres to 'cap' and protect the ends of the chromosomes. One major advantage of these G-quadruplex stabilizers is that they induce cell death quickly (*98*).

Initial TERT inhibition via long-term exposure of human cancer cells to sublethal doses of G-quadruplex stabilizers induced progressive telomere shortening and replicative senescence (*99, 100*). However, several studies showed that G-quadruplex stabilizers, including RHPS4 (3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate; Figure 3B) and BRACO-19 (3,6,9-trisubstituted acridine compound; Figure 3C) , were able to induce a short-term anti-proliferative response that cannot be explained by TERT inhibition alone (*75*). Specifically, the observation that BRACO-19 and other G-quadruplex stabilizers lead to GCRs, together with the appearance of p16-associated senescence, led to the proposal that telomeres, rather than TERT, are the targets of G-quadruplex stabilizers (*101, 102*).

G-quadruplex stabilizers were also shown to inhibit the alternative lengthening of telomeres (ALT) mechanism in tumor cell lines (*103*). The ALT mechanism is found in a minority of tumors, where it compensates for the lack of TERT, increasing the length of telomeres and allowing indefinite cell proliferation (*104*).

Both the quinoline-based 115405 and RHPS4 G-quadruplex stabilizers inhibited growth in the GM847 cell line, an SV40-immortalized human fibroblast that displays the ALT phenotype (*105, 107*). Additionally, 2,6-pyridine-dicarboxamide derivatives were strongly selective for G-quadruplex structures, inducing an anti-proliferative effect in SAOS-2, a human osteogenic sarcoma cell line that maintains telomeres through the ALT mechanism in the absence of TERT activity (*75*). These findings further corroborated the hypothesis that the anti-proliferative effects of G-quadruplex stabilizers are largely independent of TERT activity (*105*).

In this context, two G-quadruplex stabilizers deserve attention: BRACO-19 and telomestatin (Figure 3B). BRACO-19 represents the first of a "second generation" of G-quadruplex stabilizers. It possesses nanomolar potency against TERT and low non-specific cytotoxicity, and it was shown to inhibit growth and induce senescence in a human breast cancer cell line (*105*). Significant antitumor activity *in vivo* was observed when BRACO-19 was administered after paclitaxel treatment of mice bearing a human tumor xenograft carcinoma (*105*). The second compound, telomestatin, shortens telomere repeat fragments, with concomitant displacement of POT1 and TRF2 from telomere sites, in cancer but not in normal cells (*106*). Based on this evidence, a consistent mechanism of action is now emerging for G-quadruplex stabilizers in tumor cells, which initially involves alteration of G-quadruplex-overhang structure followed by degradation through a DNA damage repair (DDR) pathway and the release of POT1 from telomeres. In this regard, telomestatin is classified as a telomere-disrupting agent rather than a TERT inhibitor. Numerous studies using telomestatin in a number of cancer cells lines have demonstrated that this compound is an effective anti-proliferative agent both *in vitro* and *in vivo* (*107*).

3.3.3. The use of non-nucleoside compounds to induce senescence in tumors

A variety of non-nucleoside drugs have been shown to inhibit telomerase, including epicatechin derivatives such as epigallocatechin gallate (EGCG), which strongly and directly inhibits telomerase (*108*). In the presence of nontoxic concentrations of EGCG, two representative human cancer cell lines, U937 monoblastoid leukemia and HT29 colon adenocarcinoma, showed lifespan limitations accompanied by telomere shortening, chromosomal abnormalities, and the expression of senescence-associated β-galactosidase activity (*109-111*). Another potent and specific inhibitor of telomerase, 2,3,7-

trichloro-5-nitroquinoxaline (TNQX), causes progressive telomere attrition followed by an increased incidence of chromosome abnormalities and the induction of senescence (*112*).

Another interesting small molecule is MKT077, a toxic rhodacyanine dye analogue, which preferentially accumulates in tumor cell mitochondria and inhibits telomerase. This molecule was used as a lead structure for the development of a potent telomerase inhibitor, designated FJ5002. Long-term cultivation of U937 human leukemia cells with subacute concentrations of FJ5002 resulted in population-doubling-dependent changes characterized by progressive telomerase shortening and senescence (*97*).

However, the best-studied senescence-inducing non-nucleoside is BIBR 1532 (Figure 3B), one of the most potent TERT inhibitors discovered thus far. The exposure of human cancer cells from different histological origins to BIBR 1532 led to progressive telomere shortening and inhibition of cell proliferation, independent of p53 gene status (*75*).

BIBR 1532 has been shown to directly target TERT core components. In addition, BIBR 1532 exhibits a non-competitive mode of inhibition, which is clearly distinct from the mechanism used by nucleoside compounds or antisense oligonucleotides. Furthermore, BIBR 1532 does not cause chain termination events but rather inhibits the formation of long reaction products, reducing the number of TTAGGG repeats added during each replication event (*113*). This mechanism of action suggests that BIBR 1532 impairs the elongation of telomere DNA after its initial extension to the 5′-end of the template. These steps are most likely unique to TERT due to its high activity in tumor cells, which could explain the high selectivity of the compound (*113*). BIBR 1532 has been shown to inhibit cell proliferation in lung, breast, fibrosarcoma and prostate cancer cells (*113*). Interestingly, proliferation arrest after a sustained period of BIBR 1532 treatment was observed in combination with hallmarks of senescence, including morphological, mitotic and chromosomal aberrations and altered patterns of gene expression (*97*).

3.3.4. RNA interference technology applied to the study of senescence and cancer

Small interfering RNA (siRNA) and short-hairpin RNA (shRNA) are usually used for effective gene-specific RNA silencing. The silencing response induced by siRNA is transient because siRNAs are stable for only 3-5 days in culture, which restricts their application in gene therapy. Nevertheless, shRNA can generate a long-term gene-silencing response. The first step used to apply shRNA in gene therapy involves its expression from plasmid vectors. These gene constructs utilize the RNA polymerase III promoters H1 or U6 to transcribe shRNA that will be processed into 21-bp siRNA by the enzyme Dicer. Subsequently, this treatment results in mRNA degradation and silencing of the cognate gene. By contrast, siRNA are small oligonucleotides that are transfected into target cells (*114*).

The modulation of TERT component expression using genetic constructs (e.g., antisense RNA) is a powerful tool to induce senescence in tumor cells. The advent of siRNA and shRNA technologies at the beginning of this century has allowed the study of basic and clinical aspects of tumors and senescence. The use of shRNA and siRNA targeting hTERT has been shown to inhibit cell proliferation, decrease telomerase activity, increase the number of cells arrested at the G_0/G_1 phase of the cell cycle, and attenuate the tumor growth of xenograft mouse models (*115*).

3.3.4.1. siRNA against TERT: When transient silencing can produce long lasting effects

Telomerase siRNAs targeted against regions of human telomerase caused efficient inhibition of telomerase expression, loss of telomerase activity and severe telomere shortening. Many cells that are deficient in TERT have no compensatory mechanism to maintain their cellular viability, eventually succumbing to apoptosis. Because cancer cells are so dependent on telomerase activity, a transient drop in telomerase expression can have irreversible effects on telomere length and therefore lead to senescence induction. This type of response was observed in tumors such as Barret's adenocarcinoma (*116*), hepatic cancer (*117*) and lung cancer, with siRNA treatment combined with doxorubicin in all these cases (*118*).

However, the most realistic model with which to study senescence in cancer cells uses a vector expressing shRNA directed against hTERT. The efficiency of stable TERT silencing is sometimes greater than that of transient silencing, e.g., in bladder and oral cancer models (*119, 120* respectively).

All of the TERT inhibitors described above, together with others found in the literature, are described in Table 2.

3.3.5. The dark side of TERT inhibitors

As discussed before, telomere length and the alternative mechanisms that maintain its structure (e.g., ALT), as well as the biologic model used to study the relationship between senescence and tumor inhibition, determine the positive or negative effect of a senescence-inducing treatment. The literature contains hundreds of experiments in which TERT inhibitors displayed no effect on tumor growth, especially in those cells that possess an active ALT mechanism (*137*)

Interestingly, ALT activation was not observed in cell culture experiments in which TERT-positive cell lines were treated with TERT inhibitors or transfected with dominant-negative TERT mutants (*138*), indicating that ALT is not a preferential mechanism to preserve telomere stability. However, *in vivo*, where large tumors may contain millions or billions of cells in different microenvironments, ALT could be induced in a small fraction of cells, thus leading to the development of more aggressive and resistant clones. The development of ALT inhibitors may therefore be necessary to counteract this resistance mechanism.

- **Table 2.** Major oligonucleotides/small molecules used to induce senescence in tumor cells and their molecular targets

Target	Oligonucleotides/small molecules	Senescence-inducing approach	Clinical trial (number identification)/tumor type[a]	References
Telosome (shelterin)	siRNA, shRNA, and Cre-lox system against POT1, TRF1, and TRF2 genes	Disruption of the telosome/shelterin complex	-	(75 to 78); (121 to 124)
Telomere structure	BRACO19, telomestatin, RHPS4, and SYUIQ-5	Disruption of telomere architecture; inhibition of TERT activity	-	(75); (98 to 107); (125 to 127)
Telomerase	GRNL163	Targeting the RNA template-region of TERT; blocking TERT expression and biogenesis	NCT00124189/Chronic lymphocytic leukaemia, NCT00594126, NCT00718601, NCT01242930/Multiple myeloma, NCT00310895/Solid tumor malignancies, NCT00510445/Lung cancer, NCT01137968/Non-small cell lung cancer, NCT00732056/Breast cancer, NCT01265927/Breast neoplasms	(78 to 102); (128 to 130)
Telomerase	BIBR1532	Selective binding to TERT; blocking TERT activity	-	(113);(131)
Telomerase	siTERT/shTERT	Downregulating TERT gene expression	-	116 to 120); (132)
Telomerase	Antisense oligonucleotides	Downregulating TERT gene expression	-	(133 to 136)

[a]Sources: http://www.clinical.trials.gov; Geron Corporation (http://www.geron.com).

In addition to the potential for selection for resistant cells, TERT inhibitors may also cause side effects in normal tissues. Fortunately, telomeres are longer in normal tissues than in most cancers, and treatments can be designed to end before telomere depletion occurs in normal tissues (*138*). Further studies examining this approach must be conducted to determine how to best protect tissues, such as intestine, epidermis, and hematopoietic tissue, in which stem cells and transit cells are constantly dividing at a high rate.

4. OXIDATIVE STRESS, DNA DAMAGE, AND SENESCENCE: A PHYSIOLOGICAL LINK TO GENETIC INSTABILITY

4.1. The DNA damage response and senescence

The DNA damage response mechanism comprises a series of biochemical pathways that are activated in the presence of different types of lesions induced by chemical compounds and/or ionizing and non-ionizing radiation. Many DNA lesions are capable of altering DNA structure and function, resulting in the loss of genetic information and leading to cell senescence or even loss of viability over time (*139,140*). Moreover, the loss of specific DNA sequences or GCRs induced by DNA damage are one of the driving forces of tumorigenesis (*140*).

A variety of DNA lesions have been described, including single base or nucleotide modifications (e.g., oxidized purines and pyrimidines), single-strand breaks (SSBs), interstrand crosslinks (ICLs) and double-strand breaks (DSBs) (*141-143*). Of these lesions, ICLs and DSBs are considered to be the most damaging to the genome (*143*). One major mechanism that leads to the formation of ICLs and DSBs is oxidative damage, which is caused by reactive oxygen species (ROS) generated by an imbalance between ROS production and degradation by antioxidant systems (*144*). ROS generate mainly SSBs that can progress to DSBs. Interestingly, it was demonstrated that DSBs accumulate during senescence and aging as a consequence of decreased DDR (*145*), leading to GCR. GCR is also a leading cause of tumorigenesis, and a strong correlation between aging and tumor development has been established by different authors (*146-148*).

To protect the genome from DNA damage and the loss of genetic information, all cells use a complex network of proteins and signaling molecules termed the DNA damage response (DDR) pathway. The main function of the DDR is to sense the different types of DNA lesions and mount a cell-wide response that includes the modulation of cell cycle transitions and transcriptional processes and the stimulation of DNA repair pathways (*149*). Both responses are coordinated at the molecular level by three major classes of proteins: (i) sensor proteins that recognize abnormally structured DNA and initiate the signaling response; (ii) transducers, which amplify the signal; and (iii) effector proteins that are present in numerous downstream pathways and repair the damage in an error-free or error-prone fashion (*149*). The DDR mechanism is also necessary to evaluate potential damage that could arise during DNA replication (*150*).

The diverse nature of DNA lesions led to the evolution of different DNA repair pathways that act downstream of the DDR. In general, these DNA repair pathways are classified as (i) excision mechanisms, which encompass the base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR) pathways; (ii) recombinational mechanisms, which are normally recruited in response to SSBs and/or DSBs and include homologous recombination (HR), single-strand annealing (SSA) and non-homologous end joining (NHEJ); and (iii) direct repair, which encompasses photorepair (mediated by photolyases), DNA alkylation repair, and the

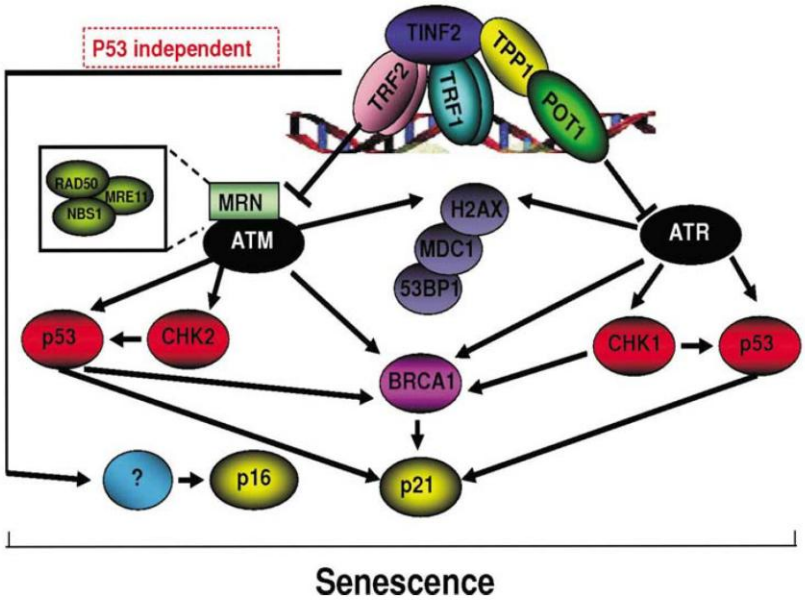


Figure 4. Schematic representation of senescence-induced DNA damage response (DDR). The progressive telomere shortening or the presence of uncapped telomeres initiates the DDR, resulting in the activation of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia (ATR) DNA-damage sensor proteins. The activation of ATM and ATR leads to the phosphorylation of the downstream kinases CHK1 and CHK2, as well as p53. Phosphorylated p53 transcriptionally upregulates genes that mediate cellular senescence and inhibit tumorigenesis. Depending on how telomeres are uncapped, the removal of telomeric-repeat binding factor 2 (TRF2) preferentially engages an ATM-dependent checkpoint, whereas removal of POT1 preferentially engages ATR. Although less well-understood, telomere dysfunction could also activate the p16 pathway and inhibit cellular proliferation.

direct reversion of damaged bases (*149*). Many of these DNA repair mechanisms are strongly interconnected. Some important DDR-associated proteins that act as sensors and/or transducers in the DNA repair pathways include the ataxia telangiectasia-mutated (ATM) and ATM-Rad3-related (ATR) protein kinases, BRCA1, BRIT1, the checkpoint kinases CHK1 and CHK2, p53, and the histone variant γ-H2AX. These sensor/transducers seem to be essential for the control of senescence, especially for regulating telomere maintenance. Thus, the discovery that DDR-associated sensors and transducers are important for normal telomere maintenance has yielded important insights into which molecules sense short telomeres and signal to modulate telomerase action and eventually induce senescence (Figure 4).

It is important to note that all telomeres are maintained in a stabilized, functional form by a process termed "telomere capping", which is promoted by the action of telomerase. However, telomeres can be converted to a non-functional state through a reverse process, termed "telomere uncapping", that is triggered by low telomerase activity and results in the activation of the DDR (Figure 4). Four distinct structural components contribute to telomere capping: (1) the higher-order telomeric DNA-protein complex, whose overall length (i.e., the number of telomeric repeats) dictates whether telomerase or nucleases can access telomeric DNA; (2) the protein complexes bound to the terminal repeats; (3) the DNA-protein complex at the single-stranded, G-rich DNA extension of the telomere, which is likely important for preventing the DNA damage response and regulating the cell cycle-dependent structure of the telomere; and (4) telomerase itself (*43*). Telomere uncapping results in a progressive shortening of telomeres over time, leading to replicative senescence by a p53-dependent mechanism (Figure 4). This p53 response to critically shortened telomeres is a result of the activation of the DDR (*151*); the shortening of telomeres results in their uncapping and subsequently their recognition as damaged DNA (*151*). Thus, under cellular conditions in which telomeres are uncapped, the cellular responses that occur are symptomatic of DNA damage, such as cell cycle arrest or cell death (*43*). Telomeric DNA is then also subjected to the molecular processes that are normally applied to DSBs within chromosomes: end-to-end fusions, degradation, and recombinational events, which in some cases fuse telomeric ends. Certain molecular changes at telomeres that compromise capping can also unleash unregulated telomerase action at the uncapped telomere, in contrast to the normally tight regulation of its action on capped telomeres (*43*).

Interestingly, cancer cells tend to have short telomeres but elevated telomerase expression, and the induction of telomere capping protects cancer cells against the DDR and, consequently, senescence (*43*). This

induction is probably related to the formation of higher-order structures at telomere sites that recruit the hTERT complex, and it blocks the DDR mechanism. However, in an uncapped telomere, the DDR is activated and sensor/transducer DNA damage-associated proteins, such as ATM and the MRN complex (MRE11-RAD50-NBS1 proteins; Figure 4), which acts together with effector proteins from recombination repair pathways (*43*), are recruited. Additionally, ATM is required to modulate telomere structure, either to facilitate telomerase access and/or to create a suitable substrate for the enzyme (*43*). In this context, telomere uncapping can cause GCRs (e.g., dicentric chromosomes) that lead to DDR activation by modulating ATM activity. Finally, activated ATM signals to the p53 and p21/SD11/C1P1 pathways, resulting in cell senescence (Figure 4) (*153*). In capped telomeres, ATM signaling is repressed by TRF2, whereas the single-stranded telomeric DNA-binding protein POT1 blocks the activation of the ATR kinase (Figure 4) (*154*). Depletion of POT1a/b induces an ATR kinase response that leads to the accumulation of DNA damage factors at chromosome ends and the activation of the effector kinases Chk1 and Chk2 (*154*). This DDR is persistent because the repair of the damaged telomeres by NHEJ is repressed by TRF2, which remains associated with telomeres despite the removal of POT1a and -b (Figure 4) (*156*).

POT1a/b deletion in cells lacking a functional p53 pathway was shown to cause polyploidization, producing cells with 4n, 8n, and 16n DNA content. In addition, it was observed that the resulting GCRs in p53-deficient cells could initiate breakage-fusion-bridge cycles that promoted the main genomic alterations observed in cancer cells: loss of heterozygosity, gene amplification, and nonreciprocal translocations (*156,157*). Polyploidization promotes the activation of telomerase, resulting in some chromosome loss (*158*) but mainly stabilizing the resulting new genome structures. Finally, the cell progeny exhibit extensively altered subtetraploid genomes on which selection for the most malignant clone can take place.

4.2. Chemotherapy and senescence

Many studies have evaluated the effects of chronic exposure to standard chemotherapeutic agents on cultured human cancer cells. The data demonstrate that cancer cells undergo senescence when exposed to a wide variety of DNA-damage drugs, especially when the cells are exposed to topoisomerase II inhibitors, such as etoposide, camptothecin, and doxorubicin (*159*).
VP-16, or etoposide, produces a senescence-like phenotype both *in vitro* (*160*) and *in vivo* (161) in a p53-dependent fashion. However, one study showed that 40–60% of p53-null lung cancer cells exposed to VP-16 also became senescent (*160*).

Camptothecin is able to induce the senescence response in tumor cells that are $p53^{+/+}$ and $p21^{waf1/cip1+/+}$ (*162*) by downregulating the expression of *CDC2*. Although the senescence response to camptothecin can be blocked in p53-null and p16-deficient human non–small cell H1299 carcinoma cells, this escape from stress-induced premature CS can be disrupted by Cdc2/Cdk1 kinase inhibitors or by knockdown of Cdc2/Cdk1 (*163*), suggesting that senescence induced by antitumor agents is strongly dependent on the genetic aspects of tumor cells.

Another antitumor drug that induces accelerated senescence in some tumor types is cisplatin. The DNA lesions generated by cisplatin are composed primarily of intrastrand crosslinks (ICLs), and it is likely that cisplatin induces senescence through both p53-dependent and -independent pathways (*164*). It is unclear whether cisplatin or other platinum-based drugs affect telomere length or TERT activity (*165*).

A possible mechanism that could connect DNA damage signaling and senescence induction by antitumor drugs is the activation of a complex cytokine network by means of promyelocytic leukemia protein (PM) in the tumor (*166*). This cytokine network includes the proinflammatory interleukins IL-6 and IL-8 and involves the reorganization and/or multiplication of a specific nuclear compartment. It has been demonstrated that cytokine signaling pathways are involved in drug-induced senescence (*167*), and chemotherapy-induced senescence can occur in neighboring cells through the so-called "bystander" effect (*168*). Work by Hubackova *et al.* (*169*) indicated that exposure of human normal and cancer cells to genotoxic drugs, including camptothecin and etoposide, results in increased PML transcript levels and activated JAK/STAT signaling. Both endogenous PML transcript levels and PML promoter-driven luciferase activity were suppressed by chemical inhibition or RNAi-mediated knockdown of JAK1 kinase, revealing a key role for JAK1-controlled signaling in PML transcription induced by genotoxic stress. Furthermore, in contrast to oncogene induced CS, in which PML expression is controlled by p53, this work demonstrated that cells expressing a dominant-negative allele of p53 also display a PML response to genotoxic drugs (*169*).

5. ONCOGENE EXPRESSION AND SENESCENCE INDUCTION IN TUMOR CELLS

5.1. Oncogene-induced senescence: primary barrier to cancer prevention and/or treatment

The early stages of cancerous transformation feature neoplastic transition followed by an accumulation of mutations that produce more aggressive cells, which are further selected by the tissue and/or tumor microenvironment (*170*). The neoplastic transition is characterized by an increase in the expression of oncogenes, which control different biological processes, such as cell proliferation and apoptosis. Oncogenes can be activated by GCRs, as a consequence of clastogenesis, or by gene structural alterations, such as fusion (*171, 172*), juxtaposition of enhancer elements (*172, 173*) or gene amplification. Translocations and mutations can occur during the initiating events that lead to tumorigenesis (*174*), whereas gene amplification is usually associated with tumor progression (*172*).

Oncogenic mutations typically cause excessive cell proliferation, leading to the disruption of normal tissue

Table 3. Oncogenes and tumor suppressor proteins that regulate senescence in tumor cells

Oncogene	Biological processes	References
H-ras^{G12V}	RAS signaling cascade	(176), (177)
K-ras^{G12V}	RAS signaling cascade	(176), (177)
N-ras^{G12D}	RAS signaling cascade	(176), (177)
BRAF^{V600E}	Promotes RAS signaling cascade	(178), (179)
c-Myc	Ras signaling effector; gene transcription inducer and chromatin remodeling factor	(180), (181)
RAF	RAS signaling cascade	(182)
AKT	PI3K/AKT signaling cascade	(183)
STAT5	Promotes JAK/STAT pathway	(184)
E2F1	Associates with retinoblastoma protein (pRB) in a cell-cycle-dependent manner; regulates cell proliferation	(185)
E2F3	Promotes G1 to S phase transition; transcription factor	(186)
Runx	Transcription inducer and chromatin remodeling factor	(187)
CDC6	Replication licensing factor; promotes S phase progression	(188)
Tumor supressor		
p53^{VP16}	Pleiotropic activity in cell cycle	(189),(190)
RB	Regulate cell cycle proliferation by selective bind to E2F family protein	(190), (191)
CDKN2A (p16INK4a)	Cyclin-dependent kinase inhibitor; inhibits G1 progression	(192), (193)
CDKN1A (p21Cip/Waf)	Cyclin-dependent kinase inhibitor; inhibits G1 progression	(194), (195)
CDKN2B (p15INK4B)	Cyclin-dependent kinase inhibitor; inhibits G1 progression	(196)
NF1	Downregulates RAS signaling	(197)
PTEN	Downregulates PI3K/Akt/mTOR signaling	(198)
VHL	Target HIF for degradation	(199)

microanatomy and impaired tissue function. Moreover, oncogenes have pleiotropic functions in the cell and can be broadly classified into seven groups (*172*): (i) transcription factors, (ii) chromatin remodelers, (iii) growth factors, (iv) growth factor receptors, (v) signal transducers, (vi) apoptosis and (vii) cell cycle regulators.

Notably, nearly three decades ago, it was observed that normal cells are refractory to oncogenic cancer initiation, indicating that non-tumor cells probably have mechanisms that override the proliferative activity of oncogenes (*16,175*) by promoting entry into senescence. Interestingly, oncogenes like Ras, Raf, E2Fs, Stat5, and AKT (Table 3) can induce cellular senescence in normal cells by activating diverse tumor supressors (Table 3). This mechanism is called oncogene-induced senescence, or oncogene induced CS. An interesting model for oncogene induced CS is the skin nevus. Nevi are characterized by the presence of slightly altered melanocytes, with variable sizes and shapes. (*200,201*). The high frequency of B-Raf mutations in common nevi is of special interest for the study of oncogene induced CS because active mitotic cells are rare or absent in this tissue, despite the potentially pro-proliferative signaling activated by B-Raf. In turn, the expression of endogenous B-raf in melanocytes resulted in growth arrest that does not require p16^{ink4a}. A murine knockin model of B-Raf targeted to cutaneous melanocytes induced benign tissue proliferation that did not generate melanomas over a period of 15 to 20 months. However, when combined with PTEN silencing, all animals developed metastasis in short periods of time, indicating that oncogene induced CS is dependent on this tumor suppressor (*202*).

5.2. Ras and B-raf: an oncogenic paradox that leads to senescence

The high complexity of the signal activation/repression mediated by oncogenes makes a full understanding of oncogene induced CS difficult. However, two major oncogenes widely studied in the context of oncogene induced CS, Ras and B-Raf, activate a common pathway (MEK/ERK) but induce senescence in tumors through different mechanisms (Figures 5A-C).

Cells that overexpress Ras consistently exhibit several signs of an activated DNA replication checkpoint, such as a high fraction of cells arrested in S phase, activation of the ATR pathway and preferential loss of heterozygosity at fragile sites. Oncogenic Ras expression leads to the overexpression of CDC6, increased numbers of active replicons and oncogene induced CS (Figure 5A). Oncogenic stress mediated by Ras has been shown to induce genome instability after a single round of DNA replication (*21*), while CDC6 overexpression is sufficient to induce DDR activation and senescence (*22*).

Another mechanism of senescence induction by Ras is mediated by the loss of the tumor suppressor NF1 (neurofibromatosis 1; Figure 5B), which results in sustained Ras activation, hypersensitivity to growth factors, and immortalization. However, in normal human diploid fibroblasts, the loss of NF1 triggers a transient activation of Ras and its effectors, followed by a dramatic suppression of these signals to lower than baseline levels. Moreover, these cells become senescent, indicating that the ultimate response to the aberrant activation of Ras pathway is a dramatic termination of Ras signaling at many levels, followed by a cellular response designed to eliminate the proliferative potential of cells that harbor oncogenic Ras (*203*). Furthermore, this sensitivity is determined not by a single biochemical event but rather by the coordinated output of cell type-specific signaling networks (*203*).

B-Raf (Figure 5C) can also results in oncogene induced CS by a different mechanism based on cytokine secretion. B-Raf induces the transcription factor C/EBP-β, which activates interleukin-6 (IL-6), forming a positive feedback loop. The C/EBP-β-IL6 feedback loop in turn activates the tumor suppressor p15^{ink4b} and an inflammatory network that includes IL-8. IL-6/IL-8 acts in concert, in a

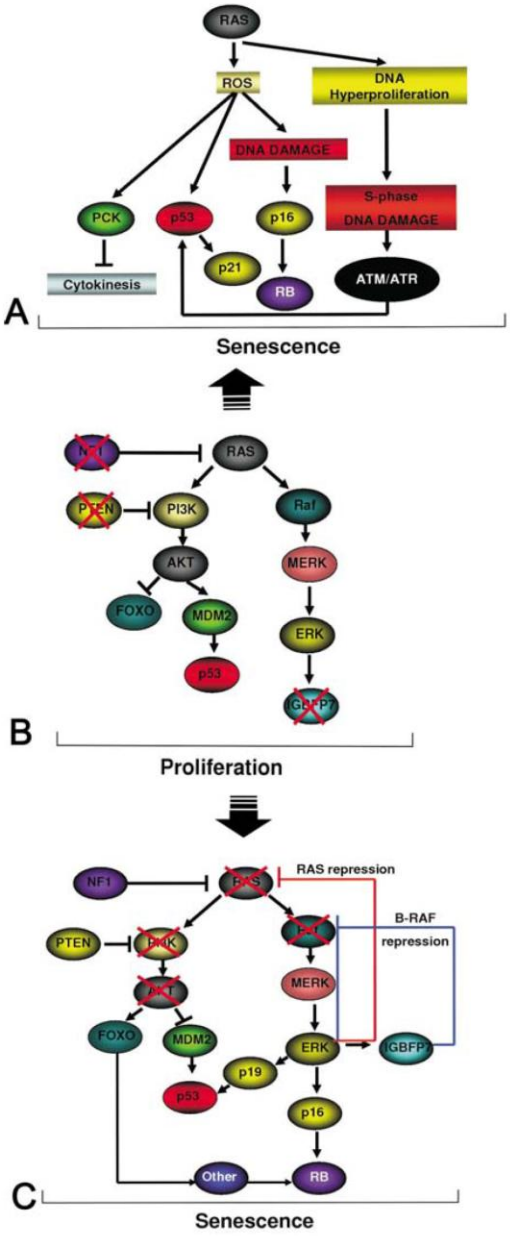


Figure 5. Model of oncogene-induced senescence (OIS) by Ras regulation. In (A), strong Ras activation can lead to DNA damage through ROS generation and/or DNA hyper-replication, which have been suggested to induce OIS by a p53-dependent mechanism. In cells that are insensitive to OIS (B), the Ras-associated pathway leads to hyperactivation of well-known effector proteins that induce cell proliferation (e.g., PI3K and RAI) and their downstream targets (e.g., AKT, MDM2, p53, MERK, ERK), thus promoting tumorigenesis. On the other hand, activated RAS leads to growth arrest as a result of potent negative feedback that abrogates ERK and PI3K signaling (C). In this sense, a negative feedback signal generated by ERK represses the RAS pathway (red line; C), while the IGFP-7 pathway becomes activated and represses B-raf, resulting in OIS via p16 (blue line; C) or p53 via p19. Despite the fact that the biochemical mechanisms of these three models differ, they are not mutually exclusive, and an overlap of pathways is observed in different biological models.

cell-autonomous manner, to leads to oncogene induced CS. IL-1a, a multifunctional cytokine and component of the "senescence-associated secretory phenotype" (SASP), was reported to be the upstream regulator of the senescence-associated IL-6/IL-8 cytokine network (*16*). Notably, DDR signaling was able to initiate and maintain cytokine secretion in a p53-independent and PRB-independent manner (*23*). The demonstration that BRAFV600E-linked oncogene induced CS displays a mosaic $p16^{ink4a}$ expression pattern indicates that $p16^{ink4a}$ is not required for senescence induction (*23*).

6. TWO DIFFERENT FACES OF SENESCENCE: INDEPENDENT MECHANISMS OF CLASSICAL PATHWAYS OF AGING IN CANCER

6.1. Premature-induced cellular senescence: Another form of senescence

Like oncogenes, the loss of tumor suppressors can also trigger senescence in mouse and human cells. While p53 is necessary for the function of several senescence inducers, its loss does not induce senescence. In contrast, the loss of PTEN induces senescence is several cell types (*204-207*). PTEN encodes a phosphatase that catalyzes the conversion of the membrane lipid PIP3 to the PI3K substrate PIP2 and negatively regulates the PI3K–AKT–mTOR pathway. This phosphatase is commonly mutated or lost in many cancer types (*208-212*).

PICS (PTEN loss-induced cellular senescence; PTEN-loss induced CS) shares several features with oncogene induced CS but is independent of the classical senescence induction that requires DDR activation and/or telomere erosion (*24*). It was shown that MEFs deficient in PTEN undergo senescence accompanied by an induction of p53 (*25*). However, in contrast to other forms of senescence, this increase in p53 was mediated mTOR-mediated translation regulation rather than the DDR (*24, 26*). Additionally, deletion of the gene encoding ARF (which promotes MDM2 degradation and leads to p53 accumulation) at the Cdkn2a locus *in vivo* fails to prevent PTEN-loss induced CS in prostate tumorigenesis, showing that ARF is dispensable for this type of senescence (*27*).

CDH1, an activator of the anaphase-promoting complex/cyclosome (APC), contributes to mitotic exit and G_1 maintenance by targeting cell cycle proteins for degradation (*20*). Nuclear PTEN activates APC–CDH1 to regulate the degradation of its targets. The observation that CDH1-null cells are refractory to PTEN-mediated growth suppression positions this protein as a mediator of PTEN-loss induced CS (*28*). However, in cancer models with PTEN loss, CDH1 is separated from the cyclosome proteins, producing an accumulation of ETS2 (substrate of CDH1). EST2 is an important transcription factor for INK4A, leading to upregulation of this CDK inhibitor and subsequent senescence induction (*29*).

Another interesting feature of PTEN-loss induced CS is that it can be induced in quiescent or growth-arrested cell types (24), whereas oncogene induced CS normally occurs only in mitotically active cells. Cancer-initiating cells (CICs) naturally arrest in a quiescent state, contribute to the maintenance of the tumour, and fail to be targeted by current therapeutic protocols (*23*) and might escape the activation of oncogene induced CS, but could be susceptible to PTEN-loss induced CS (*reviewed in 24*). An additional difference between oncogene induced CS and PTEN-loss induced CS is that the latter is activated over a much shorter time (*24*).

6.2. The SKP2 pathway induces senescence independent of p53 activation

Skp2 is a component of the Skp2–SCF complex that acts as an E3 ubiquitin ligase of the CDK inhibitor p27 and other substrates (*214, 215*). There is evidence that SKP2 may act as an oncogene (*217*), and this protein is frequently overexpressed in non-Hodgkin's lymphomas (*217*), mucosal epithelial dysplasias and squamous cell carcinomas (*216*).

The inactivation of Skp2 reduces tumorigenesis by inducing cellular senescence only under oncogenic conditions. Remarkably, this senescence response is triggered in a $p19^{Arf}$–p53-independent manner (*27*). Pharmacological inactivation of Skp2 may therefore represent a general approach towards a 'pro-senescence' therapy for cancer prevention and treatment.

7. EPIGENETICS TOOLS APPLIED TO TUMOR-INDUCED SENESCENCE: STATE-OF-THE-ART

Epigenetic modifications also play a crucial role in tumorigenesis. By definition, epigenetic modifications are heritable changes in genes and/or proteins that do not alter the primary DNA sequence. The best-known epigenetic marks are methylation, acetylation, ubiquitination, sumoylation and phosphorylation.

These marks can directly regulate gene expression by altering the chromatin state, facilitating the addition or removal of methyl and acetyl groups in chromatin. While chromatin methylation results in heterochromatin formation, acetylation leads to euchromatin formation. A special group of enzymes named DNA methyltransferases (DNMTs) are responsible for the transfer of the methyl mark to the 5′-carbon position in cytosine bases at CpG dinucleotide residues *(217)*. However, the transfer of the methyl group to the cytosine residue may occur improperly, causing DNA mutations that can lead to tumor development (*218*). It should be noticed that epigenetic mechanisms are also responsible for the repression of different sets of genes through their association with a specific complex called the polycomb repressive complex (PRC1) (*219*). As an example of the importance of these complexes, the histone methyltransferase EZH2 (enhancer of zeste homolog 2) in the PRC2 and PRC3 complexes plays a key role by serving as a recruit platform for the DNMTs and is therefore crucial to establishing gene expression silencing and epigenetic memory (*221*).

Senescence is also associated with errors in the epigenetic machinery (*218*). The loss of DNMT1 (and, therefore, global DNA methylation), particularly in

heterochromatic zones, could lead to senescence in adult organisms (*221*). Interestingly, it has been hypothesized that DNMT3B could hypermethylate DNA sequences that are not normally methylated, leading to mutations in local genes and tumor initiation (*221*). Also, during aging, some genes in CpG islands are found to be hypermethylated, whereas repetitive sequences in heterochromatin are hypomethylated (*222*). In this scenario, global gene expression could be severely compromised, again promoting tumorigenesis. Furthermore, it has been proposed that senescence can disrupt the epigenetic organization of heterochromatin, leading to disordered gene transcription and causing DNA damage (*222*). On a related note, heterochromatin loss can reduce the activity of the NURD complex, which is composed of the histone deacetylases HDAC-1 and HDAC-2 and the ATPases CHD3 and CHD4. Reduced NURD activity may cause many defects in chromatin structure and integrity (*222*) and thus activate the DDR. The DDR can lead to further rearrangement of the epigenetic mechanisms, causing alterations in chromatin integrity and, therefore, gene misregulation and cancer (*222*).

The NAD-dependent deacetylase sirtuin-1 (SIRT1) is also downregulated during aging and senescence (*221, 222*). However, in some tumor cells, SIRT1 is commonly found to be upregulated, demonstrating the interplay between senescence and cancer development (*222*). Moreover, in estrogen-dependent breast cancers, estrogen receptors can act as oncogenes by facilitating the age-related increase methylation at the promoter of the tumor suppressor RASSF1A (*223*). Likewise, in tobacco users, many tumor suppressor genes were found to be hypermethylated (*223*). However, in other types of lung cancers, the DNMT1 gene is found to be downregulated by a solution containing nicotine (*224*). Therefore, changes in methylation patterns (hypermethylation or hypomethylation) could lead to tumor progression.

Interestingly, epigenetic mechanisms are associated with the induction of cell senescence in human and mouse tumor cells (*225*). The CDK inhibitor p16 can suppress the development of spontaneous cancers by making the senescence triggered by p53 inactivation irreversible. p16 can be silenced by the methylation of its promoter (*226*). Thus, some DNMT inhibitors, such as 5-azacytidine and decitabine (5'-aza-2'-deoxycytidine), were proposed as potential treatments for cancer (*227*).

Another epigenetics-associated family of proteins, termed INGs, was found to be downregulated in many types of cancer (*228*). INGs interact with p53 in the p53-dependent response to cell senescence and apoptosis (*228*). This again implies a link between senescence and chromatin remodeling; INGs participate in the epigenetic regulation of gene expression by interacting with HDACs and proteins with histone acetyltransferase (HAT) activity such as p300, CBP, PCF, and TRRAP (*228*).

8. MICRORNAS AND THEIR ROLE IN SENESCENCE

Micro-RNAs (miRNAs) are a novel class of non-protein coding genes that play an important role in the post-transcriptional regulation of gene expression (*229*). These miRNAs are produced from PolII-transcribed primary RNA transcripts by several processing steps. The final step in the miRNA pathway is the loading of one RNA strand into the RNA-induced silencing complex (RISC) (*229*). Mature miRNAS are short non-coding sequences that range in size from 19 to 22 nucleotides and are highly conserved. miRNAs regulate protein expression by binding the 3' untranslated region (UTR) of an mRNA (*229*). The ability of miRNAs to regulate a variety of target genes allows them to induce changes in multiple pathways, and miRNAs are involved in diverse processes, including development (*230*), apoptosis, proliferation and differentiation (*231*).

Recent studies have shown that miRNA expression profiles differ between normal and tumor tissues (*232*). Interestingly, the downregulation of subsets of miRNAs is a common finding in some tumors (*233*), and the discovery that miRNA silencing could revert the tumorigenic phenotype revealed a novel regulatory mechanism in cancer proliferation (*234*). The evidence for a regulatory role for the miR-34 family of miRNAs in senescence is growing and has stemmed from the investigation of p53 and its role in senescence. p53 regulates miR-34a; members of the miR-34 family of genes contain p53-binding sites in their promoters, which are conserved among humans and rodents. In turn, miR-34a increases the activity of p53 by reducing the expression of sirtuin 1 (SIRT1), which interacts with p53 and deacetylates p53 at Lys382 in a NAD^+-dependent manner. This deacetylation decreases p53-mediated transcriptional activation and thus reduces the expression of downstream proteins, such as p21Cip1 (*235, 236*). Also, miR-34a can induce senescence and suppress cell proliferation through downregulation of the E2F pathway in human colon cancer cells regardless of p53 status, leading to the upregulation of the p53/p21Cip1 pathway (*237*). Moreover, miR-34a is upregulated after activation of the B-RAF oncogene. miR-34a also induces senescence through repression of v-myc (*238*).

Another target that induces cellular senescence mediated by miR-30 is LIN28 (a homolog of lin-28 in *Caenorhabditis elegans*) in embryonic stem cells and cancer cells. It is important to note that LIN28 functions as an oncogene to promote malignant transformation and tumor progression (*239*) Another miRNA involved in senescence, miR-449a, induces senescence by suppressing Rb phosphorylation by directly repressing the upstream regulatory factors of Rb, such as cyclin D1 (CCND1), HDAC1, cyclin-dependent kinase 6 (CDK6), and cell division cycle 25 homolog A (CDC25A) (*240*). A recent study has shown that miR-449a is downregulated in prostate cancer, indicating that this miRNA regulates cell growth and viability in part by repressing HDAC-1 expression (*241*).

A genetic screen designed to identify new miRNAs characterized by their ability to bypass senescence induced by oncogenic Ras (oncogene induced CS) led to the identification of miR-372 and miR-373 (*242*). This study was performed in partially immortalized IMR90 fibroblasts. These miRNAs are considered to be novel oncogenes, participating in the development of human testicular germ cell tumors by blocking the p53 pathway and promoting tumorigenic growth in the presence of wild-type p53. Importantly, miR-373 is able to induce the formation of foci in soft-agar assays, demonstrating its transforming capability. By contrast, the introduction of miR-34a and miR-34b/c into primary human diploid fibroblasts induces cellular senescence (*243*). Tumor cells also show signs of senescence after the introduction of ectopic miR-34a (*244*). Downregulation of miR-138 is associated with overexpression of telomerase and the acquisition of malignant behavior in human anaplastic thyroid carcinoma cell lines (*245*). Therefore, it is expected that miR-138 would be useful as a diagnostic tool and might be a target for the development of new strategic treatments for specific kinds of carcinomas, as has already been suggested for miR-378 (*246*).

The Polycomb complex is regulated by microRNAs that induce senescence, of which miR-128a directly targets the Bmi-1 oncogene (polycomb ring finger oncogene; BMI1), increasing the expression of $p16^{INK4A}$ and the production of reactive oxygen species (ROS), which promote cellular senescence in medulloblastoma cell lines (*247*). Recently, it has been reported that this tumor suppressive miRNA (miR-128a) is downregulated in medulloblastomas (*248*), glioblastomas (*249*) and acute myeloid leukemia (*250*), suggesting that this miRNA plays an important role in these types of cancer.

9. AN EVOLUTIONARY FOCUS ON SENESCENCE: AN ENDOGENOUS ANTICANCER MECHANISM?

For the past 50 years, two theories on the ultimate cause of aging, the mutation accumulation theory and the antagonistic pleiotropy theory, have dominated evolutionary discussions of senescence.

The mutation accumulation theory proposes that aging occurs due to cumulative deleterious mutations in germline cells that are only expressed during the later stages of an organism's life (*251*). Aging is thus able to occur even in an immortal population by the accumulation of these age-specific mutations over successive generations (reviewed in *252*). Antagonistic pleiotropy centers on genetic effects that enhance fitness early in life but depress fitness late in life (*253*). Such genetic alterations are able to spread because the force of selection is stronger earlier in life because more individuals are alive and, more importantly, reproductive, at this stage than at later ages. Overall, both of these theories view aging as a collateral effect of the adaptation process (*252, 253*).

The mutation accumulation theory and the antagonistic pleiotropy theories are hypothetico-deductive in nature (*254*), meaning that when first conceived they were deduced from assumed laws or premises rather than from empirical observations. These hypotheses have been adopted as gerontological paradigms largely due to a lack of alternatives rather than for any compelling evidential reasons (*255*).

The mutation accumulation theory assumes that, in the wild, most organisms die before they reach old age. For this reason, deleterious age-specific alleles can build up and eventually cause aging. In the light of current knowledge, however, this theory is untenable as an explanation of how the telomere system evolved. The telomere system is complex, hierarchical, integrated and finely regulated, and it is implausible that such sophistication could be the result of unselected mutation accumulation. Because the mutation accumulation theory is an untenable explanation of replicative senescence, the only other mainstream alternative is antagonistic pleiotropy. Here, one must argue that replicative senescence confers an adaptive advantage earlier in life, and that aging is an incidental late-life side effect of the program (*252*). Not surprisingly, then, this is the position taken by the majority of evolutionary gerontologists. It has been proposed that the primary function of the telomere system is its role as a natural defense against cancer (*256*). The basic idea here is that telomere attrition restrains tumor growth by limiting the replicative capacity of transformed cells. Once the maximum number of doublings has been reached, telomeres induce cell senescence, thereby permanently removing such cells from the cell cycle. In this way, telomerase repression, by allowing telomere attrition, acts as a barrier to uncontrolled proliferation (*257*). Under antagonistic pleiotropy, the later effects of replicative senescence (i.e., aging) are seen as secondary side effects—effects that have been allowed to persist because selection at older ages is weak (*252*).

There is abundant evidence to support the hypothesis that telomere-induced cell senescence is instrumental in the suppression of cancer, and this proposal is uncontroversial (*258*). Tumorigenic human cells often lose the functionality of key players in senescence induction, such as p53, p21, p27, and ARF, among others (*258*), and one of the hallmarks of malignancy is the ability to overcome replicative senescence by the reactivation of telomerase (reviewed in *252*). Replicative senescence (unless subverted) is an effective barrier to malignant transformation. Therefore, telomere-induced cell senescence has a function outside aging, and this function is adaptive. In mice, the deletion of one p53 allele reduces median survival to 70 weeks and homozygous deletion to 50 weeks, whereas wild type mice have a median life expectancy of around 110 weeks. Human patients with the loss of one allele of p53 have an increased incidence of several cancers from an early age (*259*).

However, there are also examples for which this prediction seems to be patently false. Telomerase activity is high in the somatic tissues of organisms that do not appear to age, such as the rainbow trout and the lobster (*252*). Libertini states (*252*) that the low cancer risk in these

organisms is evidenced by the fact they show negligible senescence. High levels of telomerase activity have also been found in several long-lived bird species, including Leach's storm petrels, again suggesting that high telomerase activity does not correlate with high cancer risk (*260*). The situation in mammals is similar; the longest-lived species within the Rodentia, such as the naked mole rat and the grey squirrel, have high telomerase activity in their somatic tissue (*261*). However, the forced overexpression of telomerase in mice reduces their lifespan due to increased cancer occurrence, but this phenotype can be reversed by the overexpression of p53, suggesting that in organisms with high telomerase activity, other genetic events may have occurred to counteract the pro-cancer effects of high telomerase activity (*262*).

When telomere uncapping occurs, telomeres from different chromosomes begin to fuse, causing genomic instability. This instability disrupts the expression of genes involved in growth control, which ultimately leads to tumorigenesis (*263*). Thus, the idea that telomerase activity removes a barrier to oncogenic risk is only half of the story; it may remove one barrier, but it simultaneously erects another. This fact is not often discussed in the mainstream literature on aging. In fact, tumors are mostly caused by the accumulation of mutations, in general due to DNA damage, which is not biologically unavoidable. Thus, a greater investment in DNA repair mechanisms can produce a much longer-lived organism. Humans, for example, live much longer than mice because, among other differences, they have much more efficient DNA maintenance and repair mechanisms, a trait which is genetically determined and thus evolutionarily malleable (*15*). Embryonic stem cells also have lower mutation frequencies, due to their superior ability to minimize oxidative stress. As soon as cells begin to differentiate, however, these repair mechanisms are downregulated (*264*). This shows that mutations are not inevitable and that organisms are able to cut the risk of cancer in other ways (commented in 2). Oncogene induced CS can co-evolve with DNA damage responses (*265*) as an antitumor mechanism to avoid cancer initiation. However, the senescence-promoting process, when it does not end in actual senescence, can lead to a trail of genetically unstable cells, which potentially can contribute to tumorigenesis (*266*). If this system was selected first and foremost as a cancer defense strategy, it is not clear why selection has not altered this post-secretory phenotype, or at least caused the immune system to efficiently remove such cells from the tissue, as it does for apoptotic cells. However, this argument is not applicable to all cancer types; for example, nevi, as described above, are a case in which senescence is clearly an endogenous anticancer mechanism.

Senescence is, in fact, an intricate process, involving the sequential activation of multiple molecular mechanisms and subjected to selective pressures that have proven necessary for the establishment and maintenance of the phenotype. New questions have been developed by evolutionary biologists together with oncologists: for example, is senescence a tissue-specific endogenous anticancer mechanism? Can cellular senescence promote or repress cancer progression dependent on specific microenvironments? Senescence as an endogenous anticancer mechanism is a mystery of aging with multiple paths remaining to be explored.

10. SUMMARY AND PERSPECTIVES

Cellular senescence is becoming a fundamental concept in tumorigenesis and cancer therapeutics. Whether telomerase-dependent replicative CS, DDR-dependent oncogene induced CS or DDR-independent PTEN-loss induced CS, all "types" of senescence seem to play an important role in preventing different types of cancers. Understanding the molecular mechanisms of senescence activation and, most importantly, escape, will allow the design of therapies aimed at (re)activating senescence in cancer cells. Many small compounds and/or genetic approaches to induce senescence in cancer cells are in development, and these senescence-associated therapies show excellent selectivity and low cytotoxicity for normal tissues. Importantly, the therapeutic potential of senescence induction strongly relies on the irreversibility of this mechanism.

Notably, many traditional anticancer therapies (e.g., alkylating anticancer drugs, ionizing radiation) have been reported to induce senescence in different tumor types, but this mechanism has never been deeply explored. Moreover, the combination of traditional anticancer treatments with senescence-inducing drugs is still in its infancy, which calls for more studies *in vitro* and *in vivo*. Thus, the direct activation or enhancement of senescence induced by classical cancer therapies has a great potential to improve cancer therapeutics.

11. ACKNOWLEDGEMENTS

This work was supported by research grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Grant Number 474117/2010-3), Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior (CAPES), the Programa Institutos Nacionais de Ciência e Tecnologia (INCT de Processos Redox em Biomedicina-REDOXOMA; Grant Number 573530/2008-4) and Pronex-Fapergs/CNPq n°: 10/0044-3. JEV is recipient of a CAPES fellowship and BCF, JFP, GL and DB are recipients of CNPq fellowships.

10. REFERENCES

1. D N Shelton, E Chang, P S Whittier, D Choi, W D Funk: Microarray analysis of replicative senescence. *Curr Biol* 9, 939-945 (1999)

2. K Nishio, A Inoue, S Qiao, H Kondo, A Mimura: Senescence and cytoskeleton: overproduction of vimentin induces senescent-like morphology in human fibroblasts. *Histochem Cell Biol* 116, 321-327 (2001)

3. D J Kurz, S Decary, Y Hong, J D Erusalimsky: Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci* 113, 3613-3622 (2000)

4. R Zhang, W Chen, P D Adams: Molecular dissection of formation of senescence-associated heterochromatin foci. *Mol Cell Biol* 27, 2343-2358 (2007)

5. P D Adams: Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. *Gene* 397, 84-93 (2007)

6. LHayflick: The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 37, 614-36 (1965)

7. W E Wright, J W Shay: Cellular senescence as a tumor-protection mechanism: the essential role of counting. *Curr Opin Genet Dev* 11, 98-103 (2001)

8. J W Shay, W E Wright: Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis* 26, 867-874 (2005)

9. J F Poyatos, A Carnero: Non-neutral role of replicative senescence in tissue homeostasis and tumorigenesis. *J Theor Biol* 230, 333-341 (2004)

10. J Campisi: Cancer and ageing: rival demons? *Nat Rev Cancer* 3, 339-349 (2003)

11. J P de Magalhaes, R G Faragher: Cell divisions and mammalian aging: integrative biology insights from genes that regulate longevity. *Bioessays* 30, 567-578 (2008)

12. K L MacKenzie, S Franco, C May, M Sadelain, M A Moore: Mass cultured human fibroblasts overexpressing hTERT encounter a growth crisis following an extended period of proliferation. *Exp Cell Res* 259, 336-350 (2000)

13. J W Shay, A F Gazdar: Telomerase in the early detection of cancer. *J Clin Pathol* 50, 106-109 (1997)

14. E H Blackburn, C W Greider, E Henderson, M S Lee, J Shampay, D Shippen-Lentz: Recognition and elongation of telomeres by telomerase. *Genome* 31, 553-560 (1989)

15. S E Artandi, R A DePinho: Telomeres and telomerase in cancer. *Carcinogenesis* 31, 9-18 (2010)

16. V G Gorgoulis, T D Halazonetis: Oncogene-induced senescence: the bright and dark side of the response. *Curr Opin Cell Biol* 22, 816-827 (2010)

17. C Chandeck, W J Mooi: Oncogene-induced cellular senescence. *Adv Anat Pathol* 17, 42-48 (2010)

18. S S Moumtzi, M L Roberts, T Joyce, M Evangelidou, L Probert, S Frillingos, T Fotsis, A Pintzas: Gene expression profile associated with oncogenic ras-induced senescence, cell death, and transforming properties in human cells. *Cancer Invest* 28, 563-587 (2010)

19. C J Sarkisian, B A Keister, D B Stairs, R B Boxer, S E Moody, L A Chodosh: Dose-dependent oncogene-induced senescence *in vivo* and its evasion during mammary tumorigenesis. *Nat Cell Biol* 9, 493-505 (2007)

20. L Mo, X Zheng, H Y Huang, E Shapiro, H Lepor, C Cordon-Cardo, T T Sun, X R Wu: Hyperactivation of Ha-ras oncogene, but not Ink4a/Arf deficiency, triggers bladder tumorigenesis. *J Clin Invest* 117, 314-325 (2007)

21. R Di Micco, M Fumagalli, A Cicalese, S Piccinin, P Gasparini, C Luise, C Schurra, M Garre, P G Nuciforo, A Bensimon, R Maestro, P G Pelicci, F d'Adda di Fagagna: Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature* 444, 638-642 (2006)

22. P Yaswen, J Campisi: Oncogene-induced senescence pathways weave an intricate tapestry. *Cell* 128, 233-234 (2007)

23. F Rodier, J P Coppe, C K Patil, W A Hoeijmakers, D P Munoz, S R Raza, A Freund, E Campeau, A R Davalos, J Campisi: Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol* 11, 973-979 (2009)

24. C Nardella, J G Clohessy, A Alimonti, P P Pandolfi: Pro-senescence therapy for cancer treatment. *Nat Rev Cancer* 11, 503-511 (2011)

25. Z Chen, L C Trotman, D Shaffer, H K Lin, Z A Dotan, M Niki, J A Koutcher, H I Scher, T Ludwig, W Gerald, C Cordon-Cardo, P P Pandolfi: Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature* 436, 725-730 (2005)

26. J Blando, M Portis, F Benavides, A Alexander, G Mills, B Dave, C J Conti, J Kim, C L Walker: PTEN deficiency is fully penetrant for prostate adenocarcinoma in C57BL/6 mice via mTOR-dependent growth. *Am J Pathol* 174, 1869-1879 (2009)

27. Z Chen, A Carracedo, H K Lin, J A Koutcher, N Behrendt, A Egia, A Alimonti, B S Carver, W Gerald, J Teruya-Feldstein, M Loda, P P Pandolfi: Differential p53-independent outcomes of p19(Arf) loss in oncogenesis. *Sci Signal* 2, ra44 (2009)

28. M S Song, A Carracedo, L Salmena, S J Song, A Egia, M Malumbres, P P Pandolfi: Nuclear PTEN regulates the APC-CDH1 tumor-suppressive complex in a phosphatase-independent manner. *Cell* 144, 187-199 (2011)

29. P Y Lin, S P Fosmire, S H Park, J Y Park, S Baksh, J F Modiano, R H Weiss: Attenuation of PTEN increases p21 stability and cytosolic localization in kidney cancer cells: a potential mechanism of apoptosis resistance. *Mol Cancer* 6, 16 (2007)

30. P Boffetta, F Nyberg: Contribution of environmental factors to cancer risk. *Br Med Bull*, 68, 71-94 (2003)

31. B C Dash, W S El-Deiry: Cell cycle checkpoint control mechanisms that can be disrupted in cancer. *Methods Mol Biol* 280, 99-161 (2004)

32. C J Sherr: Cell cycle control and cancer. *Harvey Lect* 96, 73-92 (2000)

33. C J Sherr, R A DePinho: Cellular senescence: mitotic clock or culture shock? *Cell* 102, 407-410 (2000)

34. D A Alcorta, Y Xiong, D Phelps, G Hannon, D Beach, J C Barrett: Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc Natl Acad Sci U S A* 93, 13742-13747 (1996)

35. F A Mallette, S Goumard, M F Gaumont-Leclerc, O Moieeva, G Ferbeyre: Human fibroblasts require the Rb family of tumor suppressors, but not p53, for PML-induced senescence. *Oncogene* 23, 91-99 (2004) .

