DISSERTAÇÃO DE MESTRADO

SINALIZAÇÃO AUTOFÁGICA E NÍVEIS DE MIOSTATINA EM MODELO DE HIPERTROFIA CARDÍACA FISIOLÓGICA EM CAMUNDONGOS

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Sinalização autofágica e níveis de miostatina em modelo de hipertrofia cardíaca fisiológica em camundongos

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LISTA DE ABREVIATURAS

α-MHC: Miosina de cadeia pesada tipo α

ANF: Fator natriurético atrial

AKT ou PKB: Proteína quinase B

ANG II: Angiotensina II

ANG (1-7): Angiotensina 1-7

AT1: Receptor de angiotensina II com propriedade vasoconstritora

AT2: Receptor de angiotensina II com propriedades antagonistas ao AT1

BCN1: Beclina1

ß-MHC: Miosina de cadeia pesada tipo ß

ERK: Quinase regulada por sinal extracelular

EC A: Enzima conversora da angiotensina

ECA2: Enzima conversora da angiotensina II

FGF: Fator de crescimento dos fibroblastos

FOXO: Proteína da família *forkhead box O*

IAM: Infarto agudo do miocárdio

IC: Insuficiência cardíaca

IGF: Fator de crescimento semelhante à insulina

IGFIR: Receptor tirosina quinase do fator de crescimento semelhante à insulina

GDF8: Fator de crescimento e diferenciação 8

GFP: Proteína fluorescente verde

GSK3ß: Glicogênio sintase cinase 3ß

HAS: Hipertensão arterial sistêmica

HC: Hipertrofia cardíaca

LC3I – MAP1LC3A: proteína de cadeia alfa 3 associada à microtúbulos

LC3II: Complexo lipidado formado por LC3I com PE

MAPK: Proteína quinase ativada por mitógeno

MSTN: Miostatina

mTOR: Proteína alvo da rapamicina em mamíferos

PE: Lipídeo fosfatidiletanolamina

PI3K: Fosfoinositol-3-cinase

SRA: Sistema renina angiotensina

SQSTM P62: Sequestossoma P62 regulador do clearance autofágico

TGFß: Fator transformador do crescimento ß

TNFα: Fator de necrose tumoral α

VE: Ventrículo esquerdo

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RESUMO

A hipertrofia cardíaca é caracterizada pelo aumento do músculo cardíaco devido aumento das dimensões dos cardiomiócitos. Em condições fisiológicas ou patológicas a hipertrofia está relacionada com o aumento da força do coração, provocando alterações nas células miocárdicas. O exercício ativa a via da proteína Akt relacionada à sobrevivência celular em resposta ao exercício e atua sobre a proteína quinase mTOR, ocasionando síntese proteica e hipertrofia. A mTOR participa da proliferação e crescimento celular através da regulação da tradução da proteína. Além disso, a mTOR também controla outros processos celulares como autofagia e parece estar relacionada com a sinalização de miostatina. Portanto, quando miostatina aumentada, inibe o crescimento muscular provavelmente através da inibição de mTOR. A autofagia é um mecanismo homeostático com a finalidade de degradação e reciclagem através da ação lisossomal reciclando organelas citoplasmáticas e proteínas a fim de remover esses materiais intracelulares. A ativação da autofagia ocorre em duas situações: uma em baixo nível de fluxo autofágico, devido uma baixa energética a fim de manter a sobrevivência celular e em outra situação há ativação pronunciada a fim de esgotar os elementos celulares culminando em morte celular. Esses dois extremos nas ações autofágicas são mecanismos potenciais pró ou anti-sobrevivência. Estudos mostram uma ativação da autofagia durante o exercício agudo e parece estar relacionado com a repressão da via AKT/mTOR. Por outro lado, no exercício crônico a autofagia é ativada em músculo esquelético através de aumento das proteínas autofágicas. Tal evento é explicado pela mudança do perfil muscular esquelético pós-exercício gerando maior quantidade de metabólitos que são reciclados pelo sistema, porém em músculo cardíaco ainda está sendo estudado. A miostatina é um regulador negativo do crescimento muscular esquelético. Em doenças seu aumento resulta em perda muscular, contudo, na hipertrofia fisiológica a miostatina está reduzida no músculo esquelético e no cardíaco. A miostatina aumentada bloqueia não só Akt, mas também mTOR, favorecendo a via de degradação proteica através da maquinaria autofágica. Assim, a miostatina tem ação na autofagia durante o exercício e essas vias podem estar relacionadas com o desenvolvimento da hipertrofia cardíaca.

MARCO TEÓRICO

1.1 Hipertrofia Cardíaca

A hipertrofia cardíaca (HC) desenvolve um aumento do músculo cardíaco devido o aumento das dimensões dos cardiomiócitos. Em condições fisiológicas ou patológicas a hipertrofia está relacionada com o aumento da força miocárdica, provocando alterações celulares desse tecido (1-3). As alterações estruturais dos cardiomiócitos também estão relacionadas com os tipos de hipertrofia cardíaca dependendo, então, de fatores estruturais, funcionais e bioquímicos (4). Em resposta aos fatores neuroendócrinos e fatores mecânicos, como a sobrecarga pressórica, há o desenvolvimento da dilatação cardíaca, alterações geométricas, alterações de contratilidade/relaxamento e aumento do volume sistólico e diastólico final (5, 6).

A hipertrofia cardíaca pode ser excêntrica ou concêntrica sendo classificada de acordo com o tipo de trabalho cardíaco (6), dessa forma podemos encontrá-las em modelos fisiológicos e patológicos. O exercício físico é capaz de produzir uma hipertrofia fisiológica tanto concêntrica quanto excêntrica, dependerá do objetivo do exercício seja ele isométrico ou isotônico (7). Na hipertrofia patológica encontramos uma hipertrofia concêntrica devido sobrecarga pressórica e uma hipertrofia excêntrica relacionada a uma sobrecarga de volume. (8, 9). Em suma, no modelo patológico nos confrontamos com um quadro de perda funcional do tecido cardíaco devido à substituição celular do tecido cardíaco por fibrose levando à dilatação das câmaras e insuficiência cardíaca (IC). Em contraponto, no modelo fisiológico há alargamento das câmaras de forma proporcional desencadeando uma melhora da função cardíaca sem a presença de fibrose (10). Essas observações questionam a natureza da hipertrofia fisiológica e patológica.

1.2 Hipertrofia Patológica

O estímulo não transitório causado por sobrecarga hemodinâmica é demonstrado na hipertrofia patológica a qual responde ao estresse sofrido pelo músculo cardíaco (11,12). Existem algumas características da hipertrofia patológica que direcionam para o tipo concêntrico ou excêntrico, de acordo com o estímulo estressor. A sobrecarga pressórica, conforme ocorre na hipertensão arterial sistêmica (HAS), acarreta em aumento da massa cardíaca pelo aumento do diâmetro dos miócitos e recrutamento de sarcômeros em paralelo, porém não há síntese de novos componentes como as mitocôndrias. Tais características formam o conceito de hipertrofia patológica concêntrica apresentando aumento da espessura da parede e redução do diâmetro ventricular levando para o remodelamento concêntrico (13). Na hipertrofia patológica excêntrica há sobrecarga de volume e aumento do comprimento de miócitos em relação ao diâmetro causado pela adição de sarcômeros em série (14). Os dois padrões hipertróficos foram exemplificados por Foppa et al na figura 1 (9). No modelo excêntrico a relação entre volume mitocondrial e volume do miócito se mantém, há redução da razão entre os diâmetros da parede e da cavidade ventricular demonstrando um remodelamento excêntrico da câmara cardíaca (13). Podemos evidenciar este padrão excêntrico nas cardiopatias dilatadas e no infarto agudo do miocárdio (IAM) (15).

O estímulo patológico é constituído não só por fatores hemodinâmicos, mas também neuro-humorais sendo esses importantes coadjuvantes na expressão de genes, modificações celulares que alteram o tamanho dos cardiomiócitos, além de mudanças da matriz extracelular (16). As alterações moleculares associadas ao aumento do trabalho cardíaco acarretam em desenvolvimento celular defeituoso, evoluindo para fibrose e perda de cardiomiócitos evoluindo para IC (17, 18).