36. D Hanahan, R A Weinberg: Hallmarks of cancer: the next generation. *Cell* 144, 646-674 (2011)

37. K Egawa, Y Kurihara, T Ito, M Matsumoto, K Nose: Induction of p16INK4a transcription and of cellular senescence by aclacinomycin-derivatives and cardiac glycosides. *Biol Pharm Bull* 25, 461-465 (2002)

38. J Ewald, J Desotelle, N Almassi, D Jarrard: Drug-induced senescence bystander proliferation in prostate cancer cells *in vitro* and *in vivo*. *Br J Cancer* 98, 1244-1249 (2008)

39. J Bartkova, N Rezaei, M Liontos, P Karakaidos, D Kletsas, N Issaeva, L V Vassiliou, E Kolettas, K Niforou, V C Zoumpourlis, M Takaoka, H Nakagawa, F Tort, K Fugger, F Johansson, M Sehested, C L Andersen, L Dyrskjot, T Orntoft, J Lukas, C Kittas, T Helleday, T D Halazonetis, J Bartek, V G Gorgoulis: Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 444, 633-637 (2006)

40. R Di Micco G Sulli, M Dobreva, M Liontos, O A Botrugno, G Gargiulo, R dal Zuffo, V Matti, G d'Ario, E Montani, C Mercurio, W C Hahn, V Gorgoulis, S Minucci, F d'Adda di Fagagna: Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nat Cell Biol*, 13, 292-302 (2011)

41. F A Mallette, M F Gaumont-Leclerc, G Ferbeyre: The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev* 21, 43-48 (2007)

42. S H Kim Sh, P Kaminker, J Campisi: Telomeres, aging and cancer: in search of a happy ending. *Oncogene* 21, 503-511 (2002)

43. S W Chan, E H Blackburn: New ways not to make ends meet: telomerase, DNA damage proteins and heterochromatin. *Oncogene* 21, 553-563 (2002)

44. K A Mather, A F Jorm, R A Parslow, H Christensen: Is telomere length a biomarker of aging? A review. *J Gerontol A Biol Sci Med Sci* 66, 202-213 (2011)

45. Q Yang: Cellular senescence, telomere recombination and maintenance. *Cytogenet Genome Res* 122, 211-218 (2008)

46. J Meyne, R L Ratliff, R K Moyzis: Conservation of the human telomere sequence (TTAGGG)n among vertebrates. *Proc Natl Acad Sci U S A* 86, 7049-7053 (1989)

47. R K Moyzis, J M Buckingham, L S Cram, M Dani, L L Deaven, M D Jones, J Meyne, R L Ratliff, J R Wu: A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes. *Proc Natl Acad Sci U S A* 85, 6622-6626 (1988)

48. E H Blackburn: Walking the walk from genes through telomere maintenance to cancer risk. *Cancer Prev Res* 4, 473-475 (2011)

49. S M Bailey, J P Murnane: Telomeres, chromosome instability and cancer. *Nucleic Acids Res* 34, 2408-2417 (2006)

50. M Chakhparonian, R J Wellinger: Telomere maintenance and DNA replication: how closely are these two connected? *Trends Genet* 19, 439-446 (2003)

51. M G Butler, J Tilburt, A DeVries, B Muralidhar, G Aue, L Hedges, J Atkinson, H Schwartz: Comparison of chromosome telomere integrity in multiple tissues from subjects at different ages. *Cancer Genet Cytogenet* 105, 138-144 (1998)

52. E S de Pauw, H Roelofs, A Zwinderman, J C van Houwelingen, W E Fibbe, P de Knijff, P L Pearson, H J Tanke: Studying the biological and technical sources of variation in telomere length of individual chromosomes. *Cytometry A* 65, 35-39 (2005)

53. T Suda, A Fujiyama, M Takimoto, M Igarashi, T Kuroiwa, N Waguri, H Kawai, Y Mita, Y Aoyagi: Interchromosomal telomere length variation. *Biochem Biophys Res Commun* 291, 210-214 (2002)

54. Y Deng, S S Chan, S Chang: Telomere dysfunction and tumour suppression: the senescence connection. *Nat Rev Cancer* 8, 450-458 (2008)

55. S E Artandi, R A DePinho: A critical role for telomeres in suppressing and facilitating carcinogenesis. *Curr Opin Genet Dev* 10, 39-46 (2000)

56. N Ohtani, D J Mann, E Hara: Cellular senescence: its role in tumor suppression and aging. *Cancer Sci* 100, 792-797 (2009)

57. L E Donate, M A Blasco: Telomeres in cancer and ageing. *Philos Trans R Soc Lond B Biol Sci* 366, 76-84 (2011)

58. S T Yu, L Chen, H J Wang, X D Tang, D C Fang, S M Yang: hTERT promotes the invasion of telomerase-

negative tumor cells *in vitro*. *Int J Oncol* 35, 329-336 (2009)

59. C M Heaphy, A K Meeker: The potential utility of telomere-related markers for cancer diagnosis. *J Cell Mol Med* 15, 1227-1238 (2011)

60. P Martinez, M A Blasco: Role of shelterin in cancer and aging. *Aging Cell* 9, 653-666 (2010)

61. D M Feldser, C W Greider: Short telomeres limit tumor progression *in vivo* by inducing senescence. *Cancer Cell* 11, 461-469 (2007)

62. W Cosme-Blanco, M F Shen, A J Lazar, S Pathak, G Lozano, A S Multani, S Chang: Telomere dysfunction suppresses spontaneous tumorigenesis *in vivo* by initiating p53-dependent cellular senescence. *EMBO Rep* 8, 497-503 (2007)

63. S E Artandi, R A DePinho: Telomeres and telomerase in cancer. *Carcinogenesis* 31, 9-18 (2010)

64. P Willeit, J Willeit, A Mayr, S Weger, F Oberhollenzer, A Brandstatter, F Kronenberg, S Kiechl: Telomere length and risk of incident cancer and cancer mortality. *JAMA* 304, 69-75 (2010)

65. U Svenson, B Ljungberg, G Roos: Telomere length in peripheral blood predicts survival in clear cell renal cell carcinoma. *Cancer Res* 69, 2896-2901 (2009)

66. L Mirabello, K Yu, P Kraft, I De Vivo, D J Hunter, J Prescott, J Y Wong, N Chatterjee, R B Hayes, S A Savage: The association of telomere length and genetic variation in telomere biology genes. *Hum Mutat* 31, 1050-1058 (2010)

67. E Rampazzo, R Bertorelle, L Serra, L Terrin, C Candiotto, S Pucciarelli, P Del Bianco, D Nitti, A De Rossi: Relationship between telomere shortening, genetic instability, and site of tumour origin in colorectal cancers. *Br J Cancer* 102, 1300-1305 (2010)

68. S A Stewart, I Ben-Porath, V J Carey, B F O'Connor, W C Hahn, R A Weinberg: Erosion of the telomeric single-strand overhang at replicative senescence. *Nat Genet* 33, 492-496 (2003)

69. Y Xu, T Ishizuka, K Kurabayashi, M Komiyama: Consecutive formation of G-quadruplexes in human telomeric-overhang DNA: a protective capping structure for telomere ends. *Angew Chem Int Ed Engl* 48, 7833-7836 (2009)

70. C W Greider: Telomeres do D-loop-T-loop. *Cell* 97, 419-422 (1999)

71. S Mattarocci, E D'Ambrosio, L Tafaro, V Somma, G Zannino, V Marigliano, F Ascenzioni, G Cimino-Reale: Erosion of telomeric 3'-overhangs in white blood cells of aged subjects with high frequency of very short telomeres. *Mech Ageing Dev* 132, 27-32 (2011)

72. D Rhodes, R Giraldo: Telomere structure and function. *Curr Opin Struct Biol* 5, 311-322 (1995)

73. F W Smith, J Feigon: Strand orientation in the DNA quadruplex formed from the *Oxytricha* telomere repeat oligonucleotide d(G4T4G4) in solution. *Biochemistry* 32, 8682-8692 (1993)

74. X Tian, B Chen, X Liu: Telomere and telomerase as targets for cancer therapy. *Appl Biochem Biotechnol* 160, 1460-1472 (2010)

75. M Folini, P Gandellini, N Zaffaroni: Targeting the telosome: therapeutic implications. *Biochim Biophys Acta* 1792, 309-316 (2009)

76. Q Yang, Y L Zheng, C C Harris: POT1 and TRF2 cooperate to maintain telomeric integrity. *Mol Cell Biol* 25, 1070-1080 (2005)

77. P Boukamp, N Mirancea: Telomeres rather than telomerase a key target for anti-cancer therapy? *Exp Dermatol* 16, 71-79 (2007)

78. S Gryaznov, A Asai, Y Oshima, Y Yamamoto, K Pongracz, R Pruzan, E Wunder, M Piatyszek, S Li, A Chin, C Harley, S Akinaga, Y Yamashita: Oligonucleotide N3'→ P5' thio-phosphoramidate telomerase template antagonists as potential anticancer agents. *Nucleosides Nucleotides Nucleic Acids* 22, 577-581 (2003)

79. J W Shay, W N Keith: Targeting telomerase for cancer therapeutics. *Br J Cancer* 98, 677-683 (2008)

80. L Kelland: Targeting the limitless replicative potential of cancer: the telomerase/telomere pathway. *Clin Cancer Res* 13, 4960-4963 (2007)

81. Z G Dikmen, G C Gellert, S Jackson, S Gryaznov, R Tressler, P Dogan, W E Wright, J W Shay: *In vivo* inhibition of lung cancer by GRN163L: a novel human telomerase inhibitor. *Cancer Res* 65, 7866-7873 (2005)

82. S R Jackson, C H Zhu, V Paulson, L Watkins, Z G Dikmen, S M Gryaznov, W E Wright, J W Shay: Antiadhesive effects of GRN163L--an oligonucleotide N3'→P5' thio-phosphoramidate targeting telomerase. *Cancer Res* 67, 1121-1129 (2007)

83. C B Harley: Telomerase and cancer therapeutics. *Nat Rev Cancer* 8, 167-179 (2008)

84. G C Gellert, Z G Dikmen, W E Wright, S Gryaznov, J W Shay: Effects of a novel telomerase inhibitor, GRN163L, in human breast cancer. *Breast Cancer Res Treat* 96, 73-81 (2006)

85. A E Hochreiter, H Xiao, E M Goldblatt, S M Gryaznov, K D Miller, S Badve, G W Sledge, B S Herbert: Telomerase template antagonist GRN163L disrupts telomere maintenance, tumor growth, and metastasis of breast cancer. *Clin Cancer Res* 12, 3184-3192 (2006)

86. J Gomez-Millan, E M Goldblatt, S M Gryaznov, M S Mendonca, B S Herbert: Specific telomere dysfunction induced by GRN163L increases radiation sensitivity in breast cancer cells. *Int J Radiat Oncol Biol Phys* 67, 897-905 (2007)

87. M W Djojosubroto, A C Chin, N Go, S Schaetzlein, M P Manns, S Gryaznov, C B Harley, K L Rudolph: Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human hepatoma. *Hepatology* 42, 1127-1136 (2005)

88. T Ozawa, S M Gryaznov, L J Hu, K Pongracz, R A Santos, A W Bollen, K R Lamborn, D F Deen: Antitumor effects of specific telomerase inhibitor GRN163 in human glioblastoma xenografts. *Neuro Oncol*, 6, 218-226 (2004)

89. M Akiyama, T Hideshima, M A Shammas, T Hayashi, M Hamasaki, Y T Tai, P Richardson, S Gryaznov, N C Munshi, K C Anderson: Effects of oligonucleotide N3'-->P5' thio-phosphoramidate (GRN163) targeting telomerase RNA in human multiple myeloma cells. *Cancer Res* 63, 6187-6194 (2003)

90. S K Brennan, Q Wang, R Tressler, C Harley, N Go, E Bassett, C A Huff, R J Jones, W Matsui: Telomerase inhibition targets clonogenic multiple myeloma cells through telomere length-dependent and independent mechanisms. *PLoS One* 5, e12487 (2010)

91. M Dean, T Fojo, S Bates: Tumour stem cells and drug resistance. *Nat Rev Cancer* 5, 275-284 (2005)

92. V Brower: Telomerase-based therapies emerging slowly. *J Natl Cancer Inst* 102, 520-521 (2010)

93. P E Nielsen, M Egholm, R H Berg, O Buchardt: Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* 254, 1497-1500 (1991)

94. J C Norton, M A Piatyszek, W E Wright, J W Shay, D R Corey: Inhibition of human telomerase activity by peptide nucleic acids. *Nat Biotechnol* 14, 615-619 (1996)

95. M A Shammas, C G Simmons, D R Corey, R J Shmookler Reis: Telomerase inhibition by peptide nucleic acids reverses 'immortality' of transformed human cells. *Oncogene* 18, 6191-6200 (1999)

96. S E Hamilton, C G Simmons, I S Kathiriya, D R Corey: Cellular delivery of peptide nucleic acids and inhibition of human telomerase. *Chem Biol* 6, 343-351 (1999)

97. I Tarkanyi, J Aradi: Pharmacological intervention strategies for affecting telomerase activity: future prospects to treat cancer and degenerative disease. *Biochimie* 90, 156-172 (2008)

98. H Qi, C P Lin, X Fu, L M Wood, A A Liu, Y C Tsai, Y Chen, C M Barbieri, D S Pilch, L F Liu: G-quadruplexes induce apoptosis in tumor cells. *Cancer Res* 66, 11808-11816 (2006)

99. F Riou, L Guittat, P Mailliet, A Laoui, E Renou, O Petitgenet, F Megnin-Chanet, C Helene, J L Mergny: Cell senescence and telomere shortening induced by a new series of specific G-quadruplex DNA ligands. *Proc Natl Acad Sci U S A* 99, 2672-2677 (2002)

100. T Tauchi, K Shin-Ya, G Sashida, M Sumi, A Nakajima, T Shimamoto, J H Ohyashiki, K Ohyashiki: Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways. *Oncogene* 22, 5338-5347 (2003)

101. C M Incles, C M Schultes, H Kempski, H Koehler, L R Kelland, S Neidle: A G-quadruplex telomere targeting agent produces p16-associated senescence and chromosomal fusions in human prostate cancer cells. *Mol Cancer Ther* 3, 1201-1206 (2004)

102. C Leonetti, S Amodei, C D'Angelo, A Rizzo, B Benassi, A Antonelli, R Elli, M F Stevens, M D'Incalci, G Zupi, A Biroccio: Biological activity of the G-quadruplex ligand RHPS4 (3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate) is associated with telomere capping alteration. *Mol Pharmacol* 66, 1138-1146 (2004)

103. N Temime-Smaali, L Guittat, A Sidibe, K Shin-ya, C Trentesaux, J F Riou: The G-quadruplex ligand telomestatin impairs binding of topoisomerase IIIalpha to G-quadruplex-forming oligonucleotides and uncaps telomeres in ALT cells. *PLoS One* 4, e6919 (2009)

104. S A Stewart, W C Hahn, B F O'Connor, E N Banner, A S Lundberg, P Modha, H Mizuno, M W Brooks, M Fleming, D B Zimonjic, N C Popescu, R A Weinberg: Telomerase contributes to tumorigenesis by a telomere length-independent mechanism. *Proc Natl Acad Sci U S A* 99, 12606-12611 (2002)

105. S M Gowan, J R Harrison, L Patterson, M Valenti, M A Read, S Neidle, L R Kelland: A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting *in vitro* and *in vivo* antitumor activity. *Mol Pharmacol* 61, 1154-1162 (2002)

106. H Tahara, K Shin-Ya, H Seimiya, H Yamada, T Tsuruo, T Ide: G-Quadruplex stabilization by telomestatin induces TRF2 protein dissociation from telomeres and anaphase bridge formation accompanied by loss of the 3' telomeric overhang in cancer cells. *Oncogene* 25, 1955-1966 (2006)

107. H Cheng, Z Wu, J Zheng, G Lu, J Yan, M Liu, D Huang, J Lin: Inhibition on telomerase activity and cytotoxic effects by cisplatin in cultured human choroidal melanoma cells. *Yan Ke Xue Bao* 19, 54-59 (2003)

108. D Sadava, E Whitlock, S E Kane: The green tea polyphenol, epigallocatechin-3-gallate inhibits telomerase

and induces apoptosis in drug-resistant lung cancer cells. *Biochem Biophys Res Commun* 360, 233-237 (2007)

109. S Hayakawa, K Saeki, M Sazuka, Y Suzuki, Y Shoji, T Ohta, K Kaji, A You, M Isemura: Apoptosis induction by epigallocatechin gallate involves its binding to Fas. *Biochem Biophys Res Commun* 285, 1102-1106 (2001)

110. Q Meng, C N Velalar, R Ruan: Effects of epigallocatechin-3-gallate on mitochondrial integrity and antioxidative enzyme activity in the aging process of human fibroblast. *Free Radic Biol Med* 44, 1032-1041 (2008)

111. N E Sharpless, R A DePinho: Telomeres, stem cells, senescence, and cancer. *J Clin Invest* 113, 160-168 (2004)

112. Guittat, P Alberti, D Gomez, A De Cian, G Pennarun, T Lemarteleur, C Belmokhtar, R Paterski, H Morjani, C Trentesaux, E Mandine, F Boussin, P Mailliet, L Lacroix, J F Riou, J L Mergny: Targeting human telomerase for cancer therapeutics. *Cytotechnology* 45, 75-90 (2004)

113. E Pascolo, C Wenz, J Lingner, N Hauel, H Priepke, I Kauffmann, P Garin-Chesa, W J Rettig, K Damm, A Schnapp: Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. *J Biol Chem* 277, 15566-15572 (2002)

114. G Lenz: The RNA interference revolution. *Braz J Med Biol Res* 38, 1749-1757 (2005)

115. X Qian, J Cheng, A Chen, Y Wang, Y Tao, J Cao, Z Feng: Long-term effects of short hairpin RNA-targeted human telomerase reverse transcriptase on suppression of SGC-7901 cell proliferation by inhibition of telomerase activity. *Oncol Rep* 19, 575-581 (2008)

116. A Shammas, H Koley, R B Batchu, R C Bertheau, A Protopopov, N C Munshi, R K Goyal: Telomerase inhibition by siRNA causes senescence and apoptosis in Barrett's adenocarcinoma cells: mechanism and therapeutic potential. *Mol Cancer* 4, 24 (2005)

117. P H Zhang, L Zou, Z G Tu: RNAi-hTERT inhibition hepatocellular carcinoma cell proliferation via decreasing telomerase activity. *J Surg Res* 131, 143-149 (2006)

118. X Dong, A Liu, C Zer, J Feng, Z Zhen, M Yang, L Zhong: siRNA inhibition of telomerase enhances the anti-cancer effect of doxorubicin in breast cancer cells. *BMC Cancer* 9, 133 (2009)

119. L Zou, P Zhang, C Luo, Z Tu: shRNA-targeted hTERT suppress cell proliferation of bladder cancer by inhibiting telomerase activity. *Cancer Chemother Pharmacol* 57, 328-334 (2006)

120. X Liu, H Huang, J Wang, C Wang, M Wang, B Zhang, C Pan: Dendrimers-delivered short hairpin RNA targeting hTERT inhibits oral cancer cell growth *in vitro* and *in vivo*. *Biochem Pharmacol* 82, 17-23 (2011)

121. P Spallarossa, P Altieri, C Aloi, S Garibaldi, C Barisione, G Ghigliotti, G Fugazza, A Barsotti, C Brunelli: Doxorubicin induces senescence or apoptosis in rat neonatal cardiomyocytes by regulating the expression levels of the telomere binding factors 1 and 2. *Am J Physiol Heart Circ Physiol* 297, H2169-H2181 (2009)

122. A Sfeir, S T Kosiyatrakul, D Hockemeyer, S L MacRae, J Karlseder, C L Schildkraut, T de Lange: Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. *Cell* 138, 90-103 (2009)

123. P Zhang, K Furukawa, P L Opresko, X Xu, V A Bohr, M P Mattson: TRF2 dysfunction elicits DNA damage responses associated with senescence in proliferating neural cells and differentiation of neurons. *J Neurochem* 97, 567-581 (2006)

124. L Wu, A S Multani, H He, W Cosme-Blanco, Y Deng, J M Deng, O Bachilo, S Pathak, H Tahara, S M Bailey, R R Behringer, S Chang: Pot1 deficiency initiates DNA damage checkpoint activation and aberrant homologous recombination at telomeres. *Cell* 126, 49-62 (2006)

125. A M Burger, F Dai, C M Schultes, A P Reszka, M J Moore, J A Double, S Neidle: The G-quadruplex-interactive molecule BRACO-19 inhibits tumor growth, consistent with telomere targeting and interference with telomerase function. *Cancer Res* 65, 1489-1496 (2005)

126. P Phatak, J C Cookson, F Dai, V Smith, R B Gartenhaus, M F Stevens, A M Burger: Telomere uncapping by the G-quadruplex ligand RHPS4 inhibits clonogenic tumour cell growth *in vitro* and *in vivo* consistent with a cancer stem cell targeting mechanism. *Br J Cancer* 96, 1223-1233 (2007)

127. W J Zhou, R Deng, X Y Zhang, G K Feng, L Q Gu, X F Zhu: G-quadruplex ligand SYUIQ-5 induces autophagy by telomere damage and TRF2 delocalization in cancer cells. *Mol Cancer Ther* 8, 3203-3213 (2009)

128. C O Marian, S K Cho, B M McEllin, E A Maher, K J Hatanpaa, C J Madden, B E Mickey, W E Wright, J W Shay, R M Bachoo: The telomerase antagonist, imetelstat, efficiently targets glioblastoma tumor-initiating cells leading to decreased proliferation and tumor growth. *Clin Cancer Res* 16, 154-163 (2010)

129. I Joseph, R Tressler, E Bassett, C Harley, C M Buseman, P Pattamatta, W E Wright, J W Shay, N F Go: The telomerase inhibitor imetelstat depletes cancer stem cells in breast and pancreatic cancer cell lines. *Cancer Res* 70, 9494-9504 (2010)

130. W Djojosubroto, A C Chin, N Go, S Schaetzlein, M P Manns, S Gryaznov, C B Harley, K L Rudolph: Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human hepatoma. *Hepatology* 42, 1127-1136 (2005)

131. S Mueller, U Hartmann, F Mayer, S Balabanov, J T Hartmann, T H Brummendorf, C Bokemeyer: Targeting telomerase activity by BIBR1532 as a therapeutic approach in germ cell tumors. *Invest New Drugs* 25, 519-524 (2007)

132. S M Chen, Z Z Tao, Q Q Hua, D Liu, H M Chi, Q Cai: Inhibition of human telomerase reverse transcriptase in hep-2 cells using short hairpin RNA expression vectors. *Arch Otolaryngol Head Neck Surg* 132, 200-205 (2006)

133. L Teng, M C Specht, C B Barden, T J Fahey, 3rd: Antisense hTERT inhibits thyroid cancer cell growth. *J Clin Endocrinol Metab* 88, 1362-1366 (2003)

134. L Chen, Y Hu, W P Shuai, H L Chen, W Q Liang, J Q Gao: Telomerase-targeting antisense oligonucleotides carried by polycation liposomes enhance the growth inhibition effect on tumor cells. *J Biomed Mater Res B Appl Biomater* 89B, 362-368 (2009)

135. P Paulasova, F Pellestor: The peptide nucleic acids (PNAs): a new generation of probes for genetic and cytogenetic analyses. *Ann Genet* 47, 349-358 (2004)

136. V Urquidi, D Tarin, S Goodison: Role of telomerase in cell senescence and oncogenesis. *Annu Rev Med* 51, 65-79 (2000)

137. J D Henson, A A Neumann, T R Yeager, R R Reddel: Alternative lengthening of telomeres in mammalian cells. *Oncogene* 21, 598-610 (2002)

138. K L Cann, G Dellaire: Heterochromatin and the DNA damage response: the need to relax. *Biochem Cell Biol* 89, 45-60 (2011)

139. S A Martin, M Hewish, C J Lord, A Ashworth: Genomic instability and the selection of treatments for cancer. *J Pathol* 220, 281-289 (2010)

140. M Lebel, N C de Souza-Pinto, V A Bohr: Metabolism, genomics, and DNA repair in the mouse aging liver. *Curr Gerontol Geriatr Res* 2011, 859415 (2011)

141. J McKinnon, K W Caldecott: DNA strand break repair and human genetic disease. *Annu Rev Genomics Hum Genet* 8, 37-55 (2007)

142. J Cadet, T Douki, J L Ravanat: Oxidatively generated base damage to cellular DNA *Free Radic Biol Med* 49, 9-21 (2010)

143. A Guainazzi, O D Scharer: Using synthetic DNA interstrand crosslinks to elucidate repair pathways and identify new therapeutic targets for cancer chemotherapy. *Cell Mol Life Sci* 67, 3683-3697 (2010)

144. T B Kryston, A B Georgiev, P Pissis, A G Georgakilas: Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat Res* 711, 193-201 (2011)

145. E G Seviour, S Y Lin: The DNA damage response: Balancing the scale between cancer and ageing. *Aging* 2, 900-907 (2010)

146. C Lengauer, K W Kinzler, B Vogelstein: Genetic instabilities in human cancers. *Nature* 396, 643-649 (1998)

147. J P Murnane, L Sabatier: Chromosome rearrangements resulting from telomere dysfunction and their role in cancer. *Bioessays* 26, 1164-1174 (2004)

148. P Hasty: Is NHEJ a tumor suppressor or an aging suppressor? *Cell Cycle* 7, 1139-1145 (2008)

149. F Al-Ejeh, R Kumar, A Wiegmans, S R Lakhani, M P Brown, K K Khanna: Harnessing the complexity of DNA-damage response pathways to improve cancer treatment outcomes. *Oncogene* 29, 6085-6098 (2010)

150. J W Harper, S J Elledge: The DNA damage response: ten years after. *Mol Cell* 28, 739-745 (2007)

151. J Campisi: Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 120, 513-522 (2005)

152. A Beliveau, P Yaswen: Soothing the watchman: telomerase reduces the p53-dependent cellular stress response. *Cell Cycle* 6, 1284-1287 (2007)

153. H Vaziri: Critical telomere shortening regulated by the ataxia-telangiectasia gene acts as a DNA damage signal leading to activation of p53 protein and limited life-span of human diploid fibroblasts. A review. *Biochemistry*, 62, 1306-1310 (1997)

154. E L Denchi, T de Lange: Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. *Nature* 448, 1068-1071 (2007)

155. D Hockemeyer, J P Daniels, H Takai, T de Lange: Recent expansion of the telomeric complex in rodents: Two distinct POT1 proteins protect mouse telomeres. *Cell* 126, 63-77 (2006)

156. T De Lange: Telomere-related genome instability in cancer. *Cold Spring Harb Symp Quant Biol* 70, 197-204 (2005)

157. S Maser, R A DePinho: Connecting chromosomes, crisis, and cancer. *Science* 297, 565-569 (2002)

158. T Davoli, E L Denchi, T de Lange: Persistent telomere damage induces bypass of mitosis and tetraploidy. *Cell* 141, 81-93 (2010)

159. E Michishita, K Nakabayashi, H Ogino, T Suzuki, M Fujii, D Ayusawa: DNA topoisomerase inhibitors induce reversible senescence in normal human fibroblasts. *Biochem Biophys Res Commun* 253, 667-671 (1998)

160. D A Gewirtz, S E Holt, L W Elmore: Accelerated senescence: an emerging role in tumor cell response to chemotherapy and radiation. *Biochem Pharmacol* 76, 947-957 (2008)

161. C C Chiu, C H Li, M W Ung, T S Fuh, W L Chen, K Fang: Etoposide (VP-16) elicits apoptosis following prolonged G2-M cell arrest in p53-mutated human non-

small cell lung cancer cells. *Cancer Lett* 223, 249-258 (2005)

162. Z Han, W Wei, S Dunaway, J W Darnowski, P Calabresi, J Sedivy, E A Hendrickson, K V Balan, P Pantazis, J H Wyche: Role of p21 in apoptosis and senescence of human colon cancer cells treated with camptothecin. *J Biol Chem* 277, 17154-17160 (2002)

163. R S Roberson, S J Kussick, E Vallieres, S Y Chen, D Y Wu: Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. *Cancer Res* 65, 2795-2803 (2005)

164. W Zhao, Z X Lin, Z Q Zhang: Cisplatin-induced premature senescence with concomitant reduction of gap junctions in human fibroblasts. *Cell Res* 14, 60-66 (2004)

165. H Cheng, Z Y Wu, T Xia, Q Y Hu, H F Hong, Q Y Mai, J L Zheng: Effects of cisplatin on telomerase activity of human choroidal melanoma cells. *Zhonghua Yan Ke Za Zhi* 44, 705-710 (2008)

166. Vernier, V Bourdeau, M F Gaumont-Leclerc, O Moiseeva, V Begin, F Saad, A M Mes-Masson, G Ferbeyre: Regulation of E2Fs and senescence by PML nuclear bodies. *Genes Dev* 25, 41-50 (2011)

167. Z Novakova, S Hubackova, M Kosar, L Janderova-Rossmeislova, J Dobrovolna, P Vasicova, M Vancurova, Z Horejsi, P Hozak, J Bartek, Z Hodny: Cytokine expression and signaling in drug-induced cellular senescence. *Oncogene* 29, 273-284 (2010)

168. Z Hodny, S Hubackova, J Bartek: Cytokines shape chemotherapy-induced and 'bystander' senescence. *Aging*, 2, 375-376 (2010)

169. S Hubackova, Z Novakova, K Krejcikova, M Kosar, J Dobrovolna, P Duskova, H Hanzlikova, M Vancurova, P Barath, J Bartek, Z Hodny: Regulation of the PML tumor suppressor in drug-induced senescence of human normal and cancer cells by JAK/STAT-mediated signaling. *Cell Cycle* 9, 3085-3099 (2010)

170. F Mbeunkui, D J Johann, Jr.: Cancer and the tumor microenvironment: a review of an essential relationship. *Cancer Chemother Pharmacol* 63, 571-582 (2009)

171. J B Konopka, S M Watanabe, J W Singer, S J Collins, O N Witte: Cell lines and clinical isolates derived from Ph1-positive chronic myelogenous leukemia patients express c-abl proteins with a common structural alteration. *Proc Natl Acad Sci U S A* 82, 1810-1814 (1985)

172. C M Croce: Oncogenes and cancer. *N Engl J Med* 358, 502-511 (2008)

173. Y Tsujimoto, J Gorham, J Cossman, E Jaffe, C M Croce: The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. *Science* 229, 1390-1393 (1985)

174. L R Finger, R C Harvey, R C Moore, L C Showe, C M Croce: A common mechanism of chromosomal translocation in T- and B-cell neoplasia. *Science* 234, 982-985 (1986)

175. R F Newbold, R W Overell: Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. *Nature* 304, 648-651 (1983)

176. M Elgendy, C Sheridan, G Brumatti, S J Martin: Oncogenic ras-induced expression of Noxa and Beclin-1 rootes autophagic cell death and limits clonogenic survival. *Mol Cell* 42, 23-35 (2011)

177. D X Mason, T J Jackson, A W Lin: Molecular signature of oncogenic ras-induced senescence. *Oncogene* 23, 9238-9246 (2004)

178. C Michaloglou, L C Vredeveld, M S Soengas, C Denoyelle, T Kuilman, C M van der Horst, D M Majoor, J W Shay, W J Mooi, D S Peeper: BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* 436, 720-724 (2005)

179. L A Carragher, K R Snell, S M Giblett, V S Aldridge, B Patel, S J Cook, D J Winton, R Marais, C A Pritchard: V600EBraf induces gastrointestinal crypt senescence and promotes tumour progression through enhanced CpG methylation of p16INK4a. *EMBO Mol Med* 2, 458-471 (2010)

180. D Zhuang, S Mannava, V Grachtchouk, W H Tang, S Patil, J A Wawrzyniak, A E Berman, T J Giordano, E V Prochownik, M S Soengas, M A Nikiforov: C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. *Oncogene* 27, 6623-6634 (2008)

181. J van Riggelen, D W Felsher: Myc and a Cdk2 senescence switch. *Nat Cell Biol* 12, 7-9 (2010)

182. L S Steelman, W H Chappell, S L Abrams, R C Kempf, J Long, P Laidler, S Mijatovic, D Maksimovic-Ivanic, F Stivala, M C Mazzarino, M Donia, P Fagone, G Malaponte, F Nicoletti, M Libra, M Milella, A Tafuri, A Bonati, J Basecke, L Cocco, C Evangelisti, A M Martelli, G Montalto, M Cervello, J A McCubrey: Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. *Aging* 3, 192-222 (2011)

183. T. Minamino, H Miyauchi, K Tateno, T Kunieda, I Komuro: Akt-induced cellular senescence: implication for human disease. *Cell Cycle* 3, 449-451 (2004)

184. T Eilon, I Barash: Forced activation of Stat5 subjects mammary epithelial cells to DNA damage and preferential induction of the cellular response mechanism during proliferation. *J Cell Physiol* 226, 616-26 (2011)

185. G P Dimri, K Itahana, M Acosta, J Campisi: Regulation of a senescence checkpoint response by the

E2F1 transcription factor and p14(ARF) tumor suppressor. *Mol Cell Biol* 20, 273-285 (2000)

186. E Lazzerini Denchi, C Attwooll, D Pasini, K Helin: Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol Cell Biol* 25, 2660-2672 (2005)

187. A Kilbey, A Terry, E R Cameron, J C Neil: Oncogene-induced senescence: an essential role for Runx. *Cell Cycle* 7, 2333-2340 (2008)

188. L R Borlado, J Mendez: CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. *Carcinogenesis* 29, 237-243 (2008)

189. Rillalonga-Planells, L Coll-Mulet, F Martinez-Soler, E Castano, J J Acebes, P Gimenez-Bonafe, J Gil, A Tortosa: Activation of p53 by nutlin-3a induces apoptosis and cellular senescence in human glioblastoma multiforme. *PLoS One* 6, e18588 (2011)

190. A Takahashi, N Ohtani, E Hara: Irreversibility of cellular senescence: dual roles of p16INK4a/Rb-pathway in cell cycle control. *Cell Div* 2, 10 (2007)

191. A Shamma, Y Takegami, T Miki, S Kitajima, M Noda, T Obara, T Okamoto and C Takahashi: Rb Regulates DNA damage response and cellular senescence through E2F-dependent suppression of N-ras isoprenylation. *Cancer Cell* 15, 255-269 (2009)

192. A Schmitt, J S Fridman, M Yang, S Lee, E Baranov, R M Hoffman, S W Lowe: A senescence program controlled by p53 and p16INK4a contributes to the outcome of cancer therapy. *Cell* 109, 335-346 (2002)

193. K Vijayachandra, W Higgins, J Lee, A Glick: Induction of p16ink4a and p19ARF by TGFbeta1 contributes to growth arrest and senescence response in mouse keratinocytes. *Mol Carcinog* 48, 181-186 (2009)

194. S Kagawa, T Fujiwara, Y Kadowaki, T Fukazawa, R Sok-Joo, J A Roth, N Tanaka: Overexpression of the p21 sdi1 gene induces senescence-like state in human cancer cells: implication for senescence-directed molecular therapy for cancer. *Cell Death Differ* 6, 765-772 (1999)

195. J Y Kang, J J Kim, S Y Jang, Y S Bae: The p53-p21(Cip1/WAF1) pathway is necessary for cellular senescence induced by the inhibition of protein kinase CKII in human colon cancer cells. *Mol Cells* 28, 489-494 (2009)

196. J Fuxe, G Akusjarvi, H M Goike, G Roos, V P Collins, R F Pettersson: Adenovirus-mediated overexpression of p15INK4B inhibits human glioma cell growth, induces replicative senescence, and inhibits telomerase activity similarly to p16INK4A *Cell Growth Differ* 11, 373-384 (2000)

197. S Courtois-Cox, S M Genther Williams, E E Reczek, B W Johnson, L T McGillicuddy, C M Johannessen, P E Hollstein, M MacCollin, K Cichowski: A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell* 10, 459-472 (2006)

198. H Zhao, J Dupont, S Yakar, M Karas, D LeRoith: PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGF-IR expression in prostate cancer cells. *Oncogene* 23, 786-794 (2004)

199. M Baba, S Hirai, S Kawakami, T Kishida, N Sakai, S Kaneko, M Yao, T Shuin, Y Kubota, M Hosaka, S Ohno: Tumor suppressor protein VHL is induced at high cell density and mediates contact inhibition of cell growth. *Oncogene* 20, 2727-2736 (2001)

200. C Bastian, P E LeBoit, D Pinkel: Mutations and copy number increase of HRAS in Spitz nevi with distinctive histopathological features. *Am J Pathol* 157, 967-972 (2000)

201. M Pollock, U L Harper, K S Hansen, L M Yudt, M Stark, C M Robbins, T Y Moses, G Hostetter, U Wagner, J Kakareka, G Salem, T Pohida, P Heenan, P Duray, O Kallioniemi, N K Hayward, J M Trent, P S Meltzer: High frequency of BRAF mutations in nevi. *Nat Genet* 33, 19-20 (2003)

202. X P Zhou, O Gimm, H Hampel, T Niemann, M J Walker, C Eng: Epigenetic PTEN silencing in malignant melanomas without PTEN mutation. *Am J Pathol* 157, 1123-1128 (2000)

203. S Courtois-Cox, S L Jones, K Cichowski: Many roads lead to oncogene-induced senescence. *Oncogene* 27, 2801-2809 (2008)

204. E C Chu, A S Tarnawski: PTEN regulatory functions in tumor suppression and cell biology. *Med Sci Monit* 10, RA235-RA241 (2004)

205. S Anai, S Goodison, K Shiverick, K Iczkowski, M Tanaka, C J Rosser: Combination of PTEN gene therapy and radiation inhibits the growth of human prostate cancer xenografts. *Hum Gene Ther* 17, 975-984 (2006)

206. D R Carrasco, T Fenton, K Sukhdeo, M Protopopova, M Enos, M J You, D Di Vizio, C Nogueira, J Stommel, G S Pinkus, C Fletcher, J L Hornick, W K Cavenee, F B Furnari, R A Depinho: The PTEN and INK4A/ARF tumor suppressors maintain myelolymphoid homeostasis and cooperate to constrain histiocytic sarcoma development in humans. *Cancer Cell* 9, 379-390 (2006)

207. C Nardella, J G Clohessy, A Alimonti, P P Pandolfi: Pro-senescence therapy for cancer treatment. *Nat Rev Cancer* 11, 503-511 (2011)

208. A Carracedo, A Alimonti, P P Pandolfi: PTEN level in tumor suppression: how much is too little? *Cancer Res* 71, 629-633 (2011)

209. I U Ali: Gatekeeper for endometrium: the PTEN tumor suppressor gene. *J Natl Cancer Inst* 92, 861-863 (2000)

210. T Andjelkovic, J Bankovic, J Stojsic, V Milinkovic, A Podolski-Renic, S Ruzdijic, N Tanic: Coalterations of p53 and PTEN tumor suppressor genes in non-small cell lung carcinoma patients. *Transl Res* 157, 19-28 (2011)

211. S Bandyopadhyay, S K Pai, S Hirota, S Hosobe, T Tsukada, K Miura, Y Takano, K Saito, T Commes, D Piquemal, M Watabe, S Gross, Y Wang, J Huggenvik, K Watabe: PTEN up-regulates the tumor metastasis suppressor gene Drg-1 in prostate and breast cancer. *Cancer Res* 64, 7655-7660 (2004)

212. T A Bismar, M Yoshimoto, R T Vollmer, Q Duan, M Firszt, J Corcos, J A Squire: PTEN genomic deletion is an early event associated with ERG gene rearrangements in prostate cancer. *BJU Int* 107, 477-485 (2011)

213. Y G Wen, Q Wang, C Z Zhou, G Q Qiu, Z H Peng, H M Tang: Mutation analysis of tumor suppressor gene PTEN in patients with gastric carcinomas and its impact on PI3K/AKT pathway. *Oncol Rep* 24, 89-95 (2010)

214. J. Bloom, M Pagano: Deregulated degradation of the cdk inhibitor p27 and malignant transformation. *Semin Cancer Biol* 13, 41-47 (2003)

215. K I Nakayama, K Nakayama: Regulation of the cell cycle by SCF-type ubiquitin ligases. *Semin Cell Dev Biol* 16, 323-333 (2005)

216. M Gstaiger, R Jordan, M Lim, C Catzavelos, J Mestan, J Slingerland, W Krek: Skp2 is oncogenic and overexpressed in human cancers. *Proc Natl Acad Sci U S A* 98, 5043-5048 (2001)

217. C Kim, S Friso, S W Choi: DNA methylation, an epigenetic mechanism connecting folate to healthy embryonic development and aging. *J Nutr Biochem* 20, 917-926 (2009)

218. S F Gilbert: Ageing and cancer as diseases of epigenesis. *J Biosci* 34, 601-604 (2009)

219. A Breiling, L Sessa, V Orlando: Biology of polycomb and trithorax group proteins. *Int Rev Cytol* 258, 83-136 (2007)

220. E Vire, C Brenner, R Deplus, L Blanchon, M Fraga, C Didelot, L Morey, A Van Eynde, D Bernard, J M Vanderwinden, M Bollen, M Esteller, L Di Croce, Y de Launoit, F Fuks: The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 439, 871-874 (2006)

221. M F Fraga, M Esteller: Epigenetics and aging: the targets and the marks. *Trends Genet* 23, 413-418 (2007)

222. G Pegoraro, T Misteli: The central role of chromatin maintenance in aging. *Aging* 1, 1017-1022 (2009)

223. S Gravina, J Vijg: Epigenetic factors in aging and longevity. *Pflugers Arch* 459, 247-258 (2010)

224. R Satta, E Maloku, A Zhubi, F Pibiri, M Hajos, E Costa, A Guidotti: Nicotine decreases DNA methyltransferase 1 expression and glutamic acid decarboxylase 67 promoter methylation in GABAergic interneurons. *Proc Natl Acad Sci U S A* 105, 16356-16361 (2008)

225. M E Lleonart, A Artero-Castro, H Kondoh: Senescence induction; a possible cancer therapy. *Mol Cancer* 8, 3 (2009)

226. M Narita: Cellular senescence and chromatin organization. *Br J Cancer* 96, 686-691 (2007)

227. R K Lin, H S Hsu, J W Chang, C Y Chen, J T Chen, Y C Wang: Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer. *Lung Cancer* 55, 205-213 (2007)

228. X Han, P Berardi, K Riabowol: Chromatin modification and senescence: linkage by tumor suppressors? *Rejuvenation Res* 9, 69-76 (2006)

229. R S Pillai: MicroRNA function: multiple mechanisms for a tiny RNA? *RNA*, 11(12), 1753-61 (2005)

230. K Inoue: MicroRNA function in animal development. *Tanpakushitsu Kakusan Koso* 52, 197-204 (2007)

231. M Aranha, D M Santos, J M Xavier, W C Low, C J Steer, S Sola, C M Rodrigues: Apoptosis-associated microRNAs are modulated in mouse, rat and human neural differentiation. *BMC Genomics* 11, 514 (2010)

232. K Lafferty-Whyte, C J Cairney, N B Jamieson, K A Oien, W N Keith: Pathway analysis of senescence-associated miRNA targets reveals common processes to different senescence induction mechanisms. *Biochim Biophys Acta* 1792, 341-352 (2009)

233. J Lu, G Getz, E A Miska, E Alvarez-Saavedra, J Lamb, D Peck, A Sweet-Cordero, B L Ebert, R H Mak, A A Ferrando, J R Downing, T Jacks, H R Horvitz, T R Golub: MicroRNA expression profiles classify human cancers. *Nature* 435, 834-838 (2005)

234. J S Ross, J A Carlson, G Brock: miRNA: the new gene silencer. *Am J Clin Pathol* 128, 830-836 (2007)

235. M. Yamakuchi, M Ferlito, C J Lowenstein: miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci U S A* 105, 13421-13426 (2008)

236. H Tazawa, N Tsuchiya, M Izumiya, H Nakagama: Tumor-suppressive miR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells. *Proc Natl Acad Sci U S A* 104, 15472-15477 (2007)

237. G Zauli, R Voltan, M G di Iasio, R Bosco, E Melloni, M E Sana, P Secchiero: miR-34a induces the downregulation of both E2F1 and B-Myb oncogenes in leukemic cells. *Clin Cancer Res* 17, 2712-2724 (2011)

238. R Christoffersen, R Shalgi, L B Frankel, E Leucci, M Lees, M Klausen, Y Pilpel, F C Nielsen, M Oren, A H Lund: p53-independent upregulation of miR-34a during oncogene-induced senescence represses MYC *Cell Death Differ* 17, 236-245 (2010)

239. X Zhong, N Li, S Liang, Q Huang, G Coukos and L Zhang: Identification of microRNAs regulating reprogramming factor LIN28 in embryonic stem cells and cancer cells. *J Biol Chem* 285, 41961-41971 (2010)

240. X Yang, M Feng, X Jiang, Z Wu, Z Li, M Aau, Q Yu: miR-449a and miR-449b are direct transcriptional targets of E2F1 and negatively regulate pRb-E2F1 activity through a feedback loop by targeting CDK6 and CDC25A *Genes Dev* 23, 2388-23893 (2009)

241. J Noonan, R F Place, D Pookot, S Basak, J M Whitson, H Hirata, C Giardina, R Dahiya: miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. *Oncogene* 28, 1714-24 (2009)

242. M Voorhoeve, C le Sage, M Schrier, A J Gillis, H Stoop, R Nagel, Y P Liu, J van Duijse, J Drost, A Griekspoor, E Zlotorynski, N Yabuta, G De Vita, H Nojima, L H Looijenga, R Agami: A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* 124, 1169-1181 (2006)

243. H Hermeking: The miR-34 family in cancer and apoptosis. *Cell Death Differ* 17, 193-199 (2009)

244. K Kumamoto, E A Spillare, K Fujita, I Horikawa, T Yamashita, E Appella, M Nagashima, S Takenoshita, J Yokota, C C Harris: Nutlin-3a activates p53 to both down-regulate inhibitor of growth 2 and up-regulate mir-34a, mir-34b, and mir-34c expression, and induce senescence. *Cancer Res* 68, 3193-3203 (2008)

245. S Mitomo, C Maesawa, S Ogasawara, T Iwaya, M Shibazaki, A Yashima-Abo, K Kotani, H Oikawa, E Sakurai, N Izutsu, K Kato, H Komatsu, K Ikeda, G Wakabayashi, T Masuda: Downregulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. *Cancer Sci* 99, 280-286 (2008)

246. D Y Lee, Z Deng, C H Wang, B B Yang: MicroRNA-378 promotes cell survival, tumor growth, and angiogenesis by targeting SuFu and Fus-1 expression. *Proc Natl Acad Sci U S A* 104, 20350-20355 (2007)

247. S Venkataraman, I Alimova, R Fan, P Harris, N Foreman, R Vibhakar: MicroRNA 128a increases intracellular ROS level by targeting Bmi-1 and inhibits medulloblastoma cancer cell growth by promoting senescence. *PLoS One* 5, e10748 (2010)

248. E Ferretti, E De Smaele, A Po, L Di Marcotullio, E Tosi, M S Espinola, C Di Rocco, R Riccardi, F Giangaspero, A Farcomeni, I Nofroni, P Laneve, U Gioia, E Caffarelli, I Bozzoni, I Screpanti, A Gulino: MicroRNA profiling in human medulloblastoma. *Int J Cancer* 124, 568-577 (2009)

249. Y Zhang, T Chao, R Li, W Liu, Y Chen, X Yan, Y Gong, B Yin, B Qiang, J Zhao, J Yuan, X Peng: MicroRNA-128 inhibits glioma cells proliferation by targeting transcription factor E2F3a. *J Mol Med* 87, 43-51 (2009)

250. V Havelange, N Stauffer, C C Heaphy, S Volinia, M Andreeff, G Marcucci, C M Croce, R Garzon: Functional implications of microRNAs in acute myeloid leukemia by integrating microRNA and messenger RNA expression profiling. *Cancer* (2011)

251. P. Medawar: UNSOLVED problem of biology. *Med J Aust* 1, 854-855 (1953)

252. K A Hughes: Mutation and the evolution of ageing: from biometrics to system genetics. *Philos Trans R Soc Lond B Biol Sci* 365, 1273-1279 (2010)

253. P D Williams, T Day: Antagonistic pleiotropy, mortality source interactions, and the evolutionary theory of senescence. *Evolution* 57, 1478-1488 (2003)

254. M Rose. Evolutionary Biology of Aging. New York: Oxford University Press (1991)

255. P Achinstein. Observation and theory. In: A Companion to the Philosophy of Science. Ed: W Newton-Smith, M A Malden. Blackwell Publishing Ltd (2000)

256. L A K Milewski: The evolution of ageing. *Bioscience Horizons* 3, 77-84 (2010)

257. J M Sedivy: Telomeres limit cancer growth by inducing senescence: long-sought *in vivo* evidence obtained. *Cancer Cell* 11, 389-391 (2007)

258. P A Muller, K H Vousden, J C Norman: p53 and its mutants in tumor cell migration and invasion. *J Cell Biol* 192, 209-218 (2011)

259. E M Pinto, R C Ribeiro, B C Figueiredo, G P Zambetti: TP53-associated pediatric malignancies. *Genes Cancer* 2, 485-490 (2011)

260. M F Haussmann, D W Winkler, C E Huntington, I C Nisbet, C M Vleck: Telomerase activity is maintained throughout the lifespan of long-lived birds. *Exp Gerontol* 42, 610-618 (2007)

261. V Gorbunova, A Seluanov: Coevolution of telomerase activity and body mass in mammals: from mice to beavers. *Mech Ageing Dev* 130, 3-9 (2009)

262. A Tomas-Loba, I Flores, P J Fernandez-Marcos, M L Cayuela, A Maraver, A Tejera, C Borras, A Matheu, P Klatt, J M Flores, J Vina, M Serrano, M A Blasco: Telomerase reverse transcriptase delays aging in cancer-resistant mice. *Cell* 135, 609-622 (2008)

263. D Gilley, H Tanaka, B S Herbert: Telomere dysfunction in aging and cancer. *Int J Biochem Cell Biol* 37, 1000-1013 (2005)

264. T B Kirkwood: Asymmetry and the origins of ageing. *Mech Ageing Dev* 126, 533-534 (2005)

265. G M Wahl, A M Carr: The evolution of diverse biological responses to DNA damage: insights from yeast and p53. *Nat Cell Biol* 3, E277-E286 (2001)

266. M Murga, O Fernandez-Capetillo: Genomic instability: on the birth and death of cancer. *Clin Transl Oncol* 9, 216-220 (2007)

Abbreviations: RS: replicative senescence, hTERT: catalytic subunit of the telomerase holoenzyme, HP1: heterochromatin protein 1, SAHF: senescence-associated heterochromatin foci, SIPS: stress-induced premature senescence, OIS: oncogene-induced senescence, CDKs: cyclin-dependent kinases, pRb: retinoblastoma-associated protein, TERT: telomerase reverse transcriptase, DSBs: DNA double-strand breaks, TRF1: telomeric repeat-binding factor 1, TRF2: telomeric repeat-binding factor 2, TIN2: TRF1-interacting protein 2, RAP1: repressor-activator protein 1, POT1: protection of telomeres 1, TRF2ΔBΔM: small-dominant-negative form of TRF2, CSCs: cancer stem cells, PNAs: peptide nucleic acids, BRACO-19: 3,6,9-trisubstituted acridine compound, RHPS4: 3,11-difluoro-6,8,13-trimethyl-8*H*-quino[4,3,2-*kl*]acridinium methosulfate), ALT: alternative lengthening of telomeres, DDR: DNA damage response, EGCG: epigallocatechin gallate, siRNA: small interfering RNA, shRNA: short-hairpin RNA, TNQX: trichloro-5-nitroquinoxaline, GCR: gross chromosomal rearrangements, BER: base excision repair, NER: nucleotide excision repair, MMR: mismatch repair, HR: homologous recombination, SSA: single strand annealing, NHEJ: non-homologous end joining, ATM: ataxia telangiectasia-mutated, ATR: ATM-Rad3-related, CHK1: checkpoint kinase 1, CHK2: checkpoint kinase 2, DNMTs: DNA methyltransferases, PRC1: polycomb repression complex 1, SIRT: NAD-dependent deacetylase sirtuin-1, miRNAs: micro-RNAs.

Key Words: Cancer, Replicative Senescence, Premature Senescence, Telomere Erosion, DNA Damage, Chemotherapy, Epigenetics, Evolution, Review

Send correspondence to: Diego Bonatto, Centro de Biotecnologia da UFRGS, Sala 219, Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, UFRGS, Avenida Bento Goncalves 9500, Predio 43421, Caixa Postal 15005, Porto Alegre, Rio Grande do Sul, Brazil, 91509-900, Tel: 55513308-6080, Fax: 55513308-7309, E-mail: diegobonatto@gmail.com

3.2. CAPÍTULO II - Combination of sodium butyrate with polyphenols enhance senescence-like state in glioblastoma cell lines

José E. Vargas, Eduardo C. Filippi-Chiela, Thais Suhre, Diego Bonatto, Guido Lenz

Periódico: **Cancer Science**

Estado: **Em preparação para submissão**

Combination of sodium butyrate with polyphenols enhance senescence-like state in glioblastoma cell lines

José E. Vargas[1], Eduardo C. Filippi-Chiela[1], Thais Suhre[1], Diego Bonatto[2], Guido Lenz[1*]

[1] Department of Biophysics and Center of Biotechnology, IB, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

[2] Department of Molecular Biology and Biotechnology, Av. Bento Gonçalves, , Federal University of Rio Grande do Sul (UFRGS)9500, Porto Alegre, Brazil.

[*] To whom correspondence should be addressed: Departamento de Biofísica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 9500, CEP 91501-970, Porto Alegre, RS, Brazil.
Tel: +555133087620; Fax: +555133307003 Email: lenz@ufrgs.br

Summary

Cellular senescence leads to irreversible block of cellular division ability and therefore pharmacological induction of senescence is being considered for treatment of many cancer types. Resveratrol (Resv) and quercetin (Querc), two natural polyphenols, are able to induce senescence in different cancer models, including gliomas. Resv is a molecule with pleiotropic effects which leads, among others, to the activation of Sirt1. Sirt1 is a histone deacetylase (HDAC) which has been linked positively to cancer development. Therefore we set out to evaluate if the senescence induction in cancer cells by Resv is altered in the presence of sodium butyrate (NaB), an class I and II HDAC inhibitor. Similarly, Querc act as HDAC inhibitor in different cancer cells. Results show an additive effect of sodium butyrate with Resv and Querc to induce cellular senescence in glioblastoma cell lines of two different species (human and rat) but not in normal rat astrocytes. The co-treatments induced loss of proliferative capacity and increased the number of cells positive for β-galactosidase, a classical senescence marker and increase the levels of the CKI p21. The data underline that tumor cells can be driven towards cellular senescence by this HDAC inhibitor combined with polyphenols, which may further arise as a potent possibility for tumor suppression.

Introduction

Malignant gliomas are the most common primary brain tumor, accounting for approximately 15% of all intracranial tumors. Median survival after treatment is 3 to 4 years for Grade III tumors and 10 to 12 months for Grade IV tumors [1]. Despite advances in therapy, these tumors remain fatal with a short survival for patients after diagnosis and conventional treatments, such as radiotherapy and chemotherapy [2]. Selective pharmacological activation of cellular mechanisms, such as autophagy, apoptosis and senescence are antitumor strategies that may add efficacy and specificity to current treatment regimes [3, 4].

Senescence itself is biochemically diverse, and the environmental and molecular signals that induce senescence are heterogeneous [5-8]. Moreover, senescence can be divided into two major categories: replicative senescence, which is normally induced by a large number of divisions due to telomere shortening [9], and premature senescence, which results mainly from oncogenic signals and/or DNA damage [10]. However, these mechanisms share the reduction in CDK–cyclin complex activity mainly through high expression of the CKIs p27 and p21. p27 is a negative regulator of cyclin D-Cdk4, cyclin E-CDK2 and cyclin A-CDK2, being involved in G1/S transition. Its expression is decreased or absent in glioblastomas [11]. Similarly, p21 acts as negative regulator of cyclin D/A-CDK4 being involved in G1/S or G2/M transition with decreased activity in glioblastomas [11].

Pharmacological induction of senescence recently appeared as a potential therapeutic strategy against cancer [12]. DNA damaging agents as well as autocrinous produced factors, such as IGFBP7 were shown to induce senescence in cancer cells in vitro [12-14] and in vivo [13-16]. We showed that, contrary to the effects on organisms and normal cells, Resv and Querc induce senescence in tumor cells [17].

Resveratrol (Resv; 3,5,4′-trihydroxy-trans-stilbene) is a polyphenol present in many plant species such as grapes, berries, and peanuts. The cancer chemopreventive properties of Resv were first described by Jang *et al*. (1997) [18], who demonstrated that Resv inhibited cellular events associated with tumor development. In glioblastomas, it has been shown that Resv can induce apoptosis in U251 human glioma cells [19], RT-2 rat glioma cells [20], and C6 rat glioma cell line [21] and senescence and autophagy in U87 human glioma cell [22, 23]. Resv is very pleiotropic, although few targets directly regulated by Resv have been described [24]. Recently, direct inhibition of phosphosdiesterase (PDE) activity was described to be responsible for the activation of Sirt1 and amelioration of age-related metabolic phenotypes [25]. Sirt1 is an HDAC which is over-expressed in lung [26], prostate cancer [27] and leukemia [28]. Over-expression of SIRT1 inhibits the function of p53 [29] and inhibition of Sirt1 induced p53 in leukemia cells [30].

Chemical modulation of acetylation and deacetylation of histones in chromatin plays an important role in the regulation of gene expression and senescence induction [31-33]. Whether the action of histone deacetylases inhibitors (HDACi) on gene expression is selective is not known. However, p21 and p27 genes are consistently induced by these inhibitors therefore playing a role in irreversible cell growth arrest observed in response to HDACis [34, 35]. Sodium butyrate (NaB) is a short fatty acid that acts as HDACi inducing cell cycle arrest in different cancer cells [36-38]. In human glioma cells, sodium

butyrate has been shown to inhibit proliferation through modulation of cell cycle proteins' levels [39-41]. Several HDAC inhibitors, administered intravenously or intraperitoneally, inhibit tumor growth with low citotoxicity in animal models of breast, prostate, lung and stomach cancers, besides neuroblastoma, and leukemia [42, 43].

Quercetin (Querc; 3,3′,4′,5,7-pentahydroxyflavone) is a flavonoid ubiquitous in nature that has recently been described as a potential anticancer agent 44. This compound modulates cell proliferation [45], survival, and differentiation, targeting key molecules responsible for tumor cell growth such as p53, p21, and Ras in several cancer cell types [46, 47.] In glioblastomas, Querc induced necrotic and apoptotic cell death in U138-MG, U87-MG, U251, and A172 but not in U373 glioma cells [48, 49]. Moreover, Kim et al. [50] demonstrated that Querc treatment resulted in loss of cell viability in A172 glioma cell line [51]. Quer was described to act as an HDAC inhibitor in leukemia and HBP cancer cells lines [52-54] and the additive effects of Resv and Querc on senescence induction may be due to this HDAC inhibitory effect.

Therefore, we set out to test whether HDAC inhibition could increase the senescence induction by Resv in cancer cells and whether the senescence-induction effect of Querc is through HDAC inhibition. Additionally, we explored the mechanisms of the senescence induction by these combinations.

Materials and Methods

Cell culture and treatments. Rat C6 glioma cells and human U87-MG glioma cells were obtained from ATCC (Rockville, MD,USA). Cells were cultured in DMEM low glucose supplemented with 10% FBS (5% for C6 cells), 1% penicillin/streptomycin, and 0.1% anphoterecin B at 37°C with 5% CO2 in a humidified incubator.

Primary astrocyte cultures were prepared as previously described.[260] Briefly, hippocampus of new-born Wistar rats (1–2 days old) were removed and mechanically dissociated by sequential passage through a Pasteur pipette. Cell suspension was centrifuged at 1000 g for 5 min. The supernatant was discarded, and the pellet resuspended in culture medium containing DMEM high glucose with 10% FBS,1 % penicillin/streptomycin and 0.1% anphoterecin B at 37°C with 5% CO2 in a humidified incubator. 1.5×10^5 cells/cm2 were plated in 24-well plates pretreated with poly-L-lysine, and allowed to proliferate to confluence (14 days). All animal use procedures were approved by the local Animal Care Committee and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Cell treatments. NaB, Resv and Querc were purchased from Sigma Chemical (St. Louis, MO, USA). The vehicle carrier of these polyphenols was Dimethyl sulfoxide (DMSO) (Acros Organics, NJ, USA). DMSO was present in the control wells at a maximum concentration of 0.5%.

Cell viability. The number of cells was counted in a Neubauer chamber using trypan blue. Viability of treated cells was calculated as a percentage of control.

Long-term culture of C6, U87MG and astrocytes. Cells were plated at each passage in 24-well plates at a density of 5×10^4 cells/well for C6, and 3×10^4 cells/well for

U87MG and exposed to different concentrations of NaB with medium and treatments renewal every 48 h for determination of its sub-lethal concentration of NaB in chronic treatments. Cells were passaged, and population doublings (PD) were determined according to the formula PD = [log N(t) - logN(to)]/log 2, where N(t) is the number of cells per well at time of passage, and N(to) is the number of cells seeded at the previous passage. The sum of PD was then plotted against time of culture. Similarly, PDs were performed to analyze profilaration of cell population with 10 μM Resv, 25 μM Querc, 2mM NaB and the combination 10 μM Resv with 2mM NaB and 25 μM Querc with 2mM NaB with medium and treatments renewal every 48h of medium and treatments for C6, U87MG and astrocyte culture considering N(to) of 5 x 10^4 cells/well, and 3 x 10^4 cells/well, and 1 x 10^4 cells/well, respectively.

Colony formation assay. After 4 days of treatments, C6 and U87MG, cells were trypsinized, counted, and plated at concentration of 100 cells/well using tripan blue into six-well plates. After 10 days and 16 days without treatments for C6 for and U87MG respectively, colonies were stained with 0.5 % crystal violet and manually quantified to define the number of colonies.

Senescence-associated- β -galactosidade staining. After 4 days in culture, cells were washed in PBS, fixed in 3% formaldehyde for 1 hour at room temperature, washed, and incubated with fresh senescence-associated beta-galactosidase (SA-β-gal) staining solution containing 1 mg/mL X-gal (Sigma), 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl for 12–16 h at 37°C [56]. Results are presented as ratio of SA-β-gal-positive cells to total cells of at least three fields of three independent experiments. Total cell number was determinate using 4,6-diamidino-2-phenylindole (DAPI) labeling of glioblastoma cell nuclei.

Cell cycle analysis. Cells were washed with PBS and permeabilized with 0.1% triton X. Then the cells were incubated with 40 μg/ml of propidium iodide and 0.1 mg/ml RNase A for 30 min at 37°C prior to analysis by flow cytometry Guava EasyCyte (Guava Technologies, Hayward, CA).

Nuclear Morphometric Analysis. This method was developed by our group to objectively measure size and shape of nuclei [57]. Briefly, cells were plated at 20000 cells/well in a 24-well plate, followed by treatments as indicated (4 days of treatments). After treatments, cells were fixed with 4% formaldehyde (v/v in PBS) for at least 1 h at room temperature and subsequently maintained in PBS. Next, fixed cells were stained with a solution containing 300 n M DAPI and 0.1% Triton X-100 (v/v in PBS) for 1 h at room temperature, followed by images quantification using the Software Image Pro Plus 6.0 software (IPP6; Media Cybernetics, Silver Spring, Md., USA). Roundness, aspect, radius ratio and area box were quantified and grouped in nuclear irregularity index (NII), which is composed of the sum of aspect, radius ratio and roundness, subtracted by the value of area box. Data are presented as a plot of the area versus NII, in which normal nuclei were considered within 2 standard deviations (SD) SD of the mean from un treated cells. Nuclei were considered large and regular if 1 2 SD in size

and 3 SD of NII, and irregular when 1 3 SD of the large population or 1 5 SD of the normal-sized population.

DCF (dichlorofluorescein) assay. The non-fluorescent fluorescein derivative DCFwhich is converted to highly fluorescent DCF upon oxidation was used for detection of oxidative stress. The cells grown in 24-well plates were washed with PBS 1X. Then the cells were incubated in 30 min at 37°C prior to analysis by flow cytometry Guava EasyCyte (Guava Technologies, Hayward, CA).

Immunoblotting. Protein samples preparation and western blot were performed as described previously with minor modifications [58]. Whole-cell lysate was quantified with BCA kit, electrophoresed and electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was blocked in a mixture of 5% skimmed milk and Tris-Buffered Saline and Tween 20 (TBST) for an hour and probed with Ab to phospho-retinoblastoma protein (p-Rb Ser807/811 or Ser795) (1:1000 dilution; Cell Signaling), phospho-Akt (pAkt Ser473) (1:1000; Cell Signaling), cyclin D1 (1:1000; Cell Signaling), p21 (1:500; Cell Signaling), p27 (1:500; Santa Cruz Biotechnology), gammaH2AX (1:1000; Millipore). Bound Ab was detected with appropriate HRP secondary Ab (1:1000; Cell Signaling) using ECL and X-ray films (NIH Image, Rockville, MD, USA). Stripping western membranes was realized with 1mM NaOH for 5 min. Optical density of bands was obtained with ImageJ software (NIH Image, Rockville, MD, USA).

Statistical analysis. All data were represented as mean ± standard error of the mean (SEM) and were analyzed using anova, followed by Tukey test as post-hoc test. Differences with $P < 0.05$ were considered significant. Statistical analyses of the data were performed using the GraphPad INSTAT software, (GraphPad Software, San Diego, CA, USA).

Results

NaB potentiates the long-term but not short-term effects of Resv and Querc in gliomas.

We have previously shown that the combination of Resv and Querc induce high levels of senescence in glioma cells lines [17]. However, considering that Resv leads to activation of Sirt1, a HDAC, and that growth promoting effects of activators of HDAC, we tested the hypothesis that inhibition of HDAC would potentiate the senescence-inducing effects of Resv. Firstly, we tested the effects of long-term exposure to NaB on human U87-MG and rat C6 cells. The concentration of 2mM NaB inhibits cell proliferation in C6 and U87-MG in a way that keeps the cell population stable, whereas concentrations higher than 2mM lead to a sharp reduction of cell number (Fig S1).

Combination of NaB with low doses of Querc or Resv had no effect in potentiating C6 and U87 cells over 72 h, except for the combination with Quer at 24 and 48 h (Fig. 1a and Fig. S2). With long-term exposures, NaB produced a significant potentiation of the effects of Resv or Querc on cell number in the two cell types (Fig. 1b). The same treatments did not significantly affect primary rat astrocyte growth (Fig. 1c).

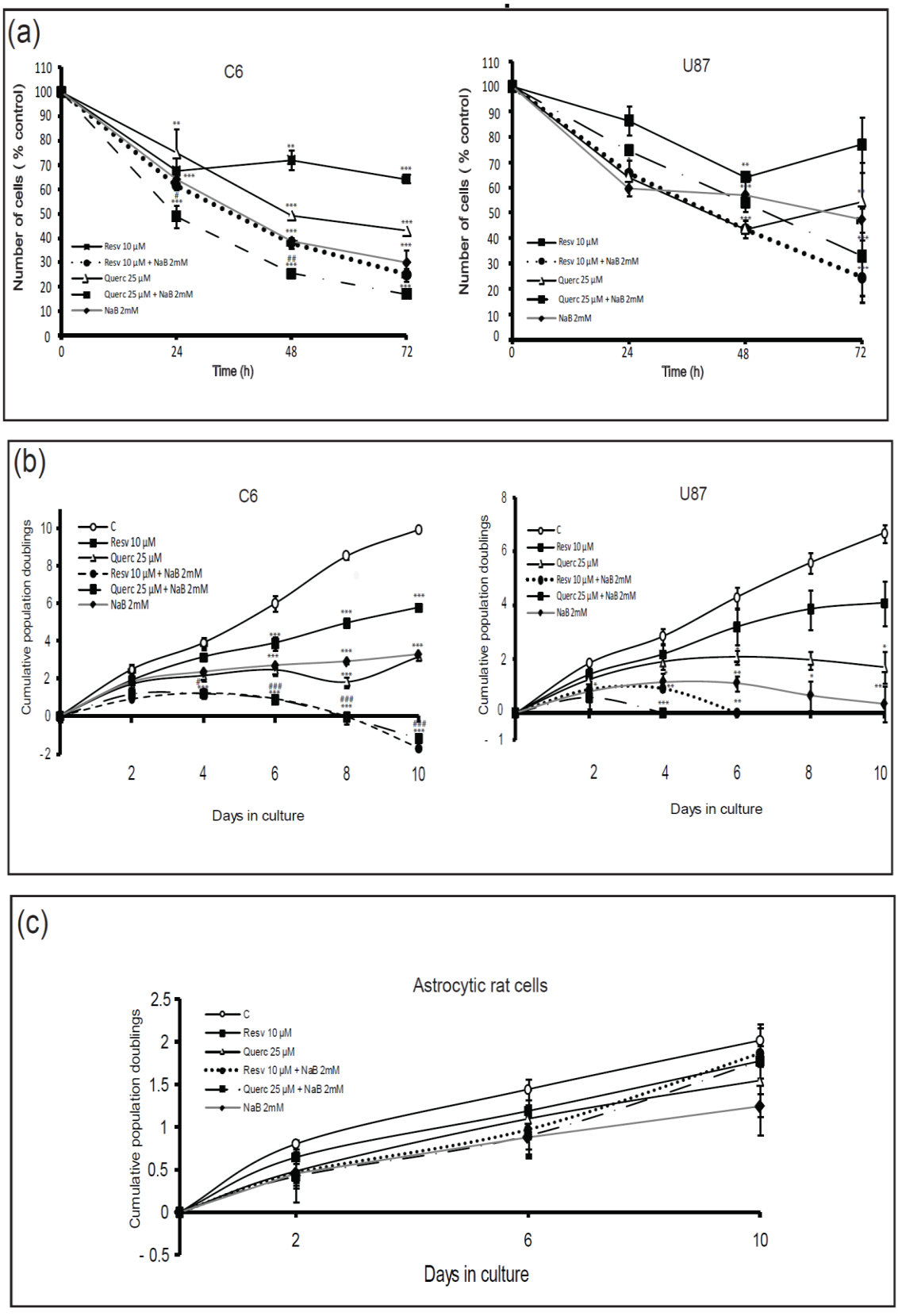


Fig. 1. Resv or Querc combined to NaB reduced the number of human and rat glioma cells in acute and chronic treatments. C6 and U87-MG cells were cultivated in continuous presence of 25 µM Querc, 10 µM Resv, 25 µM Querc/2mM NaB, 10uM Resv/2mM NaB, or 2mM NaB for 24, 48 and 72 h; cells were then counted in a Neubauer chamber (a). C6 and U87-MG were cultived in continous presence of 25 µM Querc, 10 µM Rsv, 25 µM Querc/2mM NaB, 10uM Resv/2mM NaB, or 2mM NaB for 10 days and cumulative population doublings were plotted

against time (b). Astrocytic rat cells were cultivated in similar treatment conditions above-described for 10 days and cumulative population doublings were plotted against time (c). Results are representative of three independent experiments. ANOVA with SNK as *post hoc*. *P < 0.05, **P < 0.01, ***P < 0.001 when compared to control (non treated-cells). # *P* < 0.05, ## *P* < 0.01, ###*P* < 0.001 when compared to NaB treatment.

NaB combined with Resv or Querc cooperates to induce senescence-like growth arrest in C6 and U87-MG cells.

Cell morphology alteration such as flattening, increased granularity and size, which are morphologic change associated with senescence were detected after 4 days of continuous NaB plus Resv or Querc treatment in both cell lines (Fig. 2a)**.** In order to confirm the pro-senescence phenotype, activity of β-galactosidase (E.C. 3.2.1.23), a biochemical marker for lysosome acidification, was measured (40). In C6, Resv or Querc treatment for 4 days produced about 10-20 % of senescence-associated-β-galactosidade (SA-β-gal) positive cells, whereas co-treatments (Resv/NaB or Querc/NaB) produced 80 to 90% of positive cells, and similar result was observed in U87-MG cells (Fig. 2b). It is important to note that in both cell lines, treatment with NaB alone induced around 50% of SA-B-gal positive cells (Fig. 2b).

Next we asked whether cells treated for 4 days were able to recover to form colonies. For this we plated the same number of cells after 4 days of treatment and analyzed colony formation after 10 or 16 days without treatment for C6 and U87 cells, respectively. For C6 cells, only the combination of Resv or Querc with NaB produced a reduction in clonogenic growth, whereas in U87-MG cells, single treatments also reduced clonogenic growth, although the Querc/NaB combination produced a larger reduction when compared to NaB alone (Fig. 2c).

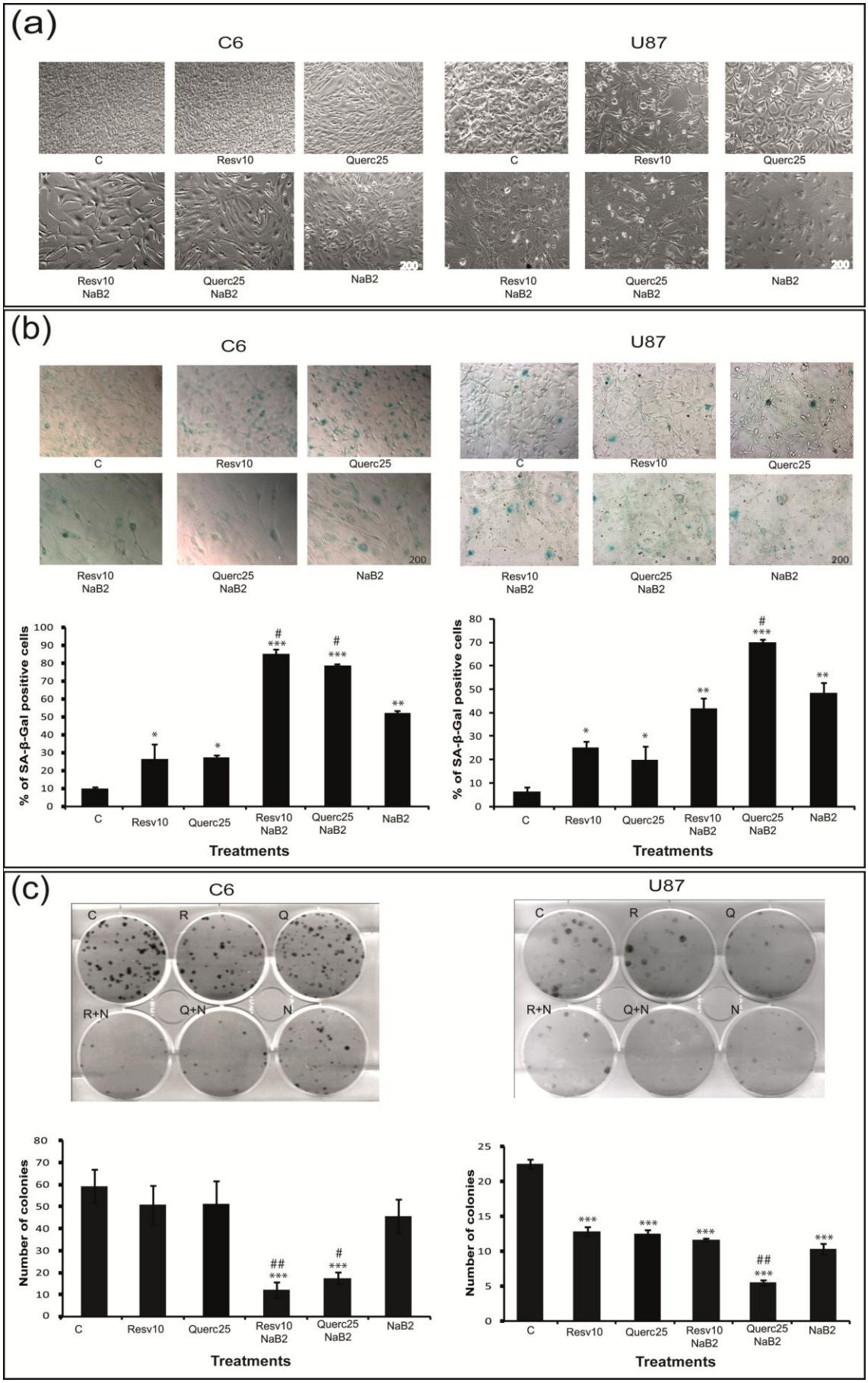


Fig. 2. Resv or Querc combined to NaB induce senescence-like state in human and rat glioma cells after 4 days of treatments. C6 and U87-MG cells were treated with 25 μM Querc

(Querc25), 10 μM Rsv (Rsv10), 25μM Querc/2mM NaB (Querc25/NaB2), 10μM Rsv/2mM NaB (Rsv10/NaB2), or 2mM NaB (NaB2), the images were obtained by Carl Zeiss inverted microscope. Representative micrographs of C6 and U87-MG (a), C6 and U87-MG cells were stained for SA-β-gal. The bar graph represents the ratio of SA-β-gal-positive cells to total cells. At least three fields of three independent experiments were analyzed (b). Colony formation efficiency of C6 and U87MG cells after 4 days treatments as in (a) followed by 10 days and 16 days in drug-free medium, respectively. Representative dishes shows clonogenic assay and colony-forming quantification. Data is presented as mean ± SEM**.** Results are representative of at least three determinations of three independent experiments. ANOVA with SNK *post hoc*. *P < 0.05, **P < 0.01, ***P < 0.001 when compared to non-treated cells. #P < 0.05, ## P < 0.01 when compared to NaB treatment.

NaB combined with Resv or Querc activates p21 expression and produces cell cycle arrest in U87-MG cells but no in C6.

According to Fig 1, transition from exponential growth to plateau in the PD graphs occurs between day 1 and 3 after treatment and therefore molecular events for senescence induction should occur at this time frame. To access molecular events potentially involved in senescence induction, we analysed levels of phosphorylated pRB, phosphorylated AKT, p21, p27 and cyclin D1 24 and 72 h after treatment. After 24h of treatment with Resv/NaB and Querc/NaB, only the reduction in the phosphorylation serine 795 of RB was detected for the co-treatments, but not the treatments alone. (Fig 3a). Moreover, 72h after the addition of co-treatments, we observed an increase in p21 and reduction in 807/811 serine phosphorylation of RB levels (Fig 3a). In U87-MG cells, Querc/NaB induced a significant increase in p21 levels.

No altered cell cycle phase distribution after treatments were observed in C6 (Fig 3b). However, Querc/NaB treatment caused cell cycle alterations in U87-MG cell line. G2/M cells population was increased (48 ± 1%) when compared to control cells (35 ± 1%) and NaB treatment (39 ± 2%). These data confirm that genetic characteristics of each cell line produce different effects on cell proliferation.

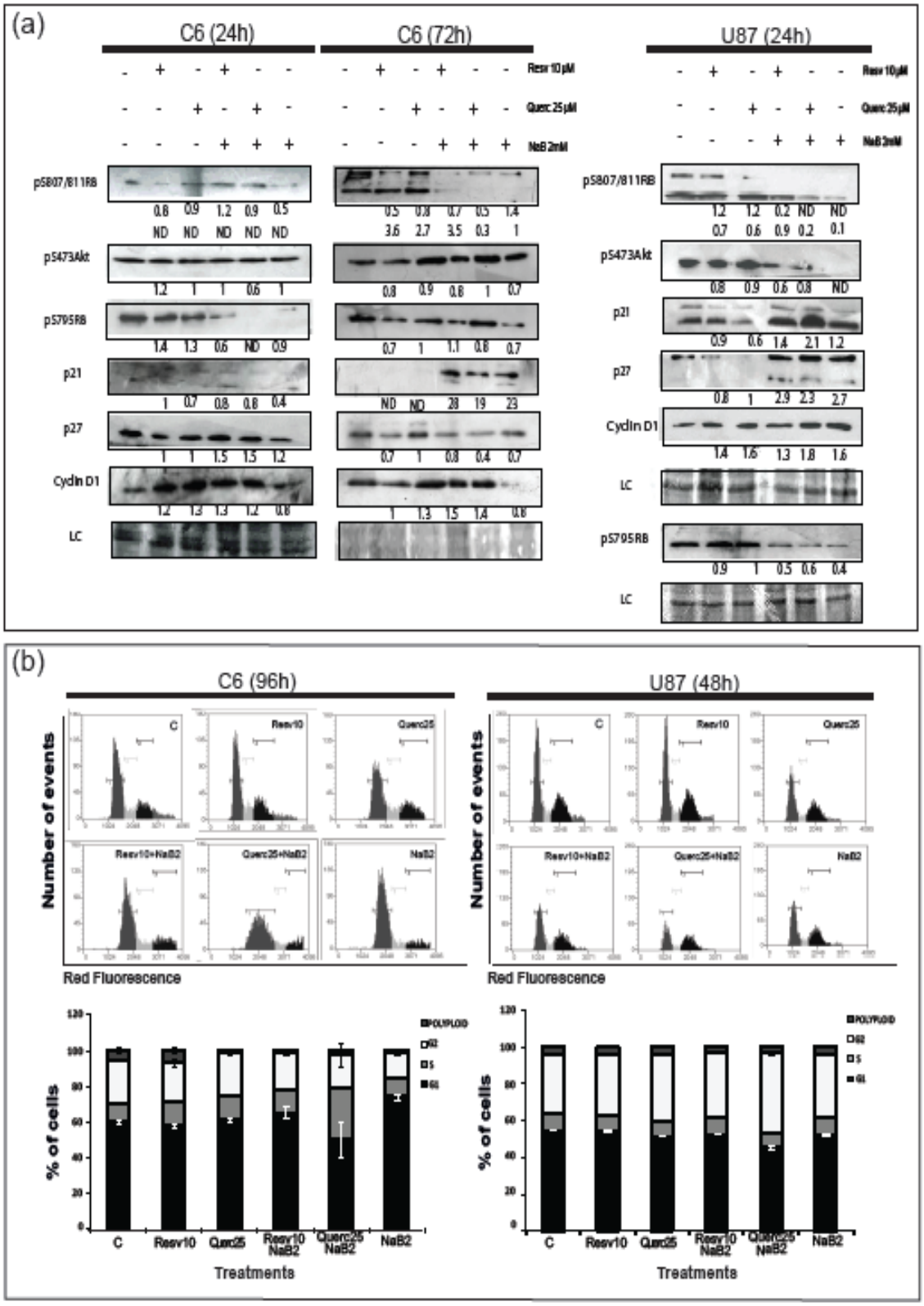


Fig. 3. Resv or Querc combined to NaB affect cell cycle regulators expression and distribution of glioma cells. Tumor cells were treated with 25 μM Querc (Querc25), 10 μM Rsv (Rsv10), 25 μM Querc/2mM NaB (Querc25 NaB2), 10uM Rsv/2mM NaB (Rsv10 NaB2), or 2mM NaB (NaB2) at 24 and 72 h for C6, and 72 h for U87-MG. Loading control (LC) represents PVDF membranes stained with Coomassie blue. Means ± SEM of two independent experiments were used to calculate band intensity. Band intensity was divided by LC intensity and compared to control (non-treated cells). (a). Cell cycle distribution analysis of C6 and U87MG cells treated

for 96 h and 48 h, respectively. The percentages of cells distribution in polyploid, G1, S and G2 phases were represented as mean ± SEM of three independent experiments (b).

NaB and polyphenols affect nuclear morphology and induce reactive oxygen species (ROS)

We have recently shown that nuclear morphology can give important insights into the populational distribution of phenotypes such as mitosis, apoptosis, senescence and mitotic catastrophe [23]. A morphometric analysis of nuclei was performed on Image Pro Plus and analysed for the distribution of nuclear size and irregularity. We observed an increase in the percentage of large nuclei (Fig. 4 and Fig. S3), which is a feature of senescence induction, supporting the findings of SA-β-galactosidase (Fig. 2b) in U87-MG cell treated with Querc/NaB. These results indicate that senescence is responsible for cell number reduction induced by co-treatments in both cell lines. Surprisingly, C6 have no differences in the nuclear size and irregularity compared to NaB treatment (Fig. 4 and Fig. S3).

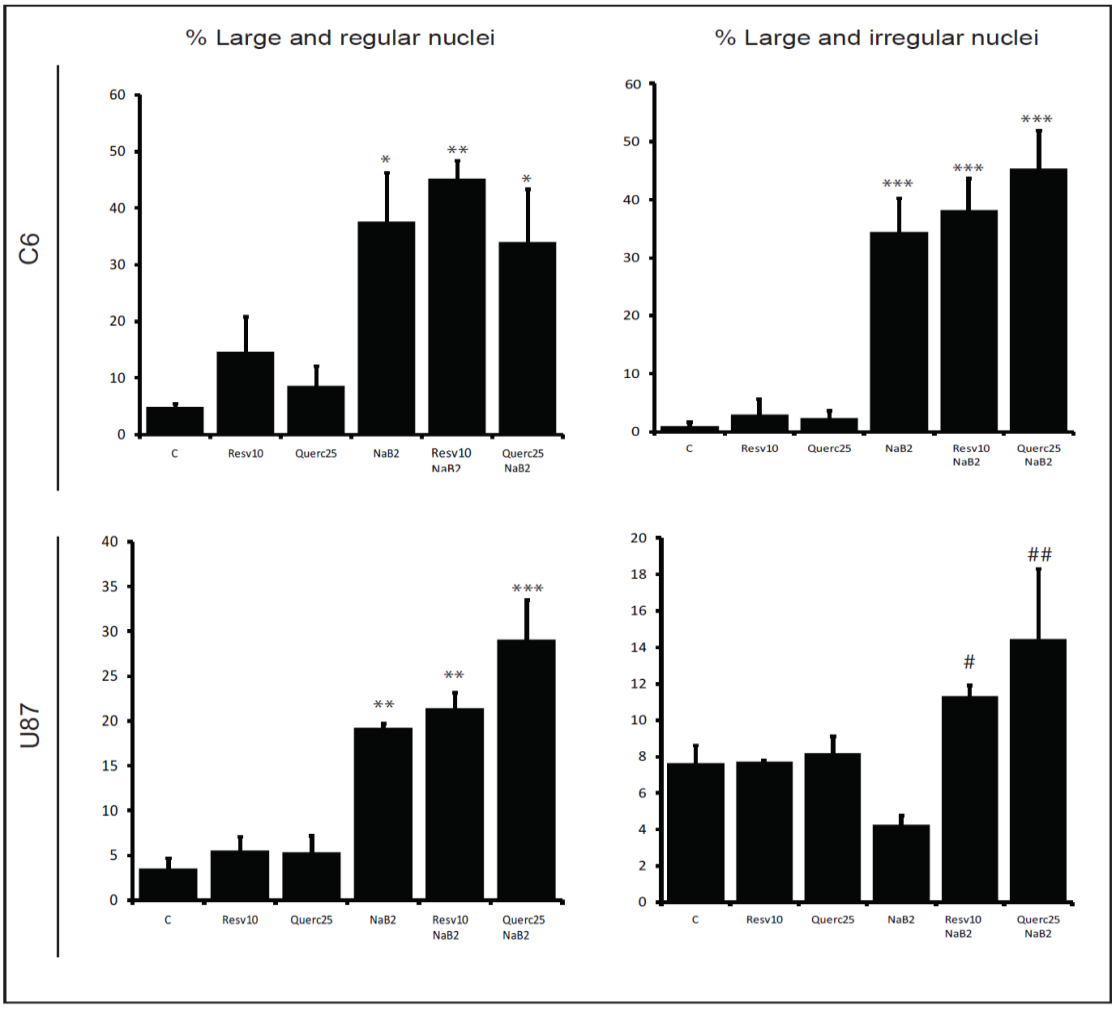


Fig.4. Querc combined to NaB induce alterations in nuclear morphology after 4 days of treatment U87-MG cell line but no in C6.. Quantification of large and regular nuclei was used as indication of senescence. Mean ± SEM of three independent experiments. ANOVA with SNK as *post hoc*. *P < 0.05 when compared to control (non treated-cells). # P < 0.05, when compared to NaB treatment.

There are many stimuli that can induce cell senescence. Among them, DNA damage and oxidative stress are the most commons [59, 60]. DNA damage signaling was analyzed focusing in gamma-H2AX (Fig. 5a), an early event of DNA double-strand break (DNA-DSB) recognition [61]. In C6 cells, NaB alone increased DNA damage, which was not further increased by Resv or Querc, and no significant increase in gamma-H2AX was observed in U87 cells, suggesting that the added senescence and growth reduction effects of the combination is not doe to increase in DNA damage induced by the combined treatments. ROS levels were verified by redox-sensitive fluorescent dye DCHF, which is able to detect H_2O_2 and superoxide. Only the Quer+NaB co-treatment significantly increased ROS production in C6 (Fig 5b), whereas the other treatments induced small or no alterations in ROS production.

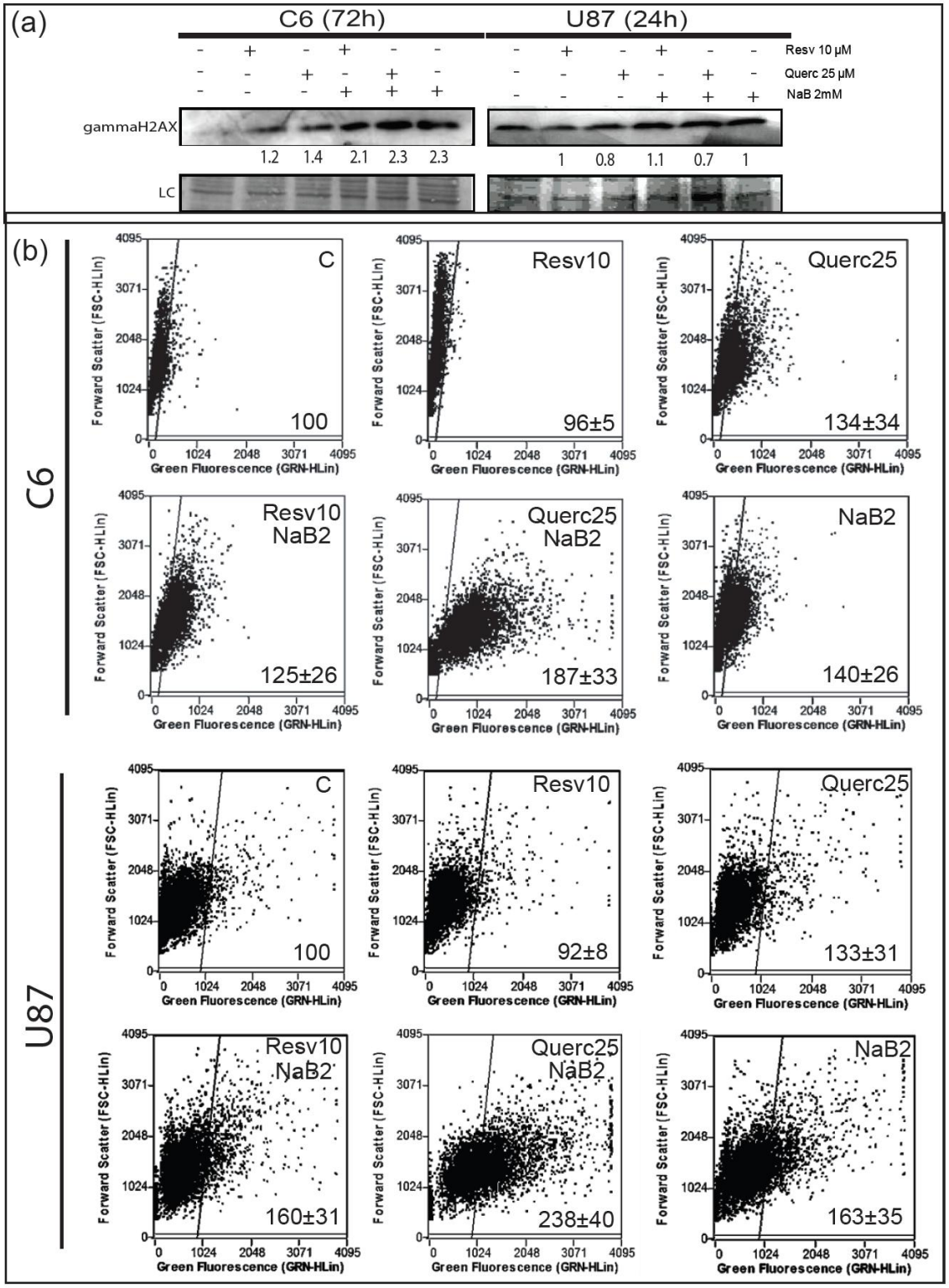


Fig.5. Querc combined to NaB induce ROS production without DNA damage signalling activation. U87-MG and C6 cells were cultivated in the presence of 25 µM Querc, 10 µM Resv, 25µM Querc/2mM NaB, 10µM Resv/2mM NaB, or 2mM NaB for 24 h and 72 h, respectively. Cell extracts were prepared for Western blotting for gamma H2AX. Loading control (LC) represents PVDF membranes stained with Coomassie blue. Mean ± SEM of two independent experiments were used to calculated band intensity. Band intensity was divided by the intensity of LC in relation to control (non-treated cells) (a). C6 and U87-MG were cultivated in similar conditions above-described. DCF fluorescence was used to detect ROS production. Mean ± SEM of three independent experiments.

Discussion

One major goal of cancer research is to block tumor cell proliferation. This aim can potentially be achieved via pharmacological induction of cellular senescence [8, 62, 63]. Plant-derived and products derived from mammalian metabolism provide natural compounds for cancer therapy. In this sense, polyphenols as Resv and Querc are a possible choice, since they have antitumor activity and are present in dietary compounds.

Resv and Querc, isolated or combined, act as cancer chemopreventive agents or adjuvants in chemotherapy [17]. Individually, high doses of Resv can induce apoptosis [64] and chronically administered in subapoptotic doses can induce senescence like growth arrest in C6, U87MG and U2-OS cancer cell lines [17, 65, 66], which is surprising, given the live extending effects of Resv in several organisms. These effects were described to depend on the indirect activation of Sirt1, and HDAC. Our rational in this study was to inhibit HDAC in order to test the hypothesis that Resv and Querc activate both pro- and anti-senescence pathways and that blocking the anti-senescence pathways a potentiation of the senescence induction could be attained.

This hypothesis was shown to be valid, since the combination of Resv plus NaB, when compared to the single treatments, induced more senescence and a greater reduction of cells in long term treatments, but not short term treatments. This increased senescence induction was not due to additive effects on DNA damage signaling, but involved induction of the p53 target p21, leading to a reduction in the phosphorylation of RB, a central mechanisms of senescence induction.

Similar results are described to Querc, which induce apoptosis in HepG2 cells by a p53-dependent increase BAX and p21 [67]. Low concentrations induce senescence as showed in C6 cell line [17]. One of the mechanisms by which Querc was proposed to induced senescence was through inhibition of HDAC, which could also explain the additive effects with Resv. Here we showed that inhibition of HDAC can increase the long term effects and senescence induction of Querc in two cells lines, suggesting that HDAC inhibition is not the only mechanism by which Querc induced senescence or increases the senescence induction by Resv.

DNA damage and ROS production are associated to cellular senescence. Passos and colleagues (2010) [68] also described this association, showing that high p21 expression is related to elevation of ROS levels, which is essential for the cellular senescence persistence [68]. These findings suggest that NaB/Querc may induce several pathways in U87-MG cells to induce cellular senescence, but NaB/Resv no. DNA damage occurrence can not be excluded; however, results show no reasons to suggest its presence, since there was no difference in gamma-H2AX between combined treatments and NaB. Importantly, the combined treatments (Resv plus NaB or Querc plus NaB) had no effects on of astrocytic rat cells proliferation, which is essential for the efficiency of an anticancer therapy.

Resv, Querc and NaB cross the intact blood–brain barrier (BBB) in rodents and reach concentrations in the range of 0.1, 0.3 and 0.4 μmol/kg on central nervous system respectively [69-71]. Glioma growth may disturb the BBB, probably increasing the concentration reachable inside the glioma. Thus, our data emphasize that tumor cells

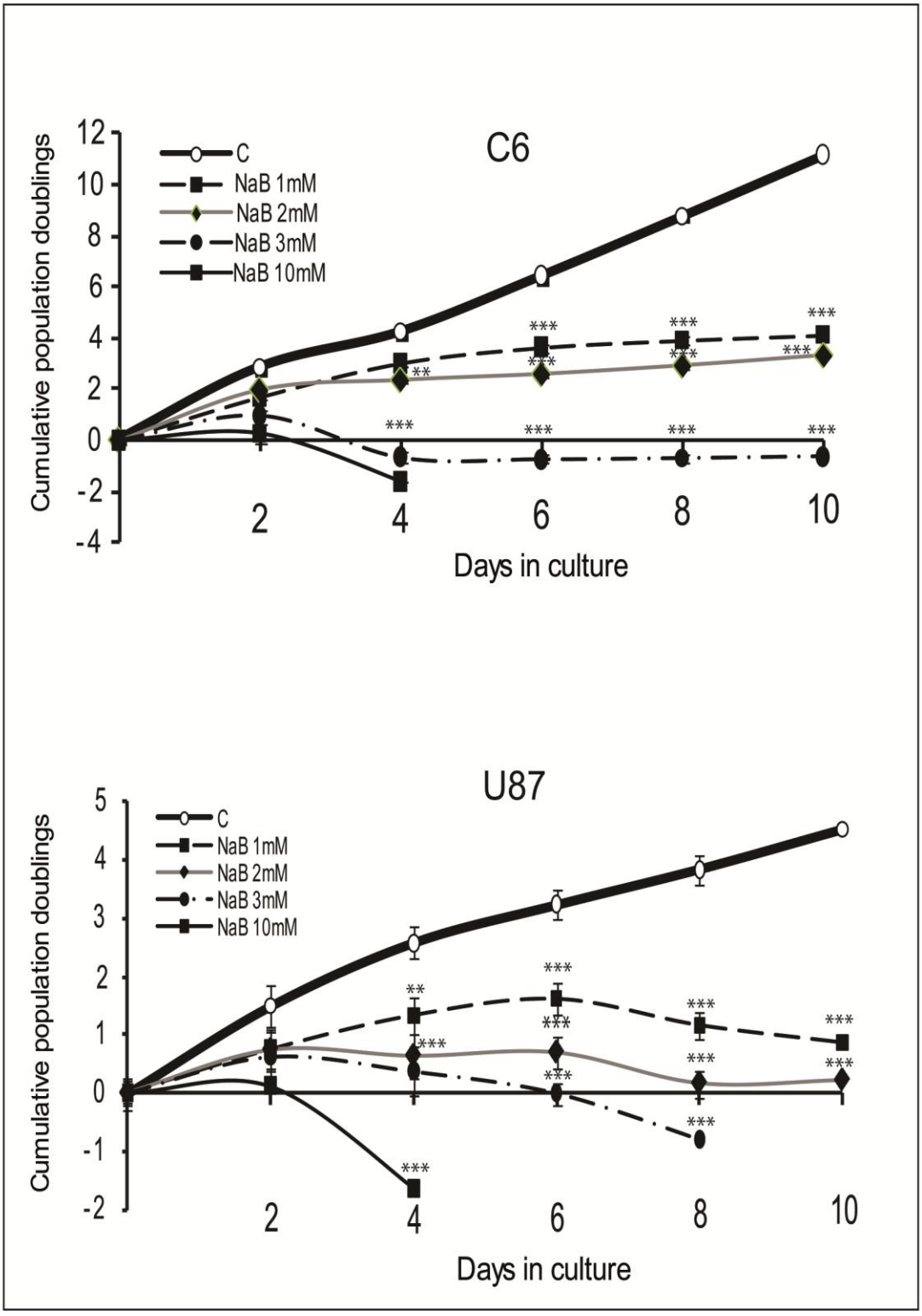
can be driven towards cellular senescence by sodium butyrate combined with polyphenols, which may further arise as a potent way for tumor suppression and guide the development of future clinical therapies.

.

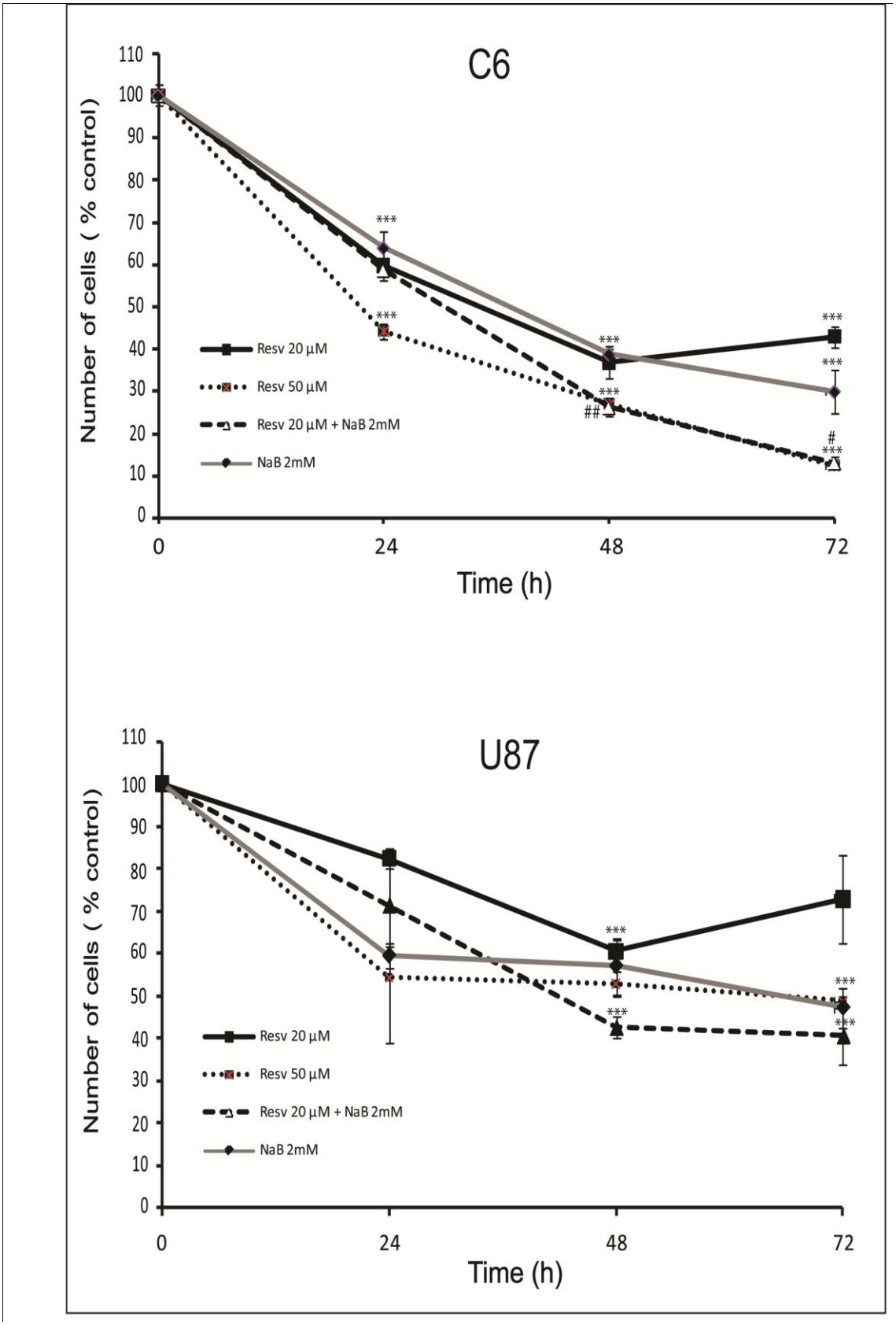
Acknowledgments

This work was supported by the Brazilian funding agencies CAPES/CNPq – Brasil.

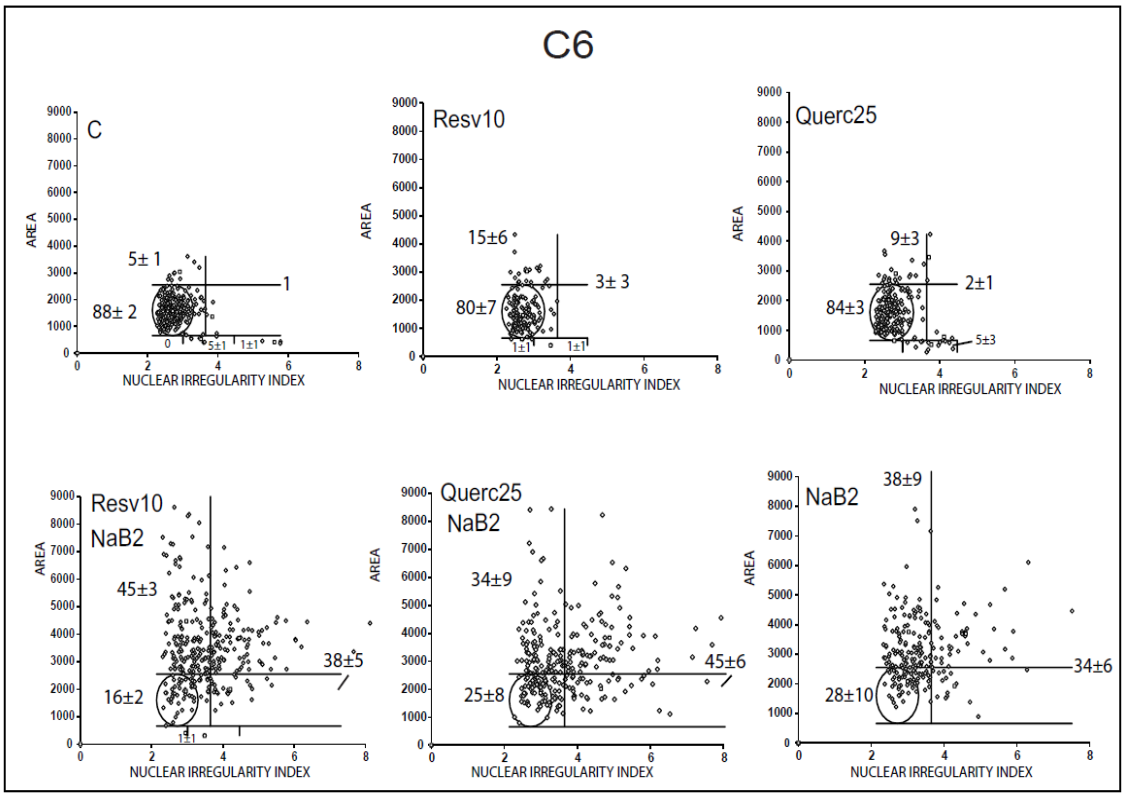
Supplementary figures

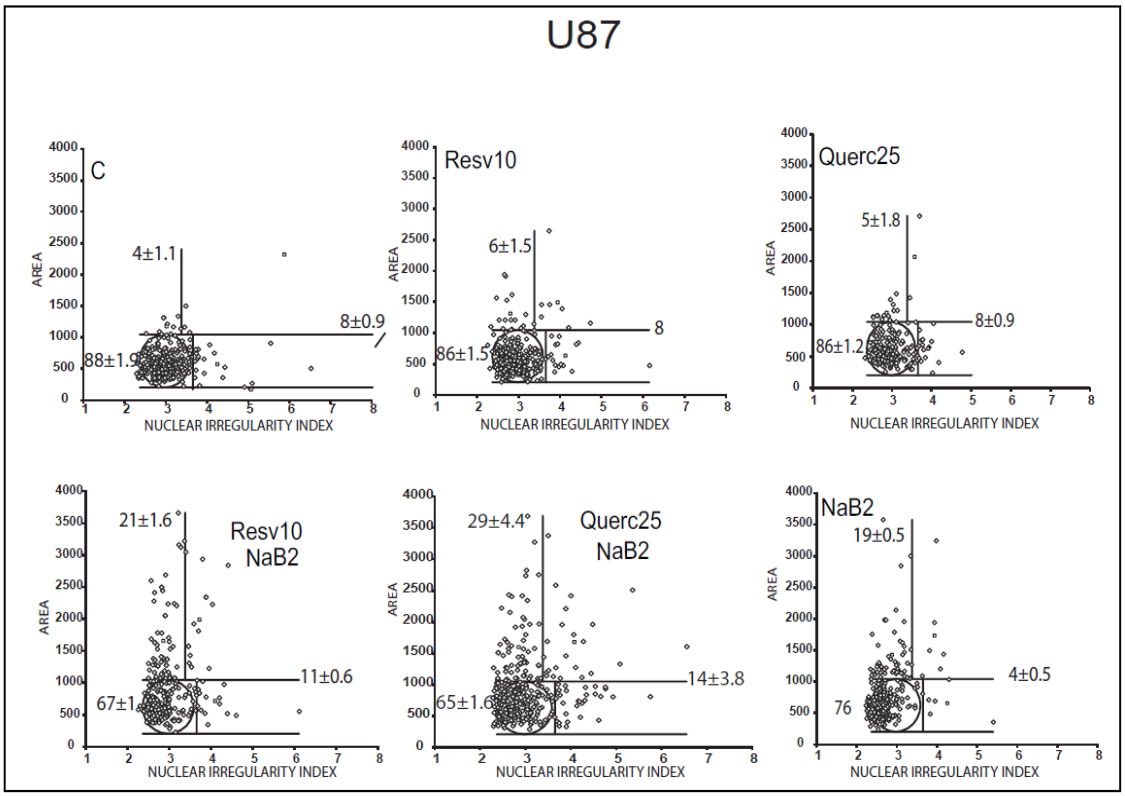


Supplementary Fig. 1. Chronic effects of NaB on human and rat glioma cells *in vitro*. C6 (A) or U87 (B) cells were cultivated at presence of 1 mM, 2 mM, 3 mM or 10 mM of NaB for 10 days and cumulative population doublings were plotted against time. Mean ± SEM of 3 independent experiments. ANOVA with SNK *post hoc*. *P < 0.05, **P < 0.01, ***P < 0.001.



Supplementary Fig. 2. Resv and Querc combined with NaB reduced the number of human and rat glioma cells in acute treatments. C6 and U87-MG cells were treated with 20 or 50 µM Rsv, 20 µM Rsv/2mM NaB, and 2mM NaB for 24, 48, and 72 h . Cells were counted in Neubauer chamber. Each time point represents mean ± SEM of three independent experiments. ANOVA with Tukey as *post hoc*. *P < 0.05, **P < 0.01, ***P < 0.001 when compared to non-treated cells.





Supplementary Fig. 3. Querc combined with sodium NaB affect nuclear morphology in U87-MG. Morphometric Nuclear Analysis of C6 and U87-MG cells after 4 days of treatment with 25 µM Querc, 10 µM Resv, 25µM Querc/2mM NaB, 10µM Resv/2mM NaB, or 2mM NaB The area (in pixels) and Nuclear Irregularity Index (NII) of each nucleus were measured from DAPI images. Graphic regions indicate nuclear morphological characteristic: Normal ; Small; Small and Regular ; Large and Regular ; Irregular. Nuclear morphometry differ in treated cells. The results represent ± SEM from three independent experiments.

References

[1] Tonn JC, Goldbrunner R. Mechanisms of glioma cell invasion. Acta Neurochir Suppl. 2003; 88: 163-7.

[2] Baskar R, Lee KA, Yeo R, Yeoh KW. Cancer and radiation therapy: current advances and future directions. Int J Med Sci. 9: 193-9.

[3] Lenz G. Endogenous anticancer mechanisms (EACMs). Front Biosci (Schol Ed). 4: 1017-30.

[4] Mukhtar H, Ahmad N. Cancer chemoprevention: future holds in multiple agents. Toxicol Appl Pharmacol. 1999; 158: 207-10.

[5] Collado M, Gil J, Efeyan A, et al. Tumour biology: senescence in premalignant tumours. Nature. 2005; 436: 642.

[6] Cosme-Blanco W, Shen MF, Lazar AJ, et al. Telomere dysfunction suppresses spontaneous tumorigenesis in vivo by initiating p53-dependent cellular senescence. EMBO Rep. 2007; 8: 497-503.

[7] Augereau A, T'Kint de Roodenbeke C, Simonet T, et al. Telomeric damage in early stage of chronic lymphocytic leukemia correlates with shelterin dysregulation. Blood. 118: 1316-22.

[8] Vargas J, Feltes BC, Poloni Jde F, Lenz G, Bonatto D. Senescence; an endogenous anticancer mechanism. Front Biosci. 2012; 17: 2616-43.

[9] Hayflick L. The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res. 1965; 37: 614-36.

[10] Chandeck C, Mooi WJ. Oncogene-induced cellular senescence. Adv Anat Pathol. 17: 42-8.

[11] Vlachostergios PJ, Voutsadakis IA, Papandreou CN. The ubiquitin-proteasome system in glioma cell cycle control. Cell Div. 7: 18.

[12] Ewald JA, Desotelle JA, Wilding G, Jarrard DF. Therapy-induced senescence in cancer. J Natl Cancer Inst. 2010; 102: 1536-46.

[13] Benatar T, Yang W, Amemiya Y, et al. IGFBP7 reduces breast tumor growth by induction of senescence and apoptosis pathways. Breast Cancer Res Treat. 2012; 133: 563-73.

[14] Hu S, Chen R, Man X, et al. Function and expression of insulin-like growth factor-binding protein 7 (IGFBP7) gene in childhood acute myeloid leukemia. Pediatr Hematol Oncol. 2011; 28: 279-87.

[15] te Poele RH, Okorokov AL, Jardine L, Cummings J, Joel SP. DNA damage is able to induce senescence in tumor cells in vitro and in vivo. Cancer Res. 2002; 62: 1876-83.

[16] Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes Dev. 2010; 24: 2463-79.

[17] Zamin LL, Filippi-Chiela EC, Dillenburg-Pilla P, Horn F, Salbego C, Lenz G. Resveratrol and quercetin cooperate to induce senescence-like growth arrest in C6 rat glioma cells. Cancer Sci. 2009; 100: 1655-62.

[18] Jang M, Cai L, Udeani GO, et al. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science. 1997; 275: 218-20.

[19] Jiang H, Zhang L, Kuo J, et al. Resveratrol-induced apoptotic death in human U251 glioma cells. Mol Cancer Ther. 2005; 4: 554-61.

[20] Tseng SH, Lin SM, Chen JC, et al. Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats. Clin Cancer Res. 2004; 10: 2190-202.

[21] Zhang W, Fei Z, Zhen HN, Zhang JN, Zhang X. Resveratrol inhibits cell growth and induces apoptosis of rat C6 glioma cells. J Neurooncol. 2007; 81: 231-40.

[22] Castino R, Pucer A, Veneroni R, et al. Resveratrol reduces the invasive growth and promotes the acquisition of a long-lasting differentiated phenotype in human glioblastoma cells. J Agric Food Chem. 59: 4264-72.

[23] Filippi-Chiela EC, Villodre ES, Zamin LL, Lenz G. Autophagy interplay with apoptosis and cell cycle regulation in the growth inhibiting effect of resveratrol in glioma cells. PLoS One. 2012; 6: e20849.

[24] Heiss EH, Schilder YD, Dirsch VM. Chronic treatment with resveratrol induces redox stress- and ataxia telangiectasia-mutated (ATM)-dependent senescence in p53-positive cancer cells. J Biol Chem. 2007; 282: 26759-66.
[25] Park SJ, Ahmad F, Philp A, et al. Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. Cell. 148: 421-33.
[26] Yeung F, Hoberg JE, Ramsey CS, et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. 2004; 23: 2369-80.
[27] Kuzmichev A, Margueron R, Vaquero A, et al. Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. Proc Natl Acad Sci U S A. 2005; 102: 1859-64.
[28] Bradbury CA, Khanim FL, Hayden R, et al. Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors. Leukemia. 2005; 19: 1751-9.
[29] Chen WY, Wang DH, Yen RC, Luo J, Gu W, Baylin SB. Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses. Cell. 2005; 123: 437-48.
[30] Li L, Wang L, Wang Z, et al. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell. 2012; 21: 266-81.
[31] Bolden JE, Peart MJ, Johnstone RW. Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov. 2006; 5: 769-84.
[32] Elknerova K, Myslivcova D, Lacinova Z, Marinov I, Uherkova L, Stockbauer P. Epigenetic modulation of gene expression of human leukemia cell lines - induction of cell death and senescence. Neoplasma. 58: 35-44.
[33] Di Bernardo G, Squillaro T, Dell'Aversana C, et al. Histone deacetylase inhibitors promote apoptosis and senescence in human mesenchymal stem cells. Stem Cells Dev. 2009; 18: 573-81.
[34] Gui CY, Ngo L, Xu WS, Richon VM, Marks PA. Histone deacetylase (HDAC) inhibitor activation of p21WAF1 involves changes in promoter-associated proteins, including HDAC1. Proc Natl Acad Sci U S A. 2004; 101: 1241-6.
[35] Matheu A, Klatt P, Serrano M. Regulation of the INK4a/ARF locus by histone deacetylase inhibitors. J Biol Chem. 2005; 280: 42433-41.
[36] Terao Y, Nishida J, Horiuchi S, et al. Sodium butyrate induces growth arrest and senescence-like phenotypes in gynecologic cancer cells. Int J Cancer. 2001; 94: 257-67.
[37] Jiang W, Guo Q, Wu J, et al. Dual effects of sodium butyrate on hepatocellular carcinoma cells. Mol Biol Rep. 39: 6235-42.
[38] Coradini D, Biffi A, Costa A, Pellizzaro C, Pirronello E, Di Fronzo G. Effect of sodium butyrate on human breast cancer cell lines. Cell Prolif. 1997; 30: 149-59.
[39] Sawa H, Murakami H, Ohshima Y, et al. Histone deacetylase inhibitors such as sodium butyrate and trichostatin A induce apoptosis through an increase of the bcl-2-related protein Bad. Brain Tumor Pathol. 2001; 18: 109-14.
[40] Ito N, Sawa H, Nagane M, Noguchi A, Hara M, Saito I. Inhibitory effects of sodium butyrate on proliferation and invasiveness of human glioma cells. Neurosurgery. 2001; 49: 430-6; discussion 6-7.
[41] dos Santos MP, Schwartsmann G, Roesler R, Brunetto AL, Abujamra AL. Sodium butyrate enhances the cytotoxic effect of antineoplastic drugs in human lymphoblastic T-cells. Leuk Res. 2009; 33: 218-21.
[42] Shankar S, Srivastava RK. Enhancement of therapeutic potential of TRAIL by cancer chemotherapy and irradiation: mechanisms and clinical implications. Drug Resist Updat. 2004; 7: 139-56.
[43] Khan O, La Thangue NB. HDAC inhibitors in cancer biology: emerging mechanisms and clinical applications. Immunol Cell Biol. 2012; 90: 85-94.
[44] Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett. 2008; 269: 315-25.

[45] Hosokawa N, Hosokawa Y, Sakai T, et al. Inhibitory effect of quercetin on the synthesis of a possibly cell-cycle-related 17-kDa protein, in human colon cancer cells. Int J Cancer. 1990; 45: 1119-24.
[46] Avila MA, Velasco JA, Cansado J, Notario V. Quercetin mediates the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468. Cancer Res. 1994; 54: 2424-8.
[47] Ranelletti FO, Maggiano N, Serra FG, et al. Quercetin inhibits p21-RAS expression in human colon cancer cell lines and in primary colorectal tumors. Int J Cancer. 2000; 85: 438-45.
[48] Braganhol E, Zamin LL, Canedo AD, et al. Antiproliferative effect of quercetin in the human U138MG glioma cell line. Anticancer Drugs. 2006; 17: 663-71.
[49] Siegelin MD, Reuss DE, Habel A, Rami A, von Deimling A. Quercetin promotes degradation of survivin and thereby enhances death-receptor-mediated apoptosis in glioma cells. Neuro Oncol. 2009; 11: 122-31.
[50] Kim EJ, Choi CH, Park JY, Kang SK, Kim YK. Underlying mechanism of quercetin-induced cell death in human glioma cells. Neurochem Res. 2008; 33: 971-9.
[51] Huang H, Lin H, Zhang X, Li J. Resveratrol reverses temozolomide resistance by downregulation of MGMT in T98G glioblastoma cells by the NF-kappaB-dependent pathway. Oncol Rep. 27: 2050-6.
[52] Priyadarsini RV, Vinothini G, Murugan RS, Manikandan P, Nagini S. The flavonoid quercetin modulates the hallmark capabilities of hamster buccal pouch tumors. Nutr Cancer. 63: 218-26.
[53] Rajendran P, Ho E, Williams DE, Dashwood RH. Dietary phytochemicals, HDAC inhibition, and DNA damage/repair defects in cancer cells. Clin Epigenetics. 2011; 3: 4.
[54] Priyadarsini RV, Vinothini G, Murugan RS, Manikandan P, Nagini S. The flavonoid quercetin modulates the hallmark capabilities of hamster buccal pouch tumors. Nutr Cancer. 2011; 63: 218-26.
[55] Lenz G, Goncalves D, Luo Z, Avruch J, Rodnight R, Neary JT. Extracellular ATP stimulates an inhibitory pathway towards growth factor-induced cRaf-1 and MEKK activation in astrocyte cultures. J Neurochem. 2001; 77: 1001-9.
[56] Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A. 1995; 92: 9363-7.
[57] Filippi-Chiela EC, Oliveira MM, Jurkovski B, Callegari-Jacques SM, da Silva VD, Lenz G. Nuclear morphometric analysis (NMA): screening of senescence, apoptosis and nuclear irregularities. PLoS One. 2012; 7: e42522.
[58] Jacques-Silva MC, Bernardi A, Rodnight R, Lenz G. ERK, PKC and PI3K/Akt pathways mediate extracellular ATP and adenosine-induced proliferation of U138-MG human glioma cell line. Oncology. 2004; 67: 450-9.
[59] Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol. 2007; 8: 729-40.
[60] van Heemst D, den Reijer PM, Westendorp RG. Ageing or cancer: a review on the role of caretakers and gatekeepers. Eur J Cancer. 2007; 43: 2144-52.
[61] Mah LJ, El-Osta A, Karagiannis TC. gammaH2AX: a sensitive molecular marker of DNA damage and repair. Leukemia. 2010; 24: 679-86.
[62] Mhaidat NM, Zhang XD, Allen J, Avery-Kiejda KA, Scott RJ, Hersey P. Temozolomide induces senescence but not apoptosis in human melanoma cells. Br J Cancer. 2007; 97: 1225-33.
[63] Yamada S, Matsuda R, Nishimura F, et al. Carnitine-induced senescence in glioblastoma cells. Exp Ther Med. 2012; 4: 21-5.
[64] Kuo PL, Chiang LC, Lin CC. Resveratrol- induced apoptosis is mediated by p53-dependent pathway in Hep G2 cells. Life Sci. 2002; 72: 23-34.
[65] Gao Z, Xu MS, Barnett TL, Xu CW. Resveratrol induces cellular senescence with attenuated mono-ubiquitination of histone H2B in glioma cells. Biochem Biophys Res Commun. 407: 271-6.

[66] Rusin M, Zajkowicz A, Butkiewicz D. Resveratrol induces senescence-like growth inhibition of U-2 OS cells associated with the instability of telomeric DNA and upregulation of BRCA1. Mech Ageing Dev. 2009; 130: 528-37.
[67] Tanigawa S, Fujii M, Hou DX. Stabilization of p53 is involved in quercetin-induced cell cycle arrest and apoptosis in HepG2 cells. Biosci Biotechnol Biochem. 2008; 72: 797-804.
[68] Passos JF, Nelson G, Wang C, et al. Feedback between p21 and reactive oxygen production is necessary for cell senescence. Mol Syst Biol. 6: 347.
[69] Asensi M, Medina I, Ortega A, et al. Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radic Biol Med. 2002; 33: 387-98.
[70] de Boer VC, Dihal AA, van der Woude H, et al. Tissue distribution of quercetin in rats and pigs. J Nutr. 2005; 135: 1718-25.
[71] Minamiyama M, Katsuno M, Adachi H, et al. Sodium butyrate ameliorates phenotypic expression in a transgenic mouse model of spinal and bulbar muscular atrophy. Hum Mol Genet. 2004; 13: 1183-92.

3.3. CAPÍTULO III - An approach to integrate cancer and senescence: a network analysis of telomerase

José Eduardo Vargas, Guido Lenz, Diego Bonatto

Periódico: **Biogerontology**

Estado: **Em preparação para submissão**

An approach to integrate cancer and senescence: a network analysis of telomerase

José Eduardo Vargas[1], Guido Lenz[1,*], and Diego Bonatto[2,*]

[1]*LABSINAL - Laboratory of Cellular Signaling and Plasticity, Department of Biophysics and Center of Biotechnology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, Porto Alegre, Brazil.*

[2]*Laboratory of Molecular Radiobiology, Department of Molecular Biology and Biotechnology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, Porto Alegre, Brazil.*

***Send correspondence to:**

Guido Lenz, Centro de Biotecnologia da UFRGS - Sala 114, Departamento de Biofisica, Universidade Federal do Rio Grande do Sul – UFRGS, Avenida Bento Goncalves 9500 - Predio 43421, Caixa Postal 15005, Porto Alegre – Rio Grande do Sul, BRAZIL, 91509-900, Phone: (+55 51) 3308-7613, Fax: (+55 51) 3308-7309, E-mail: lenz@ufrgs.br

Diego Bonatto, Centro de Biotecnologia da UFRGS - Sala 114, Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul – UFRGS, Avenida Bento Goncalves 9500 - Predio 43421, Caixa Postal 15005, Porto Alegre – Rio Grande do Sul, BRAZIL, 91509-900, Phone: (+55 51) 3308-6080, Fax: (+55 51) 3308-7309, E-mail: diegobonatto@gmail.com

Abstract

Transient and permanent cell cycle arrests are important keys to understanding the intricate signaling pathways that protect organisms against the uncontrolled proliferation of cells. One of the challenging problems in biology and medicine is to understand how pre-cancerous cells enter senescence and how cancer cells bypass cell senescence. Key to understanding senescence induction and bypass are the telomeres and the telomerase (TERT) complex that is responsible for their synthesis. In this paper, we designed a human cancer-senescence network to study the relationship between senescence and cancer, focusing on TERT. Specifically, we integrated human protein-protein interaction pathways associated with replicative senescence (RS) and oncogene-induced senescence (OIS). The objectives of this work are (i) to topologically characterize TERT between RS and OIS and (ii) to develop a model of the mechanisms that regulate TERT in cancer cells. We shown direct interaction among TERT and, oncogenes such as MYC, E2F1, AKT1 and ABL1, which can induce OIS, are directly associated with TERT. We showed a strong metabolic association between these oncogenes and OIS activity through network and gene ontology analysis. Moreover, these oncogenes also act as transcriptional and/or post-transcriptional regulators of the catalytic subunit of TERT. Additionally, the results from a centrality analysis and microarray data derived from tumors were used to design a dynamical model for TERT regulation in cancer. For these motives, we suggest a hypothetical modelo were TERT expression may be transiently and induced by reactive oxygen species (ROS), therefore increasing the survival of different tumor types. This network-based study provides new evidence for the intricate relationship between senescence and cancer with an integrative perspective.

Keywords: Systems Biology, senescence, telomerase, cancer and oxidative stress.

1. Introduction

Senescence is defined as an irreversible cell cycle arrest that occurs in normal cells after a limited number of cell divisions (Hayflick 1965; Dimri et al. 1995). Senescent cells do not respond to mitogens, and they display specific morphological changes, such as increased cytoplasmic granularity, increased size and modified nuclear structure but remain viable and metabolly active (Shelton et al. 1999). Therefore, senescence is considered part of an extensive network of mechanisms that evolved to protect multicellular organisms from cancer (Collado and Serrano 2010; Lenz 2012)

Dysfunctional telomeres play a critical role to induce senescence in normal cells and progression of human cancer. When DNA damage (DNA double-strand break or DBS) checkpoint responses (DDRs) are intact and persistent, telomere attrition active a physiological form of senescence known as replicative senescence (RS). However, telomerase (TERT) confers indefinite proliferative potential to in vitro cultured cells by promoting the maintenance of chromosome ends, and thus preventing critical telomere erosion associated with senescence (Greider and Blackburn. 1985; Bodnar et al. 1998; Kang et al. 2004). For this reason, TERT is consider as a gerontogene in the context of the organism, as illustrated by both TERT-deficient mice and by human diseases due to mutations in TERT components (Armanios et al. 2007; Blasco et al. 1997; Garcia-Cao et al. 2006; Mitchell et al. 1999; Tsakiri et al. 2007; Yamaguchi et al. 2005). Additionally, TERT appears to play a central role in tumorigenesis, as suggested by the observation that TERT activity is elevated in more than 80% of human tumors (Kim et al. 1994), while hTERT (catalytic subunit of TERT) inhibition induces senescence in multiple cancer types (Datta 2006).

On the other hand, cellular senescence can also be induced prematurely before telomere shortening due to continuous cell proliferation becomes growth limiting. Dysregulated oncogenes, for example, cause cells to undergo oncogene-induced

senescence (OIS) after a brief period of hyperproliferation (Di Micco et al. 2006). Oncogenic signals also cause high levels of DNA replication stress, which leads to the formation of DSBs and activation of a persistent DDR (Bartkova et al. 2006; Di Micco et al. 2006) but no affect the telomere regions (Hornsby 2007).

Both RS and OIS are considered different biological processes, where RS is a telomere-dependent menchanism and OIS is not telomere-dependent. However, a recent study shown that oncogenic signalling, frequently associated with initiating cancer growth in humans, dramatically affected telomere structure and function by causing telomeric replication stress, rapid and stochastic telomere attrition, and consequently telomere dysfunction in cells that lack hTERT activity. Moreover, hTERT expression also contributes to low DDR activity observed in malignant cancers by suppressing the formation of telomeric DDR foci caused by oncogene-induced telomeric replication stress and bypass OIS (Suram et al. 2012). Thus, TERT expression can be a necessary event to bypass these senescence forms. These evidences suggest that the association among RS, OIS and TERT could be an intricate process, involving the activation of multiple molecular mechanisms.Therefore, an integrative view of TERT expression and function is important to understand the role of senescence in human cancer avoidance and development.

In this paper, we applied different systems biology strategies to two major purposes: (i) to define the association of TERT and oncogenes with OIS activity based on topological analysis of networks focused in RS and OIS and (ii) to develop a hypothetical model of the mechanisms that regulate and activate TERT in human cancer cells.

2. Materials and methods

2.1. Protein-protein network design and global topological analysis

An overview of the methods is presented in Figure 1. An initial data mining screen and network design of protein–protein interaction (PPI) networks associated with senescence and TERT were carried out using Cytoscape version 2.6.3 (Shannon et al. 2003). An initial search of the principal genes/proteins associated with senescence and TERT was performed using multiple biological and literature databases, including GeneCards [http://www.genecards.org/], PubMed [http://www.pubmed.com], the Glioblastoma database (GBMbase) [http://beta.gbmbase.org/page/Welcome/display], the Breast Cancer database [http://www.breastcancerdatabase.org/], and microarray meta-analysis data stored in the GenAge database [http://genomics.senescence.info/genes/] (de Magalhães and Toussaint 2004). Numerical identification of each protein (Supplementary Table 1) was defined according to Human Aging Genomic Resources- HAGRID (de Magalhães and Toussaint. 2005; De Magalhaes et al. 2009) (Figure 1).

The Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING 9.0) and the Biological General Repository for Interaction Datasets (Biogrid) release 3.1.80 (Von Merin et al. 2003 and Stark et al. 2006, respectively) were used to design the human PPI networks. A score ≥ 0.07 (excluding the option based on text mining) was used for all PPI networks. The Cytoscape core plugin Merge was used to merge the individual PPI networks [http://www.cytoscape. org/plugins2.php] into the final PPI networks (Figure 1). Additionally, the Cytoscape core plugin Intersection was used to define node connectivity in the individual networks [http://www.cytoscape.org/plugins2.php]. To verify the presence of major clusters, we used the Molecular Complex Detection (MCODE; Bader and Hogue 2003) and Clustering with Overlapping Neighborhood Expansion (CLUSTER-ONE; Nepusz et al. 2012) software (Figure 1).

The parameters used for MCODE were as follows: loops included; degree cutoff 2; deletion of single connected nodes from cluster (haircut option enabled); expansion of cluster by one neighbor shell allowed (fluff option enable); node density cutoff 0.1; node score cutoff 0.2; kcore 2; and maximum depth of network 100 (Figure 1). CLUSTER-ONE parameters were based on the "growing" of dense regions out of small seeds guided by a quality function. The quality of a group was evaluated by the number of internal edges divided by the number of edges involving nodes of the group (Nepusz et al. 2012).

Once the major PPI network was obtained, we expanded the network data by applying the Biogrid software, which leads to a maximal saturation of TERT followed by the selection of proteins for the PPI network design.

2.2. Gene ontology analysis

To acquire information about the biological processes related to senescence in human cells, a gene ontology (GO) clustering analysis was performed for all of the proteins described in the motifs that belong in the UNION PPI network (Figure 1). This analysis was conducted using the latest version of the Biological Network Gene Ontology (BiNGO; Maere et al. 2005, Figure 1) for statistical evaluation of groups of proteins with respect to the present annotations available in the Gene Ontology Consortium [http://www.geneontology.org]. The degree of functional enrichment for a given cluster and category was quantitatively assessed (*p*-value) by hypergeometric distribution (Rivals et al. 2007) with a multiple test correction applied using the false discovery rate (FDR) algorithm in the BiNGO software (Benjamini and Hochberg 1995). Over-represented biological process categories were generated after FDR correction, with a significance level of 0.05.

2.3 Centrality parameters

For the centrality parameter analysis, the plug-in Centiscape (Scardoni et al. 2009) [http://www.cytoscape. org/plugins2.php] was used to identify the nodes that are relevant from both experimental and topological viewpoints. This algorithm also provided a Boolean logic-based tool that allows easy characterization of nodes whose topological relevance depends on more than one centrality. Moreover, different graphical outputs for each computed centrality are represented to show biological significance, which allows easy node categorization and experimental prioritization.

In this sense, all PPI networks were analyzed according to two parameters of node centrality, degree and betweenness. Node degree represents the simplest centrality measure in a given network, corresponding to the number of nodes adjacent to a given node, where adjacent nodes directly connect (Scardoni et al. 2009). The node degree represents the "popularity" of a given node in a network. The other parameter, betweenness, indicates to what extent a specific node is between all other nodes within the network (Newman 2005). By applying these two parameters, it is possible to distinguish four major groups of nodes within a network: (i) hub-bottleneck (HB), (ii) non-hub-bottleneck (NH-B), (iii) hub-non-bottleneck (H-NB), and non-hub-non-bottleneck (NH-NB) (Yu et al. 2007). Nodes that belong to the HB group tend to correspond to highly central proteins that connect several complexes or are peripheral members of central complexes. Nodes were classified based on the threshold node degree and the betweenness values. HB or bottleneck nodes have high node degree and betweenness values, H-NB nodes have high node degree values and low betweenness values, NH-B nodes have low node degree and high betweenness values, and NH-NB nodes have low node degree and betweenness values. To confirm the results obtained with CENTISCAPE, we used the Hub Objects Analyzer (CYTO-HUBBA) plug-in (Chung-Yen et al. 2008) available at [http://www.cytoscape. org/plugins2.php]. The

cyto-Hubba software allows one to predict proteins that act as bottlenecks in the network. The majority of hub proteins were identified amongst the top 30 bottlenecks (Figure 1).

2.4 Cancer microarray databases

We used a data integration framework, Anduril, for translating fragmented large-scale data, available at [http://csbl.fimm.fi/anduril/site]. This workflow automates data in order to facilitate the prospection of large-scale data results from the Cancer Genome Atlas-TCGA [http://cancergenome.nih.gov/]. It is important to note that Anduril use a robust molecular and clinical data from 600 subjects having glioblastoma, 919 patients with breast carcinoma, 423 patients with colorectal adenocarcinoma and 597 patients with ovarian cancer to analyze differential gene expression. Fold changes of genes were computed by dividing the mean tumor expression value by the mean normal tissue expression value (Ovaska et al. 2010). We consider a fold change threshold ≥ 2.0 indicative of up-regulated genes, a value of 1 indicative of neutral expression and a value ≤ 0.13 indicative of down-regulated genes. The t-test between cancer and control, followed by multiple hypothesis correction, were used for comparative analysis (Benjamin 1995; Ovaska et al. 2010). It is important to specify that the tumors types used in this work display high TERT activity, as shown by Skvortzov et al (2011).