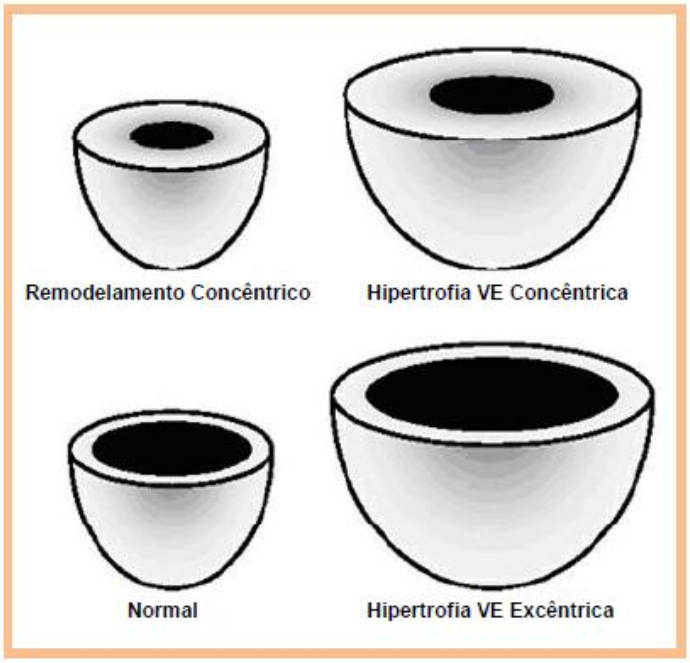

Figura 1. Diferenças do perfil morfológico na hipertrofia ventricular patológica adaptado de Foppa et al (9).

O processo patológico demonstra alterações devido aos danos causados no tecido cardíaco (como em IAM), sobrecargas crônicas de pressão (por exemplo na HAS) ou de volume (como na IC), inflamações (miocardites) ou fatores genéticos que predisponham às miocardiopatias. Esses eventos permitem a adaptação cardíaca ativando mediadores compensatórios (sistema simpático, renina-angiotensina, aldosterona e endotelina), citocinas pró-inflamatórias como TNF-α (fator de necrose tumoral) e citocinas estimuladoras de quimiotáticos como TGFß1 (fator de crescimento transformante ß1), FGF (fator de crescimento de fibroblastos), proteínas G e estresse oxidativo induzindo a hipertrofia que, por muitas vezes, progride para IC (5, 19).

O sistema renina-angiotensina (SRA) tem papel importante no modelo patológico. O SRA pode ser induzido de duas formas: 1) ativação sistêmica que ocorre através da liberação reduzida de cloreto e estímulo beta adrenérgico aumentado (20); 2) através do estiramento mecânico com sua produção local (21). A angiotensina II (ANGII) tem participação importante na hipertrofia patológica devido sua produção local após um estresse no tecido

cardíaco. Ela atua nos receptores AT1 indutores de MAPK (proteína quinase ativada por mitógeno) contribuindo para a ação de genes fetais. Os genes fetais são marcadores de hipertrofia patológica envolvidos na síntese proteica do miócito os quais induzem o crescimento celular, apoptose e necrose celular (6, 22, 23). Além disso, a ANGII produzida ativa uma cinase regulada por sinais extracelulares (ERK) o qual faz parte do complexo MAPK também ativado no exercício (13). Contudo, o complexo MAPK pode causar efeitos benéficos e deletérios, pelo fato de também ser induzido pela produção local de ANG II essa cascata pode levar à apoptose (24).

Além disso, o local lesado produz citocinas estimuladoras de células quimiotáticas (macrófagos e fibroblastos) capazes de expressar a enzima ECA que fornece ANGII local (25). O TGFß1 é uma citocina mediadora do remodelamento cardíaco capaz de aumentar a proliferação de componentes da matriz extracelular, como colágeno, e a fibrose intersticial (26). A partir dos fibroblastos há a formação de miofibroblastos que expressam pró-colágeno, receptores de ANGII, TGFß1 e endotelina. Sendo assom, ocorre auto-regulação do turnover de colágeno sob a influência de neurohormônios e citocinas contribuindo para a modulação do remodelamento (27). As terapias que tentam reverter o remodelamento cardíaco visam inibir a ECA e antagonizar os receptores de ANGII (28-30).

Ações inflamatórias também ocorrem após um estresse ou injúria cardíaca. O aumento do estresse na matriz extracelular e no citoesqueleto inicia a produção local de TNF-α e ANGII direcionando para um quadro de apoptose celular (31, 32). De fato, a ação da ANG II e níveis elevados de TNF-α sinalizam vias pró-inflamatórias acelerando o processo de fibrose e necrose (24). Assim, como foi observado, os níveis elevados TNF-α em pacientes com IC parecem estar correlacionados com grau de disfunção miocárdica (33). A participação da ANGII no do desenvolvimento de hipertrofia patológica pode ser exemplificada pela figura 2 adaptada a partir de Tunóia et al (34).

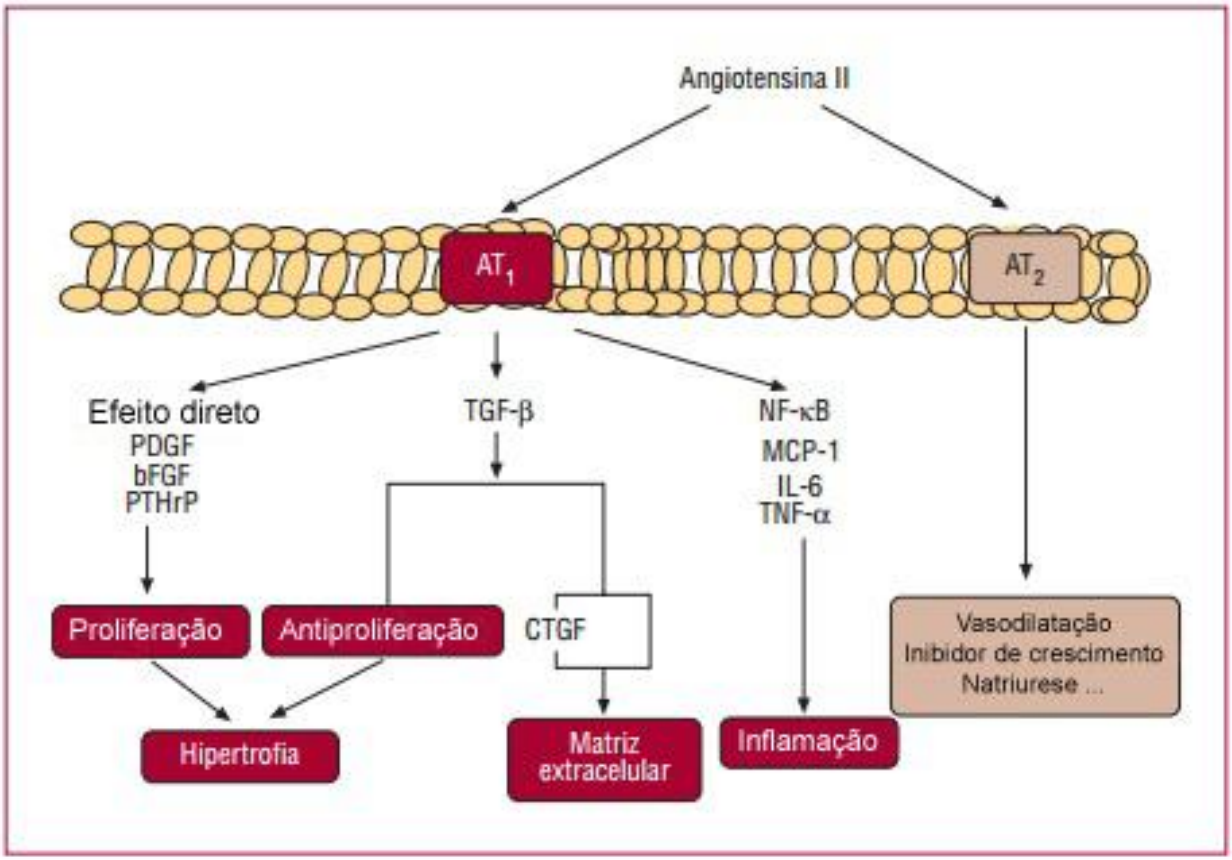

Figura 2. Sinalização celular do modelo patológico adaptado de Tuñóia et al (34).