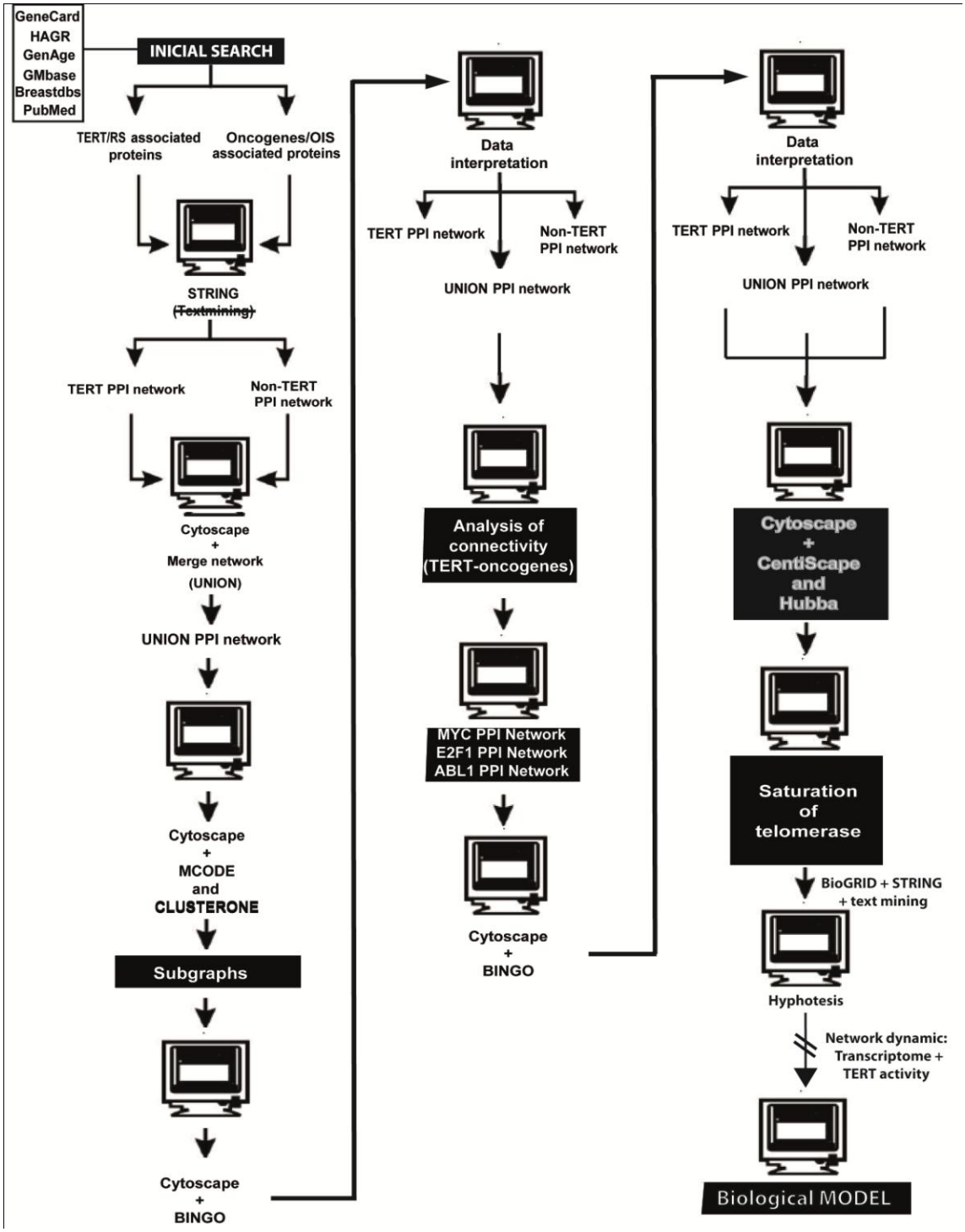


Figure 1. Experimental approach employed to retrieve cellular senescence and telomerase (TERT) from interactomic data available in different senescence and cancer databases. Human Aging Genomic Resources (HAGR-ID), cancer databases, PubMed and the Search Tool for the Retrieval of Interacting Genes/Proteins database (STRING) were used to design a PPI network. Interactome data were obtained from an initial query using RS and OIS associated proteins, resulting in a TERT protein–protein interaction (PPI) network and a non-TERT PPI network. These two networks were further analyzed using Cytoscape software, and the union function of the Merge networks plugin was used to fuse both networks into a unique PPI network. The MCODE and CLUSTER-ONE plugins were used to identify subgraphs present in the Union PPI network. GO clustering analysis was performed for all proteins described in the

graphs to acquire information about the biological processes related to TERT in RS and OIS in *homo sapiens*. Moreover, TERT-connectivity was analyzed using the intersection networks plugin together with saturation of oncogenes with OIS activity through Biogrid. Each oncogenic PPI network was analyzed by GO. Finally, principal hubs were analyzed with the CENTISCAPE and CYTO-HUBBA plugins. Oxidative genes and interactors of TERT were also analyzed using a database of gene expression signatures of four human tumor types. The information retrieved from literature data mining and network analysis was employed to design a model of how TERT functions in cancer cell homeostasis.

3. Results and discussion

To connect TERT within RS and OIS we mapped the main proteins and genes involved in senescence and designed small PPI networks. Initially, diffusible senescence factors proteins involved in major processes related to senescence (DNA damage, telomere stability -telosome/shelterin complex-, cell cycle and energy metabolism) were used to prospect networks from the STRING 90 platform based on the presence or absence of TERT (Supplementary Table 1). Thus, we constructed initial networks called TERT and non-TERT (Figure 2). The TERT PPI network contains 57 nodes and 137 connectors (Figure 2A). Similarly, oncogenes and genes associated with OIS (but not TERT) were used as the initial seeds to prospect a non-TERT PPI network containing 236 nodes and 1424 connectors (Figure 2B). This network, the non-TERT PPI network, is represented by OIS and other oncogenic proteins (Figure 2B). This network contains major oncogenes associated with cancer initiation, such as ABL1 (Sirvent et al. 2008); AKT1, 2, and 3 (Testa et al. 2008), STATs (Yu et al. 2009); MYC (Dang et al. 2012); E2F1 to 4 (Chen et al. 2009); and known oncogenes that induce OIS, such as RAS and BRAF (Serrano et al. 1997; Dhomen et al. 2009). Once generated, the individual PPI networks (TERT PPI and non-TERT PPI) were merged, producing a third network, Union PPI, containing 280 nodes and 1709 connectors (Figure 2C).

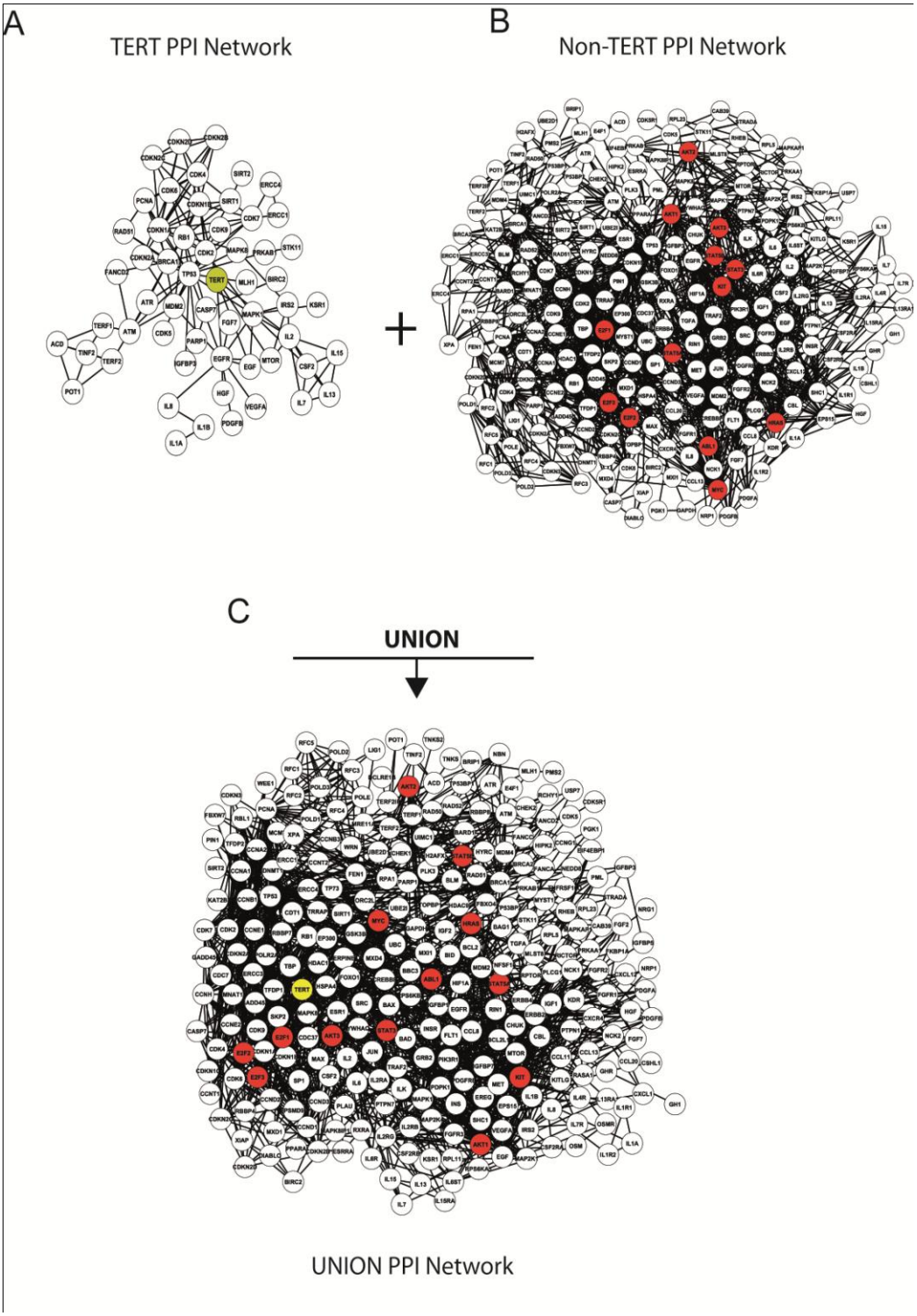


Figure 2. TERT, non-TERT and UNION graphical representation of the topological PPI networks. Telomerase (TERT) is highlighted in yellow, and oncogenes are highlighted in red.

Once the major UNION PPI network was generated, sub-network numbers and types were made using CLUSTER-ONE software (Supplementary Figure 1A). Three

sub-networks were obtained and identified by GO analysis: (i) Cell proliferation/cell signal transduction, (ii) Cell cycle and (iii) DNA repair. Interestingly, TERT was found as an unclustered protein, indicating that it does not belong to any of the identified subnetworks (Supplementary Figure 1A). Thus, TERT can be an independent protein in this topological network with different functions from the proteins present in the UNION PPI network. Additional modular analysis of the UNION PPI network with MCODE software showed similar results (Supplementary Figure. 1B).

A different strategy based on direct connectivity between TERT and oncogenes was performed to identify all oncogenes associated with TERT. Four oncogenes (MYC, E2F1, AKT1 and ABL1) showed direct connectivity with TERT (Figure 3). To understand the link among TERT, OIS and RS, we performed a literature review and topological analysis to focus on TERT, these oncogenes and their OIS activities.

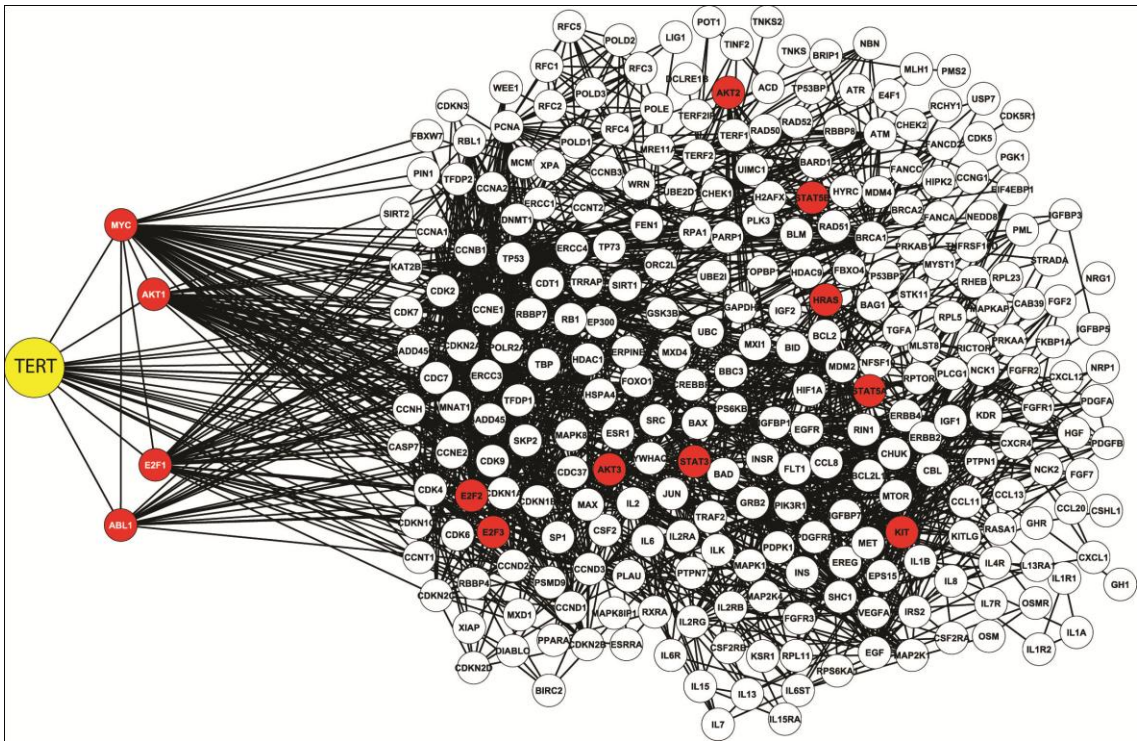


Figure 3. Connectivity analysis of TERT in the UNION PPI network. UNION PPI network oncogenes directly associated with TERT node.

3.1. TERT, RS and oncogenes: a metabolic link.

The oncogenes MYC, E2F1, AKT1 and ABL1 are directly associated with TERT and have the capacity to induce OIS (Zhuang et al. 2008; Dimri et al. 2000; Nogueira et al. 2008; Wajapeyee et al. 2012; respectively).

We chose to generate a PPI network for each TERT-associated oncogene by growing with BIOGRID software until saturation (Supplementary Figure 2). An individual analysis of these networks a indicated the biological processes where TERT is included are correlated directly with cellular metabolism. These four PPI networks represent three common biological process with lower *p*-value (major biological significance), as identified by GO analysis (Table **1**), (i) Metabolic process; (ii) nucleobase, nucleoside and nucleotide metabolic processes/DNA replication and (iii) biosynthesis processes.

Table 1. Specific GO classes derived from MYC, E2F1, ABL-1 and AKT1 PPI networks observed in the *Homo sapiens* interactome network.

ID PPI Network	**GO biological processes category**	**GO number**	***p*-value**	**Corrected *p*-value[a]**	***k*[b]**	***f*[c]**

MYC PPI	Biopolymer metabolic process	43283	1.13×10^{-25}	9.19×10^{-23}	89	108
	Nucleobase, nucleoside and nucleotide metabolic processes/DNA replication	6139	3.76×10^{-27}	1.53×10^{-24}	72	108
	Primary metabolic process	44238	4.78×10^{-20}	2.17×10^{-28}	93	108
E2F1 PPI	Biopolymer metabolic process	43283	1.06×10^{-19}	5.26×10^{-16}	52	62
	Biopolymer biosynthesis process	43284	1.12×10^{-18}	5.12×10^{-18}	38	62
	Nucleobase, nucleoside and nucleotide metabolic processes/DNA replication	6139	9.12×10^{-17}	3.55×10^{-15}	42	62
ABL1 PPI	DNA metabolic process	6259	1.56×10^{-8}	6.65×10^{-6}	15	78
	Biopolymer metabolic process	43283	1.73×10^{-7}	5.09 x 10-6	44	78
	Macromolecule metabolic process	43170	1.74×10^{-6}	3.99×10^{-5}	49	78
AKT1 PPI	Biopolymer metabolic process	43283	2.75×10^{-11}	$2.92 \times 10^{--9}$	61	102
	Macromolecule process	43170	1.64×10^{-8}	4.64×10^{-7}	65	102
	Primary metabolic process	44238	1.45×10^{-5}	1.89×10^{-4}	66	102

Evidence from studies of early-stage human tumors and animal models on OIS induced by MYC, E2F1, ABL-1 and RAS suggests that oncogene-induced replication stress activates a DDR, which in turn activates p53 and induces senescence (Bartkova et al. 2005; Robinson et al. 2009; Gonfloni 2010; Campaner et al. 2012; Di Micco et al. 2006). Thus, DNA replication stress produces an inefficient DNA replication that causes the DNA replication forks to progress slowly or stall. Factors that cause replication stress and replication stress-induced DNA damage include alterations in pools of dNTP precursors required for DNA synthesis, changes in the expression of proteins required for synthesis of dNTPs or DNA, and a decreased frequency of initiation of DNA replication (producing larger replicons), as reviewed by Burhans and Weinberger (2007). Additionally, premature stress mediated in a DDR-independent pathway was shown for ABL-1, suggesting that signals leading to senescence in ways that are not clearly described in the literature (Wajapeyee et al. 2012).

Furthermore, AKT1 was shown to induce senescence by DNA damage-independent and dependent mechanisms. Thus, AKT1 can promote rapid proliferative arrest in the absence of a hyperproliferative phase (DNA damage), principally by mTORC1 activation in PTEN-deleted cells (Astle et al. 2012). Additionally, AKT1 activation induces premature senescence in p53-proficient MEFs by DDR (Nogueira et al. 2008).

According our analysis based on gene ontology, TERT and these oncogenes have a metabolic association. Additional, OIS alter the cellular homeostase independent of DDR activation (mediated DNA replication stress).These oncogenes (MYC, E2F1, AKT1 and ABL-1) increase the production of ROS and induce genome instability (Vafa. 2002; Raimundo et al. 2012; Nogueira et al. 2008; Sattler et al. 2000,

respectively). However, hTERT subunit affects the mitochondrial homeoestase in a different manner. TERT has been showed that is transported into the mitochondrial matrix binds to mitochondrial DNA coding for complex I genes and increases complex I respiratory efficiency (Haendeler et al., 2009) and reduce the level of ROS (Indran et al. 2010)

However, the dynamics of TERT transcription and post-translation modifications necessary to avoid RS and OIS are not clear considering mitochondrial metabolism. A new strategy based on the centrality analysis was used to determine the biological relevance of TERT in network focused in senescence based on its connectivity in all senescence PPI networks.

3.2 Parameters of Centralities: Positional analysis of TERT in topological networks of senescence and cancer

The centralities of the TERT (Figures. 4A and B), non-TERT (Figures. 4C and D) and UNION PPI networks (Figures. 4E and F) were analyzed with the Cytoscape plugin CENTISCAPE considering the mathematical mean generated by each parameter of centrality, degree and betweenness (Supplementary Table 2). We also utilized the software CYTO-HUBBA to avoid false positives and to define the bottleneck status of TERT. Interestingly, TERT is positioned on the mean line that separates hub-bottleneck (H-B) from non hub-non bottleneck (NH-NB), and it is near to the mean that separates H-NB in the TERT PPI network (Figure 4A). Similarly, CYTO-HUBBA displays TERT with a mean score of centrality (Figure 4B).

The centralities of the UNION PPI network also indicated that TERT is an NH-NB protein. This result is supported by CYTO-HUBBA analysis, which showed that TERT is not found among the 30 major bottlenecks (Figures 4E and F).

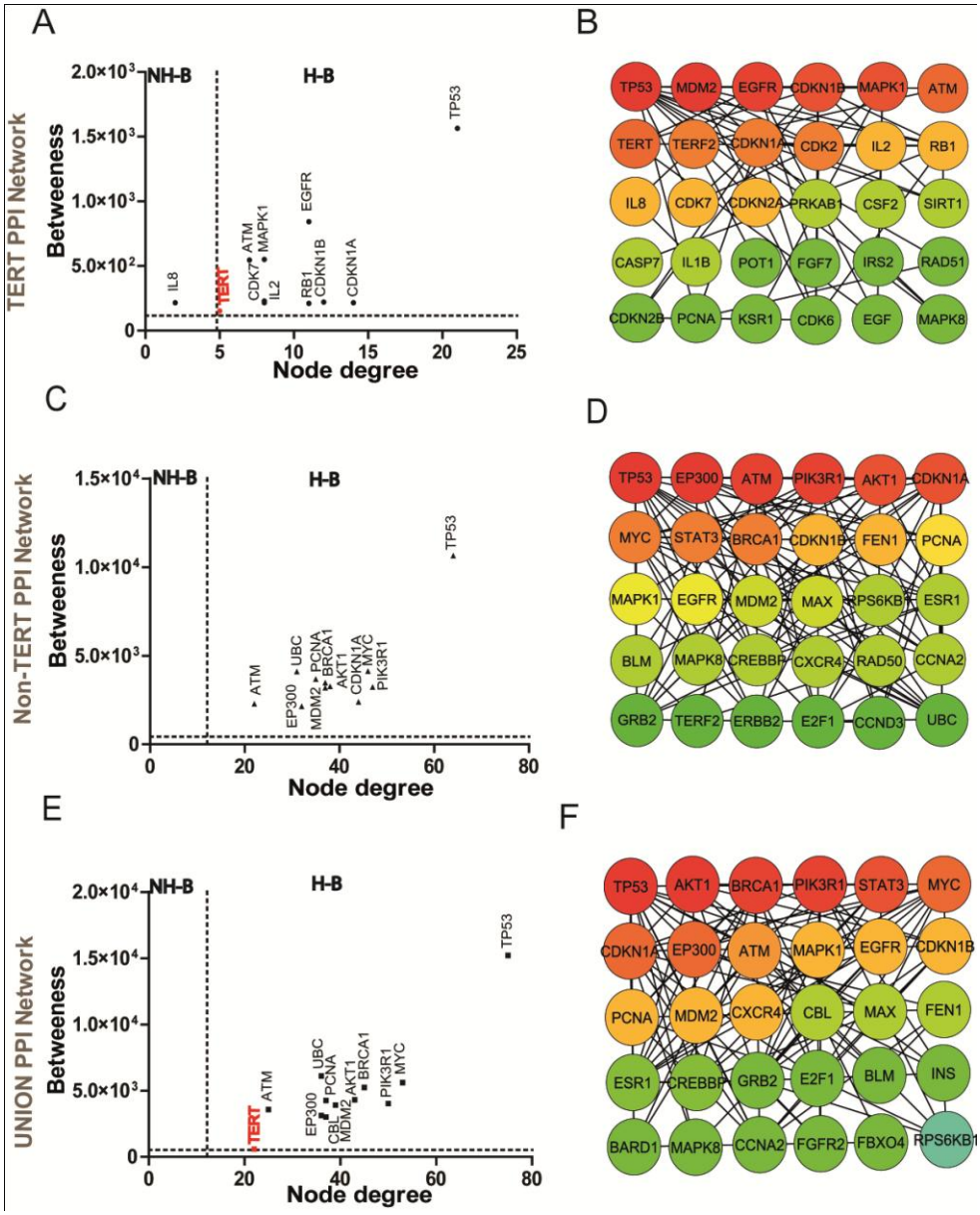


Figure 4. Centrality analysis (A to D) of all PPI networks. Dashed lines represent the threshold value (mathematical mean) calculated for each centrality. (A) and (B) represent the TERT PPI network centrality analyzed using the CENTISCAPE and CYTO-HUBBA plugins, respectively. (C) and (D) represent the non-TERT PPI network, and (E) and (F) represent the UNION PPI network. (C) and (E) show network analyses using the CENTISCAPE plugin, and (D) and (F) show network analyses using the CYTO-HUBBA plugin. This ultimate plugin represent nodes in a graphical view with a *color* scheme to show priority, red represent highest value of centrality, yellow shown intermediate value of centrality and green low centrality value. Only TERT and the first eleven proteins with HB and NH-B scores are indicated. Legend: hub-bottleneck (HB); non hub-bottleneck (NH-B).

It is important to note that we chose not to grow the network until saturation because many proteins that directly connect to TERT could have functions that are not

correlated to cellular senescence itself and could introduce a bias in the analysis. However, to analyze the H-NB condition of TERT, we assayed the maximum saturation of the TERT node by means of Biogrid and STRING databases using the TERT PPI network (Supplementary Figures 3A and B). These data indicate that even in those conditions of saturation, TERT it is still classified as H-NB (Supplementary Figure 3C).

The fact that TERT is an H-NB node indicates that this protein connects few protein complexes. These data suggest that TERT is strongly regulated by a low number of key proteins.

To understand the mechanism of TERT activation or repression and the role played by the oncogenes MYC, E2F1, AKT1 and ABL1 in these processes, it is essential to analyze the regulations that impinge on TERT. In addition, a balance between TERT transcription and translation and a gain in each terminal tract of telomeric repeats suggests a dynamic process. Insights have been gained into the cellular pathways for biogenesis of TERT ribonucleoprotein, but an integrative analysis based on interactome data has not been developed. Thus, we used different Systems Biology strategies to design a possible model of TERT regulation.

3.3. TERT: A dynamic model process to avoid RS and OIS based on oxidative stress

To build a dynamic model of how TERT and its partner proteins interact, we considered all interactors of TERT present in the UNION PPI network (Figure 5A) and a TERT-associated subnetwork using the combined analyses with Biogrid and STRING software (Figure 5B). Interestingly, the data gathered from this analysis indicated that the majority of proteins are associated with regulation of the hTERT subunit (Figure 5A and B). These sub-networks were prospected in terms of major groups of proteins and biological functions, showing the presence of five major protein groups: (i) transcriptional transactivators, (ii) transcriptional repressors, (iii) proteins associated

with telomere structure, (iv) proteins associated with posttranslational modification and (v) proteins that physically interact with hTERT (Table 2).

Table 2. Characterization of pathways and proteins associated with hTERT transcription and posttranslational modifications.

Transcriptional transactivator	**Pathways**	**References**
AKT1	PIK3/AKT/NFκB	(Kang et al. 1999; Bai et al. 2009)
ARNT	Hypoxia inducible factor 1 (HIF-1)	(Nishi et al. 2004)
CCND1		
CREBBP	Cell cycle	(Leng et al. 2002)
EGF	Complex MYC/EP300	(Cong et al. 1999; Faiola et al. 2005)
EGFR		
EP300		(Maida et al. 2002; Wang et al. 2008)
HIF1A	EGF/EGFR/AKT/ErbB	(Wang et al. 2008)
MAX	EGF/EGFR/AKT/ErbB	(Cong et al. 1999; Faiola et al. 2005)
MYC	Complex MYC/EP300/	(Anderson et al. 2006; Nishi et al. 2004)
IL-2	IL-2/AKT1	(Skvortzov et al. 2011)
NFκB	PIK3/AKT/NFkB	(Skvortzov et al. 2011)
Ruvbl1	Not described	(Baek et al. 2008)
SP1	Cell cycle	(Skvortzov et al. 2011)
WRAp53	p53	(Mahmoudi et al. 2009)
Transcriptional repressors	**Pathways**	**References**
CDKN1B	Cell cycle	(Ray et al. 2009)
E2F1	Cell cycle	(Won et al. 2002; Stanelle et al.

		2002)
FOS	Complex FOS/Jun	(Takakura et al. 2005)
HDAC1	Histone deacetylation	(Polevoda et al. 2000; Cong et al. 2000)
IFNAR2	Interferon	(Xu et al. 2000)
IFNG	Interferon	(Hussein et al. 2003)
IRF1	Interferon/CDKN1B	(Lee et al. 2005)
JUN	Complex FOS/Jun	(Takakura et al. 2005)
MXD1	MYC/MXD1	(Xu et al. 2001)
PML	Interferon/PML	(Oh et al. 2009)
RBBP4	RB/E2F1	(Nicolas et al. 2000)
RBBP7	RB/E2F1	(Nicolas et al. 2000)
SAP18	SAP18/SIN3A/HDAC1	(Zhang et al. 1997)
SAP30	SAP30/HDAC1	(Sichtig et al. 2007)
SIN3A	SAP18/SIN3A/HDAC1	(Zhang et al. 1997)
SIN3B	SAP18/SIN3A/HDAC1	(Zhang et al. 1997)
SP3	SP1/SP3	(Won et al. 2002)
WT1	Mixed	(Sitaram et al. 2010)
PTGES3	HSP90	(Woo et al. 2009)
Proteins associated to telomeres and hTERT subunit	**Functions**	**References**
HNRNPC	Regulation of telomere extension	(Ford et al. 2002)
MCRS1	Probable cell cycle/telomere relationship	(Song et al. 2004)
NCL	TERT transport	(Khurts et al. 2004)
PIF1	Removes telomerase from telomeres	(Schulz et al. 1994; Chang et al. 2009)
XRCC5	Induces the access of	(Chai et al. 2002)

	telomerase to telomeres	
XRCC6	Regulates the access of telomerase to telomeres	(Chai et al. 2002)

Posttranslational modifications	**Functions**	**References**
ABL1	Phosphorylates hTERT (inhibition TERT activity)	(Kharbanda et al. 2000)
HUS1	Phosphorylation by mTOR complex	(Francia et al. 2009)
HSP90AA1	Phosphorylation by mTOR complex	(Toogun et al. 2008)

This information was synthesized into a dynamic model of hTERT function (Figure 5C). From the many observed hTERT-associated proteins, some partners can be highlighted. For example, the histone deacetylase 1 protein (HDAC1) appears to be an important regulator of hTERT expression. Aberrant expression of HDACs has been observed in various tumor types (Sakuma et al. 2006; Polevoda et al. 2000), implicating an altered balance of protein acetylation as a major factor that contributes to cell transformation and cancer initiation/progression. Additionally, histone deacetylation leads to a decrease in hTERT expression, while overexpression of HDAC1 leads to the suppression of hTERT-promoter activity (Cong et al. 1999). On the other hand, the pharmacologic inhibition of histone deacetylation by Trichostatin A results in the activation of hTERT expression in normal cells. Considering the link between histone deacetylation and hTERT, it is reasonable to suggest that, in the case of cancer, histone deacetylation represses the hTERT promoter (Cong et al. 1999) but prevents cellular transformation in normal cells (Kyo et al. 2002). Consistent with this hypothesis, upstream chromatin modification of the hTERT gene significantly alters TERT expression (Zhu et al. 2010).

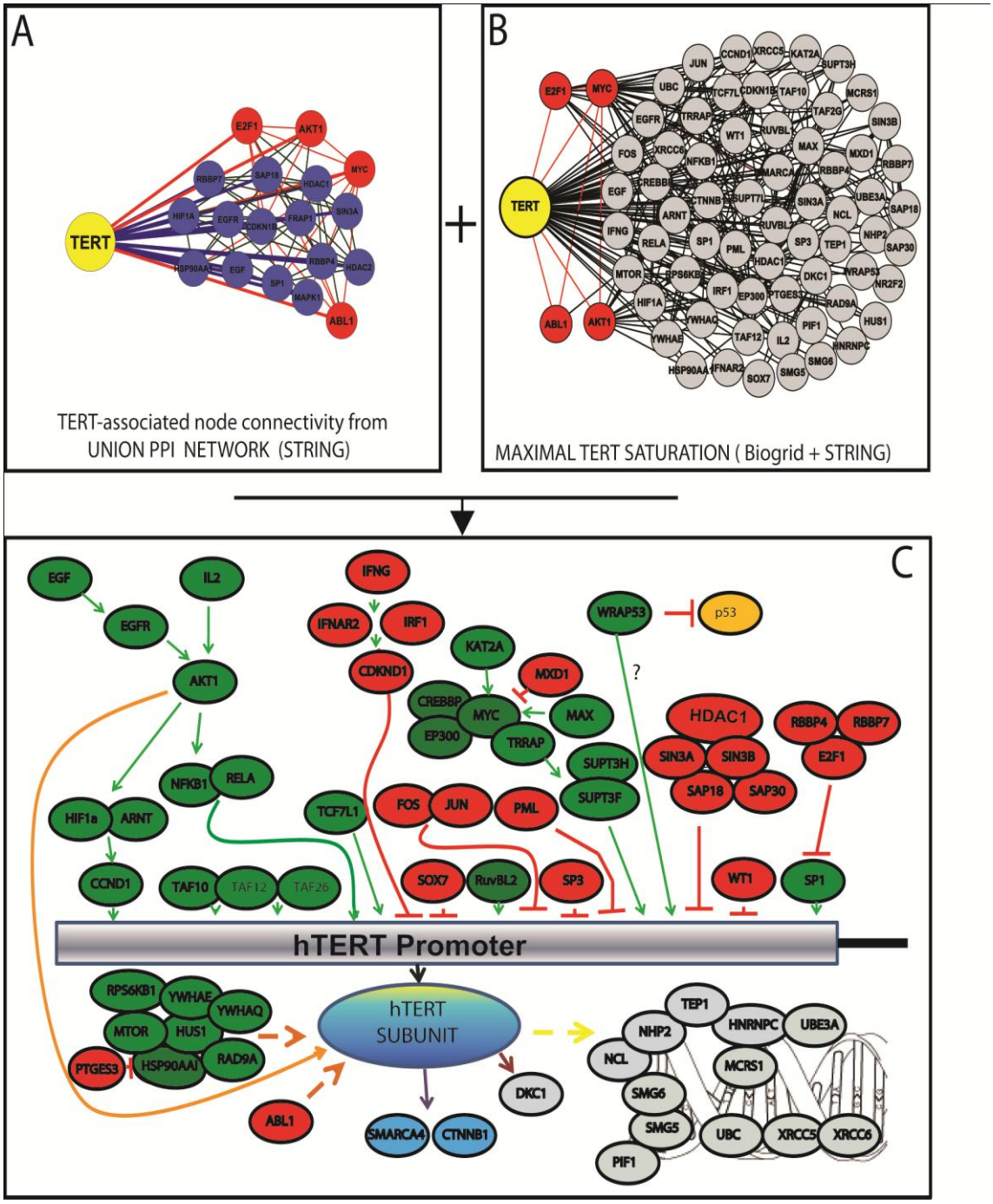


Figure 5. Regulation of the hTERT promoter according to the literature and interactome data. (A) Representation of all nodes associated with TERT in the UNION PPI network. (B) Maximal saturation of the TERT node using Biogrid and STRING. (C) Representation of pathways associated with hTERT promoter activation or repression and posttranslational modifications of hTERT subunit based on the cancer literature. Green nodes represent activators and red nodes represent repressors of hTERT activity. Gray nodes represent proteins associated with telomere regions, blue nodes represent proteins with physical associations to TERT, and orange nodes represent additional proteins. Physical association with the promoter region favoring hTERT transcription or protein-protein activation (green ⟶); Physical association with the promoter region favoring hTERT repression or protein-protein repression (red ⟝); posttranslation modifications (orange ⟶); physical association of proteins with the hTERT subunit (orange⟶); Proteins associated with telomeres and the hTERT subunit (yellow⟶).

Considering the ‘‘free radical” or “oxidative stress” theory of aging suggests that aging are the result of the accumulation of molecular damage caused by ROS during normal metabolism (Satre et al. 2003; Sedensky and Morgan 2006; Saretzki 2009).

It is noteworthy that HDAC1 activity can be modulated by ROS, leading to changes in hTERT gene expression (Trachootham et al. 2008.). The production of ROS can affect the regulation of transcription factors by direct modification of critical amino acid residues (Kregel and Zhang 2007). For example, a covalent modification of HDAC1 thiol groups (Cys261 and Cys273) by ROS attenuated histone deacetylase activity and changed the acetylation pattern of histones H3 and H4 in different transformed/tumor cell lines (Trachootham et al. 2008). Thus, HDAC1 can be defined as a redox sensor (Kato et al. 2009).

Studies in melanoma cells showed that inducible MYC expression could be used to control the expression of the GSH synthetase, and apoptosis induced by downregulation of MYC was associated with cellular depletion of reduced GSH (Biroccio et al. 2004). These data suggest that cells with active MYC may survive the ROS stress by upregulating GSH synthesis. Moreover, ROS appear not to affect the structure and function of MYC, the major activator of hTERT transcription (Biroccio et al. 2004; Bazarov et al. 2009). Thus, it is conceivable that, during malignant transformation, oncogenic signals both induce ROS generation to stimulate cell proliferation through redox-sensitive transcriptional factors and promote antioxidant adaptive mechanisms to minimize oxidative damage.

We proposed a two-part model to explain ROS-associated regulation of hTERT functions and expression and the fact that pre-cancerous and cancer cells bypass RS. Initially, the transcription/translation of the hTERT gene product leads to telomere extension, which is a consequence of activation of the hTERT promoter region

principally due to HDAC1 loss and association of transactivators. After telomere extension, hTERT translocates from the nucleus to the mitochondria in a mechanism that is dependent on Src family kinases (Haendeler et al. 2003) and acts as an antioxidative defense response to maintain the mitochondrial/cellular homeostasis and bypass RS in cancer cells (Huang et al. 2005; Passos et al. 2007; Saretzki et al. 2009; Indran et al. 2010).

It should be noted that many other protein complexes were also observed in our model (Figure 6B; Table 2). Unfortunately, how these complexes act on the hTERT gene and its products is not clear. Moreover, regulation of many of these protein complexes, such as the oncogenes AKT1, E2F1 and ABL1, and hTERT, was not studied in the context of ROS induction/protection, making the complete picture of hTERT regulation much more difficult to understand. However, to support our hypotheses, the integration of a dynamic model with microarray data is important to determine if the expression of TERT correlated with ROS. Therefore, we focused on a network biology approach using large-scale microarray expression derived from patient tumors.

3.3.1. Microarray analysis and telomerase activity in tumors: Defining a putative biological model of TERT expression

In Systems Biology studies, it is common to use a combination of knowledge mining (including to literature mining) and microarray data to elucidate biological regulatory networks (Chen et al. 2008; Jesmin et al. 2010; Chang et al. 2011; Zhang et al. 2011).

To clarify the role of ROS in TERT expression, microarray data analyses were conducted considering four different tumor types (glioblastoma multiforme, ovary cancer, breast cancer and colorectal cancer) that display a high level of ROS (Afanas´ev

2010). In this context, proteins linked to oxidative stress defense and TERT were considered (Supplementary Table 3) and we identified a subset of genes that were over-expressed in cancerous tissue (Table 3). The use of microarrays to discover differentially expressed genes confirms the umbalaced redox mechanism of these cancer types. Interestingly, TERT mRNA expression is similar in tumors and normal tissues (Table 3). This result may be explained by the heterogeneity of human solid tumors, where some express high levels of TERT and others do not (Kleinschmidt-Demasters et al. 2000; Baykal et al, 2004).

Table 3. Expression levels of genes associated with oxidative stress defense and TERT in different tumor types. Tumor gene expression databases based on four human cancers data sets were used from the Cancer Genome Atlas-TCGA (http://csbl.fimm.fi/anduril/site) – Data represent the average fold change compared to normal tissue.

		TCGA		**Literature**	
	Gene name	**Fold change**	**p-value**	**Fold change**	**Reference**
GLIOBLASTOMA MULTIFORME	CAT	2.09	6.30×10^{-7}	-	-
	CYBA	4.00	3.04×10^{-5}	-	-
	HMOX1	4.02	0.000728	3.55	(Tso et al. 2006)
	NCF2	2.39	0.00250	-	-
	PRDX4	4.20	6.97×10^{-6}	3.76	(Tso et al. 2006)
	SOD2	2.97	1.28×10^{-7}	3.23	(Tso et al. 2006)
	TERT	**1.00**	**0.762**	**0.089 to 0.202**	(Harada et al. 2000)
OVARIAN CANCER	PXDN	2.45	0.0133	-	-
	TXNRD1	2.47	0.0390	-	-

	TERT	**0.82**	**0.822**	-	-
BREAST CANCER	DUOX1	2.33	2.08×10^{-19}	-	-
	GSR	1.98	1.49×10^{-18}	-	-
	PXDNL	2.21	3.55×10^{-5}		-
	TERT	**1.06**	**1**	**0.1 to 2.26**	(Lu et al. 2011)
	TERT			**0.52**	(Hines et al. 2005)
COLORECTAL CANCER	DUOX1	2.43	0.000116	-	-
	GPR156	3.30	2.07×10^{-5}	-	-
	LPO	2.49	0.00688	-	-
	MPO	3.62	8.62×10^{-8}	-	-
	PRDX4	2.16	2.87×10^{-15}	-	-
	PTGS2	3.62	0.000237	-	-
	SOD2	3.23	3.47×10^{-7}	-	-
	TRX2	2.02	0.0004	-	-
	TERT	**1.41**	**7.15×10^{-6}**		-

An extensive review on TERT activity in these four types of cancer (Table 4) indicates a high variation of TERT activity between tumors and clearly shows that tumors have a median proportion of active samples that is far higher when compared to benign lesions or normal tissue. These observations show that TERT expression and activity varies between different tumor types and during tumor development. Our model suggests that this variation can be a result of a possible balance between TERT expression and activity and the intracellular oxidative stress of cancer cells.

Table 4. Analysis of TERT activity in different tumor types. Comparison of frecuency of TERT activity among four human cancers and normal tissues based on a literature review. The median of the entire dataset was used to show TERT activity according cancer malignancy.

	Frequency of tumors with positive TERT activity % (positive/total number)			
	Normal/adjacent tissue	**Benign/premal ignant lesion**	**Malignant lesion**	**Reference**
GLIOBLASTOMA MULTIFORME	-	-	50(3/6)	(Sano et al. 1998)
	-	-	36 (8/22)	(Harada et al. 2000)
	-	-	90 (18/20)	(Harada et al. 2000)
	-	-	75 (45/60)	(Shay and Bacchetti 1997; Skvortzov et al. 2000)
	-	-	72 (34/47)	(Hiraga et al. 1998; Skvortzov et al. 2000)
	-	-	28 (7/25)	(Carrol et al. 1999; Skvortzov et al. 2000)
	-	-	26 (7/28)	(Chong et al. 1998; Skvortzovet al. 2000)
OVARIAN CANCER	0 (0/5)	-	92 (12/13)	(Gorham et al. 1997; Chen and Chen 2011)
	-	20 (2/10)	77 (24/31)	(Murakami et al. 1997; Chen and Chen 2011)
	-	21 (5/24)	100 (37/37)	(Wan et al. 1997; Chen and Chen 2011)
	30 (3/10)	40 (6/15)	96 (24/25)	(Yokoyama et al. 1997; Chen and Chen 2011)
	33 (2/6)	31 (4/13)	74 (69/93)	(Datar et al. 1999; Chen and Chen 2011)
		27 (3/11)	85 (22/26)	(Park et al. 1999; Chen and Chen 2011)

	0 (0/12)	4 (1/28)	61 (20/33)	(Sun et al. 2007; Chen and Chen 2011)
	-	-	86 (18/21)	(Oishi et al. 1998)
	-	-	87 (7/8)	(Oishi et al. 1998)
	-	-	67 (8/13)	(Oishi et al. 1998)
BREAST CANCER	-	-	91 (21/23)	(Shay and Bacchetti, 1997); (Skvortzov et al. 2000)
	4 (2/55)	45 (9/20)	93 (130/140)	(Hiyama et al. 1996; Chen and Chen 2011)
	0 (0/6)	7 (1/15)	73 (52/71)	(Sugino et al. 1996; Chen and Chen 2011)
	0 (0/10)	20 (1/5)	95 (99/104)	(Bednarek et al. 1997; Chen and Chen 2011)
	0 (0/13)	11 (1/9)	79 (22/28)	(Nawaz et al. 1997; Chen and Chen 2011)
	-	-	75 (101/134)	(Bieche et al. 1997; Chen and Chen 2011)
COLORECTAL CANCER	0 (0/50)	53 (16/30)	90 (45/50)	(Engelhardt et al. 1997; Chen and Chen 2011)
	14 (5/35)	50 (6/12)	92 (32/35)	(Yoshida et al. 1997; Chen and Chen 2011)
	27 (3/11)	67 (14/21)	97 (33/34)	(Myung et al. 2005; Chen and Chen 2011)
	0 (0/100)	-	96 (96/100)	(Tatsumoto et al. 2000; Chen and Chen 2011)
	-	-	80 (98/122)	(Kawanishi-Tabata et al. 2002; Chen and Chen, 2011)
	45 (5/11)	57 (12/21)	94 (32/34)	(Myung et al. 2005; Chen and Chen 2011)
	-	-	93 (14/15)	(Chadeneau et al. 1995)

-	-	88 (46/55)	(Fang et al. 1999)

Median of frequency of all tumor types with positive TERT activity % (positive/total number)		
Normal/adjacent tissue	**Benign/premalignant lesion**	**Malignant lesion**
0 (0;27)	**29(20;50)**	**86 (72;92)**

3.4 An integrated model of TERT on tumor induction

Pre-tumorous cells have more ROS than normal cells (Wang and Yi 2008). Thus, mitochondrial oxidative stress causes production of ROS, which causes an increased mutation rate and tumor evolution by means of positive selection of tumor cell mutations that confer a growth advantage (Sotgia et al. 2011). In this condition, tumor suppressor genes such as RB/p16 are inactivated by ROS-induced mutations, leading to cancer initiation (Jenkins et al. 2010), a critical step of tumorigenesis. Additional, initial mutations of proto-oncogenes activate the primary antitumor barrier, OIS can ocurr in cell TERT negative (Braig et al. 2005; Vargas et al. 2012). A residual cell population can be resistant to OIS. In this sense, slow telomere erosion may act to activate RS, a secondary antitumor barrier (Parkinson et al. 2000).

Microarray expression data for TERT and genes associated with high levels of oxidative stress, as well as topological analysis and literature mining, were used to define our new model of TERT function. Our major hypothesis is that short activation "bursts" of the subunit of TERT (hTERT) may be important for cancer progression by affecting genetic stability and the ROS response to maintain metabolic homeostase and telomere stability (Figure 6). ROS derived from dysfunctional mitochondria can react with telomere regions (Liu et al. 2002; De Moura et al. 2010) and may be sufficient to activate hTERT transcription and induce telomere extension. Because we found no

evidence of constitutive TERT expression during cancer development in the literature, we believe that bursts of TERT are likely sufficient.

Additionally, it is plausible that other transitory activations can induce cancer development. Interestingly, hTERT is expressed in a manner that is independent of telomere erosion (Chiodi and Mondello 2012). As described, hTERT is post-translationally modified and exported to aberrant mitochondria, leading to reduced ROS production and increased respiratory efficiency (Kang et al., 2004; Haendeler et al. 2009). In this sense, we suggest that there is a balance between enzymatic oxidative stress defenses and hTERT export to mitochondria, leading to increased survival of tumor cells (Figure 6).

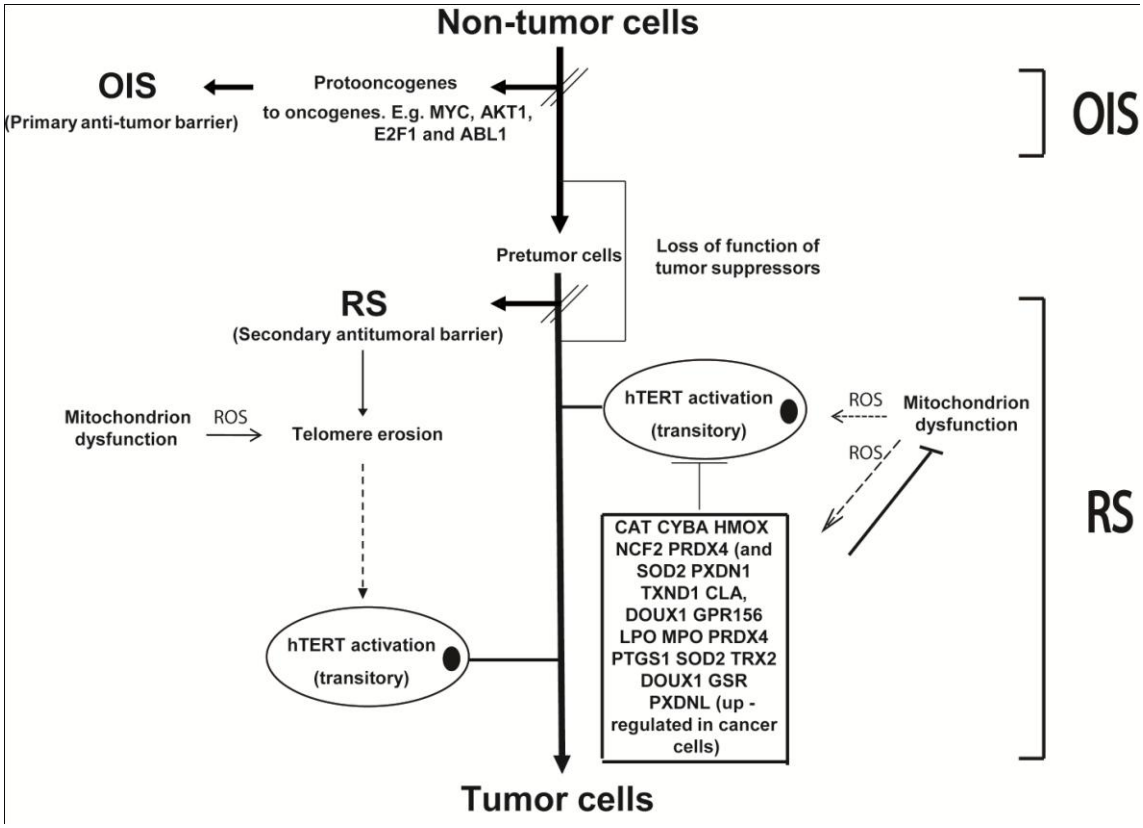


Figure 6. **Hypothetical model of hTERT subunit transcription and export to maintain tumor homeostasis**. Initial mutation of proto-oncogenes or tumor suppressor genes is the first step in cancer development. OIS appears to be a primary barrier to avoid cancer development. However, a residual cell population that is resistant to the OIS mechanism continues to

proliferate. In these cells, telomere erosion can induce RS, but this can be avoided by TERT activation. Moreover, TERT expression would play a different role than telomere extension as a response to intense intracellular stresses. Thus, the TERT subunit (hTERT) may be expressed transiently in short activation "bursts" that are important for cancer progression by affecting genetic stability and the ROS response. ROS derived from dysfunctional mitochondria in tumor cells can react with telomere regions and may be sufficient to activate hTERT transcription and induce telomere extension. However, post-translational modifications drive export hTERT to aberrant mitochondria and may decrease ROS production (independent of telomere extension). It is evidence that a possible balance among oxidative stress, oxidative genes (solid box) and TERT expression exists.

4. Conclusions

Our Systems Biology approach represents a new way to study TERT in the context of two principal senescence mechanisms (RS and OIS).We showed that oncogenes with OIS activity regulate TERT and influence the cellular metabolism based on GO analysis. Furthermore, a centrality analysis showed that TERT is an NH-NB node in major PPI network (Union PPI network) and is regulated by a small number of proteins. Moreover, we found that TERT (subunit hTERT) is regulated by proteins that are sensors of oxidative stress and is expressed heterogeneously in four human tumor types. Thus, we propose that hTERT subunit expression could act as a compensatory mechanism in tumor cells to avoid OIS and RS in response to oxidative stress (dependent and independent of telomere extension).

5. Acknowledgements

This work was supported by research grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq; Grant Number 474117/2010-3), the Programa Institutos Nacionais de Ciência e Tecnologia (INCT de Processos Redox em Biomedicina-REDOXOMA; Grant Number 573530/2008-4), Fundação de Amparo a

Pesquisa do Rio Grande do Sul FAPERGS (PRONEM Grant Number 11/2072-2) and CAPES (Cordenação de Aperfeiçoamento de Pessoal do Ensino Superior).

References

Afanas'ev I (2011). Reactive oxygen species signaling in cancer: comparison with aging. Aging Dis 2 (3):219-230

Anderson CJ, Hoare SF, Ashcroft M, Bilsland AE, Keith WN (2006) Hypoxic regulation of telomerase gene expression by transcriptional and post-transcriptional mechanisms. Oncogene 25 (1):61-69. doi:1209011 [pii]10.1038/sj.onc.1209011.

Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips JA, 3rd, Lansdorp PM, Greider CW, Loyd JE (2007) Telomerase mutations in families with idiopathic pulmonary fibrosis. N Engl J Med 356 (13):1317-1326. doi:356/13/1317 [pii] 10.1056/NEJMoa066157

Astle MV, Hannan KM, Ng PY, Lee RS, George AJ, Hsu AK, Haupt Y, Hannan RD, Pearson RB (2011). AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: implications for targeting mTOR during malignancy. Oncogene 31 (15):1949-1962. doi:onc2011394 [pii] 2012

Bader, G.D., Hogue, C.W (2003) An automated method for finding molecular complexes in large protein interaction networks BMC Bioinformatics 2003 4, 2. doi:10.1186/1471-2105-4-2

Baek SH (2008) When ATPases pontin and reptin met telomerase. Dev Cell 14 (4):459-461. doi:S1534-5807(08)00129-9 [pii]10.1016/j.devcel.2008.03.018

Bai Y, Murnane JP (2003) Telomere instability in a human tumor cell line expressing NBS1 with mutations at sites phosphorylated by ATM. Mol Cancer Res 1 (14):1058-1069.

Bai D, Ueno L, Vogt PK (2009) Akt-mediated regulation of NFkappaB and the essentialness of NFkappaB for the oncogenicity of PI3K and Akt. Int J Cancer 125 (12):2863-2870. doi:10.1002/ijc.24748

Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, Orntoft T, Lukas J, Bartek J (2005) DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature 434 (7035):864-870. doi:nature03482 [pii]10.1038/nature03482

Bavik C, Coleman I, Dean JP, Knudsen B, Plymate S, Nelson PS (2006) The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. Cancer Res 66 (2):794-802. doi:66/2/794 [pii]10.1158/0008-5472.CAN-05-1716

Baykal A, Thompson JA, Xu XC, Hahn WC, Deavers MT, Malpica A, Gershenson DM, Silva EG, Liu J (2004) In situ human telomerase reverse transcriptase expression pattern in normal and neoplastic ovarian tissues. Oncol Rep 11 (2):297-302

Bazarov AV, Hines WC, Mukhopadhyay R, Beliveau A, Melodyev S, Zaslavsky Y, Yaswen P (2009) Telomerase activation by c-Myc in human mammary epithelial cells requires additional genomic changes. Cell Cycle 8 (20):3373-3378. doi:9856 [pii]

Bednarek AK, Sahin A, Brenner AJ, Johnston DA, Aldaz CM (1997) Analysis of telomerase activity levels in breast cancer: positive detection at the in situ breast carcinoma stage. Clin Cancer Res 3 (1):11-16

Benjamini, Y., Hochberg, Y (1995).Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57, 289–300.

Benz CC, Yau C (2008) Ageing, oxidative stress and cancer: paradigms in parallax. Nat Rev Cancer 8 (11):875-879. doi:nrc2522 [pii]10.1038/nrc2522

Ben-Porath I, Weinberg RA (2005) The signals and pathways activating cellular senescence. Int J Biochem Cell Biol 37 (5):961-976. doi:S1357-2725(04)00387-5 [pii]10.1016/j.biocel.2004.10.013

Bhalla K, Hwang BJ, Dewi RE, Ou L, Twaddel W, Fang HB, Vafai SB, Vazquez F, Puigserver P, Boros L, Girnun GD (2011) PGC1alpha promotes tumor growth by inducing gene expression programs supporting lipogenesis. Cancer Res 71 (21):6888-6898. doi:0008-5472.CAN-11-1011 [pii]10.1158/0008-5472.CAN-11-1011

Bhaumik D, Scott GK, Schokrpur S, Patil CK, Orjalo AV, Rodier F, Lithgow GJ, Campisi J (2009) MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8. Aging (Albany NY) 1 (4):402-411

Bieche I, Nogues C, Paradis V, Olivi M, Bedossa P, Lidereau R, Vidaud M (2000) Quantitation of hTERT gene expression in sporadic breast tumors with a real-time reverse transcription-polymerase chain reaction assay. Clin Cancer Res 6 (2):452-459

Biroccio A, Benassi B, Fiorentino F, Zupi G (2004) Glutathione depletion induced by c-Myc downregulation triggers apoptosis on treatment with alkylating agents. Neoplasia 6 (3):195-206. doi:10.1593/neo.3370

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE (1998) Extension of life-span by introduction of telomerase into normal human cells. Science 279 (5349):349-352. DOI: 10.1126/science.279.5349.349

Bozinov O, Kalk JM, Krayenbuhl N, Woernle CM, Sure U, Bertalanffy H (2010) Decreasing expression of the interleukin-13 receptor IL-13Ralpha2 in treated recurrent malignant gliomas. Neurol Med Chir (Tokyo) 50 (8):617-621. doi:JST.JSTAGE/nmc/50.617 [pii]

Braig M, Lee S, Loddenkemper C, Rudolph C, Peters AH, Schlegelberger B, Stein H, Dorken B, Jenuwein T, Schmitt CA (2005) Oncogene-induced senescence as an initial barrier in lymphoma development. Nature 436 (7051):660-665. doi:nature03841 [pii]10.1038/nature03841

Brooks AN, Kilgour E, Smith PD (2012) Molecular pathways: fibroblast growth factor signaling: a new therapeutic opportunity in cancer. Clin Cancer Res 18 (7):1855-1862. doi:1078-0432.CCR-11-0699 [pii]

Burhans WC, Weinberger M (2007) DNA replication stress, genome instability and aging. Nucleic Acids Res 35 (22):7545-7556. doi:gkm1059 [pii]10.1093/nar/gkm1059

Caino MC, Meshki J, Kazanietz MG (2009) Hallmarks for senescence in carcinogenesis: novel signaling players. Apoptosis 14 (4):392-408. doi:10.1007/s10495-009-0316-z

Campisi J (2001) Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol 11 (11):S27-31. doi:S0962-8924(01)02151-1 [pii]

Campisi J (2004) Fragile fugue: p53 in aging, cancer and IGF signaling. Nat Med 10 (3):231-232. doi:10.1038/nm0304-231nm0304-231 [pii]

Campisi J, d'Adda di Fagagna F (2007) Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8 (9):729-740. doi:nrm2233 [pii]10.1038/nrm2233

Carvalho TL, Mota-Santos T, Cumano A, Demengeot J, Vieira P (2001) Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/)- mice. J Exp Med 194 (8):1141-1150. doi: 10.1084/jem.194.8.1141

Carroll T, Maltby E, Brock I, Royds J, Timperley W, Jellinek D (1999) Meningiomas, dicentric chromosomes, gliomas, and telomerase activity. J Pathol 188 (4):395-399. doi:10.1002/(SICI)1096-9896(199908)188:4<395::AID-PATH376>3.0.CO;2-E [pii]

Chaar I, Arfaoui TA, El Amine el HO, Mahmoud LB, Khiari M, Sammoud S, Lounis A, Amara S, Gharbi L, Hmida AB, Mzabi S, Bouraoui S (2012) Impact of MDM2 polymorphism: increased risk of developing colorectal cancer and a poor prognosis in the Tunisian population. Eur J Gastroenterol Hepatol 24 (3):320-327. doi:10.1097/MEG.0b013e32834bdf84

Chadeneau C, Hay K, Hirte HW, Gallinger S, Bacchetti S (1995) Telomerase activity associated with acquisition of malignancy in human colorectal cancer. Cancer Res 55 (12):2533-2536

Chai W, Ford LP, Lenertz L, Wright WE, Shay JW (2002) Human Ku70/80 associates physically with telomerase through interaction with hTERT. J Biol Chem 277 (49):47242-47247. doi:10.1074/jbc.M208542200M208542200 [pii]

Chang TY, Wu YH, Cheng CC, Wang HW (2011) Differentially regulated splice variants and systems biology analysis of Kaposi's sarcoma-associated herpesvirus-infected lymphatic endothelial cells. Nucleic Acids Res 39 (16):6970-6985. doi:gkr405 [pii]10.1093/nar/gkr405

Chang IH, Kim HS, Kim TH, Myoung SC, Lee SE, Kim YS (2006). The Inhibition of Human Telomerase Reverse Transcriptase Expression by Peroxiredoxin I and c-Myc in Prostatic Cancer Cells. Korean J Urol.47(4):418-425. doi:10.4111/kju.2006.47.4.418

Chang M, Luke B, Kraft C, Li Z, Peter M, Lingner J, Rothstein R (2009) Telomerase is essential to alleviate pif1-induced replication stress at telomeres. Genetics 183 (3):779-791. doi:genetics.109.107631 [pii]10.1534/genetics.109.107631

Chen CH, Chen RJ Prevalence of telomerase activity in human cancer.(2011) J Formos Med Assoc 110:275-289. DOI S0929-6646(11)60043-0 [pii]10.1016/S0929-6646(11)60043-0

Chen HZ, Tsai SY, Leone G (2009) Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer 9 (11):785-797. doi:nrc2696 [pii]10.1038/nrc2696.

Chen Z, Trotman LC, Shaffer D, Lin HK, Dotan ZA, Niki M, Koutcher JA, Scher HI, Ludwig T, Gerald W, Cordon-Cardo C, Pandolfi PP (2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature 436 (7051):725-730. doi:nature03918 [pii]

Chen BS, Yang SK, Lan CY, Chuang YJ (2008) A systems biology approach to construct the gene regulatory network of systemic inflammation via microarray and databases mining. BMC Med Genomics 1:46. doi:1755-8794-1-46 [pii]10.1186/1755-8794-1-46

Chiaradonna F, Balestrieri C, Gaglio D, Vanoni M (2008) RAS and PKA pathways in cancer: new insight from transcriptional analysis. Front Biosci 13:5257-5278. doi:3079 [pii]

Chiodi I, Mondello C (2012) Telomere-independent functions of telomerase in nuclei, cytoplasm, and mitochondria. Front Oncol 2:133. doi:10.3389/fonc.2012.00133

Cho J, Pastorino S, Zeng Q, Xu X, Johnson W, Vandenberg S, Verhaak R, Cherniack AD, Watanabe H, Dutt A, Kwon J, Chao YS, Onofrio RC, Chiang D, Yuza Y, Kesari S, Meyerson M (2011) Glioblastoma-derived epidermal growth factor receptor carboxyl-terminal deletion mutants are transforming and are sensitive to EGFR-directed therapies. Cancer Res 71 (24):7587-7596. doi:0008-5472.CAN-11-0821 [pii]10.1158/0008-5472.CAN-11-0821.

Chong EY, Lam PY, Poon WS, Ng HK (1998) Telomerase expression in gliomas including the nonastrocytic tumors. Hum Pathol 29 (6):599-603. doi: 10.1016/S0046-8177(98)80009-9

Chung-Yen Lin, Chia-Hao Chin, Hsin-Hung Wu, Shu-Hwa Chen, Chin-Wen Ho,Ming-Tat Ko (2008) Hubba: hub objects analyzer a framework of interactome hubs identification for network biology. Nucleic Acids Research, 2008 36: 438-443. doi: 10.1093/nar/gkn257

Collado M, Blasco MA, Serrano M (2007) Cellular senescence in cancer and aging. Cell 130 (2):223-233. doi:S0092-8674(07)00890-2 [pii]10.1016/j.cell.2007.07.003. doi:10.1016/j.cell.2007.07.003

Collado M, Serrano M (2010) Senescence in tumours: evidence from mice and humans. Nat Rev Cancer 10: 51–57

Collins K, Jacks T, Pavletich NP (1997) The cell cycle and cancer. Proc Natl Acad Sci U S A 94 (7):2776-2778. doi: 10.1002/path.3022

Cong YS, Wen J, Bacchetti S (1999) The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. Hum Mol Genet 8 (1):137-142. doi:ddc019 [pii]

Coppe JP, Patil CK, Rodier F, Krtolica A, Beausejour CM, Parrinello S, Hodgson JG, Chin K, Desprez PY, Campisi J (2010) A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. PLoS One 5 (2):e9188. doi:10.1371/journal.pone.0009188

Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, Nelson PS, Desprez PY, Campisi J (2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol 6 (12):2853-2868. doi:08-PLBI-RA-2566 [pii]10.1371/journal.pbio.0060301

Dang CV (2012). MYC on the Path to Cancer. Cell 149 (1):22-35. doi:S0092-8674(12)00296-6 [pii]10.1016/j.cell.2012.03.003.

Datar RH, Naritoku WY, Li P, Tsao-Wei D, Groshen S, Taylor CR, Imam SA (1999) Analysis of telomerase activity in ovarian cystadenomas, low-malignant-potential tumors, and invasive carcinomas. Gynecol Oncol 74 (3):338-345. doi:10.1006/gyno.1999.5506S0090-8258(99)95506-3 [pii]

Datta A, Bellon M, Sinha-Datta U, Bazarbachi A, Lepelletier Y, Canioni D, Waldmann TA, Hermine O, Nicot C (2006) Persistent inhibition of telomerase reprograms adult T-cell leukemia to p53-dependent senescence. Blood 108:1021-1029. DOI 2006-01-0067 [pii]10.1182/blood-2006-01-0067

Dazert E, Hall MN (2011) mTOR signaling in disease. Curr Opin Cell Biol 23 (6):744-755. doi:S0955-0674(11)00111-6 [pii]10.1016/j.ceb.2011.09.003

de Magalhães JP, Budovsky A, Lehmann G, Costa J, Li Y, Fraifeld V, Church GM (2009) The Human Aging Genomic Resources: online databases and tools for biogerontologists. Aging Cell 8:65–72. doi:10.1007/s10522-010-9268-5

de Magalhães JP, Costa J, Toussaint 0 (2005) HAGR: the Human Aging Genomic Resources. Nucleic Acids Res 33:D537–D543. doi:10.1093/nar/gki017

de Magalhães JP, Toussaint O (2004) GenAge: a genomic and proteomic network map of human aging. FEBS Lett, 2004 571:243–247. doi:10.1016/j.febslet.2004.07.006

de Moura MB, dos Santos LS, Van Houten B (1998) Mitochondrial dysfunction in neurodegenerative diseases and cancer. Environ Mol Mutagen 51 (5):391-405. doi:10.1002/em.20575

Deng Y, Chan SS, Chang S (2008) Telomere dysfunction and tumour suppression: the senescence connection. Nat Rev Cancer 8 (6):450-458. doi:nrc2393 [pii]10.1038/nrc2393

Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garre M, Nuciforo PG, Bensimon A, Maestro R, Pelicci PG, d'Adda di Fagagna F (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 444 (7119):638-642. doi:nature05327 [pii]10.1038/nature05327

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A 92 (20):9363-9367

Dimri GP (2005) What has senescence got to do with cancer? Cancer Cell 7 (6):505-512. doi:S1535-6108(05)00166-2 [pii]10.1016/j.ccr.2005.05.025

Dimri GP, Itahana K, Acosta M, Campisi J (2000) Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor. Mol Cell Biol 20 (1):273-285

Dhomen N, Reis-Filho JS, da Rocha Dias S, Hayward R, Savage K, Delmas V, Larue L, Pritchard C, Marais R Oncogenic Braf induces melanocyte senescence and melanoma in mice. Cancer Cell 15 (4):294-303. doi:S1535-6108(09)00074-9 [pii] 10.1016/j.ccr.2009.02.022

Drogaris P, Villeneuve V, Pomies C, Lee EH, Bourdeau V, Bonneil E, Ferbeyre G, Verreault A, Thibault P 9 (2012) Histone deacetylase inhibitors globally enhance h3/h4 tail acetylation without affecting h3 lysine 56 acetylation. Sci Rep 2:220. doi:10.1038/srep00220

Dudas J, Fullar A, Bitsche M, Schartinger V, Kovalszky I, Sprinzl GM, Riechelmann H (2011) Tumor-produced, active interleukin-1beta regulates gene expression in carcinoma-associated fibroblasts. Exp Cell Res 317 (15):2222-2229. doi:S0014-4827(11)00198-4 [pii]10.1016/j.yexcr.2011.05.023

Dutra-Oliveira A, Monteiro RQ, Mariano-Oliveira A (2012) Protease-activated receptor-2 (PAR2) mediates VEGF production through the ERK1/2 pathway in human glioblastoma cell lines. Biochem Biophys Res Commun. doi:S0006-291X(12)00621-3 [pii]10.1016/j.bbrc.2012.03.140

Engelhardt M, Drullinsky P, Guillem J, Moore MA (1997) Telomerase and telomere length in the development and progression of premalignant lesions to colorectal cancer. Clin Cancer Res 3 (11):1931-1941

Ewald JA, Desotelle JA, Wilding G, Jarrard DF (2010) Therapy-induced senescence in cancer. J Natl Cancer Inst 102 (20):1536-1546. doi:djq364 [pii]

Faiola F, Liu X, Lo S, Pan S, Zhang K, Lymar E, Farina A, Martinez E (2005) Dual regulation of c-Myc by p300 via acetylation-dependent control of Myc protein turnover and coactivation of Myc-induced transcription. Mol Cell Biol 25 (23):10220-10234. doi:25/23/10220 [pii]10.1128/MCB.25.23.10220-10234.2005.

Fang DC, Young J, Luo YH, Lu R, Jass J (1999) Detection of telomerase activity in biopsy samples of colorectal cancer. J Gastroenterol Hepatol 14 (4):328-332. doi:10.1046/j.1440-1746.1999.01862.x

Ford LP, Wright WE, Shay JW (2002) A model for heterogeneous nuclear ribonucleoproteins in telomere and telomerase regulation. Oncogene 21 (4):580-583. doi:10.1038/sj.onc.1205086

Frampton G, Invernizzi P, Bernuzzi F, Pae HY, Quinn M, Horvat D, Galindo C, Huang L, McMillin M, Cooper B, Rimassa L, DeMorrow S (2011) Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. Gut 61 (2):268-277. doi:gutjnl-2011-300643 [pii]10.1136/gutjnl-2011-300643

Francia S, Weiss RS, Hande MP, Freire R, d'Adda di Fagagna F (2006) Telomere and telomerase modulation by the mammalian Rad9/Rad1/Hus1 DNA-damage-checkpoint complex. Curr Biol 16 (15):1551-1558. doi:S0960-9822(06)01845-8 [pii]10.1016/j.cub.2006.06.066

Scardoni G, Petterlini M, Laudanna C (2009) Analyzing biological network parameters with CentiScaPe 25: 2857-2859. doi: 10.1093/bioinformatics/btp517

Garcia-Cao I, Garcia-Cao M, Tomas-Loba A, Martin-Caballero J, Flores JM, Klatt P, Blasco MA, Serrano M (2006) Increased p53 activity does not accelerate telomere-driven ageing. EMBO Rep 7 (5):546-552. doi:7400667 [pii]

10.1038/sj.embor.7400667

Gonfloni S (2010) DNA damage stress response in germ cells: role of c-Abl and clinical implications. Oncogene 29 (47):6193-6202. doi:onc2010410 [pii]10.1038/onc.2010.410 2010

Gorbunova V, Seluanov A, Pereira-Smith OM (2002) Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts but protects the cells from stress-induced apoptosis and necrosis. J Biol Chem 277 (41):38540-38549. doi:10.1074/jbc.M202671200M202671200 [pii]

Gorham H, Yoshida K, Sugino T, Marsh G, Manek S, Charnock M, Tarin D, Goodison S (1997) Telomerase activity in human gynaecological malignancies. J Clin Pathol 50 (6):501-504. doi:10.1136/jcp.50.6.501

Greider CW, Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 43 (2 Pt 1):405-413. doi:0092-8674(85)90170-9 [pii].

Guevara-Aguirre J, Balasubramanian P, Guevara-Aguirre M, Wei M, Madia F, Cheng CW, Hwang D, Martin-Montalvo A, Saavedra J, Ingles S, de Cabo R, Cohen P, Longo VD (2011) Growth hormone receptor deficiency is associated with a major reduction in pro-aging signaling, cancer, and diabetes in humans. Sci Transl Med 3(70):70ra13 . doi: 10.1126/scitranslmed.3001845

Gurumurthy S, Hezel AF, Sahin E, Berger JH, Bosenberg MW, Bardeesy N (2008) LKB1 deficiency sensitizes mice to carcinogen-induced tumorigenesis. Cancer Res 68 (1):55-63. doi:68/1/55 [pii]10.1158/0008-5472.CAN-07-3225

Indran IR, Hande MP, Pervaiz S (2010) Tumor cell redox state and mitochondria at the center of the non-canonical activity of telomerase reverse transcriptase. Mol Aspects Med 31 (1):21-28. doi:S0098-2997(09)00090-9 [pii] 10.1016/j.mam.2009.12.001

Haendeler J, Drose S, Buchner N, Jakob S, Altschmied J, Goy C, Spyridopoulos I, Zeiher AM, Brandt U, Dimmeler S (2009) Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage. Arterioscler Thromb Vasc Biol 29 (6):929-935. doi:ATVBAHA.109.185546 [pii]10.1161/ATVBAHA.109.185546.

Haendeler J, Hoffmann J, Brandes RP, Zeiher AM, Dimmeler S (2003) Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. Mol Cell Biol 23 (13):4598-4610. doi:10.1128/MCB.23.13.4598-4610.2003.

Hahn WC (2001) Telomerase and cancer: where and when? Clin Cancer Res 7 (10):2953-2954. doi:10.1200/JCO.2003.06.018

Harada K, Kurisu K, Tahara H, Tahara E, Ide T (2000) Telomerase activity in primary and secondary glioblastomas multiforme as a novel molecular tumor marker. J Neurosurg 93 (4):618-625. doi:10.3171/jns.2000.93.4.0618

Harry GJ, Lefebvre d'Hellencourt C, Bruccoleri A, Schmechel D (2000) Age-dependent cytokine responses: trimethyltin hippocampal injury in wild-type, APOE knockout, and APOE4 mice. Brain Behav Immun 14 (4):288-304. doi:10.1006/brbi.2000.0606

Hasan MR, Ho SH, Owen DA, Tai IT (2011) Inhibition of VEGF induces cellular senescence in colorectal cancer cells. Int J Cancer. doi:10.1002/ijc.26179. doi:10.1002/ijc.26179

Hayflick L (1965) The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res 37:614-636. doi:10.1016/0014-4827(61)90192-6

Hickman ES, Moroni MC, Helin K (2002) The role of p53 and pRB in apoptosis and cancer. Curr Opin Genet Dev 12 (1):60-66. doi:S0959437X01002659 [pii]

Hines WC, Fajardo AM, Joste NE, Bisoffi M, Griffith JK (2005) Quantitative and spatial measurements of telomerase reverse transcriptase expression within normal and malignant human breast tissues. Mol Cancer Res 3 (9):503-509. doi:3/9/503 [pii]10.1158/1541-7786.MCR-05-0031

Hiraga S, Ohnishi T, Izumoto S, Miyahara E, Kanemura Y, Matsumura H, Arita N (1998) Telomerase activity and alterations in telomere length in human brain tumors. Cancer Res 58 (10):2117-2125. doi:10.1158/1078-0432

Hornsby PJ (2007) Senescence as an anticancer mechanism. J Clin Oncol 25 (14):1852-1857. doi:25/14/1852 [pii] 10.1200/JCO.2006.10.3101

Huang XQ, Wang J, Liu JP, Feng H, Liu WB, Yan Q, Liu Y, Sun SM, Deng M, Gong L, Li DW (2005) hTERT extends proliferative lifespan and prevents oxidative stress-induced apoptosis in human lens epithelial cells. Invest Ophthalmol Vis Sci 46 (7):2503-2513. doi:46/7/2503 [pii]10.1167/iovs.05-0154

Hussein Ael S, El-Moneim NA, Mahmoud MM, Abdel -Baky MM (2003) The role of interferon-gamma and vitamin-A in modulating telomerase activity in breast cancer patients. Egypt J Immunol 10 (1):47-56

Hiyama E, Gollahon L, Kataoka T, Kuroi K, Yokoyama T, Gazdar AF, Hiyama K, Piatyszek MA, Shay JW (1996) Telomerase activity in human breast tumors. J Natl Cancer Inst 88 (2):116-122. doi: 10.1093/jnci/88.2.116

Jen EY, Poindexter NJ, Farnsworth ES, Grimm EA (2011) IL-2 regulates the expression of the tumor suppressor IL-24 in melanoma cells. Melanoma Res 22 (1):19-29. doi:10.1097/CMR.0b013e32834d2506

Jenkins NC, Liu T, Cassidy P, Leachman SA, Boucher KM, Goodson AG, Samadashwily G, Grossman D (2011) The p16(INK4A) tumor suppressor regulates cellular oxidative stress. Oncogene 30 (3):265-274. doi:onc2010419 [pii]10.1038/onc.2010.419

Jesmin J, Rashid MS, Jamil H, Hontecillas R, Bassaganya-Riera J (2010) Gene regulatory network reveals oxidative stress as the underlying molecular mechanism of type 2 diabetes and hypertension. BMC Med Genomics 3 (1):45. doi:1755-8794-3-45 [pii]10.1186/1755-8794-3-45

Kang HJ, Choi YS, Hong SB, Kim KW, Woo RS, Won SJ, Kim EJ, Jeon HK, Jo SY, Kim TK, Bachoo R, Reynolds IJ, Gwag BJ, Lee HW (2004) Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity. J Neurosci 24 (6):1280-1287. doi:10.1523/JNEUROSCI.4082-03.200424/6/1280 [pii]

Kang MK, Kameta A, Shin KH, Baluda MA, Park NH (2004) Senescence occurs with hTERT repression and limited telomere shortening in human oral keratinocytes cultured with feeder cells. J Cell Physiol 199 (3):364-370. doi:10.1002/jcp.10410

Kang SS, Kwon T, Kwon DY, Do SI (1999) Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. J Biol Chem 274 (19):13085-13090. doi: 10.1074/jbc.274.19.13085

Kato T, Shimono Y, Hasegawa M, Jijiwa M, Enomoto A, Asai N, Murakumo Y, Takahashi M (2009) Characterization of the HDAC1 complex that regulates the sensitivity of cancer cells to oxidative stress. Cancer Res 69 (8):3597-3604. doi:0008-5472.CAN-08-4368 [pii] 10.1158/0008-5472.CAN-08-4368.

Kawanishi-Tabata R, Lopez F, Fratantonio S, Kim N, Goldblum J, Tubbs R, Elson P, Lavery I, Bukowski RM, Ganapathi R, Ganapathi MK (2002) Telomerase activity in stage II colorectal carcinoma. Cancer 95 (9):1834-1839. doi:10.1002/cncr.10911

Kharbanda S, Kumar V, Dhar S, Pandey P, Chen C, Majumder P, Yuan ZM, Whang Y, Strauss W, Pandita TK, Weaver D, Kufe D (2000) Regulation of the hTERT telomerase catalytic subunit by the c-Abl tyrosine kinase. Curr Biol 10 (10):568-575. doi:S0960-9822(00)00483-8 [pii]

Khurts S, Masutomi K, Delgermaa L, Arai K, Oishi N, Mizuno H, Hayashi N, Hahn WC, Murakami S (2004) Nucleolin interacts with telomerase. J Biol Chem 279 (49):51508-51515. doi:10.1074/jbc.M407643200M407643200 [pii]

Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. Science 266 (5193):2011-2015. doi: 10.1126/science.7605428

Kim SW, Kim S, Nam EJ, Jeong YW, Lee SH, Paek JH, Kim JH, Kim JW, Kim YT (2011) Comparative proteomic analysis of advanced serous epithelial ovarian carcinoma: possible predictors of chemoresistant disease. OMICS 15 (5):281-292. doi:10.1089/omi.2010.0012

Kleinschmidt-Demasters BK, Evans LC, Bobak JB, Lopez-Uribe D, Hopper D, Shroyer AL, Shroyer KR (2000) Quantitative telomerase expression in glioblastomas shows regional variation and down-regulation with therapy but no correlation with patient outcome. Hum Pathol 31 (8):905-913. doi:S0046-8177(00)22753-6 [pii]10.1053/hupa.2000.9086

Kregel KC, Zhang HJ (2007) An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. Am J Physiol Regul Integr Comp Physiol 292 (1):R18-36. doi:00327.2006 [pii]
10.1152/ajpregu.00327.2006

Kyo S, Inoue M (2002) Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy? Oncogene 21 (4):688-697. doi:10.1038/sj.onc.1205163

Kyrylenko S, Baniahmad A (2010) Sirtuin family: a link to metabolic signaling and senescence. Curr Med Chem 17 (26):2921-2932. doi:BSP/CMC/E-Pub/ 221g [pii]

Lee MY, Kim SH, Ihm HJ, Chae HD, Kim CH, Kang BM (2011) Up-regulation of p21-activated kinase 1 by in vitro treatment with interleukin 1-beta and its increased expression in ovarian endometriotic cysts. Fertil Steril 96 (2):508-511. doi:S0015-0282(11)00868-5 [pii]10.1016/j.fertnstert.2011.05.082

Leng X, Noble M, Adams PD, Qin J, Harper JW (2002) Reversal of growth suppression by p107 via direct phosphorylation by cyclin D1/cyclin-dependent kinase 4. Mol Cell Biol 22 (7):2242-2254. doi:10.1128/MCB.22.7.2242-2254.2002

Lenz G (2012), Endogenous anticancer mechanisms (EACMs), Frontiers in Bioscience, S4, 1017-1030. doi.org/10.2741/315

Li M, Lin YM, Hasegawa S, Shimokawa T, Murata K, Kameyama M, Ishikawa O, Katagiri T, Tsunoda T, Nakamura Y, Furukawa Y (2004) Genes associated with liver metastasis of colon cancer, identified by genome-wide cDNA microarray. Int J Oncol 24 (2):305-312

Li Y, Xu W, McBurney MW, Longo VD (2008) SirT1 inhibition reduces IGF-I/IRS-2/Ras/ERK1/2 signaling and protects neurons. Cell Metab 8 (1):38-48. doi:S1550-4131(08)00148-4 [pii]10.1016/j.cmet.2008.05.004

Liu JP, Wang HT, Ou QJ, Lu YT, Lu HP, Gao JH, Chu ZH, Zhao HY (2003) [Expression of growth hormone receptor in hepatocellular carcinoma and its significance]. Ai Zheng 22:298-301.doi: 1000467X2003030298 [pii

Liu K, Hodes RJ, Weng N (2001) Cutting edge: telomerase activation in human T lymphocytes does not require increase in telomerase reverse transcriptase (hTERT) protein but is associated with hTERT phosphorylation and nuclear translocation. J Immunol 166 (8):4826-4830

Liu L, Trimarchi JR, Smith PJ, Keefe DL (2002) Mitochondrial dysfunction leads to telomere attrition and genomic instability. Aging Cell 1 (1):40-46. DOI: 10.1046/j.1474-9728.2002.00004.x

Liu M, Liberzon A, Kong SW, Lai WR, Park PJ, Kohane IS, Kasif S (2007) Network-based analysis of affected biological processes in type 2 diabetes models.PLoS Genet 2007, 3:e96. doi:10.1371/journal.pgen.0030096

Lu L, Zhang C, Zhu G, Irwin M, Risch H, Menato G, Mitidieri M, Katsaros D, Yu H (2011) Telomerase expression and telomere length in breast cancer and their associations with adjuvant treatment and disease outcome. Breast Cancer Res 13 (3):R56. doi:bcr2893 [pii]10.1186/bcr2893

Luo Y, Zou P, Zou J, Wang J, Zhou D, Liu L (2011) Autophagy regulates ROS-induced cellular senescence via p21 in a p38 MAPKalpha dependent manner. Exp Gerontol 46 (11):860-867. doi:S0531-5565(11)00192-6 [pii]10.1016/j.exger.2011.07.005

Luo Z, Saha AK, Xiang X, Ruderman NB (2005) AMPK, the metabolic syndrome and cancer. Trends Pharmacol Sci 26 (2):69-76. doi:S0165-6147(04)00340-2 [pii]10.1016/j.tips.2004.12.01

Macheda ML, Rogers S, Best JD (2005) Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 202 (3):654-662. doi:10.1002/jcp.20166

Maere, S., Heymans, K., Kuiper, M (2005) BiNGO: a Cytoscape plugin to assess overrepresentation of Gene Ontology categories in biological networks. Bioinformatics 21, 3448–3449. doi:10.1093/bioinformatics/bti551

Maggio M, Guralnik JM, Longo DL, Ferrucci L (2006) Interleukin-6 in aging and chronic disease: a magnificent pathway. J Gerontol A Biol Sci Med Sci 61 (6):575-584. doi:61/6/575 [pii]

Maida Y, Kyo S, Kanaya T, Wang Z, Yatabe N, Tanaka M, Nakamura M, Ohmichi M, Gotoh N, Murakami S, Inoue M (2002) Direct activation of telomerase by EGF through Ets-mediated transactivation of TERT via MAP kinase signaling pathway. Oncogene 21 (26):4071-4079. doi:10.1038/sj.onc.1205509

Malumbres M, Barbacid M (2009) Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 9 (3):153-166. doi:nrc2602 [pii]10.1038/nrc2602

Manfe V, Biskup E, Johansen P, Kamstrup MR, Krejsgaard TF, Morling N, Wulf HC, Gniadecki R (2012) MDM2 Inhibitor Nutlin-3a Induces Apoptosis and Senescence in Cutaneous T-Cell Lymphoma: Role of p53. J Invest Dermatol 132 (5):1487-1496. doi:jid201210 [pii]10.1038/jid.2012.10

Martinez P, Blasco MA (2011) Telomeric and extra-telomeric roles for telomerase and the telomere-binding proteins. Nat Rev Cancer 11 (3):161-176. doi:nrc3025 [pii]10.1038/nrc3025

Mahmoudi S, Henriksson S, Corcoran M, Mendez-Vidal C, Wiman KG, Farnebo M (2009) Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage. Mol Cell 33 (4):462-471. doi:S1097-2765(09)00073-2 [pii]10.1016/j.molcel.2009.01.028

McDaid JR, Loughery J, Dunne P, Boyer JC, Downes CS, Farber RA, Walsh CP (2009) MLH1 mediates PARP-dependent cell death in response to the methylating agent N-methyl-N-nitrosourea. Br J Cancer 101 (3):441-451. doi:6605186 [pii]10.1038/sj.bjc.6605186

Mengus C, Le Magnen C, Trella E, Yousef K, Bubendorf L, Provenzano M, Bachmann A, Heberer M, Spagnoli GC, Wyler S (2011) Elevated levels of circulating IL-7 and IL-15 in patients with early stage prostate cancer. J Transl Med 9:162. doi:1479-5876-9-162 [pii]

Mitchell JR, Wood E, Collins K (1999) A telomerase component is defective in the human disease dyskeratosis congenita. Nature 402 (6761):551-555. doi:10.1038/990141

Moiseeva O, Bourdeau V, Roux A, Deschenes-Simard X, Ferbeyre G (2009) Mitochondrial dysfunction contributes to oncogene-induced senescence. Mol Cell Biol 29 (16):4495-4507. doi:MCB.01868-08 [pii]

Mori MA, Liu M, Bezy O, Almind K, Shapiro H, Kasif S, Kahn CR (2010). A Systems Biology Approach Identifies Inflammatory Abnormalities between Mouse Strains Prior to Development of Metabolic Disease. Diabetes., 59. doi:10.2337/db10-0367

Myung SJ, Yang SK, Chang HS, Byeon JS, Kim KJ, Hong SS, Jeong JY, Lee SM, Hong WS, Kim JH, Min YI (2005) Clinical usefulness of telomerase for the detection of colon cancer in ulcerative colitis patients. J Gastroenterol Hepatol 20 (10):1578-1583. doi:JGH3877 [pii]

Muhlbradt E, Asatiani E, Ortner E, Wang A, Gelmann EP (2009) NKX3.1 activates expression of insulin-like growth factor binding protein-3 to mediate insulin-like growth factor-I signaling and cell proliferation. Cancer Res 69 (6):2615-2622. doi:0008-5472.CAN-08-3022 [pii]10.1158/0008-5472.CAN-08-3022nature01254 [pii]

Murakami J, Nagai N, Ohama K, Tahara H, Ide T (1997) Telomerase activity in ovarian tumors. Cancer 80 (6):1085-1092. doi:10.1002/(SICI)1097-0142(19970915)80:6<1085::AID-CNCR11>3.0.CO;2-4 [pii]

Nawaz S, Hashizumi TL, Markham NE, Shroyer AL, Shroyer KR (1997) Telomerase expression in human breast cancer with and without lymph node metastases. Am J Clin Pathol 107 (5):542-547. doi: 10.1093/jnci/89.24.1874Nepusz T, Yu H, Paccanaro A Detecting overlapping protein complexes in protein-protein interaction networks. (2012) Nat Methods. doi:nmeth.1938 [pii]10.1038/nmeth.1938

Newman, M. E. J (2005) A measure of betweenness centrality based on random walks. Soc. Networks 2005, 27, 39-54. doi:10.1016/j.socnet.2004.11.009

Nguyen H, Weng NP IL-21 preferentially enhances IL-15-mediated homeostatic proliferation of human CD28+ CD8 memory T cells throughout the adult age span. J Leukoc Biol 87 (1):43-49. doi:jlb.0209086 [pii]10.1189/jlb.0209086

Nicolas E, Morales V, Magnaghi-Jaulin L, Harel-Bellan A, Richard-Foy H, Trouche D (2000) RbAp48 belongs to the histone deacetylase complex that associates with the retinoblastoma protein. J Biol Chem 275 (13):9797-9804. doi: 10.1074/jbc.275.13.9797

Nishi H, Nakada T, Kyo S, Inoue M, Shay JW, Isaka K (2004) Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT). Mol Cell Biol 24 (13):6076-6083. doi:10.1128/MCB.24.13.6076-6083.200424/13/6076 [pii].

Nogueira V, Park Y, Chen CC, Xu PZ, Chen ML, Tonic I, Unterman T, Hay N (2008) Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. Cancer Cell 14 (6):458-470. doi:S1535-6108(08)00370-X [pii]10.1016/j.ccr.2008.11.003

Oh W, Ghim J, Lee EW, Yang MR, Kim ET, Ahn JH, Song J (2009) PML-IV functions as a negative regulator of telomerase by interacting with TERT. J Cell Sci 122 (Pt 15):2613-2622. doi:jcs.048066 [pii]

Ohanna M, Giuliano S, Bonet C, Imbert V, Hofman V, Zangari J, Bille K, Robert C, Bressac-de Paillerets B, Hofman P, Rocchi S, Peyron JF, Lacour JP, Ballotti R, Bertolotto C (2011) Senescent cells develop a PARP-1 and nuclear factor-{kappa}B-associated secretome (PNAS). Genes Dev 25 (12):1245-1261. doi:gad.625811 [pii]10.1101/gad.625811

Oishi T, Kigawa J, Minagawa Y, Shimada M, Takahashi M, Terakawa N (1998) Alteration of telomerase activity associated with development and extension of epithelial ovarian cancer. Obstet Gynecol 91 (4):568-571. doi:S0029-7844(98)00044-1 [pii]

Orjalo AV, Bhaumik D, Gengler BK, Scott GK, Campisi J (2009) Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. Proc Natl Acad Sci U S A 106 (40):17031-17036. doi:0905299106 [pii]10.1073/pnas.0905299106

Ovaska K, Laakso M, Haapa-Paananen S, Louhimo R, Chen P, Aittomaki V, Valo E, Nunez-Fontarnau J, Rantanen V, Karinen S, Nousiainen K, Lahesmaa-Korpinen AM, Miettinen M, Saarinen L, Kohonen P,

Wu J, Westermarck J, Hautaniemi S (2010) Large-scale data integration framework provides a comprehensive view on glioblastoma multiforme. Genome Med 2 (9):65. doi:gm186 [pii]10.1186/gm186

Park TW, Riethdorf S, Riethdorf L, Loning T, Janicke F (1999) Differential telomerase activity, expression of the telomerase catalytic sub-unit and telomerase-RNA in ovarian tumors. Int J Cancer 84 (4):426-431. doi:10.1002/(SICI)1097-0215(19990820)84:4<426::AID-IJC17>3.0.CO;2-1 [pii]

Park JI, Venteicher AS, Hong JY, Choi J, Jun S, Shkreli M, Chang W, Meng Z, Cheung P, Ji H, McLaughlin M, Veenstra TD, Nusse R, McCrea PD, Artandi SE (2009) Telomerase modulates Wnt signalling by association with target gene chromatin. Nature 460 (7251):66-72. doi:nature08137 [pii]

Park WY, Cho KA, Park JS, Kim DI, Park SC (2001). Attenuation of EGF signaling in senescent cells by caveolin. Ann N Y Acad Sci 928:79-84. doi: 10.1111/j.1749-6632.2001.tb05638.x

Parkinson EK, Munro J, Steeghs K, Morrison V, Ireland H, Forsyth N, Fitzsimmons S, Bryce S (2000) Replicative senescence as a barrier to human cancer. Biochem Soc Trans 28 (2):226-233

Passos JF, Saretzki G, von Zglinicki T (2007) DNA damage in telomeres and mitochondria during cellular senescence: is there a connection? Nucleic Acids Res 35 (22):7505-7513. doi:gkm893 [pii]10.1093/nar/gkm893

Phadke M, Krynetskaia N, Mishra A, Krynetskiy E (2011) Accelerated cellular senescence phenotype of GAPDH-depleted human lung carcinoma cells. Biochem Biophys Res Commun 411 (2):409-415. doi:S0006-291X(11)01178-8 [pii]10.1016/j.bbrc.2011.06.165

Polevoda B, Sherman F (2000) Nalpha -terminal acetylation of eukaryotic proteins. J Biol Chem 275 (47):36479-36482. doi:10.1074/jbc.R000023200R000023200 [pii]

Rajaraman P, Brenner AV, Butler MA, Wang SS, Pfeiffer RM, Ruder AM, Linet MS, Yeager M, Wang Z, Orr N, Fine HA, Kwon D, Thomas G, Rothman N, Inskip PD, Chanock SJ (2009) Common variation in genes related to innate immunity and risk of adult glioma. Cancer Epidemiol Biomarkers Prev 18 (5):1651-1658. doi:18/5/1651 [pii]10.1158/1055-9965.EPI-08-1041

Raimundo N, Song L, Shutt TE, McKay SE, Cotney J, Guan MX, Gilliland TC, Hohuan D, Santos-Sacchi J, Shadel GS (2012). Mitochondrial stress engages E2F1 apoptotic signaling to cause deafness. Cell 148 (4):716-726. doi:S0092-8674(12)00025-6 [pii]
10.1016/j.cell.2011.12.027

Ray A, James MK, Larochelle S, Fisher RP, Blain SW (2009) p27Kip1 inhibits cyclin D-cyclin-dependent kinase 4 by two independent modes. Mol Cell Biol 29 (4):986-999. doi:MCB.00898-08 [pii]

Raychaudhuri B, Vogelbaum MA (2011) IL-8 is a mediator of NF-kappaB induced invasion by gliomas. J Neurooncol 101 (2):227-235. doi:10.1007/s11060-010-0261-2

Reinhardt HC, Schumacher B (2012). The p53 network: cellular and systemic DNA damage responses in aging and cancer. Trends Genet 28 (3):128-136. doi:S0168-9525(11)00199-5 [pii]10.1016/j.tig.2011.12.002.

Ren JL, Pan JS, Lu YP, Sun P, Han J (2009) Inflammatory signaling and cellular senescence. Cell Signal 21 (3):378-383. doi:S0898-6568(08)00322-7 [pii]10.1016/j.cellsig.2008.10.011

Rivals I, Personnaz L, Taing L, Potier MC (2007) Enrichment or depletion of a GO category within a class of genes: which test? Bioinformatics 23, 401–407. doi: 10.1093/bioinformatics/btl633

Robinson K, Asawachaicharn N, Galloway DA, Grandori C (2009) c-Myc accelerates S-phase and requires WRN to avoid replication stress. PLoS One 4 (6):e5951. doi:10.1371/journal.pone.0005951

Ross OA, Curran MD, Meenagh A, Williams F, Barnett YA, Middleton D, Rea IM (2003) Study of age-association with cytokine gene polymorphisms in an aged Irish population. Mech Ageing Dev 124 (2):199-206. doi:S004763740200132X [pii]

Rossig L, Jadidi AS, Urbich C, Badorff C, Zeiher AM, Dimmeler S (2001) Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. Mol Cell Biol 21 (16):5644-5657. doi:10.1128/MCB.21.16.5644-5657.2001

Sahin E, Colla S, Liesa M, Moslehi J, Muller FL, Guo M, Cooper M, Kotton D, Fabian AJ, Walkey C, Maser RS, Tonon G, Foerster F, Xiong R, Wang YA, Shukla SA, Jaskelioff M, Martin ES, Heffernan TP, Protopopov A, Ivanova E, Mahoney JE, Kost-Alimova M, Perry SR, Bronson R, Liao R, Mulligan R, Shirihai OS, Chin L, DePinho RA (2011) Telomere dysfunction induces metabolic and mitochondrial compromise. Nature 470 (7334):359-365. doi:nature09787 [pii]10.1038/nature09787

Sakai N, Yoshidome H, Shida T, Kimura F, Shimizu H, Ohtsuka M, Takeuchi D, Sakakibara M, Miyazaki M CXCR4/CXCL12 expression profile is associated with tumor microenvironment and clinical outcome of liver metastases of colorectal cancer. Clin Exp Metastasis 29 (2):101-110. doi:10.1007/s10585-011-9433-5

Sakuma T, Uzawa K, Onda T, Shiiba M, Yokoe H, Shibahara T, Tanzawa H (2006) Aberrant expression of histone deacetylase 6 in oral squamous cell carcinoma. Int J Oncol 29 (1):117-124

Sano T, Asai A, Mishima K, Fujimaki T, Kirino T (1998) Telomerase activity in 144 brain tumours. Br J Cancer 77 (10):1633-1637. doi:10.1038/bjc.1998.267

Santos JH, Meyer JN, Skorvaga M, Annab LA, Van Houten B (2004) Mitochondrial hTERT exacerbates free-radical-mediated mtDNA damage. Aging Cell 3 (6):399-411. doi:ACE124 [pii]10.1111/j.1474-9728.2004.00124.x.

Sapozhnikov AM, Baev DV, Gusarova GA (2002) Involvement of heat shock proteins in the phenomenon of cell protection against apoptosis mediated by inhibitors of plasma membrane chlorine channels. Dokl Biol Sci 384:206-208

Saretzki G (2009) Telomerase, mitochondria and oxidative stress. Exp Gerontol 44 (8):485-492. doi:S0531-5565(09)00086-2 [pii]10.1016/j.exger.2009.05.004.

Sastre J, Pallardo FV, Vina J (2003) The role of mitochondrial oxidative stress in aging. Free Radic Biol Med 35 (1):1-8. doi:S0891584903001849 [pii]

Sattler M, Verma S, Shrikhande G, Byrne CH, Pride YB, Winkler T, Greenfield EA, Salgia R, Griffin JD (2000) The BCR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells. J Biol Chem 275 (32):24273-24278. doi:10.1074/jbc.M002094200
M002094200 [pii]

Shay JW, Bacchetti S (1997) A survey of telomerase activity in human cancer. Eur J Cancer 33 (5):787-791. doi:S0959-8049(97)00062-2 [pii]10.1016/S0959-8049(97)00062-2

Scheurer ME, Amirian E, Cao Y, Gilbert MR, Aldape KD, Kornguth DG, El-Zein R, Bondy ML (2008) Polymorphisms in the interleukin-4 receptor gene are associated with better survival in patients with glioblastoma. Clin Cancer Res 14 (20):6640-6646. doi:14/20/6640 [pii]10.1158/1078-0432.CCR-07-4681

Schulz VP, Zakian VA (1994) The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. Cell 76 (1):145-155. doi:0092-8674(94)90179-1 [pii]

Sedensky MM, Morgan PG (2006) Mitochondrial respiration and reactive oxygen species in C. elegans. Exp Gerontol 41 (10):957-967. doi:S0531-5565(06)00239-7 [pii]10.1016/j.exger.2006.06.056

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997, 88: 593–602. doi:10.1016/S0092-8674(00)81902-9

Shannon P, Markiel A, Ozier O, Baliga N.S, Wang J.T, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2 00313, 2498–2504. doi:10.1101/gr.1239303

Shelton DN, Chang E, Whittier PS, Choi D, Funk WD (1999) Microarray analysis of replicative senescence. Curr Biol 9 (17):939-945. doi:S0960-9822(99)80420-5 [pii]

Sichtig N, Korfer N, Steger G (2007) Papillomavirus binding factor binds to SAP30 and represses transcription via recruitment of the HDAC1 co-repressor complex. Arch Biochem Biophys 467 (1):67-75. doi:S0003-9861(07)00411-0 [pii]10.1016/j.abb.2007.08.015

Sitaram RT, Degerman S, Ljungberg B, Andersson E, Oji Y, Sugiyama H, Roos G, Li A (2010) Wilms' tumour 1 can suppress hTERT gene expression and telomerase activity in clear cell renal cell carcinoma via multiple pathways. Br J Cancer 103 (8):1255-1262. doi:6605878 [pii]10.1038/sj.bjc.6605878

Slattery ML, Lundgreen A, Bondurant KL, Wolff RK (2011).Tumor necrosis factor-related genes and colon and rectal cancer. Int J Mol Epidemiol Genet 2 (4):328-338

Skvortzov DA, Rubzova1 ME, Zvereva MP, Kiselev FL, Donzova OA. (2011). The Regulation of Telomerase in Oncogenesis. ActaNaturae 1(1): 51–67.

Song H, Li Y, Chen G, Xing Z, Zhao J, Yokoyama KK, Li T, Zhao M (2004) Human MCRS2, a cell-cycle-dependent protein, associates with LPTS/PinX1 and reduces the telomere length. Biochem Biophys Res Commun 316 (4):1116-1123. doi:10.1016/j.bbrc.2004.02.166S0006291X04004462 [pii]

Stanelle J, Stiewe T, Theseling CC, Peter M, Putzer BM (2002) Gene expression changes in response to E2F1 activation. Nucleic Acids Res 30 (8):1859-1867. doi: 10.1093/nar/30.8.1859

Stark C, Breitkreutz BJ, Reguly T, Boucher L, Breitkreutz A, Tyers M (2006) BioGRID: a general repository for interaction datasets. Nucleic Acids Res 34 (Database issue):D535-539. doi:34/suppl_1/D535 [pii]10.1093/nar/gkj109.

Steinert S, Shay JW, Wright WE (2000) Transient expression of human telomerase extends the life span of normal human fibroblasts. Biochem Biophys Res Commun 273 (3):1095-1098. doi:10.1006/bbrc.2000.3080S0006-291X(00)93080-8 [pii]

Steelman LS, Chappell WH, Abrams SL, Kempf RC, Long J, Laidler P, Mijatovic S, Maksimovic-Ivanic D, Stivala F, Mazzarino MC, Donia M, Fagone P, Malaponte G, Nicoletti F, Libra M, Milella M, Tafuri A, Bonati A, Basecke J, Cocco L, Evangelisti C, Martelli AM, Montalto G, Cervello M, McCubrey JA (2011) Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging. Aging (Albany NY) 3 (3):192-222. doi:100296 [pii]

Sun PM, Wei LH, Luo MY, et al. The telomerase activity and expression of hTERT gene can serve as indicators in the anti-cancer treatment of human ovarian cancer.Eur J Obstet Gynecol Reprod Biol 2007;130:249–57. doi: 10.1016/j.ejogrb.2006.01.028

Sugino T, Yoshida K, Bolodeoku J, Tahara H, Buley I, Manek S, Wells C, Goodison S, Ide T, Suzuki T, Tahara E, Tarin D (1996) Telomerase activity in human breast cancer and benign breast lesions: diagnostic applications in clinical specimens, including fine needle aspirates. Int J Cancer 69 (4):301-306. doi: 10.1002/(SICI)1097-0215(19960822)69:4<301::aid-ijc11>3.0.co-2-8.

Suram A, Kaplunov J, Patel PL, Ruan H, Cerutti A, Boccardi V, Fumagalli M, Di Micco R, Mirani N, Gurung RL, Hande MP, d'Adda di Fagagna F, Herbig U Oncogene-induced telomere dysfunction

enforces cellular senescence in human cancer precursor lesions. EMBO J 31 (13):2839-2851. doi:emboj2012132 [pii] 10.1038/emboj.2012.132

Takakura M, Kyo S, Inoue M, Wright WE, Shay JW (2005) Function of AP-1 in transcription of the telomerase reverse transcriptase gene (TERT) in human and mouse cells. Mol Cell Biol 25 (18):8037-8043. doi:25/18/8037 [pii]10.1128/MCB.25.18.8037-8043.2005

Tatsumoto N, Hiyama E, Murakami Y, Imamura Y, Shay JW, Matsuura Y, Yokoyama T (2000) High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. Clin Cancer Res 6 (7):2696-2701. doi: 10.1038/sj.bjc.6602054

Tchougounova E, Kastemar M, Brasater D, Holland EC, Westermark B, Uhrbom L (2007) Loss of Arf causes tumor progression of PDGFB-induced oligodendroglioma. Oncogene 26 (43):6289-6296. doi:1210455 [pii]10.1038/sj.onc.1210455

Testa JR, Tsichlis PN (2005) AKT signaling in normal and malignant cells. Oncogene 24 (50):7391-7393. doi:1209100 [pii]10.1038/sj.onc.1209100

Tjomsland V, Spangeus A, Valila J, Sandstrom P, Borch K, Druid H, Falkmer S, Falkmer U, Messmer D, Larsson M (2011) Interleukin 1alpha sustains the expression of inflammatory factors in human pancreatic cancer microenvironment by targeting cancer-associated fibroblasts. Neoplasia 13:664-675

Tomas-Loba A, Flores I, Fernandez-Marcos PJ, Cayuela ML, Maraver A, Tejera A, Borras C, Matheu A, Klatt P, Flores JM, Vina J, Serrano M, Blasco MA (2008) Telomerase reverse transcriptase delays aging in cancer-resistant mice. Cell 135 (4):609-622. doi:S0092-8674(08)01191-4 [pii]10.1016/j.cell.2008.09.034

Toogun OA, Dezwaan DC, Freeman BC (2008) The hsp90 molecular chaperone modulates multiple telomerase activities. Mol Cell Biol 28 (1):457-467. doi:MCB.01417-07 [pii]10.1128/MCB.01417-07

Trachootham D, Lu W, Ogasawara MA, Nilsa RD, Huang P (2008) Redox regulation of cell survival. Antioxid Redox Signal 10 (8):1343-1374. doi:10.1089/ars.2007.1957

Tsakiri KD, Cronkhite JT, Kuan PJ, Xing C, Raghu G, Weissler JC, Rosenblatt RL, Shay JW, Garcia CK (2007) Adult-onset pulmonary fibrosis caused by mutations in telomerase. Proc Natl Acad Sci U S A 104 (18):7552-7557. doi:0701009104 [pii] 10.1073/pnas.0701009104

Tso CL, Shintaku P, Chen J, Liu Q, Liu J, Chen Z, Yoshimoto K, Mischel PS, Cloughesy TF, Liau LM, Nelson SF (2006) Primary glioblastomas express mesenchymal stem-like properties. Mol Cancer Res 4 (9):607-619. doi:4/9/607 [pii]10.1158/1541-7786.MCR-06-0005

Ueda Y, Hiyama E, Kamimatsuse A, Kamei N, Ogura K, Sueda T (2011) Wnt signaling and telomerase activation of hepatoblastoma: correlation with chemosensitivity and surgical resectability. J Pediatr Surg 46 (12):2221-2227. doi:S0022-3468(11)00742-1 [pii]10.1016/j.jpedsurg.2011.09.003

Vafa O, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM, Wahl GM (2002) c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. Mol Cell 9 (5):1031-1044. doi:S1097276502005208 [pii]

Vargas J, Feltes B.C, de Faria Poloni J, Lenz G, Bonatto D (2012) Senescence; an endogenous anticancer mechanism. Frontiers in Bioscience 17, 2616-2643. dx.doi.org/10.2741/4074

Vaziri H, West M. D, Allsopp R C, Davison T S, Wu, Y S, Arrowsmith C H, Poirier G G, Benchimol S (1997) ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the posttranslational activation of p53 protein involving poly(ADP-ribose) polymerase. EMBO J 16: 6018–6033. doi: 10.1093/emboj/16.19.6018

Von Mering C, Huyne M, Jaeggi D, Schmidt S, Bork. P, Snel B (2003) STRING: a database of predicted functional associations between proteins.Nucleic Acids Res. 2003 31: 258–261. doi: 10.1093/nar/gkg034

Von Zglinicki T (2001) Telomeres and replicative senescence: Is it only length that counts? Cancer Lett. 168: 111–116. doi: 10.1016/S0304-3835(01)00546-8

Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green M (2008). Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. Cell132: 363–374. doi: 10.1016/j.cell.2007.12.032.

Wajapeyee N, Wang SZ, Serra RW, Solomon PD, Nagarajan A, Zhu X, Green MR (2010) Senescence induction in human fibroblasts and hematopoietic progenitors by leukemogenic fusion proteins. Blood 115 (24):5057-5060. doi:blood-2009-09-245928 [pii] 10.1182/blood-2009-09-245928 2012

Wang G, Chan CH, Gao Y, Lin HK (2011) Novel roles of Skp2 E3 ligase in cellular senescence, cancer progression, and metastasis. Chin J Cancer. doi:cjc.011.10319 [pii]10.5732/cjc.011.10319

Wan M, Li WZ, Duggan BD, Felix JC, Zhao Y, Dubeau L (1997) Telomerase activity in benign and malignant epithelial ovarian tumors. J Natl Cancer Inst 89 (6):437-441. doi: 10.1093/jnci/89.6.437

Wang J, Yi J (2008) Cancer cell killing via ROS: to increase or decrease, that is the question. Cancer Biol Ther 7 (12):1875-1884. doi:7067 [pii]

Wang J, Barnes RO, West NR, Olson M, Chu JE, Watson PH (2008) Jab1 is a target of EGFR signaling in ERalpha-negative breast cancer. Breast Cancer Res 10 (3):R51. doi:bcr2105 [pii]10.1186/bcr2105.

Wang Y, Liang Y, Vanhoutte PM (2011) SIRT1 and AMPK in regulating mammalian senescence: a critical review and a working model. FEBS Lett 585 (7):986-994. doi:S0014-5793(10)00965-8 [pii]10.1016/j.febslet.2010.11.047

Wang Y, Yang J, Yi J (2011) Redox sensing by proteins: oxidative modifications on cysteines and the consequent events. Antioxid Redox Signal 16 (7):649-657. doi:10.1089/ars.2011.4313

Wei S, Sedivy JM (1999) Expression of catalytically active telomerase does not prevent premature senescence caused by overexpression of oncogenic Ha-Ras in normal human fibroblasts. Cancer Res 59 (7):1539-1543. doi: 10.1074/jbc.M202671200

Werner T (2007) Regulatory networks: linking microarray data to systems biology. Mech Ageing Dev 128 (1):168-172. doi:S0047-6374(06)00255-7 [pii]10.1016/j.mad.2006.11.022

Wolkow CA, Iser WB (2006) Uncoupling protein homologs may provide a link between mitochondria, metabolism and lifespan. Ageing Res Rev 5 (2):196-208. doi:S1568-1637(06)00033-X [pii]10.1016/j.arr.2006.03.007

Won J, Yim J, Kim TK (2002) Opposing regulatory roles of E2F in human telomerase reverse transcriptase (hTERT) gene expression in human tumor and normal somatic cells. FASEB J 16 (14):1943-1945. doi:10.1096/fj.02-0311fje02-0311fje [pii]

Won J, Yim J, Kim TK (2002) Sp1 and Sp3 recruit histone deacetylase to repress transcription of human telomerase reverse transcriptase (hTERT) promoter in normal human somatic cells. J Biol Chem 277 (41):38230-38238. doi:10.1074/jbc.M206064200M206064200 [pii]

Woo SH, An S, Lee HC, Jin HO, Seo SK, Yoo DH, Lee KH, Rhee CH, Choi EJ, Hong SI, Park IC (2009) A truncated form of p23 down-regulates telomerase activity via disruption of Hsp90 function. J Biol Chem 284 (45):30871-30880. doi:M109.052720 [pii]10.1074/jbc.M109.052720

Wu J, Lee SW, Zhang X, Han F, Kwan SY, Yuan X, Yang WL, Jeong YS, Rezaeian AH, Gao Y, Zeng YX, Lin HK (2012) Foxo3a transcription factor is a negative regulator of Skp2 and Skp2 SCF complex. Oncogene. doi:onc201226 [pii]10.1038/onc.2012.26

Wu Y, Chen C, Sun X, Shi X, Jin B, Ding K, Yeung SC, Pan J (2012) Cyclin-dependent kinase 7/9 inhibitor SNS-032 abrogates FIP1-like-1 platelet-derived growth factor receptor alpha and bcr-abl oncogene addiction in malignant hematologic cells. Clin Cancer Res 18 (7):1966-1978. doi:1078-0432.CCR-11-1971 [pii]10.1158/1078-0432.CCR-11-1971

Wu YH, Cheng ML, Ho HY, Chiu DT, Wang TC (2009) Telomerase prevents accelerated senescence in glucose-6-phosphate dehydrogenase (G6PD)-deficient human fibroblasts. J Biomed Sci 16:18. doi:1423-0127-16-18 [pii]10.1186/1423-0127-16-18

Xu D, Erickson S, Szeps M, Gruber A, Sangfelt O, Einhorn S, Pisa P, Grander D (2000) Interferon alpha down-regulates telomerase reverse transcriptase and telomerase activity in human malignant and nonmalignant hematopoietic cells. Blood 96 (13):4313-4318

Xu D, Popov N, Hou M, Wang Q, Bjorkholm M, Gruber A, Menkel AR, Henriksson M (2001) Switch from Myc/Max to Mad1/Max binding and decrease in histone acetylation at the telomerase reverse transcriptase promoter during differentiation of HL60 cells. Proc Natl Acad Sci U S A 98 (7):3826-3831. doi:10.1073/pnas.07104319898/7/3826 [pii]

Yamaguchi H, Calado RT, Ly H, Kajigaya S, Baerlocher GM, Chanock SJ, Lansdorp PM, Young NS (2005) Mutations in TERT, the gene for telomerase reverse transcriptase, in aplastic anemia. N Engl J Med 352 (14):1413-1424. doi:352/14/1413 [pii] 10.1056/NEJMoa042980

Yan P, Benhattar J, Seelentag W, Stehle JC, Bosman FT (2004) Immunohistochemical localization of hTERT protein in human tissues. Histochem Cell Biol 121 (5):391-397. doi:10.1007/s00418-004-0645-5

Yang L, Guo S, Li Y, Zhou S, Tao S (2011) Protein microarrays for systems biology. Acta Biochim Biophys Sin (Shanghai) 43 (3):161-171. doi:gmq127 [pii]10.1093/abbs/gmq127

Yaswen P, Campisi J (2007). Oncogene-induced senescence pathways weave an intricate tapestry. Cell 128:233-4. doi:10.1016/j.cell.2007.01.005

Yoshida K, Sugino T, Goodison S, Warren BF, Nolan D, Wadsworth S, Mortensen NJ, Toge T, Tahara E, Tarin D (1997) Detection of telomerase activity in exfoliated cancer cells in colonic luminal washings and its related clinical implications. Br J Cancer 75 (4):548-553. doi:10.1038/bjc.1997.96

Yokoyama Y, Takahashi Y, Shinohara A, Lian Z, Tamaya T (1998) Telomerase activity in the female reproductive tract and neoplasms. Gynecol Oncol 68 (2):145-149. doi:S0090-8258(97)94921-0 [pii]10.1006/gyno.1997.4921

Yu H, Pardoll D, Jove R (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 9 (11):798-809. doi:nrc2734 [pii]10.1038/nrc2734

Yu, H, Kim P. M, Sprecher E, Trifonov V, Gerstein M (2007). The importance of bottlenecks in protein networks: correlation with gene essentiality and expression dynamics. PLoS Comput. Biol, 3, e59. doi:10.1371/journal.pcbi.0030059.eor

Zhang H, Pan K H, Cohen S N (2003) Senescence-specific gene expression fingerprints reveal cell-type-dependent physical clustering of up-regulated chromosomal loci. Proc Natl Acad Sci U S A 100: 3251–3256. doi: 10.1073/pnas.2627983100

Zhang Y, Iratni R, Erdjument-Bromage H, Tempst P, Reinberg D (1997) Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. Cell 89 (3):357-364. doi:S0092-8674(00)80216-0 [pii]

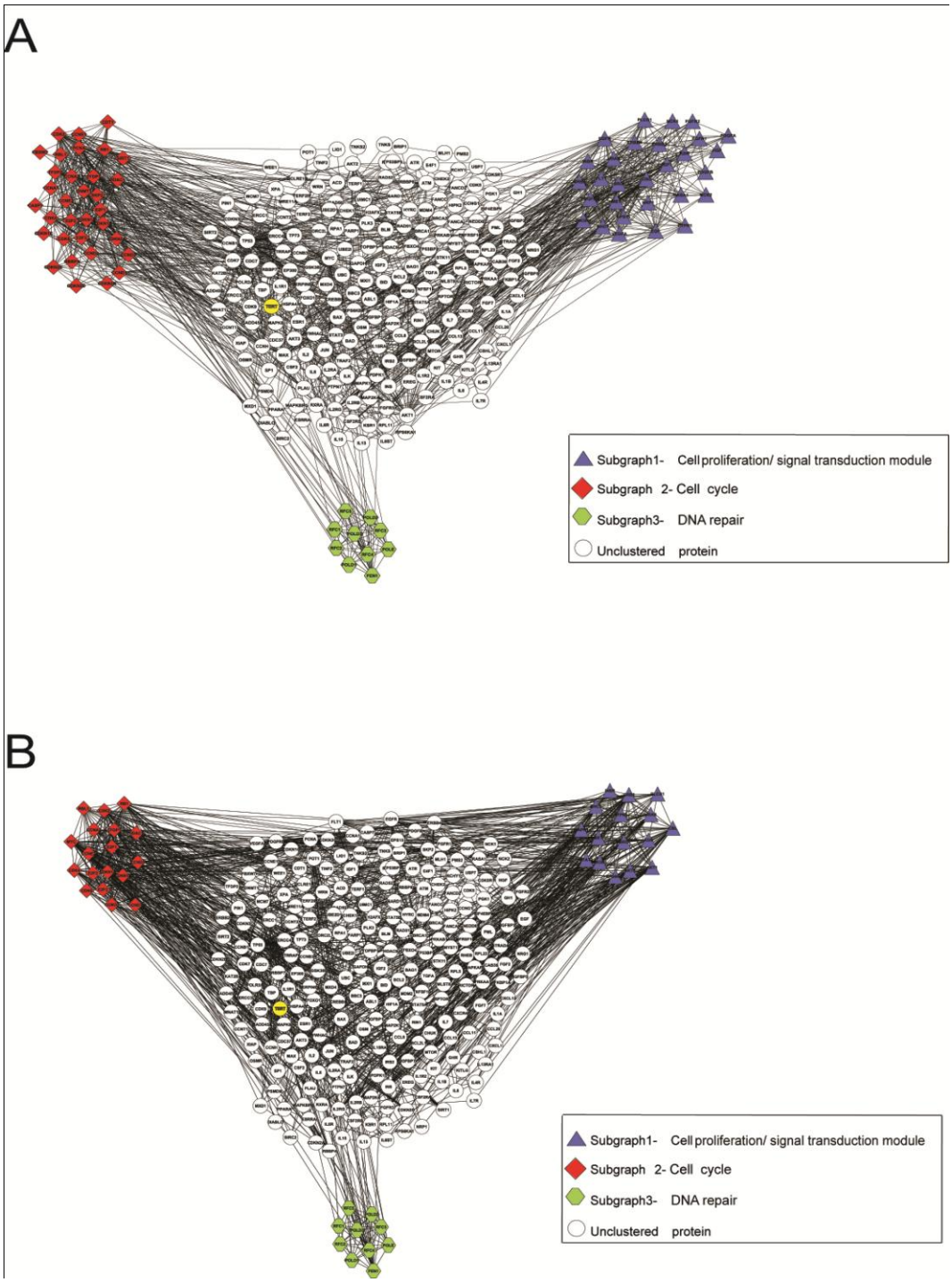
Zhang Y, Wang S, Li D, Zhnag J, Gu D, Zhu Y, He F (2011) A systems biology-based classifier for hepatocellular carcinoma diagnosis. PLoS One 6 (7):e22426. doi:10.1371/journal.pone.002242PONE-D-11-06815 [pii]

Zhu J, Zhao Y, Wang S (2010) Chromatin and epigenetic regulation of the telomerase reverse transcriptase gene. Protein Cell 1 (1):22-32. doi:10.1007/s13238-010-0014-1

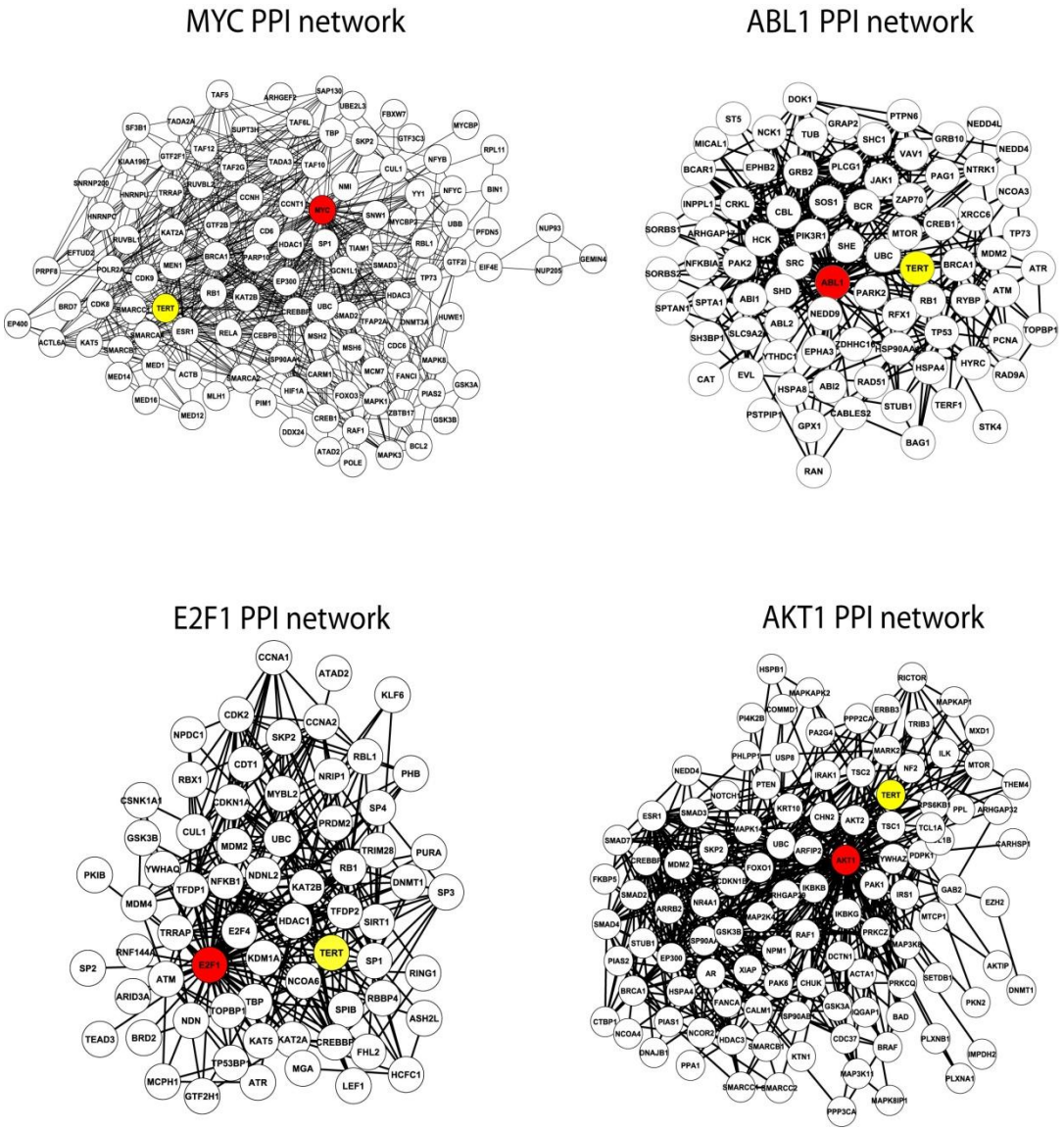
Zhu, J., Woods, D., McMahon, M., and Bishop, J. M (1998) Senescence of human fibroblasts induced by oncogenic Raf. Genes Dev., 12: 2997–3007. doi:10.1101/gad.12.19.2997

Zhuang D, Mannava S, Grachtchouk V, Tang WH, Patil S, Wawrzyniak JA, Berman AE, Giordano TJ, Prochownik EV, Soengas MS, Nikiforov MA (2008) C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. Oncogene 27 (52):6623-6634. doi:onc2008258 [pii]10.1038/onc.2008.258.

Supplementary figures

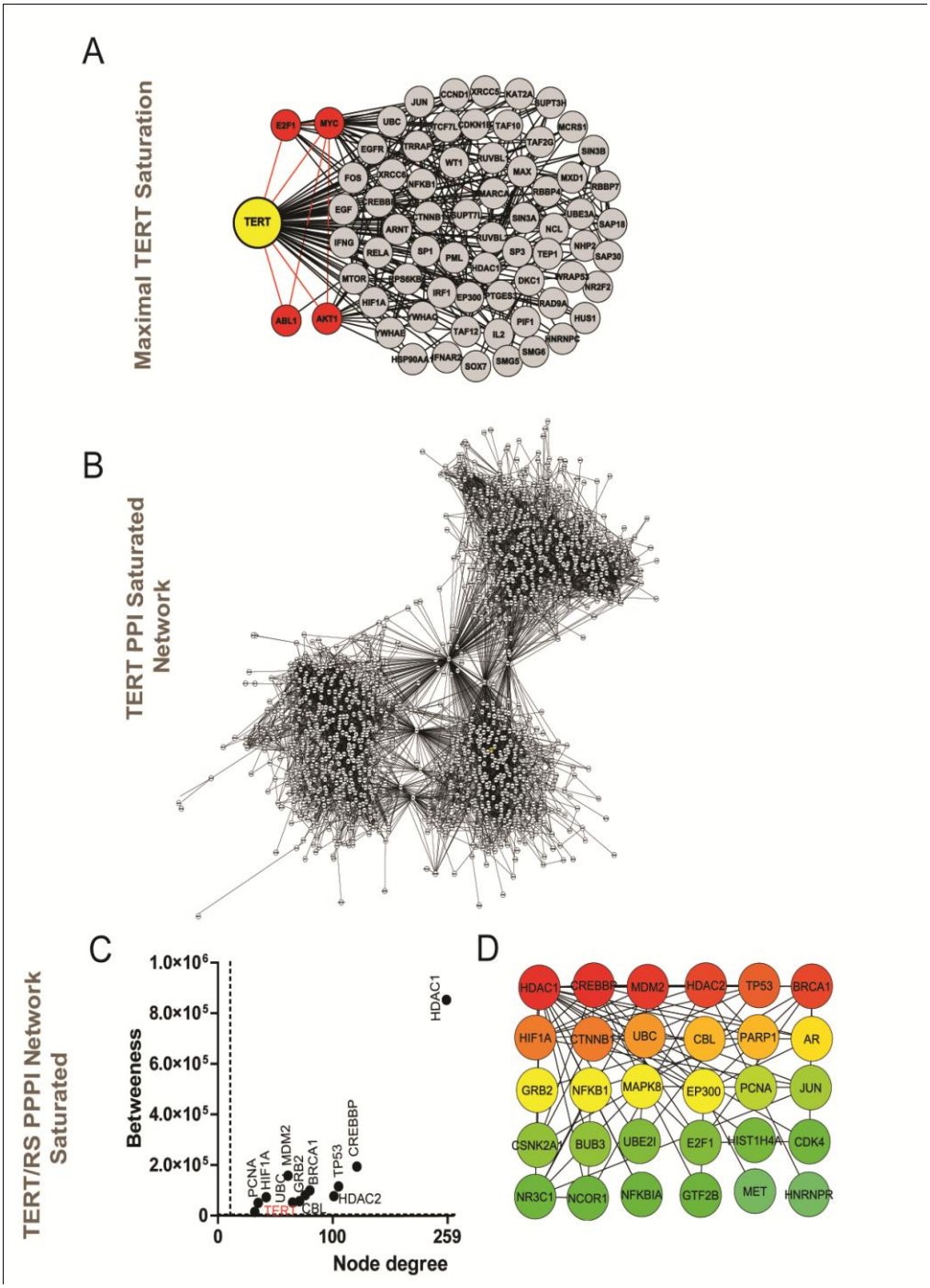


Supplementary Figure 1. Modular analysis of the UNION PPI network. (A) The highest scoring subgraphs based on CLUSTER-ONE software are represented by nodes with different shapes. GO analysis was used to determinate the major biological functions of each subgraph. (B) MCODE analysis defines the three highest scoring subgraphs.