1.3 Hipertrofia Fisiológica

A sobrecarga hemodinâmica imposta aos sistemas corporais de caráter transitório, como observado no crescimento humano ou uterino (gestação) e exercício físico, caracteriza um tipo de hipertrofia adaptativa ou fisiológica (11). O efeito do exercício físico sobre a hipertrofia fisiológica caracteriza-se por um perfil uniforme da parede ventricular e crescimento do septo sem fibrose e disfunção cardíaca (35).

Dentre os benefícios promovidos pelo o exercício físico podemos citar as adaptações causadas no sistema cardíaco, o que resultam da exposição frequente e regular às sessões de exercícios. O treinamento físico provoca mudanças morfofuncionais devido aos estímulos de sobrecarga cardíaca promovendo alterações hemodinâmicas sendo exemplificadas por bradicardia

de repouso, hipertrofia muscular assim como do ventrículo esquerdo (VE) e consumo máximo de oxigênio (36, 37). Além disso, o exercício físico estimula a angiogênese, uma vez que ocorre um aumento do aporte sanguíneo, devido neovascularização, nos músculos esquelético e cardíaco. De fato, esses parâmetros melhoram a função cardíaca e são importantes para o sistema cardiovascular suportar a demanda causada pelo exercício (38, 39).

O exercício físico isométrico tem o objetivo de desenvolver força e potência muscular como, por exemplo, durante a musculação. Portanto, é um exercício de resistência levando ao aumento pressórico, adição de sarcômeros em paralelo, aumento da largura dos cardiomiócitos e, por fim, aumento da espessura do VE sem reduzir o tamanho da cavidade interna na diástole caracterizando a hipertrofia concêntrica (39, 40). A sobrecarga pressórica causado pelo exercício é uma condição semelhante que ocorre na HAS, porém, esta, é seguida de disfunção sistólica e/ou diastólica (41) Na resposta fisiológica há aumento intermitente pressórico como mostrado em fisiculturistas em sessão de leg press acarretando em hipertrofia concêntrica (42, 43). Tais semelhanças entre os modelos pode diagnosticar equivocadamente uma hipertrofia patológica.

O estímulo gerado pelo exercício isotônico ou dinâmico como, por exemplo, a natação tem objetivo de promover mudanças hemodinâmicas como o aumento da frequência cardíaca e o volume sistólico. Ocorre incremento de sarcômeros em série desencadeando aumento no comprimento dos miócitos e, por conseguinte, aumento da massa cardíaca e volume da câmara. Portanto, tal sobrecarga de volume culmina em hipertrofia excêntrica (39, 44). Evangelista et al demonstraram este evento em modelo animal de hipertrofia cardíaca fisiológica cujos camundongos foram submetidos a duas sessões de 90min de natação por 5 dias/4semanas e o grupo treinado apresentou maior índice de hipertrofia ventricular que o grupo sedentário (45).

De modo geral os protocolos de exercício são compostos por uma combinação de exercícios estáticos e dinâmicos de forma que a HC é formada por diferentes níveis de hipertrofia concêntrica e excêntrica conforme ocorre

com triatletas (46). Diferenças entre a hipertrofia desenvolvida por exercício de resistência e aeróbico foram exemplificados conforme Figura 3 (35).

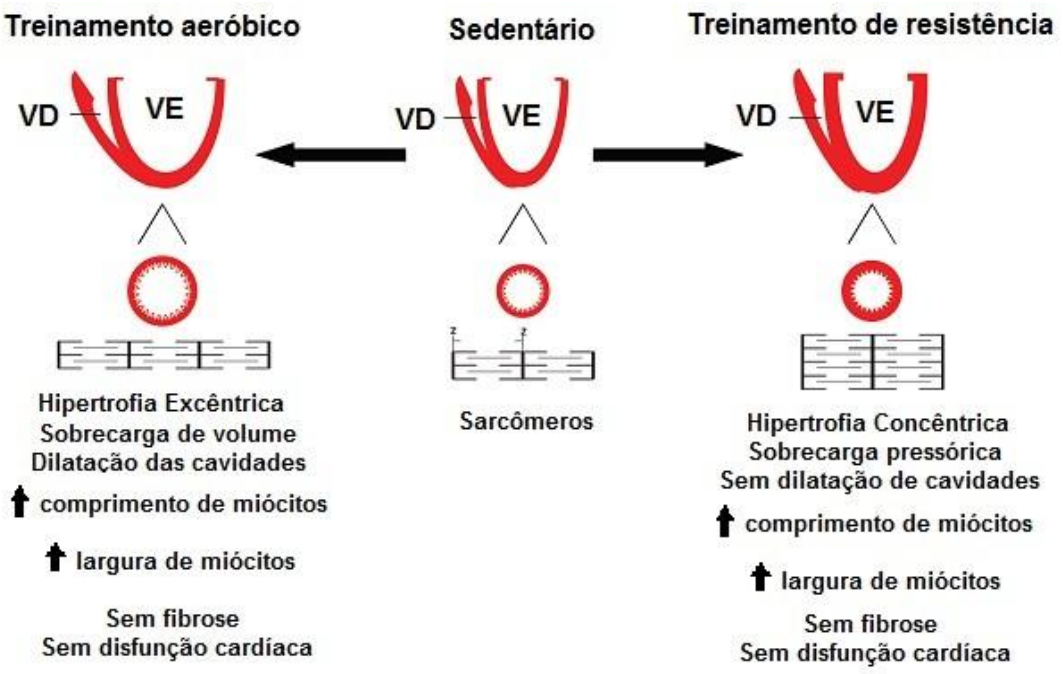

Figura 3. Diferença entre o padrão hipertrófico excêntrico e concêntrico desenvolvido por treinamento aeróbico e de resistência. Adaptado de Fernandes et al (35).

O crescimento dos miócitos na HC fisiológica ocorre pela síntese de novos componentes como, por exemplo, organelas intracelulares, aumento da espessura das miofibrilas e de sua quantidade ou aumento do seu comprimento (47). O aumento no volume dos cardiomiócitos acompanha o aumento dos componentes do estroma (fibroblastos e fibras colágenas, células endoteliais e células musculares lisas das paredes vasculares), uma vez que se adapta às novas exigências da demanda. Portanto, não ocorrem alterações das propriedades mecânicas do estroma, o que não implica em insuficiência funcional do órgão (48). Os mecanismos moleculares responsáveis pelo aumento cardíaco causado pelo exercício ainda está sendo explorado.

1.4 Sinalização molecular do fenótipo fisiológico

Diversas vias sinalizadoras têm sido estudadas como importantes transdutores de sinal da resposta hipertrófica nos cardiomiócitos (39). O treinamento físico atua como um normalizador da hipertrofia cardíaca, em nível gênico, como o ß-MHC e α-MHC (miosinas de cadeia pesada do tipo α e β), reexpressão de genes fetais e fator natriurético atrial (ANF) sendo esses marcadores de hipertrofia cardíaca patológica, portanto, no exercício, existe um perfil molecular diferente do encontrado nas doenças cardíacas (49, 50).

Há uma participação local do SRA ativado no exercício através do receptor AT2 que atua como um regulador do volume líquido, também, parece que AT2 participa da neutralização dos efeitos do receptor AT1, o que representa uma forma de proteção para o coração (51, 52). A progressão do remodelamento cardíaco pode ser mimetizada pelo exercício físico o qual exerce ação no SRA quando atua sobre a ANGII, sendo assim, após o exercício é possível observar redução dos níveis de ANGII (53). Podemos perceber que a ativação do receptor AT1 está direcionando para o perfil patológico intermediado por ANGII ao passo que o AT2 contribui para o desenvolvimento do perfil fisiológico (21, 54). O tratamento com bloqueador de AT1 (losartana) em animais submetidos à natação mostrou a ausência de hipertrofia cardíaca nesse grupo ao passo que o grupo administrado placebo e submetido à natação demonstrou hipertrofia devido o exercício (55). Em outro estudo o aumento de ANG (1-7) e ECA2 em ratos treinados por natação sugeriu aumento do receptor AT1 induzido pelo exercício, mas nesse caso sem a participação de ANGII o que direcionou o cenário para a hipertrofia fisiológica (56). Dessa forma, observamos que de acordo com o estímulo e a participação da ANGII o receptor AT1 direcionará para diferentes cenários.

O estiramento mecânico causado pelo exercício ativa a proteína quinase B (PKB) ou AKT através da ativação de AT1 (57). A via do receptor de tirosina quinase ao qual se ligam fatores de crescimento como o fator de crescimento de fibroblastos (FGF) e o fator de crescimento semelhante à insulina (IGF-I), está presente no treinamento aeróbico. O IGF-I liga-se ao seu receptor, o

receptor de tirosina quinase – IGFIR – ativando um fator de crescimento, fosfatidilinositol-3-quinase (PI3K). Este, por sua vez, inicia o recrutamento da AKT, uma via relacionada à sobrevivência celular em resposta ao exercício (58-60). Além disso, as contrações musculares durante o exercício físico são capazes de aumentar da atividade da AKT e/ou a sua fosforilação (61, 62). As vias de sobrevivência celular estão intimamente relacionadas ao processo de hipertrofia. As proteínas ativadas pela AKT fosforilam diversos substratos intracelulares responsáveis por regular crescimento, metabolismo e a sobrevivência celular.

A AKT, quando ativa, inibe a glicogênio sintase cinase 3ß (GSK3ß), cuja função é controlar negativamente a HC. Concomitantemente a AKT ativa a mTOR (proteína alvo da rapamicina em mamíferos) promovendo síntese proteica, progressão do ciclo celular e hipertrofia (63). Em contraste, a hipertrofia patológica não há ativação da AKT, muitas vezes há ausência. Estudo com uso de losartana mostrou o bloqueio dessa sinalização nos grupos treinados sugerindo que ativação de AT1 independente de ANGII interferiu na cascata AT1-AKT-mTOR após o exercício, sendo este um mecanismo potencial para hipertrófica adaptativa (64).

A mTOR é uma grande proteína quinase serina-treonina que pertence à família relacionada à cinase fosfatidilinositol (65). Ela possui diversas funções biológicas como controle do crescimento celular e proliferação através da regulação da tradução da proteína (66), responde a sinais para AKT, controla processos celulares incluindo autofagia. No músculo cardíaco exercitado a cascata AKT/mTOR induz crescimento de cardiomiócito correlacionando-se com melhora contrátil e hipertrofia ventricular (58, 67, 68). A via da mTOR também está relacionada com a sinalização de miostatina (MSTN) o qual é oposta à mTOR de forma que essa, quando aumentada, inibe o crescimento muscular provavelmente através da inibição de mTOR (69, 70). Dessa forma, o controle do crescimento muscular ocorre pela via IGF-PI3KAKT/mTOR sendo um regulador positivo da hipertrofia muscular esquelética ao passo que outra via atua como um regulador negativo, a MSTN (71). Podemos exemplificar esse raciocínio através da figura 4 adaptada e revisada por (72).

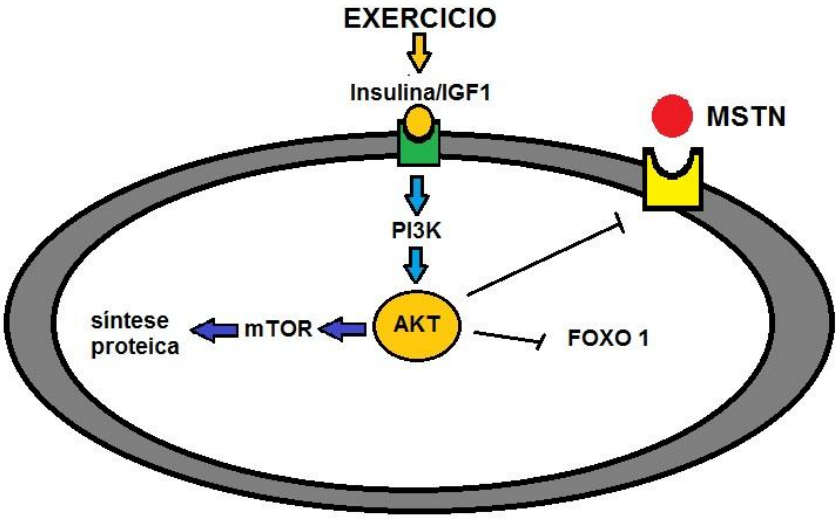

Figura 4. Sinalização exercício/mTOR/síntese proteica. Adaptado e revisado por Lebrasseur et al (72)

1.5 Via autofágica

O processo autofágico corresponde ao englobamento intracelular de componentes citosólicos como: proteínas, açúcares, lipídios assim como patógenos invasores ou organelas inteiras a fim de serem direcionados para a degradação lisossomal (73). Nos últimos anos investigou-se os mecanismos celulares nesse processo a fim de relacionar a patogênese de diversas doenças, contudo o mecanismo primário e natural é pouco compreendido e abordado.

A autofagia é um mecanismo homeostático com a finalidade de degradação e reciclagem através da maquinaria lisossomal reciclando citoplasma, proteínas, organelas (74) e, seletivamente, pode desencadear a remoção de organelas específicas como mitocôndrias danificadas promovendo mitofagia através de ubiquitinação reconhecido por p62. Sequestossoma-1 (SQSTM, também conhecida como p62) é uma proteína que auxilia tanto formação de vesículas como na degradação autofágica (75, 76). Outra proteína autofágica participa iniciação da via formando vesícula, a LC3 (MAP1LC3A proteína de cadeia alfa 3 associada à microtúbulos) interage diretamente com p62 sendo, então, os dois principais marcadores autofágicos (77, 78).

A compreensão dos mecanismos moleculares da autofagia pode estabelecer uma conexão com a patogênese de diferentes doenças como as cardíacas e aquelas relacionadas à senescência (79, 80). Sabe-se que as vias lisossomais participam da patogênese de praticamente todas as doenças cardíacas, porém recentes estudos tem observado o mecanismo autofágico a fim de avaliar as ações adaptativas ou mal adaptativas que influenciam a autofagia nessas doenças. Além disso, a autofagia “boa” ou “ruim” tem mecanismos ainda desconhecidos e levam a discussões a cerca de seus fins terapêuticos (80, 81).

A arquitetura autofágica é composta por muitas proteínas que são recrutadas pelo fagóforo a fim de participarem da formação do autofagossoma. Dentre as proteínas recrutadas para formação do autofagossoma, a Beclina (codificada pelo gene *Beclina 1*) participa na fase de iniciação (82-84). A fase de expansão do fagóforo é mediado por dois sistemas conjugados de *ubiquitina-like* que promovem a formação do complexo ATG16L e o processamento da LC3, importante para formação da membrana da vesícula (figura 5) (85). A LC3 é clivada em LC3I – forma citosólica – e conjugada com PE (fosfatidiletanolamina) formando um complexo lipidado chamado LC3II – forma encontrada na membrana do fagóforo. Os níveis dessas isoformas se correlacionam com a abundância dos autofagossomas (85).

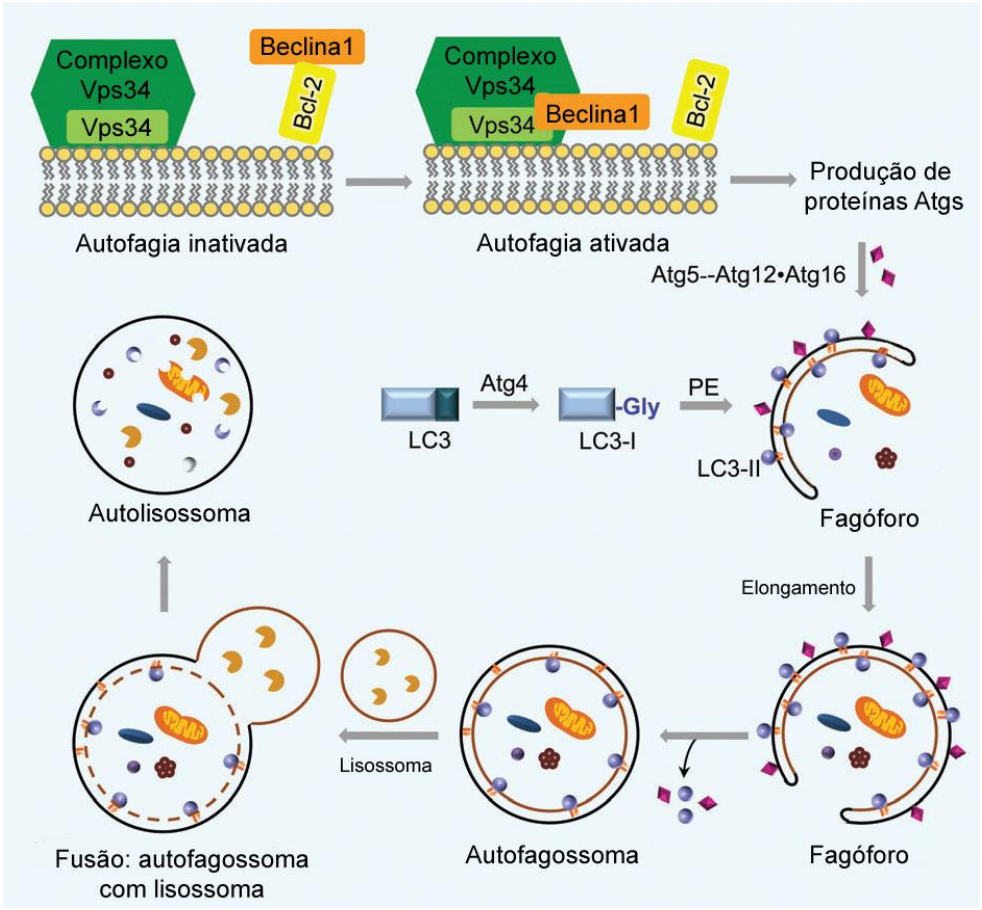

Figura 5. Via autofágica e formação do autofagossoma (86).