Supplementary Figure 2. Analysis of all connectors associated with OIS. Maximal saturation of MYC, AKT1, E2F1 and ABL1 was used to design the MYC PPI, AKT1 PPI, E2F1 PPI and ABL1 PPI network

with STRING plus Biogrid.



Supplementary Figure 3. TERT saturation and centrality analysis of the TERT saturated PPI network. (A) Saturation of TERT protein using STRING plus STING (based on experimental data). (B) Maximal saturation of TERT/RS PPI network by STRING plus Biogrid. (C) and (D) Centrality analysis of the TERT saturated PPI network by CENTISCAPE and CYTO-HUBBA, respectively. Dashed lines represent the threshold value calculated for each centrality. Futhermore, CYTO-HUBBA plugin represent *nodes* in a graphical view with a *color* scheme to show priority, red represent highest value of centrality, yellow shown intermediate value of centrality and green low centrality values.

Supplementary tables

Supplementary table 1. The main proteins and peptides described in literature associated to senescence induction or senescence bypass (malignant transformation) are represented in this table. The number of identification according Human Aging Genomic Resources (HAGR-ID) is detailed for each protein or peptide.

Receptors and diffusible factors	HAGR-ID	Senescence/Cancer references
CCL	-	(Coppe et al. 2010)
EGF	0048	(Park et al. 2001)
EGFR	0040	(Cho et al. 2011)
FGFR	0169	(Brooks et al. 2012)
GHR	001	(Guevara-Aguirre et al. 2011; Liu et al. 2003)
GM-CSF	-	(Coppe et al. 2010)
HCC-4	-	(Coppe et al. 2010)
HGF	-	(Bavik et al. 2006)
IGFBP3	0073	(Muhlbradt et al. 2009)
IGFBP7	-	(Vargas et al. 2012)
IL-13	-	(Scheurer et al. 2008; Bozinov et al. 2010; Ren et al. 2009)
IL-15	-	(Nguyen and Weng 2010; Ren et al. 2009)
IL-1a	-	(Tjomsland et al,2011; Orjalo et al, 2009; Ren et al,2009)
IL-1b	-	(Ren et al,2009; Dudas et al,2011)
IL-2	0046	(Jen et al,2011; Ross et al,2003)
IL-6	0114	(Maggio et al,2009; Frampton et al,2011)
IL-7	0167	(Carvalho et al,2001; Mengus et al,2011)
IL-8	-	(Raychaudhuri and Vogelbaum,2011; Bhaumik et al, 2009)
KGF	-	(Wang et al, 2011; Wu et al, 2012)
MCP-2	0167	(Coppe et al. 2010)
MCP-4	-	(Rajaraman et al, 2009)
MIP-1a	-	(Harry,2000; Coppe et al,2008)
MIP-3a	-	(Coppe et al,2010)
MLH1	0243	(Kim et al, 2006)
PDGFB	0047	(Tchougounova et al. 2007)

PIGF	-	(Coppe et al. 2010)
SCF	-	(Sakai et al. 2011)
SDF-1	-	(Coppe et al. 2010)
VEGF	0077	(Dutra-Oliveira et al. 2012; Hasan et al, 2011)

Oncogenes and tumor supressors	**HAGR-ID**	**Senescence/Cancer references**
AKT1	0035	(Astle et al, 2012; Vargas et al, 2012)
AKT2	-	(Steelman et al. 2011)
AKT3	-	(Steelman et al. 2011)
CDK2	-	(Collins et al.1997; Malumbres andBarbacid 2009)
CDK3	-	(Collins et al.1997; Malumbres and Barbacid 2009)
CDK4	-	(Collins et al.1997; Malumbres and Barbacid 2009)
CDK5	-	(Collins et al.1997; Malumbres andBarbacid 2009)
CDK6	-	(Collins et al,1997; Malumbres andBarbacid 2009)
CDK7	-	(Collins et al. 1997; Malumbres andBarbacid 2009)
CDK9	-	(Wu et al. 2012;Vargas et al. 2012)
CDKN1A	-	(Vargas et al. 2012)
CDKN1B	-	(Vargas et al. 2012)
CDKN2A	0226	(Wang et al,2010); (Vargas et al, 2012)
CDKN2B	-	(Vargas et al. 2012)
CDKN2C	-	(Vargas et al. 2012)
CDKN2D	-	(Vargas et al. 2012)
E2F1	0018	(Dimri et al. 2000; Chen et al. 2009)
ERK	0175	(Vargas et al. 2012)
MAPK8	0163	(Ewald et al. 2010; Slattery et al. 2011)
MDM2	0210	(Manfe et al. 2012; Chaar et al. 2012)
MEK	0179	(Vargas et al. 2012)
MYC	0039	(Zhuang et al. 2008; Dang et al. 2012)
PCNA	0113	(Rossig et al. 2001)
pRB	0120	(Hickman et al. 2002; Vargas et al. 2012)

RAS	0038	(Vargas et al. 2012)
STAT5A	0021	(Yu et al. 2009)
STAT5B	0023	(Yu et al. 2009)
TP53	0006	(Reinhardt and Schumacher 2012); (Vargas et al. 2012)
DNA repair proteins	**HAGR-ID**	**Senescence/Cancer references**
ATM	0009	(Vargas et al. 2012)
ATR	0231	(Vargas et al. 2012)
BRCA1	0061	(Vargas et al. 2012)
ERCC1	0149	(Martínez and Blasco 2011)
FANCD2	0079	(Martinez and Blasco 2011)
H2AX	0212	(Martinez and Blasco 2011)
MLH1	0243	(McDaid et al. 1994; Kim et al. 2006)
NBS1	0044	(Bai and Murnane 2003)
PARP1	0060	(Martinez and Blasco 2011)
RAD51	0084	(Martinez and Blasco 2011)
XPF	0261	(Martinez and Blasco 2011)
Telosome/shelterin complex	**HAGR-ID**	**Senescence/Cancer references**
ACD	0079	(Martinez and Blasco 2011; Vargas et al. 2012)
POT1	-	(Martinez and Blasco 2011; Vargas et al. 2012)
TERF1	0105	(Martinez and Blasco 2011; Vargas et al. 2012)
TERF2	0116	(Martinez and Blasco 2011; Vargas et al. 2012)
TINF2	-	(Martinez and Blasco 2011; Vargas et al. 2012)
Energetic metabolism	**HAGR-ID**	**Senescence/Cancer references**
AMPK	-	(Luo et al. 2005)
AMPK	-	(Wang et al. 2011)
ATG 3	-	(Kang et al. 2011)
ATG 5	-	(Luo et al. 2011)
G6PD	-	(Wu et al. 2009)
GAPDH	-	(Phadke et al. 2011)
GLUT-1	-	(Macheda et al. 2005)

GLUT-2	-	(Macheda et al. 2005)
HSP70	0160	(Sapozhnikov et al. 2002)
IGF-1	0028	(Campisi 2004)
IRS2	0034	(Li et al. 2008)
LKB1	0093	(Gurumurthy et al. 2008)
mTOR	0221	(Dazert and Hall,2011)
PARPs	-	(Ohanna et al. 2011)
PGC-1	0256	(Balha et al. 2011); (Sahin et al. 2011)
PKA	-	(Chiaradonna et al. 2008)
PPAR	0055	(Chang et al. 2008)
RISP	-	(Moiseeva et al. 2009)
SIRT1	0150	(Kyrylenko and Baniahmad 2010)
SIRT2	-	(Kyrylenko and Baniahmad 2010)
SIRT3	-	(Kyrylenko and Baniahmad 2010)
SIRT4	-	(Kyrylenko and Baniahmad 2010)
SIRT5	-	(Kyrylenko and Baniahmad 2010)
SIRT6	0239	(Kyrylenko and Baniahmad 2010)
SIRT7	-	(Kyrylenko and Baniahmad 2010)
UCP1	0258	(Wolkow and Iser 2006)
UCP2	0235	(Wolkow and Iser 2006)
UCP3	0232	(Wolkow and Iser 2006)

Telomere enlogation	**HAGR-ID**	**Senescence/Câncer references**
TERT	0008	(Kang et al. 2004; Deng et al. 2008)

Supplementary table 2. CentiScape analysis: degree and betweeness centrality scores of each node of all initial networks are plotted in tables below.

TERT PPI NETWORK		
ID	**CentiScape Betweeness**	**CentiScape Degree**
TP53	1564.087	21
EGFR	841.795	11
MAPK1	550.097	8
ATM	545.776	7
CDK7	229.211	8
CDKN1B	220.771	12
IL8	216	2
CDKN1A	215.894	14
IL2	215.821	8
RB1	210.07	11
TERT	153.285	5
OIS PPI NETWORK		
ID	**CentiScape Betweeness**	**CentiScape Degree**
TP53	10643.4	64
MYC	4134.443	46
UBC	4107.041	31
PCNA	3675.498	35
BRCA1	3475.514	37
AKT1	3262.842	38
PIK3R1	3226.814	47
MDM2	3195.085	37
CDKN1A	2397.625	44
ATM	2275.672	22
TP53	10643.4	64
UNION PPI NETWORK		
ID	**CentiScape Betweeness**	**CentiScape Degree**

TP53	15201.2	75
UBC	6101,618	36
MYC	5601,066	53
BRCA1	5246,707	45
AKT1	4310,952	43
PCNA	4255,679	37
PIK3R1	4025,417	50
MDM2	3914,568	39
ATM	3572,375	25
EP300	3131,143	36
TERT	596.1326	22

Supplementary table 3 Tumor gene expression databases based on four human cancers data sets from the Cancer Genome Atlas-TCGA (http://csbl.fimm.fi/anduril/site).

	GLIOBLASTOMA MULTIFORME		OVARIAN CANCER		COLON CANCER		BREAST CANCER	
Oxidative genes	**Fold change**	**p correction**	**Fold change**	**p correction**	**Fold change**	**p correction**	**Fold change**	**p correction**
AOX1	1,42	0,0313	0.363	0.00986	0.301	8.74e-10	1.08	1
ALB	0.921	1,00	0.975	1	-	-	-	-
ALOX12	0.986	1,00	0.775		1.42	6.35e-6	1.02	1
ANGPTL7	1	1,00	0.329	0.329	0.0161	1.56e-14	0.00822	2.20e-32
APOE	1.54	0.00157	2.41	0.0157	0.565	0.0298	0.222	5.23e-16
ATOX1	1.16	1,00E+00	0.972	1.00	1.65	0.000432	1.38	0.0257
blvra	1,36	0,011	1.12	1	0.770	0.269	1.72	8.10e-16
blvrb	1,2	1	0.606	1	0.810	8.17e-7	1.21	0.0729
CAT	2,09	6,30e-07						
CCL5	1.70	1.79e-5	0.755	0.567	0.337	1.84e-5	1.38	0.00250
CCS	1.24	0.0327	1.01	1	-	-	1.73	8.10e-6
CSDE1	1.04	1,00	1.48	0.165	0.806	1.86e-10	-	-
CYBA	4.00	3.04e-5	0.907	1.00	-	-	0.854	0.00435
CYGB	0.317	7.23e-6	0.455	0.0194	-	-	-	-
DGKK	1.00	0.114	1.00	0.219	-	-	-	-
DHCR24	0.323	2.14e-7	1.33	0.849	0.922	1	-	-
DUOX1	0.953	1,00	0.340	0.0213	2.43	0.000116	2.33	2.08e-19
DUOX	1	1,00	0.939	1	4.12	0.0068	0.616	0.051

2						4		1
DUSP1	1.83	0.199	0.478	0.479	0.430	2.09e-6	0.974	1
EPX	1	1,00	0.996	1	-	-	0.182	8.18e-25
FOXM1	9.71	2.41e-8	6.83	0.0102	-	-	-	-
GLRX1	1,54	0,000834	-	-	-	-	-	-
GLRX2	0.871	0.275	1.27	1				
GPR156	1.55	0.00431	1.34	0.0455	3.30	2.07e-5	1.80	2.72e-11
GPX1	1,41	0,114	1.01	1	1.39	0.000112	1.56	0.0119
GPX2	1,03	1	0.155	0.0897	2.65	2.93e-5	0.573	0.0309
GPX3	1,86	1,74e-06	0.775	1	0.197	1.26e-11	0.153	9.74e-17
GPX4	0,911	0,0245	0.825	0.489	-	-	-	-
GSR	0.981	1,00e+00	0.738	0.0930	0.987	1	1.98	1.49e-18
GSS	1.14	0.125	1.19	1	1.19	0.00663	1.47	2.88e-16
GSTZ1	1.32	0.0343	0.898	1	0.768	1	1.20	0.137
GTF2I	1.08	1,00e+00	1.38	0.129	1.66	0.000234	1.26	1.94e-5
Hao-1	1	1	1	1	1.35	4.75e-6	0.839	1.00
HMOX1	4,02	0,000728	0.710	1	0.295	1.53e-12	1.74	0.0192
HMOX2	0,506	2,66e-05	0.682	-	1.35	9.11e-5	1.88	6.73e-20
IPCEF1	0.178	2.63e-8	0.533	0.0935	0.483	0.000166	0.867	1
KRT1	1	1,00	0.797	0.709	0.0592	8.19e-7	0.227	2.62e-11
LPO	0.993	1,00	0.926	1	2.49	0.00688	1.47	0.00470
MBL2	0.981	1,00	1	1	1.17	1	1	1
MGST	0.525	0.041	1.12	1	0.404	8.50e-	0.896	1

3		4				22		
MPO	0,884	1	1	1	3.62	8.62e-8	0.584	2.00e-7
MSRA	0.727	0.00232	0.555	0.0326	0.937	1	0.755	9.76e-7
MT3	1.03	1,00	0.933	1	0.199	6.79e-22	1.02	1
MTL5	1	1,00	1.43	0.259	1.08	1	-	-
NCF1	-	-	-	-	-	-	-	-
NCF2	2.39	0.00250	1.09	1	0.869	0.207	1.26	0.164
NME5	0.514	8.70e-5	0.265	0.664	-	-	-	-
NOS	1,03	0,166	-	-	-	-	-	-
NOS2	0.932	0.000126	0.939	1	1.09	1	1.23	1
NOX5	1.47	1,00	-	-	0.272	0.00146	0.267	3.12e-8
NUDT1	2.28	5.74e-6	1.15	1	1.37	0.00219	1.71	1.90e-17
OXR1	0.246	5.53e-8	1.35	1	0.794	1	0.512	2.94e-9
OXSR1	1.31	0.00439	1.66	0.0268	0.801	1.76e-6	1.24	8.32e-5
PDLIM1	3.55	0.00241	0.395	0.00160	0.621	0.000241	0.652	7.46e-8
PNKP	1.16	0.281	0.737	0.286	-	-	-	-
PON1	0,862	1	1.08	1	1.05	1	0.875	0.262
PON2	1,68	0,000722	1.70	0.0143	0.892	1	1.06	1
PON3	0,643	0,243	0.578	1.00	0.867	0.751	0.248	1.41e-12
PRDX1	1,16	0,0823	1.48	0.153	-	-	-	-
PRDX2	0,952	0,385	1.25	0.216	1.54	2.62e-5	1.58	7.34e-12
PRDX3	1,01	1	1.84		1.39	0.0164	0.949	1
PRDX4	4,2	6,97e-06	0.997	1	2.16	2.87e-15	1.48	1.61e-12

PRDX5	0,85	0,0701	0.737	0.0571	0.892	1	-	-
PRDX6	1,4	0,0204	1.21	0.652	1.03	-	0.987	1
PREX1	1.36	0.183	0.932	1	0.752	0.00293	1.86	2.91e-6
PRG3	1.02	0.504	0.873	1	0.857	0.0427	0.495	5.39e-7
PRNP	0.469	2.92e-9	1.22	0.785	1.13	1	0.799	1.88e-5
PTGS1	2.40	6.71e-7	1.04	1	0.261	1.24e-13	1.13	0.111
PTGS2	0.644	0.0682	0.397	0.137	3.62	0.000237	0.0940	8.23e-23
PXDN	4.36	9.49e-9	2.45	0.0133	1.70	0.000509	1.91	3.66e-5
PXDNL	1.33	1.00e-5	0.988	0.0585	0.949	1	2.21	3.55e-5
REL	0,536	3,50E-06	1.13	0.441	1.07	1	1.62	7.93e-11
RNF7	1.35	0.490	1.29	0.358	0.655	4.47e-7	1.14	0.183
SCARA3	1.72	2.11e-5	0.421	0.833	0.917	1	0.774	0.00632
SEPP1	1.43	1.00	1.14	1	-	-	-	-
SGK2	0.496	0.00619	0.877	1	0.198	1.62e-14	0.108	3.65e-16
SIRT2	0.905	1,00	0.964	1	1.51	0.00611	1.16	1
SOD1	0,629	3,99e-16	0.875	0.189	0.587	7.13e-9	1.10	1
SOD2	2,97	1,28e-07	0.581	0.799	3.23	3.47e-7	0.611	0.000123
SOD3	0,999	0,243	0.194	0.00466	1.05	1	0.163	1.04e-25
SRXN1	0.917	1,00	1.41	0.00382	-	-	-	-
STK25	0.647	9.78e-8	0.951	1.00	1.50	2.48e-10	1.29	0.00214
TPO	0.980	1,00	0.820	1	0.104	2.49e-13	0.0781	1.31e-39
TRP14	1,84	0,002	1.07	1	0.622	6.90e-6	1.58	2.25e-

		9						10
TRX1	0,876	1	-	-	1.23	-	1.58	2.25e-10
TRX2	1,18	0,276	-	-	2.02	-	1.07	0.925
TRX3	1,02	1	1	1	1.07	1	1.07	0.925
TTN	0.708	0.0235	0.645	0.0143	0.831	1	0.477	1.60e-9
TXNDC2	1.48	0.0268	0.807	1	1.53	0.00185	1.13	1
TXNRD1	1.59	0.000220	2.47	0.0390	1.18	0.484	1.31	0.000331
TXNRD2	1.02	1,00	1	1	1.07	1	1.07	0.925
WWOX	0,963	1	0.794	1	1.15	1	0.685	4.94e-8
TERT	**1**	0.762	0.824	0.822	1.41	7.15e-6	1.06	1

4. DISCUSSÃO

O GBM é o mais comum entre os tumores cerebrais, apresentando alta taxa de mortalidade e morbidade. Este tipo tumoral representa um desafio para as atuais terapias sendo assim foco de desenvolvimento de novos tratamentos. A quimioterapia mostrou-se promissora para o tratamento de GBM. Porém, a TMZ, o quimioterápico mais utilizado tem surtido pouco efeito, com alta taxa de recorrência e progressão da doença [261]. Este composto, da mesma forma que outros quimoterápicos, induz a ativação de sinais de dano ao DNA ou aumenta de atividade de supressores tumorais, o que culmina na indução de mecanismos endógenos antitumorais como senescência (revisada no capitulo 1 desta tese), apoptose [37] ou autofagia [37].

No século XX, as plantas medicinais ocuparam um lugar relevante no tratamento e prevenção de doenças. Atualmente, elas têm voltado a ocupar este espaço [76-79]. Dois polifenois estão sendo estudados por trazerem benefícios à saúde: o resveratrol e a quercetina. Ambos estão presentes em numerosas fontes de origem vegetal, como foi descrito na seções *1.2.1* e *1.2.2*. Recentemente, foi descrito que o resveratrol, além de ativar SIRT1, participa na inibição direta da atividade da fosfosdiesterase A, retardando o envelhecimento em células de mamíferos [133]. Cabe também ressaltar que o efeito do resveratrol varia de acordo com o tipo celular e com o estado metabólico da célula. Em gliomas, o resveratrol inibe o crescimento de células RT2, U251, U87 e C6, induzindo apoptose, inibindo a angiogênese tumoral e aumenta a sobrevida dos pacientes [135]. O resveratrol interage com numerosos alvos moleculares afetando múltiplas cascatas de sinalização [208]. No entanto, o mecanismo mais descrito é a ativação em forma indireta da SIRT1 após administração deste polifenol, como descrito na seção *1.2.1.3*. Este retarda o envelhecimento celular em mamíferos [133]. No entanto, a SIRT1, quando desregulada, está relacionada ao desenvolvimento de câncer, como detalhado na seção *1.2.4*. Neste sentido, o tratamento com inibidores da SIRT1 por HDACi tem efeito antitumoral pela indução da expressão de genes supressores tumorais e aumento nos níveis de acetilação de K16-H4 e K9-H3 em linhagens celulares de câncer do cólon [216].

Por outro lado, a quercetina também pode atuar como inibidor de HDAC como demonstrado na linhagem de leucemia humana HL-60 e em um modelo tumoral de câncer de hamster (HBP) [220-222]. Assim, a eficácia na indução de senescência pela combinação de resveratrol e quercetina pode ser devido a esta inibição [140]. No

entanto, para testar de forma mais objetiva se o resveratrol poderia ter um efeito pró-tumoral pela ativação de SIRT1, a combinação de HDACi com resveratrol foi investigada, no entanto o mecanismo de inibição de butirato de sódio não seja direto sobre a atividade de SIRT1. Na mesma linha, se o efeito pró-senescência da quercetina for somente através da inibição de HDAC, um HDACi não teria efeito aditivo quando quercetina estiver presente. Neste sentido, a inibição farmacológica de HDAC combinada com o tratamento com o resveratrol ou quercetina poderia apresentar novas evidências sobre a atividade antitumoral destes compostos.

Inicialmente, foram determinadas as concentrações sub-letais de butirato de sódio em tratamentos crônicos em linhagens de GBM *in vitro* (capitulo 2 - figura suplementar 1). Como foi descrito na seção *1.1.2,* senescência pode ser induzida por exaustão replicativa ou por as células submetidas a condições de estresse [262]. Em várias linhagens tumorais, a utilização de quimioterápicos em forma crônica induz senescência após vários dias de tratamento [140, 263-265]. Assim, foi realizado o tratamento de duas diferentes linhagens comerciais de GBM - uma proveniente de humanos (U87-MG), e outra de rato (C6) - com diferentes tempos e doses. A concentração de 2 mM foi utilizada como controle experimental para ensaios posteriores, devido ao fato de que os efeitos produzidos nesta dose já foram descritos na literatura, assim como alterações na viabilidade e distribuição do ciclo celular [202]. As concentrações dos polifenois foram determinadas de acordo com dados previamente publicados pelo grupo [140, 266]. Assim, diferentes doses de resveratrol foram combinadas com butirato de sódio, e uma única dose de quercetina foi combinada com o ácido graxo em diferentes tempos (24, 48 e 72 horas). Ambos os tratamentos reduziram significativamente o número de células e a viabilidade celular quando comparados com o controle não tratado. No entanto, não houve diferença quando comparados com o butirato de sódio em ambas as linhagens celulares (Capítulo 2- figuras 1a). Como o foco do estudo é senescência, foram realizados tratamentos crônicos utilizando a concentração menor de resveratrol (10 µM) e de quercetina (25 µM) combinados com 2 mM de butirato de sódio durante 10 dias, com reposição das drogas a cada 2 dias para poder avaliar um possível efeito combinado a longo prazo. Os resultados mostraram que os co-tratamentos em forma crônica reduzem significativamente a viabilidade celular quando comparados ao tratamento controle de butirato de sódio (Capítulo 2- figura 1 b).

Uma das principais características de quimioterápicos considerados bons é a seletividade sobre células tumorais sem danificar células normais. Zamin e colaboradores (2006) mostraram o efeito neuroprotetor do resveratrol em culturas organotípicas de hipocampo de rato submetidas à privação de oxigênio e glicose (POG) [267]. Isso sugere que este composto tem potencial citotóxico para células neoplásicas e citoprotetor para células sadias. De maneira similar, Braganhol e colaboradores (2006) observaram a mesma característica para a quercetina, que mostrou um efeito neuroprotetor frente à POG em culturas organotípicas de hipocampo e, utilizando as mesmas concentrações, um efeito antitumoral em linhagens de glioma U138-MG [165]. Outros trabalhos também têm demonstrado efeitos neuroprotetores do resveratrol e da quercetina em modelos de doença de Alzheimer e Parkinson, entre outros, como já referido anteriormente (seção *1.2.1* e *1.2.2*). Além disso, vários estudos mostraram que o butirato de sódio não danifica células normais [204], mas induz senescência ou apoptose em diversas linhagens tumorais (descrito na seção *1.2.3*) [190, 268, 269]. No presente trabalho foi avaliado o efeito dos tratamentos combinados em culturas primárias de astrócitos nas mesmas doses e tempos utilizados nas linhagens de gliomas (Capítulo 2 - figura 1 c). Não houve redução da viabilidade celular, corroborando com os resultados prévios obtidos. Esta característica torna esses compostos bons candidatos à utilização em terapias antitumorais, porém os mecanismos de ação que expliquem o efeito ambíguo destes compostos ainda não foram esclarecidos.

O próximo passo foi avaliar a indução de senescência celular já que em longo prazo obteve-se um resultado diferente do curto prazo nos tratamentos combinados comparados aos tratamentos individuais. Inicialmente, foi observado que no quarto dia de tratamento com resveratrol ou quercetina combinadas com butirato de sódio, havia uma redução significativa no número de células (Capítulo 2- figura 1 c). Nesse mesmo dia foram detectadas mudanças características de CS na morfologia celular após os tratamentos (Capítulo 2 - figura 2 a). A CS foi confirmada pela atividade da enzima β-galactosidase ácida, com a qual cerca de 80-90 % das células (C6 e U87-MG) foram marcadas após o tratamento combinado. No entanto, o tratamento com butirato de sódio produziu apenas 50% das células marcadas em ambas as linhagens celulares (capítulo 2- figura 2b).

Uma das principais características que define células senescentes é a perda irreverssível da capacidade proliferativa (como descrito no Capítulo 1). Assim, a capacidade de formação de colonias após 4 dias de tratamento foi testada, deixando as

células C6 e U87-MG proliferarem em meio sem drogas durante 10 e 16 dias, respectivamente. Foi observado que, em C6, os tratamentos combinados reduziram significativamente a formação de colônias, diferente dos tratamentos individualizados (Capítulo 2- figura 2c). Porém, em U87-MG só o tratamento com quercetina e butirato de sódio mostrou-se mais efetivo que os outros (Capítulo 2 - figura 2c). Provavelmente, em ambas linhagens, as células que sobreviveram ou não ficaram senescentes após 4 dias de tratamento conseguiram formar colônias (Capítulo 2 - figura 2c). As diferentes respostas de cada linhagem frente ao mesmo tratamento podem ser explicadas por suas características específicas. Por exemplo, a linhagem U87-MG possui a proteína PTEN deletada e TP53 funcional; já a linhagem C6 possui ambas as proteínas funcionais [270, 271]. A heterogeneidade dos tumores é um dos principais desafios para quem os estuda e busca um tratamento para os pacientes acometidos dessa doença.

Posteriormente, foram estudadas as vias de sinalização que podem afetar a proliferação celular em glioblastomas. A via da PI3K/AKT está superativada em uma grande proporção dos GBM, induzindo crescimento e sobrevivência celular [272]. Os resultados obtidos mostraram que as drogas combinadas não inibiram a fosforilação de AKT em C6 após 24 e 72 horas de tratamento. Porém, o tratamento combinado e o butirato de sódio em U87-MG induziram a diminuição da forma fosforilada de AKT após 24 horas de tratamento (Capítulo 2 - figura 3 a). O papel da AKT na indução de senescência celular ainda permanece controverso. Nogueria e colaboradores (2009) demonstraram que a ativação da AKT induziu senescência prematura em cultura de fibroblastos de embrião de camundongo, e esta indução foi mediada pelo aumento de ERO induzido por AKT [273]. Porém, outro trabalho mostrou que ao restaurar o gene RASSF1A em células tumorais, houve um aumento da indução da senescência após ativação da via RAS-MEK-ERK e da inativação da via da AKT [274]. Novamente, diferentes respostas podem ser obtidas ao estimular a mesma via, dependendo do tipo celular, e muito provavelmente da intensidade do estímulo e tantos outros parâmetros que fazem a biologia celular tão diversa.

Para confirmar se os polifenois e o butirato de sódio em forma individual ou combinada afetam ou não a expressão ou atividade de proteínas envolvidas na proliferação celular, proteínas associadas ao avanço ou inibição do ciclo celular foram estudadas. Como foi descrito na seção *1.1.1,* a proteína RB regula o ciclo celular e na sua forma ativa, é capaz de paralisar a célula na fase G0/G1 do ciclo celular, bloqueando o avanço para a fase S. Como mutações no gene RB1 apresentam a mesma

consequência funcional que a amplificação CDK4/CDK6 ou mutação de p16 e p15, estes eventos costumam ocorrer isoladamente, em grande proporção, em quase todos glioblastomas [275, 276]. O loci de p16 está deletado nos genomas das linhagens U87-MG e C6 [277, 278]. No entanto, p21 e p27 mostram-se funcionais tendo participação ativa na regulação do ciclo celular nestas linhagens [279, 280].

Baseado nestas informações, os níveis de fosforilação da proteína RB e os níveis das proteínas p21, p27 e ciclina D1 foram analisados. De maneira similar, para obter um perfil completo da expressão destas proteínas, os lisados foram analisados entre o primeiro e terceiro dia após tratamento. Portanto, após 24 h de tratamento com a combinação na linhagem C6, apenas a redução da fosforilação no resíduo de serina 795 de pRB foi detectada, sem alteração significativa nos níveis das outras proteínas, como mostrado na figura 3 (Capítulo 2). No entanto, p21 é aumentada e reduções nos níveis de fosforilação das serinas de 807/811 foram detectadas após 72 h na presença dos tratamentos combinados, na mesma linhagem (Capítulo 2 - figura 3a). Do mesmo modo, as células U87-MG foram tratadas durante 24 h e os níveis de proteína foram analisados. Curiosamente, o butirato de sódio induziu efeitos agudos sobre a expressão global ou modificações nas proteínas associadas ao avanço do ciclo celular, mas apenas p21 estava significativamente aumentada quando esta linhagem foi submetida ao tratamento combinado de quercetina com butirato de sódio (Capítulo 2 - figura 3 a). Assim, confirmado o aumento dos níveis de p21 em ambas as linhagens, a distribuição no ciclo celular após tratamento foi analisada.

Como já foi discutido, se o processo de senescência acontece, uma parada irreversível no ciclo celular pode ser detectada. Portanto esta poderia estar presente em ambas as linhagens após o tratamento. A falta de alteração nos níveis de ciclina D1 sugere que esta parada poderia acontecer nas fases S ou G2/M nas linhagens (Capítulo 2 - figura 3 a). A distribuição do ciclo confirma isto, uma vez que quercetina combinada com butirato de sódio induz uma parada em G2/M na linhagem U87-MG (Capítulo 2 - figura 3 b). No entanto, o ciclo não parece ser afetado na lihagem C6 (Capítulo 2 - figura 3 b).

Alguns HDACi, incluindo o butirato de sódio, podem inibir a montagem do fuso através de alterações na função de proteínas BubRi, hBub1, Mad2, CENP-F e CENP-E, encarregadas de regular a associação e separação dos cromossomos ao fuso. Porém, este desbalanço não é suficiente para induzir parada na fase M [180, 281, 282]. Além disso, cinases mitóticas tais como Aurora B, também são afetadas através de alterações na

fosforilação da histona 3 como consequência da inibição da acetilação das histonas [180, 283]. O mecanismo para uma mitose mediado por Aurora B correta é representado na figura 18. Assim, o resultado do tratamento com HDACi é uma mitose aberrante devido a rupturas cromossômicas [180]. Isto poderia explicar o aumento de conteúdo de DNA nas células C6 tratadas com butirato de sódio, nas quais um processo de seleção celular pode ter ocorrido e um determinado grupo de células após 4 dias de tratamentos continuaram ciclando (Capítulo 2 - figura 3 b). Porém, este processo não parece ter acontecido nas células da linhagem U87-MG, nas quais não se observou aumento do conteúdo de DNA (Capítulo 2 - figura 3 b). Isto pode ter ocorrido devido às diferentes características genéticas de cada linhagem. No entanto, esta seja uma possível hipótese que explique, não foram realizados trabalhos utilizando butirato de sódioa longo prazo e por tanto não foi descrito como as células C6 metabolizam este ácido graxo. Assim antes de verificar a hipótese da indução de poliploidia é preciso realizar testes da autofluorescência de butirato de sódio, após tratamentos crônicos nesta linhagem.

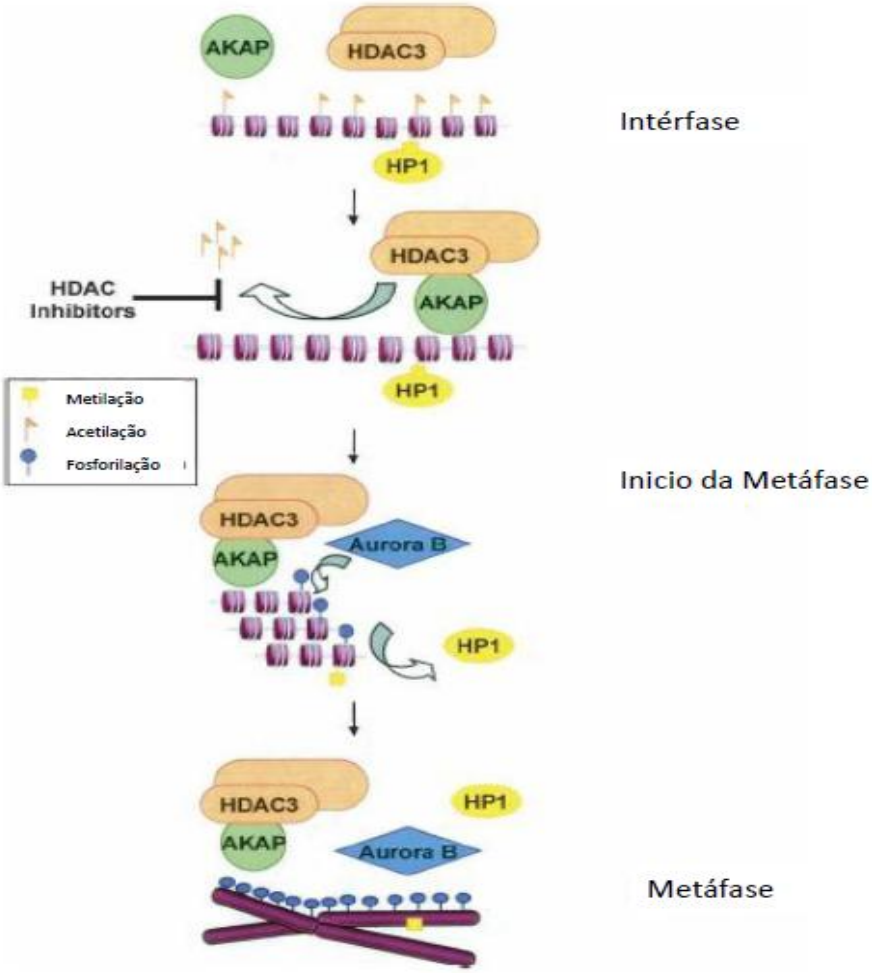


Figura 18. A cascata de regulação de HDAC3-AKAP95/HA95-Aurora B na mitose. Nas células em interfase, AKAP95/HA95 liga-se à matriz nuclear [283, 284]. Proteínas HP1 são recrutadas para a heterocromatina para interagir com H3K9 metiladas. Quando as células entram em mitose, AKAP95/HA95 recruta HDAC3 juntamente com Aurora B. HDAC3 media a desacetilação de histona e aumenta a atividade quinase de Aurora B na H3S10. Este passo é bloqueado por HDACi. Aurora B catalisa a fosforilação de H3S10 e dissocia HP1 de cromossomos mitóticos através do "*meth-phos switch*" [283, 285, 286]. Esta via é necessária para a progressão normal através de mitose.

No entanto, células da linhagem C6 tratadas com quercetina também apresentaram aumento do conteúdo de DNA (Capítulo 2 - figuras 3 b). Uma possibilidade baseia-se nas propiedades da molécula de quercetina, a qual interage diretamente com o DNA, ligando-se a ele como um possível agente intercalante [287-289]. Assim, a quercetina poderia se colocar entre as bases do DNA, levando a uma mudança na conformação desta molécula e podendo gerar rupturas cromossômicas. Isto poderia ocasionar um processo de seleção celular permitindo que algumas células ciclem, ainda que possuindo um número anormal de cromossomos. A morfologia

nuclear de ambas as linhagens celulares foi analisada com o intuito de obter uma inferência quantidicavél destas possivéis instabilidades genéticas após os tratamentos (Capítulo 2 - figura 4). O tratamento com quercetina nas linhagens C6 e U87-MG não resultou em aumento no número de núcleos irregulares (capitulo 2 - figuras 4).

Quando a quercetina foi combinada com butirato de sódio em ambas as linhagens celulares observou-se o aumento de núcleos grandes e regulares característicos de células senescentes, apenas em células da linhagem U87-MG (Capítulo 2 - figura 4). Se o efeito pró-senescência da quercetina for somente através da inibição da HDAC, um HDACi não teria efeito aditivo em presença de quercetina. Este último dado confirma o aumento da senescência celular mostrado na figura 2 (Capítulo 2 - figura 2) pelo tratamento combinado com quercetina e butirato de sódio, sugirindo que outros mecanismos além da inibição de HDAC podem estar envolvidos.

O presente trabalho demonstrou que o tratamento combinado pode diminuir a proliferação celular de ambas as linhagens, aumentar o número de células marcadas positivamente para β-galactosidade e aumentar os níveis de p21. No entanto, a morfologia nuclear é afetada de maneira diferente em ambas as linhagens. Este tratamento também induz alteração no ciclo celular de U87-MG, mas não de C6. No entanto, a capacidade proliferativa é fortemente afetada (como sugerido pelo ensaio clonogenico) o que confirma que a senescência celular é o principal mecanismo induzido pela combinação destas drogas.

A combinação de resveratrol e HDACi confirmou nossa hipótese em quanto à possível indução de senescência pela inibição de desacetilases. No entanto, quercetina combinada com butirato de sódio mostrou que este polifenol pode induzir aumento da senescência de maneira independente da ativação de SIRT1 pelo resveratrol. O próximo passo é determinar um mecanismo alternativo pelo qual a senescência pode ser induzida pela combinação dos polifenóis estudados com butirato de sódio.

Diversos estímulos podem conduzir à senescência e dentre eles, a indução de dano ao DNA [36], que pode ser inferida pelo aumento de γH2AX , um indicador de dano na dupla fita do DNA (DBS) [36]. As combinações das drogas não levaram ao aumento dos níveis de γH2AX em C6 e U87-MG após 72 ou 24 horas de tratamento, respectivamente (Capítulo 2 - figura 5 a). Isto pode dever-se à ativação precoce dos sinais de dano [290].

Outro estímulo indutor de senescência é o aumento de ERO [291], que pode induzir senescência diretamente, ou por danos oxidativos nas bases do DNA [292].

Estes danos podem ocorrer devido à oxidação direta dos ácidos nucleicos ou, muitas vezes, podem levar à formação de quebras em uma das cadeias do DNA ou quebras simples em posições aproximadamente simétricas nas duas cadeias do DNA [292]. Além disso, foi observado um aumento dos níves de p21, que está associado à produção de ERO [293]. De maneira similar, o butirato de sódio aumenta os níveis de ERO em várias linhagens tumorais, dependente ou independentemente do aumento dos níveis de p21 [294]. Embora predominem na literatura relatos de que os polifenois utilizados neste trabalho têm uma forte ação antioxidante [295], foi investigado se eles estariam levando a um aumento na produção de ERO (Capítulo 2 - figura 5 c). Esta possibilidade foi confirmada. A quercetina combinada com butirato de sódio induz aumento de ERO nas duas linhagens testadas. No entanto, o resveratrol e o butirato de sódio não aumentaram a produção de ERO quando combinados, o que sugere que esta combinação pode induzir senescência possivelmente pela inibição indireta de SIRT1, por butirato de sódio. No nosso conhecimento, este foi o primeiro estudo a demonstrar o efeito pró-senescência do resveratrol ou quercetina em combinação com butirato de sódio em GBM.

Os avanços sobre a compreensão da biologia celular e molecular dos GBM têm definido alvos candidatos críticos para iniciação, progressão e manuntenção destes tumores [296], sendo a telomerase (TERT), um deles. Nas células somáticas, o conceito de mortalidade está relacionado à erosão das regiões teloméricas. O encurtamento destas regiões atua como regulador da proliferação celular, limitando assim o número de divisões celulares, proposto como limite de Hayflick (descrito no Capítulo 1). No entanto, células neoplásicas perdem este limite. Cerca 80 % destas conseguem manter o comprimento das regiões telomericas pela ação da TERT, um mecanismo compensatório para evitar RS nestas células [297].

Como foi descrito na seção *1.1.2.3*, existe outra forma de senescência que pode ser induzida prematuramente antes de encurtamento dos telômeros. Oncogenes desregulados, por exemplo, levam as células a sofrer OIS, após um breve período de hiperproliferação [298]. Sinais oncogênicos causam níveis elevados de estresse na replicação do DNA, o que leva à formação de DBS e ativação de persistente de DDR [73, 298] Por varias décadas foi sugerido que OIS não afetavam as regiões dos telômeros [299] e por este motivo, RS e OIS são clasificados como processos biológicos diferentes, nos quais RS é um mecanismo dependente dos telômeros e OIS é independente do encurtamento destes.

No entanto, um estudo recente mostrou que a sinalização por oncogenes afeta a estrutura dos telômeros dramaticamente, causando estresse na replicação das regiões teloméricas de forma rápida e estocástica, e consequentemente causando disfunção dos telômeros em células que não possuem TERT. Além disso, a expressão da hTERT também contribui para a diminuir a DDR nas regiões teloméricas, evitando que a senescência aconteça como observado em cânceres malignos na presença de estresse oncogênico [300]. Assim, a expressão TERT pode ser um evento necessário para ultrapassar as duas formas de senescência. Essas evidências sugerem que a associação entre RS, OIS e TERT pode ser possível, mas de acordo a literatura, pode ser complexa envolvendo multiplas vias de sinalização (como revisado no capitulo 1).

No terceiro capítulo desta tese, foi realizada uma análise topológica e integrativa da TERT em redes e interação de proteinas que contém os principais nós que podem levar à ativação de vias que induzem ambas as formas de senescência (RS e OIS), sendo que estas vias desreguladas favorecem a iniciação e progressão de câncer (references). Para atingir este objetivo foram desenvolvidas diferentes estratégias *in silico* como apresentada na figura 1 (capítulo 3). Inicialmente foi realizada uma mineração de dados considerando proteínas envolvidas na indução de RS e OIS, o que poderia permitir a integração de TERT dentro destas vias. Assim, foi utilizada biologia de sistemas para entender o envolvimento de TERT com estas formas de senescência

Para a geração de redes de interações proteína-proteína (PPI) foi utilizado o banco de interação STRING 9.0. Este banco considera as interações entre proteínas tanto de células normais como cancerígenas, o que é uma vantagem, já que é possível obter em uma rede todas as interações possíveis entre duas proteínas nessas duas circunstâncias. A estratégia para utilizar este banco e obter redes pequenas, mas com informação suficiente para realizar análises topologicas posteriores, basou-se em gerar redes de interação entre protéinas considerando os dados obtidos na tabela suplementar 1 (capítulo 3) e na presença ou ausência da proteina TERT. Assim, obtiveram-se **redes de interação PPI** chamadas de **TERT** e **não-TERT** (Capítulo 3 – Figura 2 a e 2 b, respectivamente). A **rede de interação PPI TERT** que contém 57 nós e 137 conectores, incluindo a proteína TERT (Capítulo 3- Figura 2 a). Por outro lado, considerando oncogenes e proteínas com atividade de OIS (mas sem considerar a presenca da TERT) foi obtida a **rede de interação PPI não-TERT**. Esta rede contém 236 nós e 1424 conectores (Capítulo 3- Figura 2 a). Na **rede de interação PPI não-TERT** além de oncogenes com atividade de OIS como RAS e BRAF [301, 302], outras

proteínas que atuam como oncogenes, tais como ABL1 [303], AKT1, 2 e 3 [304], STAT [305], MYC [306] e E2F1 a 4 [307] foram incluidas,. É importante notar que ao utilizar o banco de interação STRING 9.0 para prospectar estas redes e redes posteriores se utilizou um escore de interação $\geq$ 0,07 (excluindo a possibilidade interação entre protéinas pelo acaso na literatura científica, sem comprovação experimental). Isto evita a obtenção de redes com falsas interações que possam influenciar as análises topológicas subsequentes.

A análise topológica de uma rede de interação de protéinas implica estudar uma série de propiedades incluídas na teoria dos grafos, como modularidade, centralidade e ontogenia (descrito na seção *1.3.1*). Com o objetivo de estudar a posição topológica de TERT dentro das **redes de interação PPI TERT e não-TERT**, foi preciso gerar uma terceira rede a partir destas, chamada **rede de interação UNION**. Esta última rede, ficou constituida por 280 nós e 1709 conectores incluindo a proteína telomerase (Capítulo 3 - figuras 2 c). Para determinar a relação de entre OIS e TERT dentro da **rede de interação UNION**, foi realizada análise de modularidade com dois *softwares* diferentes (CLUSTER-ONE e MCODE) como mostrado na figura suplementar 1 a e 1 b (Capítulo 3). Três sub-redes (módulos) foram obtidas e identificadas por análise de ontologia gênica (GO): (i) proteínas associadas à transdução de sinais intracelulares, (ii) proteínas envolvidas no ciclo celular e (iii) proteínas envolvidas na reparação do DNA. Surpreendentemente, TERT foi encontrada como uma proteína não-agrupada, ou seja, sem módulo, o que indica que não faz parte de um grupo de proteínas altamente associadas com uma função definida. Assim, pode-se sugerir que TERT é uma proteína com uma função independente nesta rede topológica.

Uma estratégia diferente foi realizada com base em conectividade direta entre TERT e oncogenes. Assim, quatro oncogenes (MYC, E2F1, AKT1 e ABL1) mostraram ligação direta com TERT (Capítulo 3 - figura 3). Curiosamente, todos estes oncogenes têm capacidade de induzir OIS [273, 303, 308, 309]. Para determinar qual é a função biológica mais relevante entre TERT e cada um destes oncogenes, optou-se por gerar redes de interação para cada um deles incluido TERT na rede. Assim, para conseguir a máxima saturação (todas as proteínas associadas a uma proteína, nas quais cada conexão está experimentalmente comprovada) do oncogene foi utilizada a base de PPI, BIOGRID (Capítulo 3 - figura suplementar 2). Três processsos comuns e com significância biológica elevada foram encontrados ao analisar as redes de interação de proteínas para cada um dos oncogenes (capitulo 3- Tabela 1): (i) proteínas envolvidas

em processos metabólicos (ii) proteínas envolvidas no metabolismo de nucleosídeos, nucleotídeos e replicação do DNA e (iii) protéinas envolvidas em processos de biossíntese.

Desta forma, MYC, E2F1 e RAS ativam DDR, a qual por sua vez ativa a p53 e induz a senescência como mostrado em diferentes modelos tumorais [73, 298, 310, 311]. Assim, o estresse na replicação do DNA parece ser o fenômeno em comun produzido por estes oncogenes. No estresse replicativo acontecem alterações nos níveis dos precursores de dNTP requeridos para a síntese de DNA, o que ocasiona uma diminuição na frequência de iniciação da replicação de DNA, como revisado por Burhans e Weinberger (2007) [312]. Estas evidências poderiam explicar a associação entre o metabolismo de dNTP e estes oncogenes. No entanto, outro oncogene AKT1 associado a TERT pode induzir OIS por dois mecanismos dependentes e independentes de DDR. Assim, AKT1 pode promover uma rápida parada na proliferação em células na ausência de uma fase de hiperproliferação (dano de DNA), principalmente pela ativação de mTORC1 em células com PTEN-deletadas [74]. Outro mecanismo que ativa DDR pela ativação AKT1 induz OIS em fibroblastos (MEF) p53 proficientes [273]. De maneira similar, ABL-1 induz senescência em células independente de DDR [303]. Isto sugere que a ativação de DDR, não necessariamente é a única via de sinalização a considerar para mostrar a associação metabólica entre estes oncogenes e o DNA. Assim foi demonstrado que todos estes oncogenes (MYC, E2F1, AKT1 e ABL-1) podem aumentar a produção de ERO e induzir instabilidade no genoma das células [273, 313-315], respectivamente. Neste contexto, se pergunta qual a relação metabólica entre OIS, RS e TERT. Nos últimos cinco anos, foram descritas propriedades diferentes da clássica extensão dos telômeros por TERT, como a regulação da homeostase mitocondrial mediada por esta mesma enzima. A subunidade hTERT afeta a homeoestase mitocondrial. Foi demonstrado que ela é transportada para a matriz mitocondrial, ligando-se ao DNA mitocondrial, aumentando a eficiência respiratória [63] e reduzindo o nível de ERO intracelular [316].

Considerando estas informações e a importância biológica de TERT (descrita na seção e no capitulo 1 desta tese), foi desenvolvida uma análise de centralidade de redes baseada em dois príncipios, grau e intermedialidade utilizando os *softwares* CENTISCAPE e CYTO-HUBBA (capitulo 3 – figura 4 e. tabela suplementar 2). Curiosamente, TERT está posicionada abaixo da linha média que separa *Hub-Bottleneck* (HB) de *Hub non-Bottleneck* (H-NB), na **rede de interação PPI UNION**

(capitulo 3- figuras 4 e). Para confirmar se TERT é H-NB, foi utilizado o *software* CYTO-HUBBA, o qual mostrou que TERT não é encontrada entre as 30 principais proteínas gargalos (capitulo 3- figuras 4 d). O fato de que o nó TERT seja H-NB indica que esta proteína liga-se a poucos complexos protéicos (pouca popularidade), mas possui alta intermedialidade. Estes dados sugerem que TERT pode estar regulada por um pequeno número de proteínas-chaves.

Como foi descrito anteriormente, a adição de repetições teloméricas ou a exportação da subunidade catalítca hTERT para regular a funcionalidade da mitocôndria sugere um equilíbrio entre a transcrição, tradução e atividade pós-traducional de TERT de maneira dinâmica. Assim, todos os nós, incluindo oncogenes, foram curados pela literatura em função de seu envolvimento na regulação de TERT e sua associação com ERO, considerando o metabolismo de células tumorais, como mostrado na figura 5 e na tabela 2 (Capítulo 3). A análise mostrou que a maioria das proteínas regula a subunidade catalítica hTERT. De todos os nós analisados, HDAC1 e MYC parecem cumprir um papel central na regulação de TERT [317-319]. Curiosamente, HDAC1 pode ser modulada por ERO, levando a alterações na expressão do gene hTERT [320]. A produção de ERO pode afetar a regulação dos fatores de transcrição por modificação direta de resíduos de aminoácidos [320]. Por exemplo, uma modificação covalente dos grupos tiol na HDAC1 (Cys261 e Cys273) altera o padrão de acetilação de histonas H3 e H4 em diferentes linhas de células tumorais transformadas [320].

Por outro lado, estudos em células de melanoma demonstraram que a indução da expressão de MYC poderia controlar a expressão da (glutationa reduzida) GSH sintetase. Da mesma maneira, apoptose pode ser induzida por *downregulation* de MYC, o que induz a redução da atividade de GSH [321]. Estes dados sugerem que as células ativam MYC para sobreviver ao estresse oxidativo mediado por ERO. Além disso, ERO parecem não afectar a estrutura e função de MYC [321, 322]. AKT, ABL-1 e E2F1, como já discutido, induzem ERO, mas ativam a transcrição de hTERT (capitulo 3 – tabela 2). Além disto, foi descrito que durante a transformação maligna, os sinais oncogênicos podem induzir a produção de ERO para estimular a proliferação celular através de mecanismos redox-sensíveis [323]. Porém, mecanismos antioxidantes podem ser ativados para minimizar os danos oxidativos [324].

Assim como outros tipos tumorais, os GBM possuem elevada produção de ERO intracelular pela presença de mitocôndrias defeituosas (references). Para entender o papel de TERT na regulação da produção de ERO por oncogenes e sua associação com

OIS e RS, é necessária a complementação com dados de microarranjos de tumores de pacientes, o que permite mostrar a peculiaridade de cada tumor e sugerir uma hipótese, *comum* para estes. Além disso, Hernandez e colaboradores (2007) [325] sugeriram que para uma transformação de células normais para cancerosas, mudanças dinâmicas na sinalização, modifcações nas alças de regulação, alterações na modularidade além de mutações em proteínas individuais são requeridas (descrito na seção *1.3.2*). Neste mesmo estudo, foi realizada uma análise integrativa de genes diferencialmente expressos em câncer a partir de microarranjos de câncer de próstata, pulmão e cólon. Independentemente do tipo de tumor, genes do cancer *downregulated* têm propriedades comuns: podem codificar proteínas que interagem mais, com elevada intermediação, sugerindo que estes genes estão envolvidos em vários processos biológicos [325]. Os dados do presente estudo confirmam que TERT tem alto valor de intermedialidade (Capítulo 3 – figura 4 e tabela suplementar 2). É possível que TERT possa ter sua expressão baixa durante o desenvolvimento tumoral? Sua associação com o metabolismo celular (regulação de ERO) poderia estar relacionada à sua transcrição?

Assim, dados de microarranjos de GBM, câncer de ovário, câncer de mama e câncer colorretal comparados com tecidos sadios foram considerados (como detalhado na seção matériais e métodos do capítulo 3). Neste contexto, proteínas relacionadas à defesa do estresse oxidativo e sua expressão foram analisadas em forma conjunta com TERT (capitulo 3 - Tabela Suplementar 3). Foi identificado um subconjunto de genes superexpressos em tecidos cancerosos (Capítulo 3 - Tabela 3), o que confirma níves elevados de ERO intracelular. Interessantemente, a expressão gênica de TERT é semelhante em tumores e tecidos normais (Capítulo 3- Tabela 3). Este resultado pode ser explicado pela heterogeneidade de tumores sólidos humanos, onde algumas células expressam níveis elevados de TERT e outras não [326, 327] e também confirma o sugerido por Hernandez e colaboradores (2007) que sugerem que TERT pode estar envolvida em vários processos biológicos [325].

A atividade TERT nos tipos de câncer estudados neste trabalho (Tabela 4) foi verificada na literatura. Novamente, foi observada uma alta variação de atividade TERT entre tumores, mas à medida que aumenta a malignidade, a mediana de amostras mostrando TERT ativa é maior quando comparado a lesões benignas ou tecidos normais. Estas observações confirman que a expressão e atividade de TERT variam entre diferentes tipos de tumores e, durante o desenvolvimento, dentro de cada tipo de tumores.

Fazendo a interpretação global destes dados (análises topológicas e dados de obtidos por microarranjos), é possível elaborar uma hipótese comum sobre o que acontece dento de célula à medida que ela evolui à malignidade. Assim, é conhecido a priori, que células pré-tumorais possuem um aumento da taxa de mutação. Uma selecção positiva de mutações confere uma vantagem no crescimento destas células [328]. Algumas destas mutações iniciais podem agir sobre proto-oncogenes ativando a barreira antitumoral primária em células nas quais TERT ainda não foi expressa [36, 329]. A população celular residual pode ser resistente à OIS pela ativação de TERT, que ao mesmo tempo evita a erosão lenta dos telômeros, uma barreira antitumoral secundária [330].

Pelas análises do presente estudo, TERT mostrou não estar sendo parte de nenhum módulo dentro das redes de PPI TERT e non-TERT considerando as proteínas presentes nas redes. Além disso, ela atua como H-NB de acordo com a análise de centralidade, o que sugere que sua participação em várias funções biológicas. A expressão global de genes associados à regulação do estresse oxidativo foi utilizada para definir o novo modelo de função de TERT. Sugere-se que a expressão de hTERT acontece na forma de "bursts" sendo fundamental para a iniciação de câncer por afetar a estabilidade genética e níves de ERO necessários para manter a homeostase metabólica e estabilidade dos telômeros. Neste sentido, sugere-se que existe um equilíbrio entre OIS, RS e defesas enzimáticas de estresse oxidativo, que conduzem a um aumento de sobrevivência de células tumorais (figura 6 - capitulo 3).

5. CONCLUSÕES

5.1. Conclusão geral

A senescência celular atua como mecanismo antitumoral em glioblastomas. *In vitro*, a utilização de resveratrol ou quercetina com butirato de sódio pode induzir senescência em duas linhagens celulares de espécies diferentes de glioblastoma. *In silico*, foi possível gerar a hipótese de que o escape da senescência depende do equilíbrio de mecanismos de resposta ao estresse oxidátivo, sendo a ativação da telomerase um deles e fundamental para manter a homeostase de células tumorais, incluindo os glioblastomas.

5.2. Conclusões específicas

- Os tratamentos agudos com resveratrol e quercetina combinados com butirato de sódio não reduziram a viabilidade de linhagens de glioblastoma. Porém, tratamentos crônicos com estes compostos combinados reduziram a viabilidade

após 10 dias. Isto sugere que o efeito mediado pela combinação de butirato de sódio e os polifenois ocorre em longo prazo, o que aumenta a possibilidade de ocorrência da senescência.;

- O resveratrol, quercetina, butirato de sódio e suas combinações não causaram efeito tóxico nem afetaram a proliferação celular em cultura primária de astrócitos (capítulo 2).

- Os tratamentos combinados induzem senescência após 4 dias de tratamento (capítulo 2). Isto pode confirmar a hipótese de que a utilização de um HDACi combinado com resveratrol pode induzir senescência celular. Porém, quercetina combinada a butirato de sódio também induz senescência, o que sugere a possibilidade de outros mecanismos estarem envolvidos, independentes da atividade de SIRT1.

- Os tratamentos combinados afetam a capacidade de formação de colônias após 4 dias de tratamento (capítulo 2). Estes dados confirmam o envolvimento da senescência celular como mecanismo antitumoral em ambos os tratamentos;
- O co-tratamento crônico de resveratrol com butirato de sódio aumenta o nível de p21 na linhagem C6 após 72 h de tratamento. De maneira similar, a quercetina combinada com butirato de sódio aumenta o nível do mesmo supressor tumoral após 24 h de tratamento em U87-MG (capítulo 2). Isto confirma que p21 pode estar atuando como principal supressor tumoral para reter as células em alguma fase específica do ciclo celular;
- A quercetina combinada com butirato de sódio induz alterações na distribuição do ciclo celular U87-MG, mas não C6. O co-tratamento de resveratrol e butirato de sódio não induz modificações nas fases do ciclo celular em ambas as linhagens. Isto sugere que a inibição de SIRT1 pelo resveratrol pode não produzir alteração no ciclo celular no tempo de análise (capítulo 2);
- A quercetina combinada com butirato de sódio aumenta a produção de ERO em C6 e U87-MG após 48 h e 96 h de tratamento respectivamente (capítulo 2). Este dado apoia a possibilidade de que a inibição da ativação de SIRT1 pelo tratamento com resveratrol através da utilização de butirato de sódio seja o principal mecanismo de indução de senescência nestas linhagens. Sendo a

produção de ERO, um possível mecanismo de indução de senescência pelo tratamento de quercetina e butirato de sódio.

- TERT não está integrada em nenhum módulo especifico na rede de interação PPI UNION (capítulo 3). O que pode sugerir uma atividade independente destas proteínas;
- TERT é um H-NB, sugerindo que é uma proteína pouco popular, mas com elevada intermedialidade (capítulo 3). Este resultado sugere que TERT está regulada por um número baixo de proteínas, mas importantes do ponto de vista da fisiologia tumoral;
- TERT é regulada transcricionalmente por proteínas sensoras de ERO (capítulo 3);
- TERT possui expressão e atividade variáveis nos tecidos tumorais (capítulo 3).
- Pelas inferências anteriores, pode-se hipotetizar que TERT pode atuar como um mecanismo compensatório para manter a homeostase tumoral (capítulo 3).

6. PERSPECTIVAS

- Verificar a instabilidade genética induzida pelos tratamentos combinados de quercetina e butiraro de sódio através da contagem total de cromossomos em C6 e U87-MG;
- Verificar se o resveratrol ou quercetina combinados com butirato de sódio estão modulando o sistema de reparo.
- Verificar parada no ciclo celular pelo tratamento combinado de resveratrol e butirato de sódio em tempos superiores aos avaliados na linhagem C6.
- Avaliar a expressão gênica de SIRT1 após tratamento com resveratrol, quercetina, butirato de sódio e suas combinações.
- Avaliar a atividade de SIRT1 após tratamento com resveratrol, quercetina, butirato de sódio e suas combinações.
- Oferecer suficiente embasamento experimental *in vitro* para a elaboração de um modelo *in vivo*, para confirmar a possibilidade de utilizar resveratrol ou quercetina em combinação com HDACi como um possível tratamento para glioblastomas.

7. REFERÊNCIAS BIBLIOGRÁFICAS

[1] G. Minniti, R. Muni, G. Lanzetta, P. Marchetti, R.M. Enrici, Chemotherapy for glioblastoma: current treatment and future perspectives for cytotoxic and targeted agents Anticancer Res 29 (2009) 5171-5184.

[2] Q. Zhou, [WHO classification of tumors of central nervous system (2007): an introduction] Zhonghua Bing Li Xue Za Zhi 37 (2008) 5-7.

[3] E.A. Maher, F.B. Furnari, R.M. Bachoo, D.H. Rowitch, D.N. Louis, W.K. Cavenee, R.A. DePinho, Malignant glioma: genetics and biology of a grave matter Genes Dev 15 (2001) 1311-1333.

[4] E.C. Holland, Gliomagenesis: genetic alterations and mouse models Nat Rev Genet 2 (2001) 120-129.

[5] T.A. Dolecek, J.M. Propp, N.E. Stroup, C. Kruchko, CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009 Neuro Oncol 14 Suppl 5 (2012) v1-49.

[6] D.G. Flores, Receptor do peptídeo liberador de gastrina (GRPR) em gliomas: expressão, influência na proliferação e mecanismos de sinalização UFRGS (2009) 1-196.

[7] P.Y. Wen, S. Kesari, Malignant gliomas in adults N Engl J Med 359 (2008) 492-507.

[8] H.A. Fine, K.B. Dear, J.S. Loeffler, P.M. Black, G.P. Canellos, Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults Cancer 71 (1993) 2585-2597.

[9] L.A. Stewart, Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials Lancet 359 (2002) 1011-1018.

[10] Y.R. Lawrence, M.V. Mishra, M. Werner-Wasik, D.W. Andrews, T.N. Showalter, J. Glass, X. Shen, Z. Symon, A.P. Dicker, Improving prognosis of glioblastoma in the 21st century: who has benefited most? Cancer 118 (2011) 4228-4234.

[11] H. Ohgaki, Epidemiology of brain tumors Methods Mol Biol 472 (2009) 323-342.

[12] C. Krakstad, M. Chekenya, Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics Mol Cancer 9 (2010) 135.

[13] C.H. McGowan, Regulation of the eukaryotic cell cycle Prog Cell Cycle Res 5 (2003) 1-4.

[14] E. Filipi Chiela, C, A autofagia e o ciclo celular desempenham papel central no mecanismo de ação do resveratrol e do co-tratamento com temozolamida em células de linhagens de glioblastoma huamno UFRGS (2011) 1-172.

[15] J.M. Enserink, R.D. Kolodner, An overview of Cdk1-controlled targets and processes Cell Div 5 (2010) 11.

[16] J. Lukas, J. Bartkova, J. Bartek, Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb-controlled G1 checkpoint Mol Cell Biol 16 (1996) 6917-6925.

[17] A. Carnero, G.J. Hannon, The INK4 family of CDK inhibitors Curr Top Microbiol Immunol 227 (1998) 43-55.

[18] C.J. Sherr, J.M. Roberts, CDK inhibitors: positive and negative regulators of G1-phase progression Genes Dev 13 (1999) 1501-1512.

[19] S. Lu, C. Ren, Y. Liu, D.E. Epner, PI3K-Akt signaling is involved in the regulation of p21(WAF/CIP) expression and androgen-independent growth in prostate cancer cells Int J Oncol 28 (2006) 245-251.

[20] I. Shin, F.M. Yakes, F. Rojo, N.Y. Shin, A.V. Bakin, J. Baselga, C.L. Arteaga, PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization Nat Med 8 (2002) 1145-1152.