A ativação das vias de fluxo autofágico ocorre em duas situações: em baixo nível de fluxo autofágico devido uma baixa energética a fim de manter a sobrevivência celular (87) e ativação pronunciada a fim de esgotar os elementos celulares necessários para a vida daquela célula, culminando em morte celular (88). Esses dois extremos nas ações autofágicas são mecanismos potenciais pró ou anti-sobrevivência, respectivamente. A sobrevivência dos cardiomiócitos está relacionada com níveis fisiológicos de autofagia, no qual a reciclagem normal dos componentes danificados constitui um meio de controle da qualidade das organelas. Com efeito, a participação das vias autofágicas no coração resulta em hipertrofia, dilatação do ventrículo esquerdo e débito cardíaco reduzido (80, 89). Tanto estudos com humanos quanto com animais mostram presença de vacúolos autofágicos em cardiomiócitos isquêmicos caracterizando falência cardiomiopática (90-92). Em modelo cirúrgico de sobrecarga pressórica *in vivo* o fluxo autofágico foi

correlacionado com o grau de estresse pressórico (93, 94). Uma vez que a autofagia é induzida pelo estresse de carga há uma superexpressão de BCN1, um elemento molecular importante para o fluxo autofágico e observado na transição para a IC (95). Por outro lado, outros estudos demonstram uma redução da resposta autofágica em ratos com baixa expressão de BCN1 e, por conseguinte, um remodelamento cardíaco atenuado (93). Esse dualismo da autofagia pode ser explicado pela evidencia de que a autofagia excessiva pode levar ao esgotamento de organelas e moléculas celulares, provocando morte celular (96-98).

Estudos mais recentes mostram uma participação da autofagia durante o exercício. Camundongos GFP submetidos a exercício agudo (30 e 80min) mostram aumento de LC3II em músculo vasto lateral e cardíaco, porém o aumento da relação LC3II/I e níveis reduzidos de p62 ocorre apenas no músculo esquelético (99). Em uma sessão de exercício é possível aumentar a autofagia durante as primeiras 3h pós-exercício a fim de repor a demanda energética exigida durante esse stress fisiológico (100). Animais em estado de jejum submetidos a uma sessão de corrida apresentam aumento tanto da proteína LC3II/I como a expressão do gene LC3 em músculo esquelético, porém o mesmo não ocorre nos animais em estado alimentado e treinados. Essa ativação autofágica se dá em consequência da repressão aguda da via AKT/mTOR (101). No exercício crônico observamos a autofagia ativada em músculo sóleo através de aumento em LC3II/I e BCN seguido da redução de p62. Tal ativação pode ser explicada pela mudança do perfil muscular pós-exercício de forma que um músculo misto (composto de fibras oxidativas e fibras glicolíticas) aumenta o recrutamento de fibras oxidativas gerando maior quantidade de metabólitos e capacidade oxidativa que são reciclados pelo sistema autofágico (102).

De fato, a autofagia parece estar relacionada tanto nas respostas fisiológicas como patológicas, porém seu papel e mecanismo molecular na hipertrofia cardíaca ainda não estão bem estabelecidos. Em um primeiro momento o estudo de seu mecanismo no cenário fisiológico é essencial para entender esse processo como alvo na hipertrofia cardíaca a fim de, no futuro, alcançar entendimento no cenário patológico.

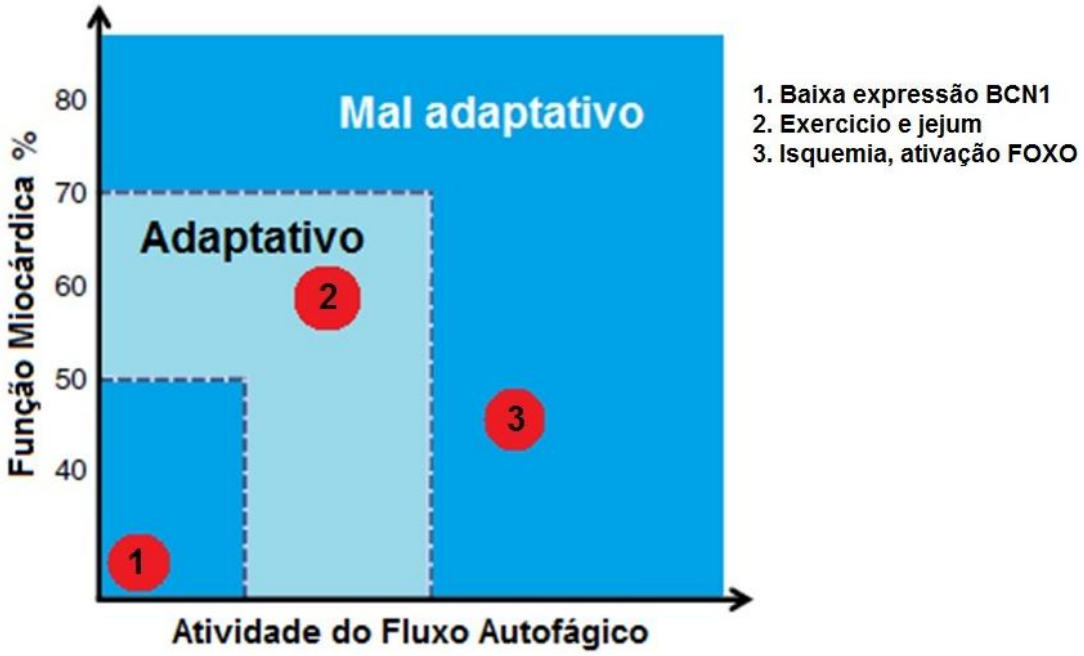

Figura 6. Relação entre atividade do fluxo autofágico e função miocárdica adaptado de Nemchencko et al (103). 1) relacionado à baixa expressão de BCN1 (93), 2) relacionado ao exercício e jejum (99, 101), 3) relacionado à isquemia e ativação de FOXO (92, 104).

1.6 Relação miostatina e autofagia

A miostatina é um regulador negativo do crescimento muscular esquelético. É conhecida como um fator de crescimento e diferenciação 8 (GDF-8) importante para o músculo e pertencente à superfamília do fator transformador do crescimento ß (TGFß). Além disso, sua função é regular o crescimento de músculos esqueléticos na fase embrionária e adulta bem como limitar o crescimento do músculo (69). Com relação às outras proteínas da superfamília do TGFß a miostatina difere das demais devido sua exclusividade em músculo esquelético na fase adulta (69). Muitos estudos se interessam nas ações da MSTN quando em condições patológicas como IC, neoplasias, cirrose, distrofias musculares, uremia e desnervação (105-107). Nas patologias como as doenças cardiovasculares observamos uma redução da musculatura esquelética mediada pela ação de MSTN aumentada (108, 109). Por outro

lado, nos modelos de hipertrofia fisiológica, como induzidos por exercício físico, a MSTN parece estar reduzida tanto no músculo esquelético quanto no cardíaco (110, 111).

A MSTN participa da ativação do sistema ubiquitina proteossoma e outros sistemas proteolíticos, uma vez que medeia o aumento da degradação de proteínas musculares. Essa degradação proteica ocorre pela inibição de PI3K/AKT e consequente fosforilação de *forkhead Box O1* (FoxO1) levando a ação de genes relacionados à atrofia (atrogenes) relacionados à ativação de autofagia. Esses atrogenes estão ativados quando a via da AKT estiver inibida, portanto a via da AKT ativada provoca inibição dos atrogenes (112-114). No músculo, o sistema ubiquitina proteossoma tem a função de remover proteínas sarcoméricas em resposta às alterações na atividade muscular. A AKT regula tanto o sistema ubiquitina proteossoma quanto a autofagia lisossomal, uma vez que em estado inativo libera a via para ações mediadas por fatores de transcrição da FOXO1 (115-117).

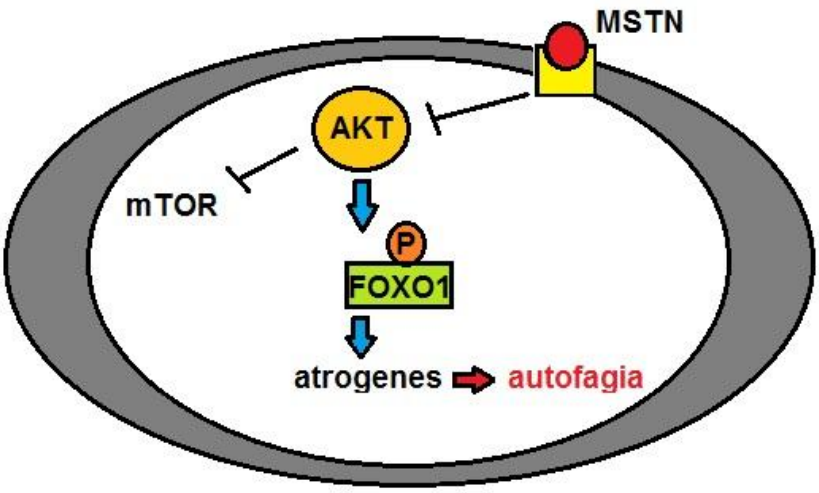

Figura 7. Relação entre sinalização da miostatina e autofagia. Adaptado e revisado por Lebrasseur et al (72).

A MSTN aumentada bloqueia não só AKT, mas também mTOR que sinaliza a via hipertrófica, portanto quando a via de síntese está inativada ocorre ativação da via promovendo degradação proteica através da maquinaria

autofágica (104, 118, 119). Estudo com pacientes mecanicamente ventilados com presença de atrofia do diafragma mostrou ativação proteolítica e autofagia através da inibição da AKT (117). Outro estudo, porém *in vitro* mostrou que a administração de MSTN em cultura de células musculares promoveu aumento do fluxo autofágico através da expressão de LC3II (120). Por outro lado, a redução de RNAm de MSTN é observada em humanos exercitados por 6 meses (121), já em camundongos deficientes de MSTN e exercitados cronicamente (natação e corrida) observamos mudança do fenótipo muscular esquelético expressando mais marcadores oxidativos e genes autofágicos upregulados (122). Dessa forma, é plausível que a MSTN aumentada participe da ativação de autofagia em músculo esquelético assim como a redução de MSTN tenha efeito inibitório da autofagia. Um resumo dos últimos estudos avaliando autofagia, MSTN e exercício físico podem ser observados na tabela 1.

Tabela 1. Principais estudos abordando autofagia e miostatina

	Modelo	Local	Gene LC3	LC3II/I	Ref.
Autofagia	Patológico Envelhecimento	Células cardíacas		↓ velhos	(123)
Autofagia	Exercício 30min, 80min	Vasto lateral e cardíaco		↑ Vasto Lateral	(99)
Autofagia	Fisiológico Exercício agudo	Cardíaco		↑ apenas de LC3II	(100)
Autofagia	Fisiológico Exercício e Jejum 90min	Gastrocnêmio	↑ jejum e exercício	↑ jejum	(101)
Autofagia	Fisiológico Exercício voluntário 4 semanas	Músculos Sóleo Plantar		↑sóleo	(102)
Autofagia miostatina	Patológico Células tratadas com miostatina	Células musculares esqueléticas		↑ autofagia via outras proteínas autofágicas	(120)
Autofagia miostatina	Fisiológico Patológico Natação Exercício voluntária Camundongos $MSTN^{-/-}$ e $MSTN^{+/+}$	Músculo esquelético	↑ natação em $MSTN^{-/-}$ Não difere em $MSTN^{+/+}$		(122)
Miostatina	Fisiológico Humanos 6 meses exercício	Músculo esquelético ↓ miostatina			(121)

1.7 Modelos Experimentais

Modelos animais são importantes para estudar processos patofisiológicos de doenças assim como compreender mecanismos fisiológicos dos sistemas. Sendo assim é necessário escolher um modelo animal que possa reproduzir e responder a hipótese a ser testada em um estudo.

Os modelos animais podem ser espontâneos, estes apresentando mutações específicas para determinada condição a exemplo temos o diabetes, por outro lado os modelos induzidos que podem ser criados por manipulação mecânica, genética ou química como, por exemplo, modelos de insuficiência cardíaca estabelecidos por bandeamento aórtico (124-126).

Animais de pequeno porte como ratos e camundongos são muito utilizados em estudos devido seu curto ciclo de vida, curto período de gestação, além do baixo custo para a pesquisa. Em relação à hipertrofia cardíaca fisiológica é necessário avaliar o objetivo do trabalho para melhor escolha do modelo animal (tabela 2). Diferentes protocolos de treinamento físico foram testados em modelos de pequeno porte considerando algumas variantes como a continuidade do treinamento, progressão, inclinação e duração da sessão a fim de alcançar a hipertrofia cardíaca fisiológica.

No caso de camundongos os protocolos de corrida com diferentes intensidades e duração induzem apenas um grau limitado na hipertrofia cardíaca quando não a induzem, entretanto a razão desse evento ainda está desconhecida (127). Os programas de treinamento voluntário demonstram uma robusta hipertrofia cardíaca, contudo não há um controle ideal sobre o exercício tornando o modelo heterogêneo (128). O modelo de hipertrofia cardíaca por natação parece demonstrar melhores resultados em camundongos chegando a 15% de hipertrofia nesses animais. Alguns estudos mostram uso de carga adicionada durante o nado desses animais desenvolvendo uma efetiva hipertrofia (129, 130). Portanto, o uso ou não de peso na cauda, o aumento da frequência e duração de sessão diária, assemelham-se à hipertrofia demonstrada nos protocolos de exercício voluntário (45).

Tabela 2. Modelos animais de hipertrofia cardíaca induzido por exercício adaptado de Wang (131).

Programa de treinamento físico em camundongos	Comentários	Ref.
Treinamento de corrida **Machos e fêmeas adultas, 5 vezes/semana, 120 min/dia, 8 semanas, intervalo de 8min 85-90% e 2 min 50-60% do VO2máx.**	Protocolo de alta intensidade: VE/PC↑, VD/PC↑, peso coração/PC↑, dimensão de cardiomiócitos↑	(127)
Treinamento voluntário de corrida **Adultos de várias linhagens, livre acesso as rodinhas de corrida por 2 semanas.** **Machos adultos, livre acesso as rodinhas de 2-4 semanas**	Resposta linhagem específica: menor resposta quanto ao índice de hipertrofia (coração/PC) em C3H/HeJ e maior resposta em DBA/2J. Duração e exercício: média de 4,3h/dia, 6,8km/dia por 4 semanas, coração/PC↑.	(132) (133)
Treinamento em natação **Machos adultos, temperatura da água 30-32ºC, 1*vs* 2 vezes/dia, 5 dias/semana, 60 *vs* 90min/sessão, 4 *vs* 6 semanas, 2 *vs* 4% do peso corporal para o peso carregado na cauda ou sem peso.**	Efeito de diferentes durações, frequências e pesos: VE/PC↑, Coração/PC↑ dependendo do protocolo.	(45)

2. Justificativa

O desenvolvimento da hipertrofia cardíaca fisiológica requer ativação de sinalizadores intracelulares, assim como proteínas citoplasmáticas a fim de aumentar o diâmetro dos miócitos e das fibras cardíacas. Através do exercício crônico, induzido por natação, é possível atingir o limiar hipertrófico de camundongos em até 28 dias de treinamento. Os mecanismos envolvidos na via hipertrófica vêm sendo estudado. A miostatina é um regulador negativo muscular, em contraponto a mTOR tem ação oposta encaminhando a via para o crescimento do músculo. Nesse âmbito, a hipertrofia cardíaca apresenta influência desses dois componentes durante a síntese proteica.