[21] M. Welcker, J. Singer, K.R. Loeb, J. Grim, A. Bloecher, M. Gurien-West, B.E. Clurman, J.M. Roberts, Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation Mol Cell 12 (2003) 381-392.
[22] M. Xu, K.A. Sheppard, C.Y. Peng, A.S. Yee, H. Piwnica-Worms, Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation Mol Cell Biol 14 (1994) 8420-8431.
[23] A.L.D.S. CRUZ, PAPEL DOS CORPÚSCULOS LIPÍDICOS NA PROGRESSÃO DO CICLO CELULAR Instituto Nacional de Câncer Programa de Pós-Graduação em Oncologia (2010) 1-139.
[24] F. Ramirez-Valle, M.L. Badura, S. Braunstein, M. Narasimhan, R.J. Schneider, Mitotic raptor promotes mTORC1 activity, G(2)/M cell cycle progression, and internal ribosome entry site-mediated mRNA translation Mol Cell Biol 30 (2011) 3151-3164.
[25] M. Nakamura, T. Watanabe, U. Klangby, C. Asker, K. Wiman, Y. Yonekawa, P. Kleihues, H. Ohgaki, p14ARF deletion and methylation in genetic pathways to glioblastomas Brain Pathol 11 (2001) 159-168.
[26] L. Zamin, RESVERATROL E QUERCETINA: AVALIAÇÃO DA ATIVIDADE ANTITUMORAL E DOS MECANISMOS DE AÇÃO EM LINHAGENS DE GLIOMAS IN VITRO E NUM MODELO DE IMPLANTE DE GLIOMAS IN VIVO UFRGS (2009).
[27] CANCER GENOME ATLAS RESEARCH NETWORK. Comprehensive genomic characterization defines human glioblastoma genes and core pathways Nature 455. (2008) 1061-1068.
[28] P.L.d.C. Lopez, Influência das proteínas XIAP e Survivina na resistência de gliomas Tese de doutorado (2012) 1-171.
[29] L. Hayflick, The Limited in Vitro Lifetime of Human Diploid Cell Strains Exp Cell Res 37 (1965) 614-636.
[30] K. Nishio, A. Inoue, S. Qiao, H. Kondo, A. Mimura, Senescence and cytoskeleton: overproduction of vimentin induces senescent-like morphology in human fibroblasts Histochem Cell Biol 116 (2001) 321-327.
[31] D.N. Shelton, E. Chang, P.S. Whittier, D. Choi, W.D. Funk, Microarray analysis of replicative senescence Curr Biol 9 (1999) 939-945.
[32] A.T.b. Kurz T., Ulf T. Brunk, Autophagy, ageing and apoptosis: The role of oxidative stress and lysosomal iron Archives of Biochemistry and Biophysics 462 (2007) 220-230.
[33] J.A.D. Ewald J.A., George Wilding, David F. Jarrard, Therapy-Induced Senescence in Cancer JNCI J Natl Cancer Inst (2010) 102 (20): . 102 (2010) 1536-1546.
[34] C.W. Lawless C., Diana Jurk, Alina Merz, Thomas von Zglinicki, João F. Passos, Quantitative assessment of markers for cell senescence Experimental Gerontology 45 (2010) 772-778.
[35] P. Arivazhagan, E. Mizutani, M. Fujii, D. Ayusawa, Cardiolipin induces premature senescence in normal human fibroblasts Biochem Biophys Res Commun 323 (2004) 739-742.
[36] J. Vargas, B.C. Feltes, F. Poloni Jde, G. Lenz, D. Bonatto, Senescence; an endogenous anticancer mechanism Front Biosci 17 (2012) 2616-2643.
[37] G. Lenz, Endogenous anticancer mechanisms (EACMs) Front Biosci (Schol Ed) 4 (2012) 1017-1030.
[38] a.F.d.A.d.F. Campisi J, Cellular senescence: when bad things happen to good cells Nature Reviews Molecular Cell Biology 8 (2007) 729-740.
[39] W.E. Wright, J.W. Shay, Cellular senescence as a tumor-protection mechanism: the essential role of counting Curr Opin Genet Dev 11 (2001) 98-103.
[40] T. von Zglinicki, Telomeres and replicative senescence: Is it only length that counts? Cancer Lett 168 (2001) 111-116.
[41] S.H. Kim Sh, P. Kaminker, J. Campisi, Telomeres, aging and cancer: in search of a happy ending Oncogene 21 (2002) 503-511.

[42] R.K. Moyzis, J.M. Buckingham, L.S. Cram, M. Dani, L.L. Deaven, M.D. Jones, J. Meyne, R.L. Ratliff, J.R. Wu, A highly conserved repetitive DNA sequence, (TTAGGG)n, present at the telomeres of human chromosomes Proc Natl Acad Sci U S A 85 (1988) 6622-6626.
[43] E.H. Blackburn, Walking the walk from genes through telomere maintenance to cancer risk Cancer Prev Res (Phila) 4 (2011) 473-475.
[44] X. Tian, B. Chen, X. Liu, Telomere and telomerase as targets for cancer therapy Appl Biochem Biotechnol 160 (2010) 1460-1472.
[45] R. Ofir, A.C. Wong, H.E. McDermid, K.L. Skorecki, S. Selig, Position effect of human telomeric repeats on replication timing Proc Natl Acad Sci U S A 96 (1999) 11434-11439.
[46] G.C. Smith, R.B. Cary, N.D. Lakin, B.C. Hann, S.H. Teo, D.J. Chen, S.P. Jackson, Purification and DNA binding properties of the ataxia-telangiectasia gene product ATM Proc Natl Acad Sci U S A 96 (1999) 11134-11139.
[47] K.O. Hartley, D. Gell, G.C. Smith, H. Zhang, N. Divecha, M.A. Connelly, A. Admon, S.P. Lees-Miller, C.W. Anderson, S.P. Jackson, DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product Cell 82 (1995) 849-856.
[48] J.H. Lee, T.T. Paull, ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex Science 308 (2005) 551-554.
[49] K. Savitsky, A. Bar-Shira, S. Gilad, G. Rotman, Y. Ziv, L. Vanagaite, D.A. Tagle, S. Smith, T. Uziel, S. Sfez, M. Ashkenazi, I. Pecker, M. Frydman, R. Harnik, S.R. Patanjali, A. Simmons, G.A. Clines, A. Sartiel, R.A. Gatti, L. Chessa, O. Sanal, M.F. Lavin, N.G. Jaspers, A.M. Taylor, C.F. Arlett, T. Miki, S.M. Weissman, M. Lovett, F.S. Collins, Y. Shiloh, A single ataxia telangiectasia gene with a product similar to PI-3 kinase Science 268 (1995) 1749-1753.
[50] C. Redon, D. Pilch, E. Rogakou, O. Sedelnikova, K. Newrock, W. Bonner, Histone H2A variants H2AX and H2AZ Curr Opin Genet Dev 12 (2002) 162-169.
[51] A. Celeste, S. Petersen, P.J. Romanienko, O. Fernandez-Capetillo, H.T. Chen, O.A. Sedelnikova, B. Reina-San-Martin, V. Coppola, E. Meffre, M.J. Difilippantonio, C. Redon, D.R. Pilch, A. Olaru, M. Eckhaus, R.D. Camerini-Otero, L. Tessarollo, F. Livak, K. Manova, W.M. Bonner, M.C. Nussenzweig, A. Nussenzweig, Genomic instability in mice lacking histone H2AX Science 296 (2002) 922-927.
[52] J.H. Back, H.R. Rezvani, Y. Zhu, V. Guyonnet-Duperat, M. Athar, D. Ratner, A.L. Kim, Cancer cell survival following DNA damage-mediated premature senescence is regulated by mammalian target of rapamycin (mTOR)-dependent Inhibition of sirtuin 1 J Biol Chem 286 (2011) 19100-19108.
[53] J.H. Back, H.R. Rezvani, Y. Zhu, V. Guyonnet-Duperat, M. Athar, D. Ratner, A.L. Kim, Cancer cell survival following DNA damage-mediated premature senescence is regulated by mammalian target of rapamycin (mTOR)-dependent Inhibition of sirtuin 1 J Biol Chem 286 19100-19108.
[54] J.D. Henson, A.A. Neumann, T.R. Yeager, R.R. Reddel, Alternative lengthening of telomeres in mammalian cells Oncogene 21 (2002) 598-610.
[55] C.B. Harley, Telomerase and cancer therapeutics Nat Rev Cancer 8 (2008) 167-179.
[56] J.W. Shay, A.F. Gazdar, Telomerase in the early detection of cancer J Clin Pathol 50 (1997) 106-109.
[57] L.E. Donate, M.A. Blasco, Telomeres in cancer and ageing Philos Trans R Soc Lond B Biol Sci 366 (2011) 76-84.
[58] S.T. Yu, L. Chen, H.J. Wang, X.D. Tang, D.C. Fang, S.M. Yang, hTERT promotes the invasion of telomerase-negative tumor cells in vitro Int J Oncol 35 (2009) 329-336.
[59] D.M. Feldser, C.W. Greider, Short telomeres limit tumor progression in vivo by inducing senescence Cancer Cell 11 (2007) 461-469.
[60] W. Cosme-Blanco, M.F. Shen, A.J. Lazar, S. Pathak, G. Lozano, A.S. Multani, S. Chang, Telomere dysfunction suppresses spontaneous tumorigenesis in vivo by initiating p53-dependent cellular senescence EMBO Rep 8 (2007) 497-503.

[61] K.B. Beckman, B.N. Ames, The free radical theory of aging matures Physiol Rev 78 (1998) 547-581.
[62] R.S. Balaban, S. Nemoto, T. Finkel, Mitochondria, oxidants, and aging Cell 120 (2005) 483-495.
[63] J. Haendeler, S. Drose, N. Buchner, S. Jakob, J. Altschmied, C. Goy, I. Spyridopoulos, A.M. Zeiher, U. Brandt, S. Dimmeler, Mitochondrial telomerase reverse transcriptase binds to and protects mitochondrial DNA and function from damage Arterioscler Thromb Vasc Biol 29 (2009) 929-935.
[64] F. Mbeunkui, D.J. Johann, Jr., Cancer and the tumor microenvironment: a review of an essential relationship Cancer Chemother Pharmacol 63 (2009) 571-582.
[65] C.M. Croce, Oncogenes and cancer N Engl J Med 358 (2008) 502-511.
[66] J.B. Konopka, S.M. Watanabe, J.W. Singer, S.J. Collins, O.N. Witte, Cell lines and clinical isolates derived from Ph1-positive chronic myelogenous leukemia patients express c-abl proteins with a common structural alteration Proc Natl Acad Sci U S A 82 (1985) 1810-1814.
[67] B.C. Bastian, P.E. LeBoit, D. Pinkel, Mutations and copy number increase of HRAS in Spitz nevi with distinctive histopathological features Am J Pathol 157 (2000) 967-972.
[68] P.M. Pollock, U.L. Harper, K.S. Hansen, L.M. Yudt, M. Stark, C.M. Robbins, T.Y. Moses, G. Hostetter, U. Wagner, J. Kakareka, G. Salem, T. Pohida, P. Heenan, P. Duray, O. Kallioniemi, N.K. Hayward, J.M. Trent, P.S. Meltzer, High frequency of BRAF mutations in nevi Nat Genet 33 (2003) 19-20.
[69] C. Wellbrock, L. Ogilvie, D. Hedley, M. Karasarides, J. Martin, D. Niculescu-Duvaz, C.J. Springer, R. Marais, V599EB-RAF is an oncogene in melanocytes Cancer Res 64 (2004) 2338-2342.
[70] H. Davies, G.R. Bignell, C. Cox, P. Stephens, S. Edkins, S. Clegg, J. Teague, H. Woffendin, M.J. Garnett, W. Bottomley, N. Davis, E. Dicks, R. Ewing, Y. Floyd, K. Gray, S. Hall, R. Hawes, J. Hughes, V. Kosmidou, A. Menzies, C. Mould, A. Parker, C. Stevens, S. Watt, S. Hooper, R. Wilson, H. Jayatilake, B.A. Gusterson, C. Cooper, J. Shipley, D. Hargrave, K. Pritchard-Jones, N. Maitland, G. Chenevix-Trench, G.J. Riggins, D.D. Bigner, G. Palmieri, A. Cossu, A. Flanagan, A. Nicholson, J.W. Ho, S.Y. Leung, S.T. Yuen, B.L. Weber, H.F. Seigler, T.L. Darrow, H. Paterson, R. Marais, C.J. Marshall, R. Wooster, M.R. Stratton, P.A. Futreal, Mutations of the BRAF gene in human cancer Nature 417 (2002) 949-954.
[71] C. Michaloglou, L.C. Vredeveld, W.J. Mooi, D.S. Peeper, BRAF(E600) in benign and malignant human tumours Oncogene 27 (2008) 877-895.
[72] E. Urbano, Detecção da mutação T1799 do gene BRAF em células de carcinoma papilífero obtidas por punção aspirativa com agulha fina Faculdade de Medicina da Universidade de São Paulo (2012) 1-125.
[73] J. Bartkova, N. Rezaei, M. Liontos, P. Karakaidos, D. Kletsas, N. Issaeva, L.V. Vassiliou, E. Kolettas, K. Niforou, V.C. Zoumpourlis, M. Takaoka, H. Nakagawa, F. Tort, K. Fugger, F. Johansson, M. Sehested, C.L. Andersen, L. Dyrskjot, T. Orntoft, J. Lukas, C. Kittas, T. Helleday, T.D. Halazonetis, J. Bartek, V.G. Gorgoulis, Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints Nature 444 (2006) 633-637.
[74] M.V. Astle, K.M. Hannan, P.Y. Ng, R.S. Lee, A.J. George, A.K. Hsu, Y. Haupt, R.D. Hannan, R.B. Pearson, AKT induces senescence in human cells via mTORC1 and p53 in the absence of DNA damage: implications for targeting mTOR during malignancy Oncogene 31 (2012) 1949-1962.
[75] M.L. Rodriguez, Mulheres da Rede Fitovida:ervas medicinais, envelhecimento e associativismo Universidade do estado de Rio de Janeiro 1-164 (2007).
[76] F.J.A. Matos, O formulário fitoterápico do professor Dias da Rocha: informações sobre emprego na medicina caseira, de plantas do Nordeste, especialmente no Ceará, Fortaleza: EUFC., 1997, pp. 219.
[77] F. Oliveira, G. Akisue, Fundamentos de farmacobotânica. 2ª ed. , São Paulo: Atheneu, 1997, pp. 178.

[78] A. Resende, Preparo e cuidados com as ervas Ervas medicinais e terapias alternativas. São Paulo (2003) 4,5.
[79] V.F. Veiga Junior, A.C. Pinto, M.A.M. Maciel, Plantas medicinais: cura segura? Química Nova 28 (2005) 519-528.
[80] P. Jeandet, A.C. Douillet-Breuil, R. Bessis, S. Debord, M. Sbaghi, M. Adrian, Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic plants, antifungal activity, and metabolism J Agric Food Chem 50 (2002) 2731-2741.
[81] D.M. Goldberg, S.E. Hahn, J.G. Parkes, Beyond alcohol: beverage consumption and cardiovascular mortality Clin Chim Acta 237 (1995) 155-187.
[82] C.D.S. Moreno, Estudo do efeito radioprotetor do resveratrol Autarquia associada á Universidade de São Paulo (2009) 1-93.
[83] A.S. St Leger, A.L. Cochrane, F. Moore, Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine Lancet 1 (1979) 1017-1020.
[84] E.H. Siemann, L.L. Creasy, Concentration of phytoalexin resveratrol in wine American jounal of Enology and Viticulture 43 (1992) 49-52.
[85] B.F. Ruan, X.Q. Lu, J. Song, H.L. Zhu, Derivatives of resveratrol: potential agents in prevention and treatment of cardiovascular disease Curr Med Chem 19 (2012) 4175-4183.
[86] S. He, X. Yan, From Resveratrol to Its Derivatives: New Sources of Natural Antioxidant Curr Med Chem (2012).
[87] N.Y. Elmadhun, A.D. Lassaletta, L.M. Chu, C. Bianchi, F.W. Sellke, Vodka and wine consumption in a swine model of metabolic syndrome alters insulin signaling pathways in the liver and skeletal muscle Surgery 152 (2012) 414-422.
[88] L. Frémont, Minireview: Biological effects of resveratrol Life Sciences 66 (2000) 663-673.
[89] A.I. Romero-Perez, R.M. Lamuela-Raventos, C. Andres-Lacueva, M.C. de La Torre-Boronat, Method for the quantitative extraction of resveratrol and piceid isomers in grape berry skins. Effect of powdery mildew on the stilbene content J Agric Food Chem 49 (2001) 210-215.
[90] M. Careri, C. Corradini, L. Elviri, I. Nicoletti, I. Zagnoni, Direct HPLC analysis of quercetin and trans-resveratrol in red wine, grape, and winemaking byproducts J Agric Food Chem 51 (2003) 5226-5231.
[91] A.A. Souto, M.C. Carneiro, M. Serefin, M.J.H. Senna, A. Conz, K. Obbi, Determination of trans-resveratrol concentrations in Brazilian red wine by HPLC Journal of Food Composition and Analysis 14 (2001) 441-445.
[92] C.K. Sautter, S. Denardin, A.O. Alvees, C.A. Mallmann, N.G. Penna, L.H. Heckttheuer, Determinação de resveratrol em sucos de uva no Brasil Ciência e Tecnologia de Alimentos 25 (2005) 437-442.
[93] M. Jang, L. Cai, G.O. Udeani, K.V. Slowing, C.F. Thomas, C.W. Beecher, H.H. Fong, N.R. Farnsworth, A.D. Kinghorn, R.G. Mehta, R.C. Moon, J.M. Pezzuto, Cancer chemopreventive activity of resveratrol, a natural product derived from grapes Science 275 (1997) 218-220.
[94] T. Walle, F. Hsieh, M.H. DeLegge, J.E. Oatis, Jr., U.K. Walle, High absorption but very low bioavailability of oral resveratrol in humans Drug Metab Dispos 32 (2004) 1377-1382.
[95] B. Jannin, M. Menzel, J.P. Berlot, D. Delmas, A. Lancon, N. Latruffe, Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake Biochem Pharmacol 68 (2004) 1113-1118.
[96] C. Yu, Y.G. Shin, A. Chow, Y. Li, J.W. Kosmeder, Y.S. Lee, W.H. Hirschelman, J.M. Pezzuto, R.G. Mehta, R.B. van Breemen, Human, rat, and mouse metabolism of resveratrol Pharm Res 19 (2002) 1907-1914.
[97] M. Asensi, I. Medina, A. Ortega, J. Carretero, M.C. Bano, E. Obrador, J.M. Estrela, Inhibition of cancer growth by resveratrol is related to its low bioavailability Free Radic Biol Med 33 (2002) 387-398.

[98] J.K. Kundu, Y.J. Surh, Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives Cancer Lett 269 (2008) 243-261.
[99] V. De Ledinghen, A. Monvoisin, V. Neaud, S. Krisa, B. Payrastre, C. Bedin, A. Desmouliere, P. Bioulac-Sage, J. Rosenbaum, Trans-resveratrol, a grapevine-derived polyphenol, blocks hepatocyte growth factor-induced invasion of hepatocellular carcinoma cells Int J Oncol 19 (2001) 83-88.
[100] Y. Kozuki, Y. Miura, K. Yagasaki, Resveratrol suppresses hepatoma cell invasion independently of its anti-proliferative action Cancer Lett 167 (2001) 151-156.
[101] Q.B. She, W.Y. Ma, M. Wang, A. Kaji, C.T. Ho, Z. Dong, Inhibition of cell transformation by resveratrol and its derivatives: differential effects and mechanisms involved Oncogene 22 (2003) 2143-2150.
[102] C. Huang, W.Y. Ma, A. Goranson, Z. Dong, Resveratrol suppresses cell transformation and induces apoptosis through a p53-dependent pathway Carcinogenesis 20 (1999) 237-242.
[103] G. Lanzilli, M.P. Fuggetta, M. Tricarico, A. Cottarelli, A. Serafino, R. Falchetti, G. Ravagnan, M. Turriziani, R. Adamo, O. Franzese, E. Bonmassar, Resveratrol down-regulates the growth and telomerase activity of breast cancer cells in vitro Int J Oncol 28 (2006) 641-648.
[104] M.P. Fuggetta, G. Lanzilli, M. Tricarico, A. Cottarelli, R. Falchetti, G. Ravagnan, E. Bonmassar, Effect of resveratrol on proliferation and telomerase activity of human colon cancer cells in vitro J Exp Clin Cancer Res 25 (2006) 189-193.
[105] T. Sundin, P. Hentosh, InTERTesting association between telomerase, mTOR and phytochemicals Expert Rev Mol Med 14 (2012) e8.
[106] E. Pozo-Guisado, A. Alvarez-Barrientos, S. Mulero-Navarro, B. Santiago-Josefat, P.M. Fernandez-Salguero, The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle Biochem Pharmacol 64 (2002) 1375-1386.
[107] M. Fontecave, M. Lepoivre, E. Elleingand, C. Gerez, O. Guittet, Resveratrol, a remarkable inhibitor of ribonucleotide reductase FEBS Lett 421 (1998) 277-279.
[108] K. Subbaramaiah, A.J. Dannenberg, Resveratrol inhibits the expression of cyclooxygenase-2 in mammary epithelial cells Adv Exp Med Biol 492 (2001) 147-157.
[109] K. Subbaramaiah, P. Michaluart, W.J. Chung, T. Tanabe, N. Telang, A.J. Dannenberg, Resveratrol inhibits cyclooxygenase-2 transcription in human mammary epithelial cells Ann N Y Acad Sci 889 (1999) 214-223.
[110] J.R. Stewart, C.A. O'Brian, Resveratrol antagonizes EGFR-dependent Erk1/2 activation in human androgen-independent prostate cancer cells with associated isozyme-selective PKC alpha inhibition Invest New Drugs 22 (2004) 107-117.
[111] O.P. Mgbonyebi, J. Russo, I.H. Russo, Antiproliferative effect of synthetic resveratrol on human breast epithelial cells Int J Oncol 12 (1998) 865-869.
[112] Y.C. Liang, S.H. Tsai, L. Chen, S.Y. Lin-Shiau, J.K. Lin, Resveratrol-induced G2 arrest through the inhibition of CDK7 and p34CDC2 kinases in colon carcinoma HT29 cells Biochem Pharmacol 65 (2003) 1053-1060.
[113] C. Tian, R. Zhang, X. Ye, C. Zhang, X. Jin, Y. Yamori, L. Hao, X. Sun, C. Ying, Resveratrol ameliorates high-glucose-induced hyperpermeability mediated by caveolae via VEGF/KDR pathway Genes Nutr (2012).
[114] S.R. Bhatt, M.F. Lokhandwala, A.A. Banday, Resveratrol prevents endothelial nitric oxide synthase uncoupling and attenuates development of hypertension in spontaneously hypertensive rats Eur J Pharmacol 667 (2011) 258-264.
[115] B. Agarwal, M.J. Campen, M.M. Channell, S.J. Wherry, B. Varamini, J.G. Davis, J.A. Baur, J.M. Smoliga, Resveratrol for primary prevention of atherosclerosis: Clinical trial evidence for improved gene expression in vascular endothelium Int J Cardiol.
[116] F.C. Liu, L.F. Hung, W.L. Wu, D.M. Chang, C.Y. Huang, J.H. Lai, L.J. Ho, Chondroprotective effects and mechanisms of resveratrol in advanced glycation end products-stimulated chondrocytes Arthritis Res Ther 12 R167.

[117] F.C. Liu, L.F. Hung, W.L. Wu, D.M. Chang, C.Y. Huang, J.H. Lai, L.J. Ho, Chondroprotective effects and mechanisms of resveratrol in advanced glycation end products-stimulated chondrocytes Arthritis Res Ther 12 (2012) R167.
[118] S. Davinelli, N. Sapere, D. Zella, R. Bracale, M. Intrieri, G. Scapagnini, Pleiotropic protective effects of phytochemicals in Alzheimer's disease Oxid Med Cell Longev 2012 (2012) 386527.
[119] H. Li, N. Xia, U. Forstermann, Cardiovascular effects and molecular targets of resveratrol Nitric Oxide 26 (2012) 102-110.
[120] Y.E. Lee, J.W. Kim, E.M. Lee, Y.B. Ahn, K.H. Song, K.H. Yoon, H.W. Kim, C.W. Park, G. Li, Z. Liu, S.H. Ko, Chronic Resveratrol Treatment Protects Pancreatic Islets against Oxidative Stress in db/db Mice PLoS One 7 (2012) e50412.
[121] H. Capiralla, V. Vingtdeux, H. Zhao, R. Sankowski, Y. Al-Abed, P. Davies, P. Marambaud, Resveratrol mitigates lipopolysaccharide- and Abeta-mediated microglial inflammation by inhibiting the TLR4/NF-kappaB/STAT signaling cascade J Neurochem 120 (2012) 461-472.
[122] B. Dasgupta, J. Milbrandt, Resveratrol stimulates AMP kinase activity in neurons Proc Natl Acad Sci U S A 104 (2007) 7217-7222.
[123] M. Lagouge, C. Argmann, Z. Gerhart-Hines, H. Meziane, C. Lerin, F. Daussin, N. Messadeq, J. Milne, P. Lambert, P. Elliott, B. Geny, M. Laakso, P. Puigserver, J. Auwerx, Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha Cell 127 (2006) 1109-1122.
[124] K. Zarse, S. Schmeisser, M. Birringer, E. Falk, D. Schmoll, M. Ristow, Differential effects of resveratrol and SRT1720 on lifespan of adult Caenorhabditis elegans Horm Metab Res 42 (2010) 837-839.
[125] C. Wang, C.T. Wheeler, T. Alberico, X. Sun, J. Seeberger, M. Laslo, E. Spangler, B. Kern, R. de Cabo, S. Zou, The effect of resveratrol on lifespan depends on both gender and dietary nutrient composition in Drosophila melanogaster Age (Dordr) (2011).
[126] M. Kaeberlein, T. McDonagh, B. Heltweg, J. Hixon, E.A. Westman, S.D. Caldwell, A. Napper, R. Curtis, P.S. DiStefano, S. Fields, A. Bedalov, B.K. Kennedy, Substrate-specific activation of sirtuins by resveratrol J Biol Chem 280 (2005) 17038-17045.
[127] J.L. Barger, T. Kayo, T.D. Pugh, T.A. Prolla, R. Weindruch, Short-term consumption of a resveratrol-containing nutraceutical mixture mimics gene expression of long-term caloric restriction in mouse heart Exp Gerontol 43 (2008) 859-866.
[128] J.M. Wu, Z.R. Wang, T.C. Hsieh, J.L. Bruder, J.G. Zou, Y.Z. Huang, Mechanism of cardioprotection by resveratrol, a phenolic antioxidant present in red wine (Review) Int J Mol Med 8 (2001) 3-17.
[129] M. Pacholec, J.E. Bleasdale, B. Chrunyk, D. Cunningham, D. Flynn, R.S. Garofalo, D. Griffith, M. Griffor, P. Loulakis, B. Pabst, X. Qiu, B. Stockman, V. Thanabal, A. Varghese, J. Ward, J. Withka, K. Ahn, SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1 J Biol Chem 285 (2010) 8340-8351.
[130] D. Beher, J. Wu, S. Cumine, K.W. Kim, S.C. Lu, L. Atangan, M. Wang, Resveratrol is not a direct activator of SIRT1 enzyme activity Chem Biol Drug Des 74 (2009) 619-624.
[131] H. Schirmer, Análise do padrão transcricional de sirtuínas influenciado por trans-resveratrol, etanol e restrição calórica utilizando modelo de zebrafish (Danio rerio) PUCRS (2011) 1-168.
[132] J.L. Barger, T. Kayo, J.M. Vann, E.B. Arias, J. Wang, T.A. Hacker, Y. Wang, D. Raederstorff, J.D. Morrow, C. Leeuwenburgh, D.B. Allison, K.W. Saupe, G.D. Cartee, R. Weindruch, T.A. Prolla, A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice PLoS One 3 (2008) e2264.
[133] S.J. Park, F. Ahmad, A. Philp, K. Baar, T. Williams, H. Luo, H. Ke, H. Rehmann, R. Taussig, A.L. Brown, M.K. Kim, M.A. Beaven, A.B. Burgin, V. Manganiello, J.H. Chung, Resveratrol ameliorates aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases Cell 148 (2012) 421-433.

[134] C.X. Deng, SIRT1, is it a tumor promoter or tumor suppressor? Int J Biol Sci 5 (2009) 147-152.
[135] S.H. Tseng, S.M. Lin, J.C. Chen, Y.H. Su, H.Y. Huang, C.K. Chen, P.Y. Lin, Y. Chen, Resveratrol suppresses the angiogenesis and tumor growth of gliomas in rats Clin Cancer Res 10 (2004) 2190-2202.
[136] Y. Chen, S.H. Tseng, H.S. Lai, W.J. Chen, Resveratrol-induced cellular apoptosis and cell cycle arrest in neuroblastoma cells and antitumor effects on neuroblastoma in mice Surgery 136 (2004) 57-66.
[137] H. Jiang, L. Zhang, J. Kuo, K. Kuo, S.C. Gautam, L. Groc, A.I. Rodriguez, D. Koubi, T.J. Hunter, G.B. Corcoran, M.D. Seidman, R.A. Levine, Resveratrol-induced apoptotic death in human U251 glioma cells Mol Cancer Ther 4 (2005) 554-561.
[138] H. Jiang, X. Shang, H. Wu, S.C. Gautam, S. Al-Holou, C. Li, J. Kuo, L. Zhang, M. Chopp, Resveratrol downregulates PI3K/Akt/mTOR signaling pathways in human U251 glioma cells J Exp Ther Oncol 8 (2009) 25-33.
[139] Z. Gao, M.S. Xu, T.L. Barnett, C.W. Xu, Resveratrol induces cellular senescence with attenuated mono-ubiquitination of histone H2B in glioma cells Biochem Biophys Res Commun 407 (2011) 271-276.
[140] L.L. Zamin, E.C. Filippi-Chiela, P. Dillenburg-Pilla, F. Horn, C. Salbego, G. Lenz, Resveratrol and quercetin cooperate to induce senescence-like growth arrest in C6 rat glioma cells Cancer Sci 100 (2009) 1655-1662.
[141] A.M. Pereira Alves, E.P. Belato Alves, N.C. Buttow, J.V. Colombo Martins Perles, J. Nelisis Zanoni, S.R. Stabille, Aspectos gerais e abordagem terepêuticas da quercetina sobre as complicações do diabetes causadas pelos estresse oxidativo Arq. Ciênc. Saúde UNIPAR 14 (2010) 179-186.
[142] Y.J. Moon, L. Wang, R. DiCenzo, M.E. Morris, Quercetin pharmacokinetics in humans Biopharm Drug Dispos 29 (2008) 205-217.
[143] J.B. Harborne, Flavonoids in the environment: structure-activity relationships Prog Clin Biol Res 280 (1988) 17-27.
[144] F.A. Tomas-Barberan, M. Maillard, K. Hostettmann, Antifungal flavonoids from the leaf surfaces of Helichrysum nitens and from the stem bark of Erythrina berteroana Prog Clin Biol Res 280 (1988) 61-65.
[145] M. Hashimoto, T. Suzuki, T. Iwashina, New acylated anthocyanins and other flavonoids from the red flowers of Clematis cultivars Nat Prod Commun 6 (2011) 1631-1636.
[146] A.A. Franke, R.V. Cooney, L.J. Custer, L.J. Mordan, Y. Tanaka, Inhibition of neoplastic transformation and bioavailability of dietary flavonoid agents Adv Exp Med Biol 439 (1998) 237-248.
[147] M.G.L. Hertog, P.C.H. Hollman, M.B. Katan, Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands J Agric Food Chem 40 (1992) 2379-2383.
[148] V. Crespy, C. Morand, C. Manach, C. Besson, C. Demigne, C. Remesy, Part of quercetin absorbed in the small intestine is conjugated and further secreted in the intestinal lumen Am J Physiol 277 (1999) G120-126.
[149] P.C. Hollman, J.H. de Vries, S.D. van Leeuwen, M.J. Mengelers, M.B. Katan, Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers Am J Clin Nutr 62 (1995) 1276-1282.
[150] J.M. Gee, M.S. DuPont, A.J. Day, G.W. Plumb, G. Williamson, I.T. Johnson, Intestinal transport of quercetin glycosides in rats involves both deglycosylation and interaction with the hexose transport pathway J Nutr 130 (2000) 2765-2771.
[151] V.C. de Boer, A.A. Dihal, H. van der Woude, I.C. Arts, S. Wolffram, G.M. Alink, I.M. Rietjens, J. Keijer, P.C. Hollman, Tissue distribution of quercetin in rats and pigs J Nutr 135 (2005) 1718-1725.

[152] M. Harwood, B. Danielewska-Nikiel, J.F. Borzelleca, G.W. Flamm, G.M. Williams, T.C. Lines, A critical review of the data related to the safety of quercetin and lack of evidence of in vivo toxicity, including lack of genotoxic/carcinogenic properties Food Chem Toxicol 45 (2007) 2179-2205.
[153] M. Oren, V. Rotter, Mutant p53 gain-of-function in cancer Cold Spring Harb Perspect Biol 2 (2011) a001107.
[154] E. Obinaju Blessing, F.L. a Martin, Dose-related effects of Quercetin in the human breast carcinoma MCF-7 cell line AMERICAN JOURNAL OF SCIENTIFIC AND INDUSTRIAL RESEARCH 1 (2010) 242-261.
[155] M.A. Avila, J.A. Velasco, J. Cansado, V. Notario, Quercetin mediates the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468 Cancer Res 54 (1994) 2424-2428.
[156] M. Yoshida, M. Yamamoto, T. Nikaido, Quercetin arrests human leukemic T-cells in late G1 phase of the cell cycle Cancer Res 52 (1992) 6676-6681.
[157] S. Madhusudan, T.S. Ganesan, Tyrosine kinase inhibitors and cancer therapy Recent Results Cancer Res 172 (2007) 25-44.
[158] D.R. Ferry, A. Smith, J. Malkhandi, D.W. Fyfe, P.G. deTakats, D. Anderson, J. Baker, D.J. Kerr, Phase I clinical trial of the flavonoid quercetin: pharmacokinetics and evidence for in vivo tyrosine kinase inhibition Clin Cancer Res 2 (1996) 659-668.
[159] M. Mouria, A.S. Gukovskaya, Y. Jung, P. Buechler, O.J. Hines, H.A. Reber, S.J. Pandol, Food-derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome C release and apoptosis Int J Cancer 98 (2002) 761-769.
[160] S. Tanigawa, M. Fujii, D.X. Hou, Stabilization of p53 is involved in quercetin-induced cell cycle arrest and apoptosis in HepG2 cells Biosci Biotechnol Biochem 72 (2008) 797-804.
[161] I.K. Wang, S.Y. Lin-Shiau, J.K. Lin, Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells Eur J Cancer 35 (1999) 1517-1525.
[162] L. Gibellini, M. Pinti, M. Nasi, J.P. Montagna, S. De Biasi, E. Roat, L. Bertoncelli, E.L. Cooper, A. Cossarizza, Quercetin and cancer chemoprevention Evid Based Complement Alternat Med 2011 (2011) 591356.
[163] E.J. Kim, C.H. Choi, J.Y. Park, S.K. Kang, Y.K. Kim, Underlying mechanism of quercetin-induced cell death in human glioma cells Neurochem Res 33 (2008) 971-979.
[164] T.J. Chen, J.Y. Jeng, C.W. Lin, C.Y. Wu, Y.C. Chen, Quercetin inhibition of ROS-dependent and -independent apoptosis in rat glioma C6 cells Toxicology 223 (2006) 113-126.
[165] E. Braganhol, L.L. Zamin, A.D. Canedo, F. Horn, A.S. Tamajusuku, M.R. Wink, C. Salbego, A.M. Battastini, Antiproliferative effect of quercetin in the human U138MG glioma cell line Anticancer Drugs 17 (2006) 663-671.
[166] M.D. Siegelin, D.E. Reuss, A. Habel, A. Rami, A. von Deimling, Quercetin promotes degradation of survivin and thereby enhances death-receptor-mediated apoptosis in glioma cells Neuro Oncol 11 (2009) 122-131.
[167] E.I. Campos, D. Reinberg, Histones: annotating chromatin Annu Rev Genet 43 (2009) 559-599.
[168] Editorial, Histone modifications., in: M. 2003 (Ed.), 2003.
[169] M. Iizuka, M.M. Smith, Functional consequences of histone modifications Curr Opin Genet Dev 13 (2003) 154-160.
[170] H. Santos-Rosa, C. Caldas, Chromatin modifier enzymes, the histone code and cancer Eur J Cancer 41 (2005) 2381-2402.
[171] S.B. Hake, A. Xiao, C.D. Allis, Linking the epigenetic 'language' of covalent histone modifications to cancer Br J Cancer 90 (2004) 761-769.
[172] J. Mellor, Dynamic nucleosomes and gene transcription Trends Genet 22 (2006) 320-329.

[173] T. Kouzarides, Histone acetylases and deacetylases in cell proliferation Curr Opin Genet Dev 9 (1999) 40-48.
[174] J. Schwartzentruber, A. Korshunov, X.Y. Liu, D.T. Jones, E. Pfaff, K. Jacob, D. Sturm, A.M. Fontebasso, D.A. Quang, M. Tonjes, V. Hovestadt, S. Albrecht, M. Kool, A. Nantel, C. Konermann, A. Lindroth, N. Jager, T. Rausch, M. Ryzhova, J.O. Korbel, T. Hielscher, P. Hauser, M. Garami, A. Klekner, L. Bognar, M. Ebinger, M.U. Schuhmann, W. Scheurlen, A. Pekrun, M.C. Fruhwald, W. Roggendorf, C. Kramm, M. Durken, J. Atkinson, P. Lepage, A. Montpetit, M. Zakrzewska, K. Zakrzewski, P.P. Liberski, Z. Dong, P. Siegel, A.E. Kulozik, M. Zapatka, A. Guha, D. Malkin, J. Felsberg, G. Reifenberger, A. von Deimling, K. Ichimura, V.P. Collins, H. Witt, T. Milde, O. Witt, C. Zhang, P. Castelo-Branco, P. Lichter, D. Faury, U. Tabori, C. Plass, J. Majewski, S.M. Pfister, N. Jabado, Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma Nature 482 (2012) 226-231.
[175] G. Wu, A. Broniscer, T.A. McEachron, C. Lu, B.S. Paugh, J. Becksfort, C. Qu, L. Ding, R. Huether, M. Parker, J. Zhang, A. Gajjar, M.A. Dyer, C.G. Mullighan, R.J. Gilbertson, E.R. Mardis, R.K. Wilson, J.R. Downing, D.W. Ellison, S.J. Baker, Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas Nat Genet 44 (2012) 251-253.
[176] M. Gessi, G.H. Gielen, J. Hammes, E. Dorner, A.Z. Muhlen, A. Waha, T. Pietsch, H3.3 G34R mutations in pediatric primitive neuroectodermal tumors of central nervous system (CNS-PNET) and pediatric glioblastomas: possible diagnostic and therapeutic implications? J Neurooncol (2013).
[177] S.Y. Archer, R.A. Hodin, Histone acetylation and cancer Curr Opin Genet Dev 9 (1999) 171-174.
[178] S. Thiagalingam, K.H. Cheng, H.J. Lee, N. Mineva, A. Thiagalingam, J.F. Ponte, Histone deacetylases: unique players in shaping the epigenetic histone code Ann N Y Acad Sci 983 (2003) 84-100.
[179] T. Abel, R.S. Zukin, Epigenetic targets of HDAC inhibition in neurodegenerative and psychiatric disorders Curr Opin Pharmacol 8 (2008) 57-64.
[180] O. Khan, N.B. La Thangue, HDAC inhibitors in cancer biology: emerging mechanisms and clinical applications Immunol Cell Biol 90 (2012) 85-94.
[181] M.S. Finnin, J.R. Donigian, A. Cohen, V.M. Richon, R.A. Rifkind, P.A. Marks, R. Breslow, N.P. Pavletich, Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors Nature 401 (1999) 188-193.
[182] R.B. Canani, M.D. Costanzo, L. Leone, M. Pedata, R. Meli, A. Calignano, Potential beneficial effects of butyrate in intestinal and extraintestinal diseases World J Gastroenterol 17 (2011) 1519-1528.
[183] H.V. Lin, A. Frassetto, E.J. Kowalik, Jr., A.R. Nawrocki, M.M. Lu, J.R. Kosinski, J.A. Hubert, D. Szeto, X. Yao, G. Forrest, D.J. Marsh, Butyrate and propionate protect against diet-induced obesity and regulate gut hormones via free fatty acid receptor 3-independent mechanisms PLoS One 7 (2012) e35240.
[184] J. Ordonez, A. Garcia de Lorenzo, J. Lopez, J.A. Rodriguez, [Enteral nutrition and fiber in intensive care] Nutr Hosp 9 (1994) 355-363.
[185] T.G. Machinsky, Efeito da adição de ácido butírico e da fitase na digestibilidade de nutrientes de suínos na fase de crescimento UFRGS (2008) 1-126.
[186] W. Scheppach, H. Sommer, T. Kirchner, G.M. Paganelli, P. Bartram, S. Christl, F. Richter, G. Dusel, H. Kasper, Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis Gastroenterology 103 (1992) 51-56.
[187] J.L. Thompson, M. Hinton, Antibacterial activity of formic and propionic acids in the diet of hens on Salmonellas in the crop Br Poult Sci 38 (1997) 59-65.
[188] F. Van Immerseel, V. Fievez, J. de Buck, F. Pasmans, A. Martel, F. Haesebrouck, R. Ducatelle, Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with Salmonella enteritidis in young chickens Poult Sci 83 (2004) 69-74.

[189] Y. Terao, J. Nishida, S. Horiuchi, F. Rong, Y. Ueoka, T. Matsuda, H. Kato, Y. Furugen, K. Yoshida, K. Kato, N. Wake, Sodium butyrate induces growth arrest and senescence-like phenotypes in gynecologic cancer cells Int J Cancer 94 (2001) 257-267.
[190] Y.F. Wang, N.S. Chen, Y.P. Chung, L.H. Chang, Y.H. Chiou, C.Y. Chen, Sodium butyrate induces apoptosis and cell cycle arrest in primary effusion lymphoma cells independently of oxidative stress and p21(CIP1/WAF1) induction Mol Cell Biochem 285 (2006) 51-59.
[191] S. Xue, P.N. Rao, Sodium butyrate blocks HeLa cells preferentially in early G1 phase of the cell cycle J Cell Sci 51 (1981) 163-171.
[192] J. Yang, Y. Kawai, R.W. Hanson, I.J. Arinze, Sodium butyrate induces transcription from the G alpha(i2) gene promoter through multiple Sp1 sites in the promoter and by activating the MEK-ERK signal transduction pathway J Biol Chem 276 (2001) 25742-25752.
[193] S. Siavoshian, J.P. Segain, M. Kornprobst, C. Bonnet, C. Cherbut, J.P. Galmiche, H.M. Blottiere, Butyrate and trichostatin A effects on the proliferation/differentiation of human intestinal epithelial cells: induction of cyclin D3 and p21 expression Gut 46 (2000) 507-514.
[194] C.P. Tran, M. Familari, L.M. Parker, R.H. Whitehead, A.S. Giraud, Short-chain fatty acids inhibit intestinal trefoil factor gene expression in colon cancer cells Am J Physiol 275 (1998) G85-94.
[195] Y. Lu, R. Lotan, Transcriptional regulation by butyrate of mouse galectin-1 gene in embryonal carcinoma cells Biochim Biophys Acta 1444 (1999) 85-91.
[196] G.E. Walker, E.M. Wilson, D. Powell, Y. Oh, Butyrate, a histone deacetylase inhibitor, activates the human IGF binding protein-3 promoter in breast cancer cells: molecular mechanism involves an Sp1/Sp3 multiprotein complex Endocrinology 142 (2001) 3817-3827.
[197] J.R. Davie, Inhibition of histone deacetylase activity by butyrate J Nutr 133 (2003) 2485S-2493S.
[198] W. Jiang, Q. Guo, J. Wu, B. Guo, Y. Wang, S. Zhao, H. Lou, X. Yu, X. Mei, C. Wu, S. Qiao, Y. Wu, Dual effects of sodium butyrate on hepatocellular carcinoma cells Mol Biol Rep 39 (2012) 6235-6242.
[199] Q. Wang, Y. Zhou, X. Wang, B.M. Evers, p27 Kip1 nuclear localization and cyclin-dependent kinase inhibitory activity are regulated by glycogen synthase kinase-3 in human colon cancer cells Cell Death Differ 15 (2008) 908-919.
[200] D.A. Litvak, K.O. Hwang, B.M. Evers, C.M. Townsend, Jr., Induction of apoptosis in human gastric cancer by sodium butyrate Anticancer Res 20 (2000) 779-784.
[201] J. Watanabe, H. Sato, T. Kanai, Y. Kamata, T. Jobo, H. Hata, T. Fujisawa, E. Ohno, T. Kameya, H. Kuramoto, Paradoxical expression of cell cycle inhibitor p27 in endometrioid adenocarcinoma of the uterine corpus - correlation with proliferation and clinicopathological parameters Br J Cancer 87 (2002) 81-85.
[202] H.H. Engelhard, H.A. Duncan, S. Kim, P.S. Criswell, L. Van Eldik, Therapeutic effects of sodium butyrate on glioma cells in vitro and in the rat C6 glioma model Neurosurgery 48 (2001) 616-624; discussion 624-615.
[203] H. Kamitani, S. Taniura, K. Watanabe, M. Sakamoto, T. Watanabe, T. Eling, Histone acetylation may suppress human glioma cell proliferation when p21 WAF/Cip1 and gelsolin are induced Neuro Oncol 4 (2002) 95-101.
[204] E.H. Kim, H.S. Kim, S.U. Kim, E.J. Noh, J.S. Lee, K.S. Choi, Sodium butyrate sensitizes human glioma cells to TRAIL-mediated apoptosis through inhibition of Cdc2 and the subsequent downregulation of survivin and XIAP Oncogene 24 (2005) 6877-6889.
[205] G.J. Soleas, E.P. Diamandis, D.M. Goldberg, Resveratrol: a molecule whose time has come? And gone? Clin Biochem 30 (1997) 91-113.
[206] H.J. Park, J.Y. Yang, S. Ambati, M.A. Della-Fera, D.B. Hausman, S. Rayalam, C.A. Baile, Combined effects of genistein, quercetin, and resveratrol in human and 3T3-L1 adipocytes J Med Food 11 (2008) 773-783.

[207] A. Schlachterman, F. Valle, K.M. Wall, N.G. Azios, L. Castillo, L. Morell, A.V. Washington, L.A. Cubano, S.F. Dharmawardhane, Combined resveratrol, quercetin, and catechin treatment reduces breast tumor growth in a nude mouse model Transl Oncol 1 (2008) 19-27.
[208] V. Granados-Soto, Pleiotropic effects of resveratrol Drug News Perspect 16 (2003) 299-307.
[209] H.Y. Cohen, C. Miller, K.J. Bitterman, N.R. Wall, B. Hekking, B. Kessler, K.T. Howitz, M. Gorospe, R. de Cabo, D.A. Sinclair, Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase Science 305 (2004) 390-392.
[210] E. Nisoli, C. Tonello, A. Cardile, V. Cozzi, R. Bracale, L. Tedesco, S. Falcone, A. Valerio, O. Cantoni, E. Clementi, S. Moncada, M.O. Carruba, Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS Science 310 (2005) 314-317.
[211] A.E. Civitarese, S. Carling, L.K. Heilbronn, M.H. Hulver, B. Ukropcova, W.A. Deutsch, S.R. Smith, E. Ravussin, Calorie restriction increases muscle mitochondrial biogenesis in healthy humans PLoS Med 4 (2007) e76.
[212] F. Yeung, J.E. Hoberg, C.S. Ramsey, M.D. Keller, D.R. Jones, R.A. Frye, M.W. Mayo, Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase EMBO J 23 (2004) 2369-2380.
[213] A. Kuzmichev, R. Margueron, A. Vaquero, T.S. Preissner, M. Scher, A. Kirmizis, X. Ouyang, N. Brockdorff, C. Abate-Shen, P. Farnham, D. Reinberg, Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation Proc Natl Acad Sci U S A 102 (2005) 1859-1864.
[214] C.A. Bradbury, F.L. Khanim, R. Hayden, C.M. Bunce, D.A. White, M.T. Drayson, C. Craddock, B.M. Turner, Histone deacetylases in acute myeloid leukaemia show a distinctive pattern of expression that changes selectively in response to deacetylase inhibitors Leukemia 19 (2005) 1751-1759.
[215] A. Vaquero, M. Scher, D. Lee, H. Erdjument-Bromage, P. Tempst, D. Reinberg, Human SirT1 interacts with histone H1 and promotes formation of facultative heterochromatin Mol Cell 16 (2004) 93-105.
[216] K. Pruitt, R.L. Zinn, J.E. Ohm, K.M. McGarvey, S.H. Kang, D.N. Watkins, J.G. Herman, S.B. Baylin, Inhibition of SIRT1 reactivates silenced cancer genes without loss of promoter DNA hypermethylation PLoS Genet 2 (2006) e40.
[217] M.F. Fraga, E. Ballestar, A. Villar-Garea, M. Boix-Chornet, J. Espada, G. Schotta, T. Bonaldi, C. Haydon, S. Ropero, K. Petrie, N.G. Iyer, A. Perez-Rosado, E. Calvo, J.A. Lopez, A. Cano, M.J. Calasanz, D. Colomer, M.A. Piris, N. Ahn, A. Imhof, C. Caldas, T. Jenuwein, M. Esteller, Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer Nat Genet 37 (2005) 391-400.
[218] W.Y. Chen, D.H. Wang, R.C. Yen, J. Luo, W. Gu, S.B. Baylin, Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses Cell 123 (2005) 437-448.
[219] L. Li, L. Wang, Z. Wang, Y. Ho, T. McDonald, T.L. Holyoake, W. Chen, R. Bhatia, Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib Cancer Cell 21 (2012) 266-281.
[220] R.V. Priyadarsini, G. Vinothini, R.S. Murugan, P. Manikandan, S. Nagini, The flavonoid quercetin modulates the hallmark capabilities of hamster buccal pouch tumors Nutr Cancer 63 218-226.
[221] P. Rajendran, E. Ho, D.E. Williams, R.H. Dashwood, Dietary phytochemicals, HDAC inhibition, and DNA damage/repair defects in cancer cells Clin Epigenetics 3 (2011) 4.
[222] R.V. Priyadarsini, G. Vinothini, R.S. Murugan, P. Manikandan, S. Nagini, The flavonoid quercetin modulates the hallmark capabilities of hamster buccal pouch tumors Nutr Cancer 63 (2011) 218-226.
[223] D.G. Knowles, A. McLysaght, Recent de novo origin of human protein-coding genes Genome Res 19 (2009) 1752-1759.

[224] J. Rosado, Análise de redes de interação de drogas quimioterápicas usadas no tratamentos de câncer gástrico: explorando portéinas e processos biológicos por medio de ferramentas de farmacologia de sistemas UFRGS (2010).
[225] K. Komurov, M.H. Gunes, M.A. White, Fine-scale dissection of functional protein network organization by statistical network analysis PLoS One 4 (2009) e6017.
[226] S. Clarke, Aging as war between chemical and biochemical processes: protein methylation and the recognition of age-damaged proteins for repair Ageing Res Rev 2 (2003) 263-285.
[227] F. Rodier, J. Campisi, Four faces of cellular senescence J Cell Biol 192 (2011) 547-556.
[228] L. Freeman, Going the Wrong Way on a One Way Street: Centrality in Physics and Biology Journal of Social Structure 9 (2008).
[229] J.A. Papin, T. Hunter, B.O. Palsson, S. Subramaniam, Reconstruction of cellular signalling networks and analysis of their properties Nat Rev Mol Cell Biol 6 (2005) 99-111.
[230] R.S. Wang, R. Albert, Elementary signaling modes predict the essentiality of signal transduction network components BMC Syst Biol 5 (2011) 44.
[231] S.I. Berger, R. Iyengar, Network analyses in systems pharmacology Bioinformatics 25 (2009) 2466-2472.
[232] M. Ashburner, C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski, S.S. Dwight, J.T. Eppig, M.A. Harris, D.P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J.C. Matese, J.E. Richardson, M. Ringwald, G.M. Rubin, G. Sherlock, Gene ontology: tool for the unification of biology. The Gene Ontology Consortium Nat Genet 25 (2000) 25-29.
[233] L. Pu, S. Brady, Systems biology update: cell type-specific transcriptional regulatory networks Plant Physiol 152 (2010) 411-419.
[234] K. Jonas, Y.E. Chen, M.T. Laub, Modularity of the bacterial cell cycle enables independent spatial and temporal control of DNA replication Curr Biol 21 1092-1101.
[235] F. Barea, D. Bonatto, Aging defined by a chronologic-replicative protein network in Saccharomyces cerevisiae: an interactome analysis Mech Ageing Dev 130 (2009) 444-460.
[236] R. Milo, S. Shen-Orr, S. Itzkovitz, N. Kashtan, D. Chklovskii, U. Alon, Network motifs: simple building blocks of complex networks Science 298 (2002) 824-827.
[237] S.P. Borgatti, Centrality and network flow. Soc networks 27 (2005) 55-71.
[238] T. Mori, D.D. Ikeda, Y. Yamaguchi, M. Unoki, NIRF/UHRF2 occupies a central position in the cell cycle network and allows coupling with the epigenetic landscape FEBS Lett 586 (2012) 1570-1583.
[239] U. de Lichtenberg, L.J. Jensen, S. Brunak, P. Bork, Dynamic complex formation during the yeast cell cycle Science 307 (2005) 724-727.
[240] J.K. Sicklick, Y.X. Li, A. Jayaraman, R. Kannangai, Y. Qi, P. Vivekanandan, J.W. Ludlow, K. Owzar, W. Chen, M.S. Torbenson, A.M. Diehl, Dysregulation of the Hedgehog pathway in human hepatocarcinogenesis Carcinogenesis 27 (2006) 748-757.
[241] R.A. Clark, M. Pavlis, Dysregulation of the mTOR pathway secondary to mutations or a hostile microenvironment contributes to cancer and poor wound healing J Invest Dermatol 129 (2009) 529-531.
[242] K.Q. Liu, Z.P. Liu, J.K. Hao, L. Chen, X.M. Zhao, Identifying dysregulated pathways in cancers from pathway interaction networks BMC Bioinformatics 13 (2012) 126.
[243] T. Sjoblom, S. Jones, L.D. Wood, D.W. Parsons, J. Lin, T.D. Barber, D. Mandelker, R.J. Leary, J. Ptak, N. Silliman, S. Szabo, P. Buckhaults, C. Farrell, P. Meeh, S.D. Markowitz, J. Willis, D. Dawson, J.K. Willson, A.F. Gazdar, J. Hartigan, L. Wu, C. Liu, G. Parmigiani, B.H. Park, K.E. Bachman, N. Papadopoulos, B. Vogelstein, K.W. Kinzler, V.E. Velculescu, The consensus coding sequences of human breast and colorectal cancers Science 314 (2006) 268-274.
[244] T.R. Yeager, S. DeVries, D.F. Jarrard, C. Kao, S.Y. Nakada, T.D. Moon, R. Bruskewitz, W.M. Stadler, L.F. Meisner, K.W. Gilchrist, M.A. Newton, F.M. Waldman, C.A. Reznikoff, Overcoming cellular senescence in human cancer pathogenesis Genes Dev 12 (1998) 163-174.

[245] D. Rambaldi, F.M. Giorgi, F. Capuani, A. Ciliberto, F.D. Ciccarelli, Low duplicability and network fragility of cancer genes Trends Genet 24 (2008) 427-430.
[246] J. Soh, N. Okumura, W.W. Lockwood, H. Yamamoto, H. Shigematsu, W. Zhang, R. Chari, D.S. Shames, X. Tang, C. MacAulay, M. Varella-Garcia, T. Vooder, Wistuba, II, S. Lam, R. Brekken, S. Toyooka, J.D. Minna, W.L. Lam, A.F. Gazdar, Oncogene mutations, copy number gains and mutant allele specific imbalance (MASI) frequently occur together in tumor cells PLoS One 4 (2009) e7464.
[247] N.B. Kiss, A. Muth, A. Andreasson, C.C. Juhlin, J. Geli, M. Backdahl, A. Hoog, B. Wangberg, O. Nilsson, H. Ahlman, C. Larsson, Acquired hypermethylation of the P16INK4A promoter in abdominal paraganglioma: relation to adverse tumor phenotype and predisposing mutation Endocr Relat Cancer (2012).
[248] H. Liang, W.H. Li, Gene essentiality, gene duplicability and protein connectivity in human and mouse Trends Genet 23 (2007) 375-378.
[249] B.Y. Liao, J. Zhang, Mouse duplicate genes are as essential as singletons Trends Genet 23 (2007) 378-381.
[250] P.F. Jonsson, T. Cavanna, D. Zicha, P.A. Bates, Cluster analysis of networks generated through homology: automatic identification of important protein communities involved in cancer metastasis BMC Bioinformatics 7 (2006) 2.
[251] Q. Cui, Y. Ma, M. Jaramillo, H. Bari, A. Awan, S. Yang, S. Zhang, L. Liu, M. Lu, M. O'Connor-McCourt, E.O. Purisima, E. Wang, A map of human cancer signaling Mol Syst Biol 3 (2007) 152.
[252] G. Deblois, G. Chahrour, M.C. Perry, G. Sylvain-Drolet, W.J. Muller, V. Giguere, Transcriptional control of the ERBB2 amplicon by ERRalpha and PGC-1beta promotes mammary gland tumorigenesis Cancer Res 70 10277-10287.
[253] A. Awan, H. Bari, F. Yan, S. Moksong, S. Yang, S. Chowdhury, Q. Cui, Z. Yu, E.O. Purisima, E. Wang, Regulatory network motifs and hotspots of cancer genes in a mammalian cellular signalling network IET Syst Biol 1 (2007) 292-297.
[254] A.A. Prinz, D. Bucher, E. Marder, Similar network activity from disparate circuit parameters Nat Neurosci 7 (2004) 1345-1352.
[255] A.G. Jegga, L. Schneider, X. Ouyang, J. Zhang, Systems biology of the autophagy-lysosomal pathway Autophagy 7 477-489.
[256] A.C. Ng, Integrative systems biology and networks in autophagy Semin Immunopathol 32 (2010) 355-361.
[257] I.N. Lavrik, Systems biology of apoptosis signaling networks Curr Opin Biotechnol 21 551-555.
[258] I.N. Lavrik, R. Eils, N. Fricker, C. Pforr, P.H. Krammer, Understanding apoptosis by systems biology approaches Mol Biosyst 5 (2009) 1105-1111.
[259] R. Tacutu, A. Budovsky, H. Yanai, V.E. Fraifeld, Molecular links between cellular senescence, longevity and age-related diseases - a systems biology perspective Aging (Albany NY) 3 (2011) 1178-1191.
[260] G. Lenz, D. Goncalves, Z. Luo, J. Avruch, R. Rodnight, J.T. Neary, Extracellular ATP stimulates an inhibitory pathway towards growth factor-induced cRaf-1 and MEKK activation in astrocyte cultures J Neurochem 77 (2001) 1001-1009.
[261] D.N. Louis, Molecular pathology of malignant gliomas Annu Rev Pathol 1 (2006) 97-117.
[262] J. Chen, S. Patschan, M.S. Goligorsky, Stress-induced premature senescence of endothelial cells J Nephrol 21 (2008) 337-344.
[263] L.W. Elmore, C.W. Rehder, X. Di, P.A. McChesney, C.K. Jackson-Cook, D.A. Gewirtz, S.E. Holt, Adriamycin-induced senescence in breast tumor cells involves functional p53 and telomere dysfunction J Biol Chem 277 (2002) 35509-35515.
[264] Y.W. Eom, M.A. Kim, S.S. Park, M.J. Goo, H.J. Kwon, S. Sohn, W.H. Kim, G. Yoon, K.S. Choi, Two distinct modes of cell death induced by doxorubicin: apoptosis and cell death

through mitotic catastrophe accompanied by senescence-like phenotype Oncogene 24 (2005) 4765-4777.
[265] A.M. Tejera, D.F. Alonso, D.E. Gomez, O.A. Olivero, Chronic in vitro exposure to 3'-azido-2', 3'-dideoxythymidine induces senescence and apoptosis and reduces tumorigenicity of metastatic mouse mammary tumor cells Breast Cancer Res Treat 65 (2001) 93-99.
[266] E.C. Filippi-Chiela, E.S. Villodre, L.L. Zamin, G. Lenz, Autophagy interplay with apoptosis and cell cycle regulation in the growth inhibiting effect of resveratrol in glioma cells PLoS One 6 e20849.
[267] L.L. Zamin, P. Dillenburg-Pilla, R. Argenta-Comiran, A.P. Horn, F. Simao, M. Nassif, D. Gerhardt, R.L. Frozza, C. Salbego, Protective effect of resveratrol against oxygen-glucose deprivation in organotypic hippocampal slice cultures: Involvement of PI3-K pathway Neurobiol Dis 24 (2006) 170-182.
[268] V. Chopin, R.A. Toillon, N. Jouy, X. Le Bourhis, Sodium butyrate induces P53-independent, Fas-mediated apoptosis in MCF-7 human breast cancer cells Br J Pharmacol 135 (2002) 79-86.
[269] K.Y. Fung, G.V. Brierley, S. Henderson, P. Hoffmann, S.R. McColl, T. Lockett, R. Head, L. Cosgrove, Butyrate-induced apoptosis in HCT116 colorectal cancer cells includes induction of a cell stress response J Proteome Res 10 (2011) 1860-1869.
[270] N. Ishii, D. Maier, A. Merlo, M. Tada, Y. Sawamura, A.C. Diserens, E.G. Van Meir, Frequent co-alterations of TP53, p16/CDKN2A, p14ARF, PTEN tumor suppressor genes in human glioma cell lines Brain Pathol 9 (1999) 469-479.
[271] L. Yang, S. Wang, B. Sung, G. Lim, J. Mao, Morphine induces ubiquitin-proteasome activity and glutamate transporter degradation J Biol Chem 283 (2008) 21703-21713.
[272] C.K. Cheng, Q.W. Fan, W.A. Weiss, PI3K signaling in glioma--animal models and therapeutic challenges Brain Pathol 19 (2009) 112-120.
[273] V. Nogueira, Y. Park, C.C. Chen, P.Z. Xu, M.L. Chen, I. Tonic, T. Unterman, N. Hay, Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis Cancer Cell 14 (2008) 458-470.
[274] S. Thaler, P.S. Hahnel, A. Schad, R. Dammann, M. Schuler, RASSF1A mediates p21Cip1/Waf1-dependent cell cycle arrest and senescence through modulation of the Raf-MEK-ERK pathway and inhibition of Akt Cancer Res 69 (2009) 1748-1757.
[275] K. Ichimura, E.E. Schmidt, H.M. Goike, V.P. Collins, Human glioblastomas with no alterations of the CDKN2A (p16INK4A, MTS1) and CDK4 genes have frequent mutations of the retinoblastoma gene Oncogene 13 (1996) 1065-1072.
[276] K. Ueki, M.P. Rubio, V. Ramesh, K.M. Correa, J.L. Rutter, A. von Deimling, A.J. Buckler, J.F. Gusella, D.N. Louis, MTS1/CDKN2 gene mutations are rare in primary human astrocytomas with allelic loss of chromosome 9p Hum Mol Genet 3 (1994) 1841-1845.
[277] G.W. Xu, C.L. Nutt, M.C. Zlatescu, M. Keeney, I. Chin-Yee, J.G. Cairncross, Inactivation of p53 sensitizes U87MG glioma cells to 1,3-bis(2-chloroethyl)-1-nitrosourea Cancer Res 61 (2001) 4155-4159.
[278] J. Schlegel, G. Piontek, M. Kersting, M. Schuermann, R. Kappler, H. Scherthan, C. Weghorst, G. Buzard, H. Mennel, The p16/Cdkn2a/Ink4a gene is frequently deleted in nitrosourea-induced rat glial tumors Pathobiology 67 (1999) 202-206.
[279] C. Gomez-Manzano, J. Fueyo, A.P. Kyritsis, T.J. McDonnell, P.A. Steck, V.A. Levin, W.K. Yung, Characterization of p53 and p21 functional interactions in glioma cells en route to apoptosis J Natl Cancer Inst 89 (1997) 1036-1044.
[280] C.H. Brandts, B. Bilanges, G. Hare, F. McCormick, D. Stokoe, Phosphorylation-independent stabilization of p27kip1 by the phosphoinositide 3-kinase pathway in glioblastoma cells J Biol Chem 280 (2005) 2012-2019.
[281] A. Taddei, C. Maison, D. Roche, G. Almouzni, Reversible disruption of pericentric heterochromatin and centromere function by inhibiting deacetylases Nat Cell Biol 3 (2001) 114-120.

[282] F.E. Stevens, H. Beamish, R. Warrener, B. Gabrielli, Histone deacetylase inhibitors induce mitotic slippage Oncogene 27 (2008) 1345-1354.
[283] Y. Li, G.D. Kao, B.A. Garcia, J. Shabanowitz, D.F. Hunt, J. Qin, C. Phelan, M.A. Lazar, A novel histone deacetylase pathway regulates mitosis by modulating Aurora B kinase activity Genes Dev 20 (2006) 2566-2579.
[284] A. Mikhailov, M. Shinohara, C.L. Rieder, Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway J Cell Biol 166 (2004) 517-526.
[285] W. Fischle, Y. Wang, C.D. Allis, Binary switches and modification cassettes in histone biology and beyond Nature 425 (2003) 475-479.
[286] T. Hirota, J.J. Lipp, B.H. Toh, J.M. Peters, Histone H3 serine 10 phosphorylation by Aurora B causes HP1 dissociation from heterochromatin Nature 438 (2005) 1176-1180.
[287] N.K. Alvi, R.Y. Rizvi, S.M. Hadi, Interaction of quercetin with DNA Biosci Rep 6 (1986) 861-868.
[288] M.R. Webb, S.E. Ebeler, Comparative analysis of topoisomerase IB inhibition and DNA intercalation by flavonoids and similar compounds: structural determinates of activity Biochem J 384 (2004) 527-541.
[289] C.D. Kanakis, P.A. Tarantilis, M.G. Polissiou, S. Diamantoglou, H.A. Tajmir-Riahi, DNA interaction with naturally occurring antioxidant flavonoids quercetin, kaempferol, and delphinidin J Biomol Struct Dyn 22 (2005) 719-724.
[290] O.A. Sedelnikova, D.R. Pilch, C. Redon, W.M. Bonner, Histone H2AX in DNA damage and repair Cancer Biol Ther 2 (2003) 233-235.
[291] R. Colavitti, T. Finkel, Reactive oxygen species as mediators of cellular senescence IUBMB Life 57 (2005) 277-281.
[292] G. Slupphaug, B. Kavli, H.E. Krokan, The interacting pathways for prevention and repair of oxidative DNA damage Mutat Res 531 (2003) 231-251.
[293] I. Masgras, S. Carrera, P.J. de Verdier, P. Brennan, A. Majid, W. Makhtar, E. Tulchinsky, G.D. Jones, I.B. Roninson, S. Macip, Reactive oxygen species and mitochondrial sensitivity to oxidative stress determine induction of cancer cell death by p21 J Biol Chem 287 (2012) 9845-9854.
[294] Q. Liu, T. Shimoyama, K. Suzuki, T. Umeda, S. Nakaji, K. Sugawara, Effect of sodium butyrate on reactive oxygen species generation by human neutrophils Scand J Gastroenterol 36 (2001) 744-750.
[295] G.L. Russo, Ins and outs of dietary phytochemicals in cancer chemoprevention Biochem Pharmacol 74 (2007) 533-544.
[296] S. Sathornsumetee, J.N. Rich, Designer therapies for glioblastoma multiforme Ann N Y Acad Sci 1142 (2008) 108-132.
[297] G. Pellegrini, E. Dellambra, P. Paterna, O. Golisano, C.E. Traverso, P. Rama, P. Lacal, M. De Luca, Telomerase activity is sufficient to bypass replicative senescence in human limbal and conjunctival but not corneal keratinocytes Eur J Cell Biol 83 (2004) 691-700.
[298] R. Di Micco, M. Fumagalli, A. Cicalese, S. Piccinin, P. Gasparini, C. Luise, C. Schurra, M. Garre, P.G. Nuciforo, A. Bensimon, R. Maestro, P.G. Pelicci, F. d'Adda di Fagagna, Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication Nature 444 (2006) 638-642.
[299] P.J. Hornsby, Senescence as an anticancer mechanism J Clin Oncol 25 (2007) 1852-1857.
[300] A. Suram, J. Kaplunov, P.L. Patel, H. Ruan, A. Cerutti, V. Boccardi, M. Fumagalli, R. Di Micco, N. Mirani, R.L. Gurung, M.P. Hande, F. d'Adda di Fagagna, U. Herbig, Oncogene-induced telomere dysfunction enforces cellular senescence in human cancer precursor lesions EMBO J 31 (2012) 2839-2851.

[301] M. Serrano, A.W. Lin, M.E. McCurrach, D. Beach, S.W. Lowe, Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a Cell 88 (1997) 593-602.
[302] N. Dhomen, J.S. Reis-Filho, S. da Rocha Dias, R. Hayward, K. Savage, V. Delmas, L. Larue, C. Pritchard, R. Marais, Oncogenic Braf induces melanocyte senescence and melanoma in mice Cancer Cell 15 (2009) 294-303.
[303] N. Wajapeyee, S.Z. Wang, R.W. Serra, P.D. Solomon, A. Nagarajan, X. Zhu, M.R. Green, Senescence induction in human fibroblasts and hematopoietic progenitors by leukemogenic fusion proteins Blood 115 (2010) 5057-5060.
[304] J.R. Testa, P.N. Tsichlis, AKT signaling in normal and malignant cells Oncogene 24 (2005) 7391-7393.
[305] H. Yu, D. Pardoll, R. Jove, STATs in cancer inflammation and immunity: a leading role for STAT3 Nat Rev Cancer 9 (2009) 798-809.
[306] C.V. Dang, MYC on the path to cancer Cell 149 (2012) 22-35.
[307] H.Z. Chen, S.Y. Tsai, G. Leone, Emerging roles of E2Fs in cancer: an exit from cell cycle control Nat Rev Cancer 9 (2009) 785-797.
[308] D. Zhuang, S. Mannava, V. Grachtchouk, W.H. Tang, S. Patil, J.A. Wawrzyniak, A.E. Berman, T.J. Giordano, E.V. Prochownik, M.S. Soengas, M.A. Nikiforov, C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells Oncogene 27 (2008) 6623-6634.
[309] G.P. Dimri, K. Itahana, M. Acosta, J. Campisi, Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14(ARF) tumor suppressor Mol Cell Biol 20 (2000) 273-285.
[310] K. Robinson, N. Asawachaicharn, D.A. Galloway, C. Grandori, c-Myc accelerates S-phase and requires WRN to avoid replication stress PLoS One 4 (2009) e5951.
[311] S. Gonfloni, DNA damage stress response in germ cells: role of c-Abl and clinical implications Oncogene 29 (2010) 6193-6202.
[312] W.C. Burhans, M. Weinberger, DNA replication stress, genome instability and aging Nucleic Acids Res 35 (2007) 7545-7556.
[313] O. Vafa, M. Wade, S. Kern, M. Beeche, T.K. Pandita, G.M. Hampton, G.M. Wahl, c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability Mol Cell 9 (2002) 1031-1044.
[314] N. Raimundo, L. Song, T.E. Shutt, S.E. McKay, J. Cotney, M.X. Guan, T.C. Gilliland, D. Hohuan, J. Santos-Sacchi, G.S. Shadel, Mitochondrial stress engages E2F1 apoptotic signaling to cause deafness Cell 148 (2012) 716-726.
[315] M. Sattler, S. Verma, G. Shrikhande, C.H. Byrne, Y.B. Pride, T. Winkler, E.A. Greenfield, R. Salgia, J.D. Griffin, The BCR/ABL tyrosine kinase induces production of reactive oxygen species in hematopoietic cells J Biol Chem 275 (2000) 24273-24278.
[316] I.R. Indran, M.P. Hande, S. Pervaiz, Tumor cell redox state and mitochondria at the center of the non-canonical activity of telomerase reverse transcriptase Mol Aspects Med 31 (2010) 21-28.
[317] M. Takakura, S. Kyo, Y. Sowa, Z. Wang, N. Yatabe, Y. Maida, M. Tanaka, M. Inoue, Telomerase activation by histone deacetylase inhibitor in normal cells Nucleic Acids Res 29 (2001) 3006-3011.
[318] S. Kyo, M. Inoue, Complex regulatory mechanisms of telomerase activity in normal and cancer cells: how can we apply them for cancer therapy? Oncogene 21 (2002) 688-697.
[319] K.J. Wu, C. Grandori, M. Amacker, N. Simon-Vermot, A. Polack, J. Lingner, R. Dalla-Favera, Direct activation of TERT transcription by c-MYC Nat Genet 21 (1999) 220-224.
[320] D. Trachootham, W. Lu, M.A. Ogasawara, R.D. Nilsa, P. Huang, Redox regulation of cell survival Antioxid Redox Signal 10 (2008) 1343-1374.

[321] A. Biroccio, B. Benassi, F. Fiorentino, G. Zupi, Glutathione depletion induced by c-Myc downregulation triggers apoptosis on treatment with alkylating agents Neoplasia 6 (2004) 195-206.
[322] A.V. Bazarov, W.C. Hines, R. Mukhopadhyay, A. Beliveau, S. Melodyev, Y. Zaslavsky, P. Yaswen, Telomerase activation by c-Myc in human mammary epithelial cells requires additional genomic changes Cell Cycle 8 (2009) 3373-3378.
[323] S.J. Ralph, S. Rodriguez-Enriquez, J. Neuzil, E. Saavedra, R. Moreno-Sanchez, The causes of cancer revisited: "mitochondrial malignancy" and ROS-induced oncogenic transformation - why mitochondria are targets for cancer therapy Mol Aspects Med 31 (2010) 145-170.
[324] A. Acharya, I. Das, D. Chandhok, T. Saha, Redox regulation in cancer: a double-edged sword with therapeutic potential Oxid Med Cell Longev 3 (2010) 23-34.
[325] P. Hernandez, J. Huerta-Cepas, D. Montaner, F. Al-Shahrour, J. Valls, L. Gomez, G. Capella, J. Dopazo, M.A. Pujana, Evidence for systems-level molecular mechanisms of tumorigenesis BMC Genomics 8 (2007) 185.
[326] B.K. Kleinschmidt-Demasters, L.C. Evans, J.B. Bobak, D. Lopez-Uribe, D. Hopper, A.L. Shroyer, K.R. Shroyer, Quantitative telomerase expression in glioblastomas shows regional variation and down-regulation with therapy but no correlation with patient outcome Hum Pathol 31 (2000) 905-913.
[327] A. Baykal, J.A. Thompson, X.C. Xu, W.C. Hahn, M.T. Deavers, A. Malpica, D.M. Gershenson, E.G. Silva, J. Liu, In situ human telomerase reverse transcriptase expression pattern in normal and neoplastic ovarian tissues Oncol Rep 11 (2004) 297-302.
[328] L.R. Yates, P.J. Campbell, Evolution of the cancer genome Nat Rev Genet 13 (2012) 795-806.
[329] M. Braig, S. Lee, C. Loddenkemper, C. Rudolph, A.H. Peters, B. Schlegelberger, H. Stein, B. Dorken, T. Jenuwein, C.A. Schmitt, Oncogene-induced senescence as an initial barrier in lymphoma development Nature 436 (2005) 660-665.
[330] E.K. Parkinson, J. Munro, K. Steeghs, V. Morrison, H. Ireland, N. Forsyth, S. Fitzsimmons, S. Bryce, Replicative senescence as a barrier to human cancer Biochem Soc Trans 28 (2000) 226-233.

8. ANEXO

pLR: A lentiviral backbone series to stable transduction of bicistronic genes and exchange of promoters

José Eduardo Vargas, Gabrielle Salton, Andressa Sodré de Castro Laino, Tiago Dalberto Pires, Martin Bonamino, Guido Lenz, Andrés Delgado-Cañedo

Periódico: **Plasmid**

Estado: **Publicado**

Referência: **J.E. Vargas, G. Salton, A. Sodre de Castro Laino, T.D. Pires, M. Bonamino, G. Lenz, A. Delgado-Canedo, pLR: a lentiviral backbone series to stable transduction of bicistronic genes and exchange of promoters Plasmid 68 (2012) 179-185.**

Plasmid 68 (2012) 179–185

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Short Communication

pLR: A lentiviral backbone series to stable transduction of bicistronic genes and exchange of promoters

José Eduardo Vargas [a,b], Gabrielle Salton [a], Andressa Sodré de Castro Laino [c], Tiago Dalberto Pires [d], Martin Bonamino [c], Guido Lenz [a], Andrés Delgado-Cañedo [b,e,*]

[a] Laboratório de Sinalização e Plasticidade Celular, Departamento de Biofísica, Universidade Federal do Rio Grande do Sul (UFRGS), 91501-970 Porto Alegre, RS, Brazil
[b] Laboratório de Cardiologia Molecular e Celular, Fundação Universitária de Cardiologia/Instituto de Cardiologia – RS, 90620-000, Porto Alegre, RS, Brazil
[c] Programa de Medicina Experimental, Instituto Nacional de Câncer (INCA), Rio de Janeiro, RJ, Brazil
[d] Laboratório de Imunogenética, Departamento de Genetica, Universidade Federal do Rio Grande do Sul (UFRGS), 91501-970 Porto Alegre, RS, Brazil
[e] Grupo de estudo da expressão gênica em eucariotas, Universidade Federal do Pampa (Unipampa),Campus São Gabriel, 93000-000 São Gabriel, RS, Brazil

ARTICLE INFO

Article history:
Received 9 October 2011
Accepted 5 June 2012
Available online 20 June 2012
Communicated by Grzegorz Wegrzyn

Keywords:
Gene transfer
Stable expression
Bicistronic reporter
Promoter exchange
HIV based vectors

ABSTRACT

Gene transfer based on lentiviral vectors allow the integration of exogenous genes into the genome of a target cell, turning these vectors into one of the most used methods for stable transgene expression in mammalian cells, *in vitro* and *in vivo*. Currently, there are no lentivectors that allow the cloning of different genes to be regulated by different promoters. Also, there are none that permit the analysis of the expression through an IRES (internal ribosome entry site) – reporter gene system.

In this work, we have generated a series of lentivectors containing: (1) a malleable structure to allow the cloning of different target genes in a multicloning site (mcs); (2) unique site to exchange promoters, and (3) IRES followed by one of two reporter genes: eGFP or DsRed. The series of the produced vectors were named pLR (for lentivirus and RSV promoter) and were fairly efficient with a strong fluorescence of the reporter genes in direct transfection and viral transduction experiments. This being said, the pLR series have been found to be powerful biotechnological tools for stable gene transfer and expression.

1. Introduction

Gene transfer vectors based on retroviruses, including oncogenic retroviruses and lentiviruses, provide effective means for the delivery, integration, and expression of exogenous genes in mammalian cells, converting these vectors into a powerful biotechnological tool that allows stable transgene expression both *in vitro* and *in vivo* (Yi et al., 2011). In contrast to oncogenic retroviruses, which are dependent on transduction of mitotic cells (Miller et al., 1990), lentiviruses are independent of cell division in completing their replicative cycle (Lewis and Emerman, 1994). Therefore, they provide attractive gene delivery vehicles for non-dividing cells and are therefore widely used as gene vectors (Miller et al., 1990; Frecha et al., 2010, 2011).

Lentiviral-based vectors have also showed promise for long-term gene expression due to their stable integration into the host genome and low degree of silencing. These vectors were used to transduce cells *in vivo* from several organs and tissues from, for example, the central nervous system (Anliker et al., 2010), hematopoietic system (Biffi et al., 2011; Anliker et al., 2010), retina (Miyoshi et al., 1997), muscle and liver (Kafri et al., 1997), lung (Johnson et al., 2000; Shenoy et al., 2010), pancreatic islets (Hynes et al., 2011) and cochlea and corneal tissue (Duan and Mi, 2010) and are being increasingly used in basic and applied research (Dropulic, 2011).

* Corresponding author at: Universidade Federal do Pampa (Unipampa), Campus São Gabriel, Av. Antônio Trilha 1847, São Gabriel 97300-000, RS, Brazil. Fax: +55 55 32326075.
E-mail address: andres@unipampa.edu.br (A. Delgado-Cañedo).

0147-619X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.plasmid.2012.06.001

Development of systems based on self-inactivating HIV continues to receive modifications, especially in regard to the improvement of genetic constructs and security in such systems (Naldini et al., 1996; Lois et al., 2002; Fux and Fussenegger, 2003; Koldej and Anson, 2009; Escors and Breckpot, 2010; Durand and Cimarelli, 2011).

Many lentivectors are available; however, most of them use strong viral promoters to ensure the expression of the transgene of interest without the possibility of exchanging them to obtain the desired expression level or to study a specific promoter activity. At the same time, the need to produce viral systems coding one, two, or more proteins under regulation of the same promoter continues to be a strategic demand requiring constructs specific for multicistronic expression (Vagner et al., 2001; Ngoi et al., 2004; Pacheco and Martinez-Salas, 2010).

In this work, we created a lentivector series containing: structural plasticity to allow the cloning of several genes in a multicloning site (mcs); unique cleavage sites to interchange different promoters, and an IRES sequence for the generation of bicistronic mRNA that allows, at the same time, to check the transfection efficiency and quantify the transgene expression through the use of reporter genes such as eGFP or DsRed.

2. Methods

2.1. PCR

To construct the series of vectors, sequences were amplified by PCR using different plasmids as templates and primers containing restriction sites to facilitate the cloning strategies. Primers and plasmids that were used together the time used to amplify each fragment are specified in Table 1.

2.2. Cloning strategies

The PCR products were cloned using the commercial TOPO TA cloning system, following the manufacturer's recommendations (Invitrogen, USA).

All ligations and transformation of *Epicurian coli* XL1 blue cells were made as described elsewhere (Sambrook and Russell, 2005). After transformation, cells were plated in LB-Agar medium containing ampicillin (50 μg/ml) for the selection of transformants.

Plasmid extraction was made by alkaline lysis as previously described (Sambrook and Russell, 2005) and analyzed through cleavage with restriction enzymes and sequencing to confirm the integrity of the sequence.

The constructs confirmed by enzyme restriction and sequencing analysis were purified using PureLink™ HiPure Plasmid Midiprep Kit following the protocol of the manufacturers (Invitrogen).

Plasmid maps, were built using the program pDRAW32.1.1.88 (http://www.acaclone.com). The sequencing results were analyzed through the software BioEdit 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.3. Cell transfection

HEK-293T cells were grown in DMEM (Invitrogen) supplement with 10% FCS (Cultilab, Brazil). Cells were seeded at 70% confluence in 24 well plates ($3–5 \times 10^5$ cells) and transfected, after 24 h of culture, using 2μL of lipofectamine 2000 (Invitrogen) and 0.8 μg of the different expression vectors. Transfected cells were analyzed 24, 48 and 72 h post-transfection.

2.4. Production and titration of lentiviral vectors

Supernatant lentiviral vectors were produced from HEK-293T packaging cell line and transfection was performed by calcium phosphate precipitation on 2.5×10^6 HEK-293T cells in a T75 culture bottle as previously described (Bonamino et al., 2004). Transfection was performed the day after cell seeding in fresh medium by mixing HEPES buffered saline solution 1×, calcium chloride (12.5 mM), lentiviral vectors (20 μg) encoding pRL2, pLR3, pRL2-DsRed, or pRL3-eGFP, and the packaging vectors pMDLg/RRE (10 μg), pRSV-REV (5 μg) and pMD2-G (6 μg). Supernatants containing the lentivirus were collected 48 h after transfection, filtered on 0.22 μm low protein binding filters and frozen at −80 °C. Titrations of vector stocks were performed by transducing 3×10^5 HEK-293T cells in a six plate well and reading eGFP or DsRed expression 48 h later by FACS. Titers were determined by the formula: positive cell% × number of cells/

Table 1
Primers used to amplify the fragments IRES-eGFP and IRES-DsRed from plasmids. Together, sequences of primers and probes used in real-time PCR from genomic DNA of transduced cells.

Primer	Sequence (5′ to 3′)	Product size	PCR template	Extension time (min)
IRES-eGFP (F)	TGAATTCCGAGAGATCCGTGGC	1331	pIRES-eGFP	2
IRES-eGFP (R)	TCCCACATGTTACTTGTACAGCTCG	1331	pIRES-eGFP	2
IRES-DsRed (F)	TAACCCGGGTGAATTCCGCC	1280	pTtrKRAB-IRESDsRED	2
IRES-DsRed (R)	GCCACATGTTACAGGAACAGGTGGTGG	1280	pTtrKRAB-IRESDsRED	2
eGFP (F)	CAACAGCCACAACGTCTATATCATG	79	Genomic DNA	1
eGFP (P)	CAAGCAGAAGAACGGCATCAAGGTGA	-	Genomic DNA	
eGFP (R)	ATGTTGTGGCGGATCTTGAAG	79	Genomic DNA	1
DsRed (F)	GCAGCTGCCCGGCTACT	79	Genomic DNA	1
DsRed (P)	CGTGGACTCCAAGCTGGACATCACCT	-	Genomic DNA	
DsRed (R)	CGATGGTGTAGTCCTCGTTGTG	79	Genomic DNA	1

Table shows the sequences of each primer, the product size, template used and the time used in the extension stage during the PCR. (F) forward, (R) reverse and (P) probe. Taqman systems to amplify GFP and DsRed were obtained from (Ji et al., 2005).

volume of virus stock. For the generation of transgene expressing cells, 2×10^5 HEK-293T cells were transduced with Multiplicities of Infection (MOIs) of 0.62, 0.31, and 1.08 for pRL2, pLR2-dsRed and pLR3-eGFP constructs. The transduction protocol was repeated twice for HEK-293T with pLR3 and pLR3-eGFP constructs and three times with pLR2-DsRED construct. After *in vitro* expanding, transduced cell lines were analyzed by flow cytometry with the BD FACSCalibur™ platform and cell sorting was performed with the BD FACSAria™ cell sorter, based on expression of GFP and/or dsRED fluorescent proteins.

2.5. Fluorescence analysis

Transfected and transduced cells were analyzed by fluorescence microscopy and flow cytometry to study the functionality of the different structures cloned into the lentivector series. The gene expression pattern of eGFP and DsRed was analyzed initially by fluorescence microscopy on an AxioVert 40 CFL inverted microscope (Zeiss, Germany) equipped with filters 09 and 20 (FT 510; BP 450/90; LP 520 and FT 560; BP 546/12; BP 575–640, respectively). Microphotographs were obtained using a camera AxioCam MRC (Zeiss). Transfected cells were fixed in 4% paraformaldehyde for 15 min. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

The intensity of fluorescence and transduction percentage of cells were analyzed in a FACS Calibur cytometer (Becton Dickinson, USA), using FL-1 PMT to detect the eGFP protein and FL-2 PMT to detect the DsRed protein. Ten thousand events were collected. Off-line analysis was performed by WinMDI 2.9 software (http://facs.scripps.edu/software.html).

2.6. Quantitative real-time PCR (qPCR)

TaqMan reactions were performed using 10 ng of HEK-293T genomic DNA after transduction with pLR3-GFP, pLR2-dsRED, pLR2 or pLR3 plasmids, in a mixture containing 1× TaqMan Universal PCR Master Mix (Invitrogen), 300 nM of each (forward and reverse) primer and 200 nM of TaqMan probe. The standard curve for quantification of the number of integrations of the plasmids in the genome was based on dilution of a known number of copies of the pLR3-GFP plasmid in 10 ng of HEK-293T genomic DNA. TaqMan PCR was performed on an CFX96Real-Time system (BIO-RAD) with the following protocol: 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min (Ji et al., 2005). Primers, probes used are specified in Table 1.

3. Results and discussion

3.1. Development of the lentiviral vector series with different expression cassettes

Lentivectors are a valorous biotechnological tool because of their natural mechanism to transduce many kinds of cells in a stable manner, including non-dividing cells. However, there are currently only a few plasmids that allow biological assays to be easily performed in ways that take advantage of different promoters and reporter genes. Taking this into account, we developed the pLR series considering three important points: strong and exchangeable promoters, unique cleavage sites strategically located, and IRES activity followed by fluorescent reporter genes.

To develop the lentivector series we used commercial plasmid pCR3.1 (Invitrogen), pREP9 (Invitrogen), pUC18 (Amersham Pharmacia) and pIRES-eGFP (Stratagene) along with the plasmids, pTtrKRAB-IRES-DsRED and pLL3.7, generously provided by Dr. Didier Trono (EPA, Lausanne, Switzerland) and Dr. Luc van Parijs, (MIT, Cambridge, MA, USA), respectively.

First, the DNA fragments containing the sequence IRES-eGFP and IRES-DsRED were amplified by PCR using plasmids pIRES-eGFP or pTTR-KRAB-DsRed as the template, respectively. The PCR products were cloned into the plasmid pCR2.1 of the TOPO TA cloning system and they were named pCR2.1-IRES-eGFP and pCR2.1-IRES-DsRed.

Next, the fragment located between the restriction sites for HpaI and EcoRI of pLL3.7 was replaced by the multicloning site (MCS) of pUC18, located between the restriction sites of EcoRI and PvuII. This plasmid was named pLLmcs18. The presence of a unique cloning site for EcoRI in pLLmcs18 was used to perform the cloning of IRES-RED or IRES-eGFP sequences, obtained from pCR2.1-IRES-DsRED and pCR2.1-IRES-eGFP plasmids and the new lentiviral plasmids were named pLLmcs18-IRES-DsRED and pLLmcs18-IRES-eGFP, respectively.

To create the first lentiviral expression plasmid of the series, which we named pLR1, pLR2 and pLR3, the fragment located between XbaI and BamHI sites in pLLmcs18, pLLmcs18-IRES-eGFP and pLLmcs18-IRES-DsRed were cleaved with BamHI and XbaI and replaced by the fragment located in pREP9 between the same enzymes. This fragment contains the RSV (Rous sarcoma virus) promoter and a multi-cloning site. Cloning strategies and the pLR lentiviral plasmid maps are shown in Fig. 1.

3.2. Polylinker plasticity

The pLR series was planned to offer a plastic multi cloning site to facilitate the transgene cloning. Thus, one can choose between nine unique endonuclease recognition sites in pLR1 lentivector and eight unique sites in pLR2 and pLR3; however, taking into account the isoschizomers and compatible overhangs, the endonuclease number can be elevated to more than thirty enzymes, facilitating the cloning of a wide range of DNA fragments. Altogether, the unique site for XbaI is close to the 5′ end of the promoter sequence and it allows the promoter to be exchanged. Unique sites of each lentivector are shown in Supplementary Table 1.

3.3. Molecular and cellular testing of the vectors

To determine the correct cloning of the sequences in the different lentiviral plasmids of the pLR series, we analyzed the different endonuclease-based restriction patterns. The pLR1 plasmid was analyzed using KpnI or KpnI plus BamHI endonucleases and pLR2 and pLR3 plasmids were con-

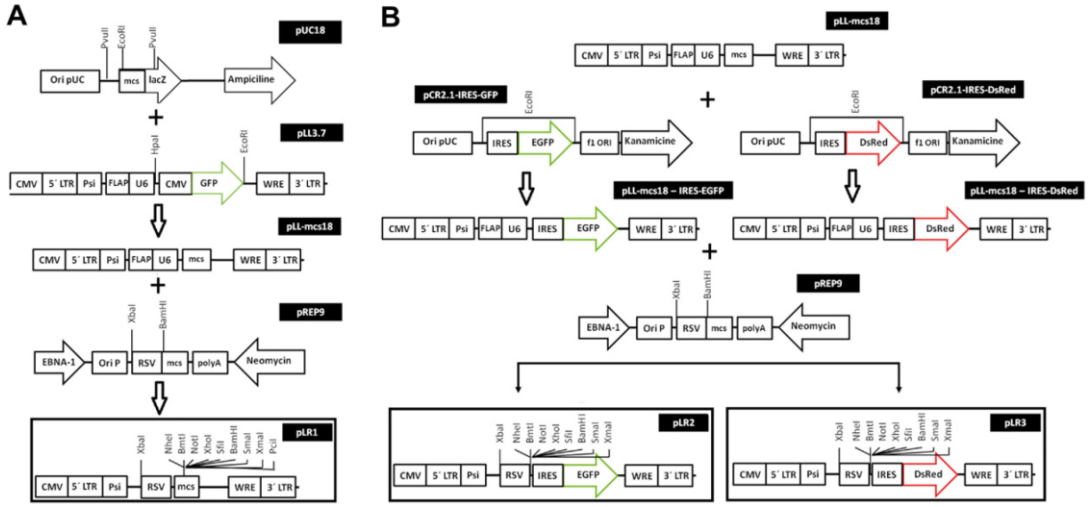


Fig. 1. Schematic representation for the engineering of lentiviral plasmids of pLR series. Diagram depicting cloning strategy and the expression cassette of (A) pLR1 lentivector, (B) lentiviral constructs pLR2 and pLR3.

firmed by cleavage with ApaI, HindIII or PvuII endonucleases (data not shown). The positive clones, three of each plasmid, were further sequenced and the expression cassettes of all the plasmids showed the expected sequences without any mutation (data not shown). These experiments confirmed the sequence integrity of the genetic constructs. Finally, the functionality of promoter and IRES sequences of the pLR2 and pLR3 lentivectors was tested, cloning DsRed coding sequence of the plasmid pTA-DsRed into lentiviral construct pLR2. Similarly, the sequence encoding eGFP, obtained from pEGFP-N1 plasmid, was cloned into pLR3 lentivector. Promoter and IRES activities of pLR2, pLR2-Dsred, pLR3 and pLR3-eGFP were analyzed 72 h after transfection in HEK-293T. Both cloning strategies showed optimal promoter and IRES expression of fluorescent proteins coded by the sequences cloned upstream and downstream of this sequence (Fig. 2A–C).

These results demonstrated that the chosen promoter was functional in our constructs, producing high reporter gene expression. This was expected because it is well known that RSV promoter has strong activity in many types of cells (Majhen et al., 2010; Jang et al., 2011; Yu et al., 2011).

On the other hand, according the literature, the IRES sequence chosen initiate translation efficiently in reticulocyte lysates and in many other cell types (Borman et al., 1997). Moreover, it maintains efficient expression of Yamanaka factors to produce induced pluripotent stem cells (iPS) (Yu et al., 2009), making it a popular and effective biotechnological sequence choice of many researchers.

3.4. Transduction: expression and stability analysis

After confirming that all structures were functional, we tested the construct for viral vector production. Lentivectors pLR2, pLR2-DsRed and pLR3-eGFP produced high viral titers, ranging from 9.3×10^5 particles/ml for pLR2 to 6.47×10^6 particles/ml for pLR3-eGFP, showing that the fragments cloned upstream to IRES did not interfere with the transduction efficiency of the vectors.

The transduction efficiency reached in this work was higher than 40% for pLR2, pLR2-DsRed and pLR3-eGFP. The pLR3 transduction efficiency could not be determined because potentially transduced cells did not shown red fluorescence. When mean fluorescence intensity (MFI) was analyzed for eGFP and DsRed in the transduced cells two points came to light. First, the MFI of the fluorescent cells relative to the MFI of the negative cells was higher for eGFP than DsRed and, second, when relative MFI were analyzed comparing the reporter genes position from the IRES sequence, it was not possible to observe any difference between the MFI of the reporter genes when these were cloned upstream or downstream of the IRES sequence (Table 2). Regarding stability of the insert, it was observed that the pool of fluorescent cells sorted 48 h post-transduction maintained the fluorescence throughout the time analyzed (10 passages, approximately 5 weeks).

3.5. IRES fold structure could explain the non-fluorescence in pLR3

In this paper, we used a variant 2 of DsRed reporter gene in pLR3 lentivector (downstream of the IRES sequence) by reducing the tendency to form aggregates and decreasing the time from transfection to detection (Terskikh et al., 2002; Yanushevich et al., 2002). However, an unexpected lack of expression of DsRed occurred with the pLR3 lentivector (Fig. 2B and C). When this construct was analyzed by transfection the fluorescence became very weakly visible at 24 and 48 h after transfection (Supplementary Fig. 1A). It could be explained by two interrelated points. First, it is known that DsRed have an extended mat-

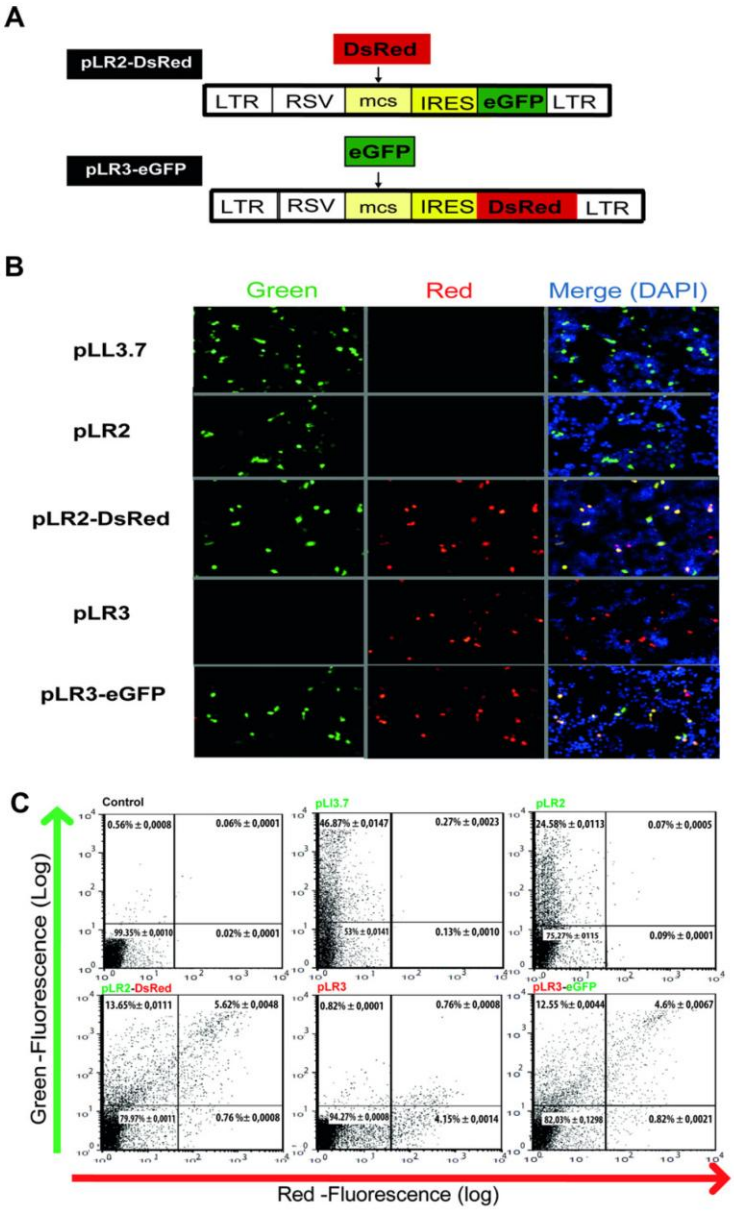


Fig. 2. Functional analyze of IRES-mediated bicistronic protein expression in transient transfection. (A) Schematic of the coding region of bicistronic constructs containing eGFP and DsRed. (B) Fluorescent microscopic image of HEK-293T cells transfected with bicistronic constructs. The images used to examine fluorescence were obtained with the same exposure time for each filter set (72 h). (D) Flow cytometry showing the percentage of fluorescence cells 72 h after transfection. Transfection experiments were realized in triplicate (mean ± SD).

uration time (Bevis and Glick, 2002; Yanushevich et al., 2002) when compared with to eGFP. Thus, DsRed fluorescence in pLR3 is significantly detected at 72 h whereas eGFP is visible at 24 h after transfection (Fig. 2B and C; Supplementary Fig. 1A). To confirm this data, we created a new plasmid design (pC-DsRed2) by cloning a fragment containing an expression cassette of the DsRed gene from pREP9-DsRed (available in our laboratory) into the pCR3.1 plasmid (map is shown in Supplementary Fig. 1B). This plasmid was used to analyze RSV promoter-mediated DsRed expression without the presence of IRES sequence. Interestingly, red fluorescence was detected at

Table 2
Tranduction efficiencies and MFI of the different constructs.

Constructs	Tranduction efficiency (mean) (%)	MFI				Relative MFI (+ve/−ve)		Ratio between relative MFI (eGFP/DsRed)
		eGFP		DsRed				
		−ve cells	+ve cells	−ve cells	+ve cells	eGFP	DsRed	
pLR2	75.56	4.1	152.2			38.05		
pLR3	0.0							
pLR2-DsRed	42.38	4.33	131.67	7.27	66.67	30.38	9,17	3.31
pLR3-eGFP	48.29	3.95	175.00	6.30	72.05	44.30	11.43	3.87

Table shows the efficiency of transduction (mean) of each construct, the mean fluorescence intensity (MFI) of each reporter gene in de different constructs. Ratio between MFI values are shown too.

24 h after transfection (Supplementary Fig. 1C and D). This data about DsRed maturation is in agreement with data described in the literature using similar experimental design (Maruyama et al., 2004; Vallier et al., 2004; Duvernay et al., 2009). It shows that neither the RSV promoter nor DsRed maturation is a limiting factor of low expression at pLR3 after transfection.

On the other hand, a second point is the influence of IRES sequence on mRNA conformation. It was suggested that a high level of translation of the gene located downstream to the IRES is very difficult to achieve (López de Quinto and Martínez-Salas., 1998, 1999). However, when eGFP was cloned upstream to the IRES sequence, the DsRed protein could be promptly visualized as expected.

These data suggest that IRES sequence could be influencing the DsRed expression in the pLR3 lentivector. Thus, this behavior led us to compare the secondary structures of each transcript, suggesting that this could be the cause of the low expression of DsRed in pLR3 but normal expression when eGFP was cloned upstream of IRES. Nevertheless, when pLR3 was used to transfect HEK-293T cells it was proved to be functional producing red fluorescence. To understand this incongruence, we analyzed if the lack of DsRed expression in transduced cells is a consequence of the IRES-directed translation associated to low titer of the virus or a limitation in the assembly of viral vector particles. To respond this question, we evaluated by q-PCR the number of provirus copies in transduced 293T DNA. As shown in Table 3, we were able to detect the lentiviral genome in the DNA of cells transducted with pLR2, pLR2-DsRed or pLR3-eGFP, but it was not possible to obtain signal when we tested for the detection of the pLR3 construct lacking cloned sequences upstream to the IRES element. Thus, there are two possibilities to consider: the genomic RNA have some secondary structure that impairs the efficient the packaging into the capsid or it can be packaged and the RNA introduced into de target cell cannot be retrotranscribed. We did not further exploited these possibilities at this time since this is not the focus of this paper and this limitation does not decrease the value of this tool because the IRES sequence used works well when some sequence is cloned upstream to it.

Table 3
Presence/absence of lentiviral genome DNA in transducted cells analyzed by quantitative real time PCR.

Plasmid	eGFP	DsRed
Negative control (no DNA)	–	–
DNA from Non-transducted cells	–	–
pLR2	+	–
pLR3	–	–
pLR2-DsRed	+	+
pLR3-GFP	+	+

(+) Presence of lentiviral genome DNA above the detection limit (>947 lentiviral genome copies/10 ng of total DNA).
(–) Absence of lentiviral genome DNA or presence below the detection limit (>947 lentiviral genome copies /10 ng of total DNA).

4. Conclusions

In conclusion, using an efficient cloning strategy, we have constructed a versatile lentiviral vector series. These can be used for cloning a wide number of transgenes to be selectively expressed with optional promoters upon promoter exchange. On the other hand, the created lentivector series allows the expression of the reported transgenes taking advantage of IRES elements. To the best of our knowledge, the pLR series on lentiviral backbone is the first proposed series including this broad promoter, MCS and transgene options. Moreover, using alternative cloning strategy, this plastic vector series is amenable to the development of additional vectors to be used in several other proposals, transforming the pLR lentiviral series into a powerful biotechnological tool for stable gene transfer into mammalian cells.

Acknowledgments

This work was supported by the Rio Grande do Sul State Research Foundation (FAPERGS), the National Council for Research and Development (CNPq), Fundação do Câncer, INCA, Instituto do Milênio – Terapia Gênica (CNPq), and Fundo de Apoio à Pesquisa do Instituto de Cardiologia (FAPPIC) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We thank, Dr. Melissa Markowsky for assistance finishing this work in LCMC laboratory and Dr. Bruno Paredes from UFRJ for technical assistance in FACS cell sorting experiments.

We thank Kyle Sunshein for the English review of this paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plasmid.2012.06.001.

References

Anliker, B., Abel, T., Kneissl, S., Hlavaty, J., Caputi, A., et al., 2010. Specific gene transfer to neurons, endothelial cells and hematopoietic progenitors with lentiviral vectors. Nat. Methods 7, 929–935.

Bevis, B.J., Glick, B.S., 2002. Rapidly maturing variants of the Discosoma red fluorescent protein (DsRed). Nat. Biotechnol. 20, 83–87.

Biffi, A., Aubourg, P., Cartier, N., 2011. Gene therapy for leukodystrophies. Hum. Mol. Genet. 20, R42–R53.

Bonamino, M., Serafini, M., D'Amico, G., Gaipa, G., Todisco, E., et al., 2004. Functional transfer of CD40L gene in human B-cell precursor ALL blasts by second-generation SIN lentivectors. Gene Ther. 11, 85–93.

Borman, A.M., Le Mercier, P., Girard, M., Kean, K.M., 1997. Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins. Nucleic Acids Res. 25, 925–932.

Dropulic, B., 2011. Lentiviral vectors: their molecular design, safety, and use in laboratory and preclinical research. Hum. Gene Ther. 22, 649–657.

Duan, M., Mi, Q., 2010. Local delivery of reporter gene to the cochlea does not spread to brain tissue in an animal model. Acta Otolaryngol. 130, 25–30.

Durand, S., Cimarelli, A., 2011. The inside out of lentiviral vectors. Viruses 3, 132–159.

Duvernay, M.T., Dong, C., Zhang, X., Robitaille, M., Hébert, T.E., Guangyu, W., 2009. A single conserved leucine residue on the first intracellular loop regulates ER export of G protein-coupled receptors. Traffic 10, 552–566.

Escors, D., Breckpot, K., 2010. Lentiviral vectors in gene therapy: their current status and future potential. Arch. Immunol. Ther. Exp. (Warsz) 58, 107–119.

Frecha, C., Lévy, C., Cosset, F.L., Verhoeyen, E.L., 2010. Advances in the field of lentivector-based transduction of T and B lymphocytes for gene therapy. Mol. Ther. 18, 1748–1757.

Frecha, C., Lévy, C., Costa, C., Nègre, D., Amirache, F., Buckland, R., Russell, S.J., Cosset, F.L., Verhoeyen, E., 2011. Measles virus glycoprotein-pseudotyped lentiviral vector-mediated gene transfer into quiescent lymphocytes requires binding to both SLAM and CD46 entry receptors. J. Virol. 85, 5975–5985.

Fux, C., Fussenegger, M., 2003. Toward higher order control modalities in mammalian cells-independent adjustment of two different gene activities. Biotechnol. Prog. 19, 109–205.

Hynes, S.O., McCabe, C., O'Brien, T., 2011. Beta cell protection by inhibition of iNOS through lentiviral vector-based strategies. Methods Mol. Biol. 704, 153–168.

Jang, H.J., Choi, J.W., Kim, Y.M., Shin, S.S., Lee, K., et al., 2011. Reactivation of transgene expression by alleviating CpG methylation of the rous sarcoma virus promoter in transgenic quail cells. Mol. Biotechnol. 49, 222–228.

Ji, W., Zhou, W., Abruzzese, R., Guo, W., Blake, A., Davis, S., Davis, S., Polejaeva, I., 2005. A method for determining zygosity of transgenic zebrafish by TaqMan real-time PCR. Anal. Biochem. 344, 240–246.

Johnson, L.G., Olsen, J.C., Naldini, L., Boucher, R.C., 2000. Pseudotyped human lentiviral vector-mediated gene transfer to airway epithelia in vivo. Gene Ther. 7, 568–574.

Kafri, T., Blomer, U., Peterson, D.A., Gage, F.H., Verma, I.M., 1997. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat. Genet. 17, 314–317.

Koldej, R.M., Anson, D.S., 2009. Refinement of lentiviral vector for improved RNA processing and reduced rates of self inactivation repair. BMC Biotechnol. 9, 86.

Lewis, P.F., Emerman, M., 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. J. Virol. 68, 510–516.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., Baltimore, D., 2002. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science 295, 868–872.

López de Quinto, S., Martinez-Salas, E., 1998. Parameters influencing translational efficiency in aphthovirus IRES-based bicistronic expression vectors. Gene 217, 51–56.

López de Quinto, S., Martínez-Salas, E., 1999. Involvement of the aphthovirus RNA region located between the two functional AUGs in start codón selection. Virology 255 (2), 324–336.

Majhen, D., Brozovic, A., Buger, T., Gabrilovac, J., Osmak, M., et al., 2010. Vincristine-resistant human laryngeal carcinoma cells demonstrate increased Rous sarcoma virus promoter activity. Life Sci. 87, 468–474.

Maruyama, M., Teruko, N., Toyokazu, Y., Chisaki, I., Kayo, I., Yoshihiko, W., Makiya, N., Yoshinobu, T., 2004. Simultaneous detection of DsRed2-tagged and EGFP-tagged human beta-interferons in the same single cells. J. Cell. Biochem. 93, 497–502.

Miller, D.G., Adam, M.A., Miller, A.D., 1990. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. Mol. Cell. Biol. 10, 4239–4242.

Miyoshi, H., Takahashi, M., Gage, F.H., Verma, I.M., 1997. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. Proc Natl. Acad. Sci. USA 94, 10319–10323.

Naldini, L., Blomer, U., Gage, F.H., Trono, D., Verma, I.M., 1996. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. Proc Natl. Acad. Sci. USA 93, 11382–11388.

Ngoi, S.M., Chien, A.C., Lee, C.G., 2004. Exploiting internal ribosome entry sites in gene therapy vector design. Curr. Gene Ther. 4, 15–31.

Pacheco, A., Martinez-Salas, E., 2010. Insights into the biology of IRES elements through riboproteomic approaches. J. Biomed. Biotechnol. 2010, 458927.

Sambrook, J., Russell, D.W., 2005. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Shenoy, V., Ferreira, A.J., Qi, Y., Fraga-Silva, R.A., Díez-Freire, C., Dooies, A., Jun, J.Y., Sriramula, S., Mariappan, N., Pourang, D., Venugopal, C.S., Francis, J., Reudelhuber, T., et al., 2010. The angiotensin-converting enzyme 2/angiogenesis-(1–7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. Am. J. Respir. Crit. Care Med. 182, 1065–1072.

Terskikh, A.V., Fradkov, A.F., Zaraisky, A.G., Kajava, A.V., Angre, S.B., 2002. Analysis of DsRed Mutants. Space around the fluorophore accelerates fluorescence development. J. Biol. Chem. 277, 7633–7666.

Vagner, S., Gensac, M.C., Maret, A., Bayard, F., Amalric, F., Prats, H., Prats, A.C., 2001. Irresistible IRES. Attracting the translation machinery to internal ribosome entry sites. EMBO Rep. 2, 893–898.

Vallier, L., Rugg-Gunn, P.J., Bouhon, I.A., Andersson, F.K., Sadler, A.J., Pedersen, R.A., 2004. Enhancing and diminishing gene function in human embryonic stem cells. Stem cells 22, 2–11.

Yanushevich, Y.G., Staroverov, D.B., Savitsky, A.P., Fradkov, A.F., Gurskaya, N.G., Bulina, M.E., Lukyanov, K.A., Lukyanov, S.A., 2002. A strategy for the generation of non-aggregating mutants of Anthozoa fluorescent proteins. FEBS Lett. 511, 11–14.

Yi, Y., Noh, M.J., Lee, K.H., 2011. Current advances in retroviral gene therapy. Curr. Gene Ther. 11, 218–228.

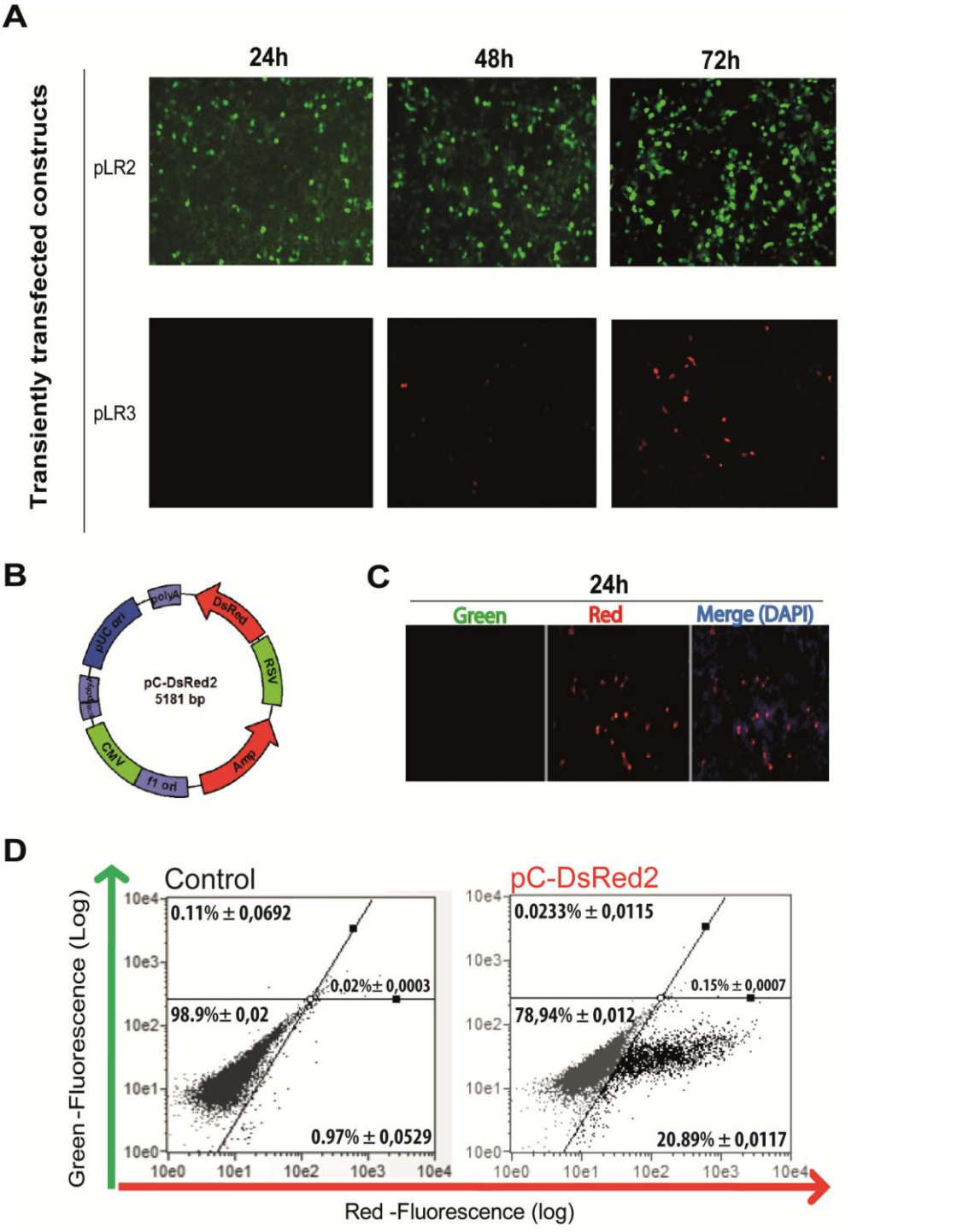
Yu, D.L., Linnerth-Petrik, N.M., Halbert, C.L., Walsh, S.R., Miller, A.D., et al., 2011. Jaagsiekte sheep retrovirus and enzootic nasal tumor virus promoters drive gene expression in all airway epithelial cells of mice but only induce tumors in the alveolar region of the lungs. J. Virol. 85, 7535–7545.

Yu, J., Hu, K., Smuga-Otto, K., Tian, S., Stewart, R., Slukvin, I.I., Thomson, J.A., 2009. Human induced pluripotent stem cells free of vector and transgene sequences. Science 324, 797–801.

Web references

<http://www.acaclone.com> (pDRAW32.1.1.88 program).
<http://www.mbio.ncsu.edu/BioEdit/bioedit.html> (BioEdit 7.0.5.3 software).
<http://facs.scripps.edu/software.html> (WinMDI 2.9 software).

Supplementary figure



Supplementary Fig. 1. Kinetic analysis of pLR lentivector series. (A) Microphotographies of HEK-293T cell line transfected with pLR2 plasmid and pLR3 plasmid. Expression of fluorescent proteins was analyzed 24, 48 and 72 h after transfection. (B) Representation of pC-DsRed2 plasmid map. (C) Microphotographies of HEK-293T cell transfected with pC-DsRed2 after 24 h. (D) Flow cytometry showing the percentage of fluorescent cell (DsRed positive) 24 h after transfection with pC-DsRed2. Transfection experiments were realized in triplicate (mean ± SD)

Supplementary table 1

Multicloning site structure.

Enzyme	Compatible ends	Isoschizomers
NheI (G/CTAGC)	AvrII, SpeI, StyI (C/CTAGG), XbaI	AsuNHI, BmtI, BspOI
BmtI (GCTAG/C)	BmtI	AsuNHI, NheI
NotI (GC/GGCCGC)	PspOMI, EagI	CciNI
XhoI (C/TCGAG)	PspXI,	PaeR7I, Sfr274I, SlaI, StrI, TliI
XmaI (C/CCGGG)	AgeI, BsaWI, BspEI, BsrFI, NgoMIV, SgrAI, AvaI	Cfr9I, SmaI, TspMI, XmaCI
BamHI (G/GATCC)	BclI, DpnII, BglII, BstYI	
SmaI (CCC/GGG)	Any blunt	Cfr9I, TspMI, XmaI, XmaCI
PciI * (A/CATGT)	BspHI, FatI, NcoI	PscI

Sites present in the multicloning site structure 5' to 3', are indicated in bold type. Compatible and recleaved site are shown on the right. (*)This site belongs to pLR1.

José Eduardo Vargas

Curriculum Vitae

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Genética.

2. Grande área: Ciências Biológicas / Área: Genética / Subárea: Biologia de sistemas.

3. Grande área: Ciências Biológicas / Área: Bioquímica / Subárea: Biologia Molecular.

Formação acadêmica/titulação

2009- atual
Doutorado em andamento em Biologia Celular e Molecular (Conceito CAPES 6).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
Título: Estudos in vitro e in silico para determinar os mecanismos moleculares associados á senescência celular em glioblastomas,
Orientador: Guido Lenz.
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

2007 - 2009
Mestrado em Biologia Celular e Molecular (Conceito CAPES 6).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
Título: Creação d euma série de lentivetores de expressão genica regulada,Ano de Obtenção: 2009.
Orientador: Guido Lenz; Andrés Delgado Cañedo
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico.

2001 - 2007
Graduação em licenciatura en genética.
Universidad Nacional de misiones, UNM, Argentina.
Título: " Criação de uma serie de lentivetores para tranferência gênica estavél".

Formação Complementar

2012 - 2012
EXTENSÃO UNIVERSITÁRIA EM 1 INTERNATIONAL MEETING OF LABORATORY ANIMALS. (Carga horária: 10h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2010 - 2010
EXTENSÃO UNIVERSITÁRIA EM INTRODUÇÃO A MICROSCOPIA CONFOCAL FLUORESCÊNCIA. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2010 - 2010

EXTENSÃO UNIVERSITÁRIA EM VERDADES, FALÁCIAS, ANGUSTIAS -RADICAIS LIVRES. (Carga horária: 15h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2010 - 2010
EXTENSÃO UNIVERSITÁRIA EM LATIN AMERICAN SCHOOL OF HUMAN/MEDICAL GENETICS. (Carga horária: 40h).
Hospital de Clínicas de Porto Alegre.

2008 - 2008
SIMPÓSIO GAÚCHO DE TERAPIA GÊNICA E CELULAR. (Carga horária: 9h).
Hospital de Clínicas de Porto Alegre.

2007 - 2007
EXTENSÃO UNIVERSITÁRIA EM CICLO DE PALESTRAS EM TERAPIA GÊNICA. (Carga horária: 20h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2006 - 2006
ASESORAMIENTO GENÉTICO Y CÁLCULO DE RIESGO. (Carga horária: 12h).
Universidad Nacional de misiones, UNM, Argentina.

2006 - 2006
PESQUISA PRÉ E CLÍNICA EM TERAPIA GÊNICA E CELULAR. (Carga horária: 30h).
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2006 - 2006
DESENVOLVIMIENTO EN EL LABORATORIO QCA BIOLOGICA. (Carga horária: 60h).
Universidad Nacional de misiones, UNM, Argentina.

2006 - 2006
II CURSO DE EPIDEMIOLOGIA E APLICAÇÃO CLÍNICA.
Instituto de Cardiologia do Rio Grande do Sul.

2004 - 2004
INT. A LA GENÓMICA, TRANSCRIPTÓMICA Y PROTEÓMICA. (Carga horária: 20h).
Universidad Nacional de misiones, UNM, Argentina.

2002 - 2002
HERRAMIENTAS BIOTECNOLOGICAS Y MAPEAMIENTO GENICO. (Carga horária: 12h).
Universidad Nacional de misiones, UNM, Argentina.

Atuação Profissional

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

2012 - 2012
Vínculo: Colaborador, Enquadramento Funcional: Tutor na disciplina Atividade Orientada II
Outras informações
José Eduardo Vargas colaborou na ministração da disciplina Atividade Orientada II (BTC99003) do curso de Biotecnologia da UFRGS, sob orientação do professor Diego Bonatto.

2008 - 2008
Vínculo: Bolsista, Enquadramento Funcional: Tutor na disciplina Biologia Molecular Básica, Carga horária: 5
Outras informações
Esta atividade está incluída dentro de Programa de integração entre Graduação e Pós-graduação (PROIN). Nesta atividade, o tutor ministrou todo o conteúdo teórico e prático da disciplina para 03 (três) alunos de graduação do curso de Ciências Biológicas da UFRGS, o que apresentou um total de 60 horas/aula presenciais teórico-práticas, além de 10 horas/aula destinadas á preparação dos procedimentos práticos.

Instituto de Cardiologia do Rio Grande do Sul.

2008 - 2008
Vínculo: Colaborador, Enquadramento Funcional: Co-orientação

Universidad Nacional de misiones, UNM, Argentina.

2005 - 2006
Vínculo: Colaborador, Enquadramento Funcional: AUXILIAR DOCENTE DE SEGUNDA, Regime: Dedicação exclusiva.
Outras informações
Nesta atividade José Eduardo Vargas ajudou na ministração das disciplinas Química Biológica dos cursos: Profesorado en Biologia y Licenciatura en Genética na Universidad Nacional de Misiones (Argentina)

Atividades
05/2005 - 05/2006
Ensino, licenciatura en genética y profesorado em Biologia, Nível: Graduação

Disciplinas ministradas
QUIMICA BIOLOGICA

Projetos de pesquisa

2011 - 2012
Efeito da transdução da atividade NTPDásica2 sobre a morfologia e proliferação in vitro do glioma U87

Situação: Concluído; Natureza: Pesquisa.

Integrantes: José Eduardo Vargas / Guido Lenz - Coordenador / Franciele Cristina Kipper - Integrante / Marcia Wink - Integrante.

2009 - 2012
Efeito dos polifenóis no crescimento e morte de glioma em modelo in vivo

Situação: Concluído; Natureza: Pesquisa.
Integrantes: José Eduardo Vargas / Guido Lenz - Coordenador / Fillipi-Chiela eduardo - Integrante / Lauren Zamin - Integrante.

2009 - Atual
Estudos in vitro e in silico para determinação de mecanismos moleculares associados à senescência celular em glioblastoma

2008 - 2012
Criação de um plasmídeo para estudo de promotores in vitro através do uso de proteínas fluorescentes

Integrantes: José Eduardo Vargas / Andrés Delgado Cañedo - Integrante.

2007 – 2012 Criação de uma série de lentivetores para transferência genica estável e troca de promotores

Situação: Concluído; Natureza: Pesquisa.

Integrantes: José Eduardo Vargas / Guido Lenz - Integrante / Salton - Integrante / Sódre, A - Integrante / Dalberto - Integrante / Bonamino - Integrante / Andrés Delgado Cañedo - Coordenador

Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Espanhol Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Francês Compreende Razoavelmente, Fala Razoavelmente, Lê Razoavelmente, Escreve Razoavelmente.
Português Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.

José Eduardo Vargas possui o nível intermediário de Proficiência em Língua Portuguesa para Estrangeiros (Celpe-Bras), com validade em todo o território brasileiro, adjudicado pelo Ministério da Educação (MEC) no ano 2007.

Prêmios e títulos

2008
Melhor apresentação da Sessão, Instituto Universitário de cardiologia.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

1. **Vargas, José Eduardo** ; FELTES, B. C. ; POLONI, J. F. ; Lenz G ; Bonatto,D . Senescence; an endogenous anticancer mechanism. Frontiers in Bioscience (Print) JCR, v. 17, p. 2616-2643, 2012.

2. **Vargas, José Eduardo** ; Salton, Gabrielle ; Sodré de Castro Laino, Andressa ; Pires, Tiago Dalberto ; Bonamino, Martin ; Lenz, Guido ; Delgado-Cañedo, Andrés **.** pLR: A lentiviral backbone series to stable transduction of bicistronic genes and exchange of promoters. Plasmid (San Diego. Print) JCR, v. 68, p. 179-185, 2012.

Resumos publicados em anais de congressos

1. **Vargas, José Eduardo** ; Lenz G ; Delgado C. A . CRIAÇÃO DE UMA SÉRIE DE LENTIVETORES COMO FERRAMENTA PARA TRANSFERÊNCIA GÊNICA ESTÁVEL. In: FEIRA DE INICIAÇÃO CIENTÍFICA E SALÃO DE EXTENSÃO - 2006, 2006, NOVO HAMBURGO. FEIRA DE INICIAÇÃO CIENTÍFICA E SALÃO DE EXTENSÃO - 2006, 2006.

2. **Vargas, José Eduardo** ; Lenz G ; Delgado C. A . CRIAÇÃO DE UMA SÉRIE DE LENTIVETORES COMO FERRAMENTA PARA TRANSFERÊNCIA GÊNICA ESTÁVEL. In: XVIII SALÃO DE INICIAÇÃO CIENTÍFICA - XV FEIRA DE INICIAÇÃO CIENTÍFICA - I SALÃO DE UFRGS JOVEM, 2006, PORTO ALEGRE. XVIII SALÃO DE INICIAÇÃO CIENTÍFICA - XV FEIRA DE INICIAÇÃO CIENTÍFICA - I SALÃO DE UFRGS JOVEM, 2006.

3. **Vargas, José Eduardo** ; Lenz G ; Delgado C. A . CRIAÇÃO DE UMA SÉRIE DE LENTIVETORES COMO FERRAMENTA PARA TRANSFERÊNCIA GÊNICA ESTÁVEL. In: 26 SEMANA CIENTÍFICA DO HCPA - 5º REUNIÃO DA REDE NACIONAL DE PESQUISA CLÍNICA EM HOSPITAL DE ENSINO - 13ºCONGRESSO DE PESQUISA E DESENVOLVIMENTO EM SAÚDE DO MERCOSUL, 2006, PORTO ALEGRE. 26 SEMANA CIENTÍFICA DO HCPA - 5º REUNIÃO DA REDE NACIONAL DE PESQUISA CLÍNICA EM HOSPITAL DE ENSINO - 13ºCONGRESSO DE PESQUISA E DESENVOLVIMENTO EM SAÚDE DO MERCOSUL, 2006.

Apresentações de Trabalho

1. Vargas, José Eduardo . Criação de uma série de lentivectores estáveis para estudo de doenças geneticas. 2008. (Apresentação de Trabalho/Congresso).

2. Vargas, José Eduardo . PROGRAMA DE ARTICULACION UNIVERSIDAD ESCUELA MEDIA II. 2005. (Apresentação de Trabalho/Conferência ou palestra).

Demais tipos de produção técnica

1. Lenz G ; Lopez, L ; Silva, A.O ; Fillipi-Chiela, E ; Ledur, P ; Kipper, F ; **Vargas, José Eduardo** . Curso Estudo da Sinalização Celular no Câncer. 2011. (Curso de curta duração ministrado/Outra).

2. **Vargas, José Eduardo** . Microorganismos: Mocinhos ou Bandidos. 2009. (Desenvolvimento de material didático ou instrucional - Curso de férias).

Educação e Popularização de C & T

Entrevistas, mesas redondas, programas e comentários na mídia

Vargas, José Eduardo ; KIRKLAND, J. ; LEMAITRE, J. . Avanços científicos podem eliminar células danificadas e prevenir doenças. 2011. (Entrevista para o jornal Correio Braziliense).

FONTES DE FINANCIAMENTO UTILIZADAS PARA DESENVOLVER ESTA TESE

PRONEX/FAPERS

PRONEM/FAPERGS

Universal/CNPq

PROBITEC/CAPES

ICGEB

É importante mencionar que o autor deste trabalho é **bolsista da CAPES –Brasil.**