Outras vias sinalizadoras presentes em patologias cardíacas são estudadas a fim de entender seu mecanismo mal adaptativo e identificar possíveis métodos terapêuticos para combater a ativação dessas vias. Torna-se importante estudar os mecanismos fisiológicos para compreender a base mecanística dessas vias. Nesse cenário a maquinaria autofágica atua em diversas doenças encaminhando a via para a anti-sobrevivência celular. Dessa forma, é importante entender o mecanismo fisiológico dessa via, assim como vem sendo explorado o mecanismo patológico. Além disso, a miostatina parece estar envolvida na inativação da maquinaria autofágica. Entretanto, poucos estudos relacionam os mecanismos da miostatina durante na hipertrofia cardíaca e a sua interação com a via autofágica. Explorar a interação dessas rotas entre si e a participação delas no processo de hipertrofia fisiológica é importante para o entendimento do modelo fisiológico e, posteriormente, relacionar essas vias com o modelo patológico.

3. Hipótese

A hipertrofia cardíaca fisiológica iniciada em 7 dias é estabelecida em 28 dias de natação em camundongos e o desenvolvimento hipertrófico no coração está relacionado com a redução dos níveis de miostatina e da sinalização autofágica.

4. Objetivos

4.1 Objetivo Principal

Desenvolver a hipertrofia cardíaca fisiológica, avaliar níveis de miostatina e a sinalização da via autofágica em camundongos *Balb/c* submetidos à natação.

4.2 Objetivos Específicos

- Avaliar a presença de hipertrofia cardíaca em 1 e 28 dias após a natação, avaliados pela relação do peso do ventrículo esquerdo corrigido pelo comprimento da tíbia de cada animal além de avaliar o diâmetro dos cardiomiócitos;
- Avaliar a expressão gênica de LC3, p62, Beclina-1, mTOR e miostatina através de PCR em tempo real em tecido cardíaco;
- Avaliar a expressão proteica de LC3I, LC3II, p62, Beclina-1 e mTOR através de western blot em tecido cardíaco;
- Comparar os níveis de proteínas e RNAm entre os grupos treinado e sedentário;

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ORIGINAL ARTICLE

REDUCED AUTOPHAGY AND MYOSTATIN LEVELS IN A MODEL OF PHYSIOLOGIC CARDIAC HYPERTROPHY IN MICE

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Abstract

Myostatin and autophagy are involved in the control of muscle growth in both physiological and pathological conditions, but their role in the development of cardiac hypertrophy induced by physiologic stimulus is not elucidated. The aim of this study was to evaluated myostatin levels, as well as genes and proteins related to autophagy, in mouse with physiological cardiac hypertrophy. To achieve cardiac hypertrophy, mice (balb/c) were subjected to a swimming protocol for 7 or 28 days (to represent initial and established hypertrophy, respectively), and compared to sedentary controls. Left ventricular weight/tibial length (LW/TL) and cardiomyocite diameter were measured in order to evaluate cardiac hypertrophy. Myostatin, mTOR and, autophagy gene expression were evaluated by RT-qPCR. Western blot was performed to analyze autophagy proteins and the phosphorylation status of mTOR. The protocol resulted in cardiac hypertrophy as evidenced by progressive increments of the LW/TL and cardiomyocyte diameters in trained groups at both 7 and 28 days. By contrast, myostatin expression was reduced in trained animals during initial hypertrophy, but it was similar to controls in established hypertrophy. Autophagic genes showed decreased expression while protein levels remained stable compared to sedentary controls. mTOR gene expression was reduced, and phosphorylation increased, in established hypertrophy, with no change in the early-time point. Both reduced myostatin levels during initial hypertrophy and increased mTOR phosphorylation observed with the established hypertrophic phenotype might be important mechanisms favoring muscular growth in this scenario and seems to favor reduced basal autophagy.

Keywords: Autophagy, Swimming, Left Ventricular Hypertrophy, Myostatin, mTOR

INTRODUCTION

Cardiac hypertrophy develops as a response to a variety of stimulus to compensate body needs. [1-3] Physiological hypertrophy is characterized by a uniform increase of cardiomyocytes size, synthesis of new components and organelles without fibrosis. [4, 5] On the other hand, pathological hypertrophy is constituted by changes in the extracellular matrix and fibrosis that result in reduced cardiac function. [6] Autophagy is a catabolic mechanism that occurs in most cells, and is required in several homeostatic processes such as protein and organelle turnover. [7, 8 7, 8] During fed state it is maintained at low levels to keep cell survival; [8, 9] however, when cells are submitted to stress conditions autophagy is activated [10-12] and the autophagossome (double-membrane vesicle) is formed to enhance energy generation.[13-15]

Autophagy seems to participate in pathological situations [16, 17] however its relation to physiological cardiac hypertrophy is yet to be elucidated. In the skeletal muscle, autophagy seems to be induced in response to either acute or chronic exercise. [18, 19]

Myostatin, a member of the TGF-β superfamily, is a negative regulator of muscle growth which is expressed in the skeletal muscle as well as in the heart and adipose tissue. [20] Animal studies demonstrated the role of myostatin in muscle hypertrophy, involving the Akt/mTOR signaling pathway. [21-24] Recently, it has been shown that myostatin increases autophagic signaling in the skeletal muscle, reinforcing its participation in this pathway. [25] Moreover, in pathological conditions, elevated levels of myostatin are associated to muscle wasting probably due to stimulation of autophagy. [26, 27] Therefore, the role of myostatin in autophagy pathways is well established in skeletal muscle, mostly in the pathological scenario. However, few studies have addressed its role during the physiological adaptation of cardiac tissue to exercise. Our aim was to evaluate myostatin levels and autophagy during the development of physiological hypertrophy in mice submitted to a swimming protocol.

RESULTS

The effects of exercise training on cardiac hypertrophy and body weight are shown on Table 1. The animals had similar weights at prior to the protocol and at initial hypertrophy (day 7), but the trained group had lower weight compared to the sedentary at day 28. We therefore used left ventricular weight corrected to tibia length ratio for hypertrophy analysis.

Left ventricular weight/tibia length (LVW/TL) ratio was higher in trained animals compared to sedentary controls both at 7 (increased by 9%, p= 0.17) and 28 days (increased by 13%, p= 0.0001) (Figure 1A). In addition, we observed a progressive increase in cardiomyocyte diameter in both trained groups (20% in T7 and 30% in T28 as compared to their sedentary controls, both p value < 0.01) (Figure 1B).

Myostatin - Figure 2 shows the gene expression of myostatin (*Mstn*) during initial (7 days) and established (28 days) cardiac hypertrophy. There was a significant reduction in *Mstn* gene levels at initial hypertrophy (p=0.01). This reduction was no longer observed at 28 days.

mTOR gene expression and protein phosphorylation – Since myostatin is known to act through AKT/mTOR pathway to inhibit muscle growth, we further evaluated *mTOR* gene expression and protein phosphorylation. Figure 3 shows that *mTOR* gene expression was reduced at day 28 (Fig 3.A), while mTOR phosphorylation was increased at this same time point (Fig 3B). No changes were observed at initial hypertrophy.

Autophagic genes expression – Gene expression of *Lc3*, *P62* and *Beclin-1* are shown in Figure 4. There was a reduction in *Lc3*, *P62* and *Beclin-1* in trained mice compared with sedentary, and this reduction was significant at both initial (T7) and established hypertrophy (T28) evaluations (Figure 4 A, B, and C respectively).

Autophagic proteins – The protein expressions in cardiac muscle is shown in Figure 5. There was a reduction in LC3I protein, only on T7 group (Figure 5A). Regarding LC3II (Figure 5B), LC3II/I ratio (Figure 5C), P62 (Figure 5D) and

BECLIN-1 (Figure 5E), there were no differences between sedentary and trained groups, neither at early nor at late evaluation.

DISCUSSION

The present model of physiological cardiac hypertrophy resulted in an initial phenotype at 7 days and established hypertrophy after 28 days, and our mains findings are as follow: 1) Myostatin gene expression was reduced during initial hypertrophy, and returned to baseline levels at day 28, when established hypertrophic phenotype was present; 2) mTOR phosporylation increased only late in this model, and 3) the development of hypertrophy favor a reduction on the autophagic genes expression in cardiac muscle. The regulation of myostatin autophagic activity seem to be important components of the hypertrophic response, and additional players might be involved in the regulation of these pathways.

The swimming protocol resulted in the development of cardiac hypertrophy that was established after 28 days. This model was indeed effective to induce early muscle growth. At the early-time point (T7) we observed a 9% increase in ventricular mass (by LVW/TL) but without statistical difference to sedentary control (S7), nevertheless the tendency to hypertrophy was confirmed after 28 days of training (T28) that showed 13% increase in cardiac mass with statistical difference to sedentary control (S28). The cellular hypertrophy was more pronounced than the ventricular hypertrophy; cardiomyocyte diameter increased 20% and 30% in T7 and T28, respectively. Thus, our data corroborate to previous findings that showed cardiac hypertrophy in between 13% and 25% induced by controlled exercise, wheel running or swimming. [28-30] We used the ratio of the left ventricle weight correct by tibia to evaluate hypertrophy because exercised mice had a decrease in their body weight at the end of the study.

In the present study, myostatin levels were reduced at the early-time point and this reduction was no longer observed after 28 days. Myostatin is an important regulator of muscle growth, with negative effects for both hypertrophy and hyperplasia. [20] In mouse model of heart failure, myostatin was described as mediator of the skeletal muscle atrophy. [31-33] In an animal model of heart failure,

cardiac deletion in *Myostatin* gene resulted in reduced skeletal muscle atrophy while transgenic overexpression of myostatin in the heart induced muscle wasting.[31] On the other hand, previous studies in physiological hypertrophy/exercise corroborate our findings by showing a reduction in myostatin mRNA expression in skeletal muscle after chronic exercise, possibly related to increased muscle mass. [34, 35]

Another interesting finding was the observed increase in phospho-mTOR regardless of decreased mTOR mRNA levels after 28 days of training. Our data shows that *Myostatin* gene levels and mTOR phosphorylation follow opposite directions, while myostatin decreases the mTOR phosphorylation increases. Previous studies argue that mTOR signaling is opposite to myostatin, which inhibits muscle growth [20, 36] possibly because myostatin has inhibitory effects on mTOR signaling. [24] Our findings are consistent with studies that evaluate increase of phosphorylated mTOR in endurance or strength exercise after 2h or 22h of the last session showing its relation with protein synthesis and hypertrophy. [37-39] The signal transduction that drives muscle growth metabolism occurs through PI3K/AKT pathway. [40] Activation of this pathway leads to protein synthesis in response to stimuli such as insulin, growth factors, resistance exercise and nutritional inputs. [40-42]. The PI3K/AKT pathway signaling activates the mTOR complex resulting in cell proliferation and growth. [43-45] Recent works have shown that myostatin inhibits the AKT pathway, thus affecting protein synthesis. [23, 46] In fact, when AKT pathway is inactive, the FOXO pathway is activated leading the transcription factor MuRF1 and MAFbx to induce protein degradation and muscle atrophy by activating the autophagic system. [47-49 10, 50]

Increased levels of myostatin in pathological models seem to activate the autophagyc machinery, while mTOR is a negative regulator of autophagy. In nutrient-rich conditions mTOR is activated and results in the suppresision of autophagy. [51-53] As we observed decreased myostatin and increased pmTOR, we could expect reduced autophagy in the exercised mice. On the other hand, acute exercise is a potent inducer of autophagy in both skeletal and cardiac muscle [54-56] and when combined with fasting the autophagy is also enhanced. [41] In fact, increased autophagy has been observed in skeletal muscle in chronic exercise and attributed to increased muscle oxidative capacity with increased

mitochondrial biogenesis and glucose metabolism. [16, 17, 56] In the present study, we did not observe major differences in autophagic proteins, except for LC3I protein. The LC3I protein level was reduced at day 7 and the LC3II/I ratio showed 46% increased with training at day 28, with no statistical significance. The LC3I is the cytosolic form of the protein; during the autophagossoma formation the LC3I is cleaved and attached to phosphatidylethanolamine (PE) to form LC3II which is then attached to phagophore membrane. [57, 58] Thus, when autophagy is activated the LC3I concentration must decrease, which provoke LC3II concentrations and LC3II/I ratio increase. Since we did expect at least 24 hours after exercise to evaluate gene expression and protein levels, we did not evaluate acute reponses but probably the chronic adaptation the cardiac hypertrophy. Further, the mRNA expression of autophagic proteins was reduced compared to their sedentary controls at both time-points. This reduction in autophagic gene expression was not associated to a decrease in the autophagic flux when the animals were not exercised for the previous 24 hours. Unchanged basal authopahty might reflect the physiological and reversible changes observed in these models, in contrast to changes observed in pathological models, in which we observe persistent activation of autophagy.

CONCLUSIONS

This effective model of physiological cardiac hypertrophy was associated with reduced myostatin levels when hypertrophy was developing, which returned to baseline levels at day 28, when hypertrophy phenotype was already established. Increased mTOR phosporylation might be an important mechanism favoring muscular growth in this scenario. The effects in autophagy pathway seem to favor reduced basal autophagy in this model. Taken together, these data suggest both myostatin and autophagy downregulation might be involved in the development of physiological cardiac hypertrophy, where a proliferative/survival pathway seems to take place over catabolic processes.

MATERIAL AND METHODS

All protocols were approved by the Animal Care and Use Committee of Hospital de Clínicas de Porto Alegre under the number 12-0250. The present study is in accordance with Guide for the Care and Use of Laboratory Animals [42] and the ARRIVE guidelines. [43]

Animals

A total of 52 male BALB/C mice aged 8 weeks were included in this study. The animals were housed in controlled temperature (22±2ºC) with 12:12 light-dark cycle, and received normal chow and water *ad libitum*. Animals were randomly allocated to sedentary or exercise training groups, and to evaluation at early (7 days) or late (28 days) of follow-up, generating four groups: S7 (sedentary 7 days, *n*=12), S28 (sedentary 28 days, *n*=12), T7 (trained 7 days, *n*=13) and T28 (trained 28 days, *n*=15).

Swimming

The objective of physical training was to promote cardiac hypertrophy and the protocol was adapted from Evangelista et al [3]. The training consisted of twice 90 min daily sessions of swimming training 5 days/wk, during a total of 7 (T7 group) or 28 days (T28 group) with water temperature 30-32ºC. The first swimming session had a duration of 20 min; the time was increased by 10 min every day until day 5, and by 15 min in days 6 and 7. All remaining sessions had 90 min duration. To minimize the influence of water stress, sedentary animals were placed in the pool for 5 minutes, twice a week during experimental protocol. An adapted pool from Evangelista et al summarized in supplementary figure. [3]

Tissues harvesting and euthanasia

Hearts were harvested 24h after the last training session under deep anesthesia (ketamine 100mg/kg and xylazine 10mg/kg). Immediately, the atriums and the right ventricle were excised in order to isolate the left ventricle.

The left ventricle was weighed to calculate weight ratio of the left ventricle/tibial length (LV/TL in mg/cm). Left ventricle was sliced in three sections, immediately 1) frozen in liquid nitrogen; 2) stored in RNA later solution (Qiagen) or 3) formalin fixed (10% buffered formalin) with posterior paraffin-embedded.

Morphometric Analysis

Paraffin-embedded heart sections (4-5 µm thick) were prepared and were stained with hematoxylin-eosin (HE). LV samples were photographed in microscope (magnification) and cardiomyocytes were measured using public software Image J. A single transverse measurement of width, passing through the nucleus, was made in 10 cardiomyocytes per animal, the values were averaged of the pixels of the slides photographed for each animal and were transformed in µm.

Western Blot

The samples were weighed and to each 100 mg of tissue 900 µL of homogenization buffer (50 mM Tris, pH 7.5, 1% Triton, 1 mM PMSF, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 tablet of SigmaFast protease inhibitor cocktail for every 100 mL of buffer) was added, homogenized, and immediately centrifuged (1000xg). The supernatant was collected and had its protein concentration assessed by Bradford method. [44] Samples were diluted in 0.1 M Tris, 6.8 pH, achieving final concentrations of protein 2µg/µl in Laemmli buffer (250mM Tris, 8% SDS, 40% glycerol, 0.008% bromophenol blue, pH 6.8%, 20% ß-mercaptoetanol), samples were heated to 70°C for 10min SDS-PAGE (12%) gel was loaded with 30µg of protein sample and run for 90 min at 120V before transference to PVDF membranes (Bio-Rad Laboratorioes Inc.) by using transfer buffer (48mM Tris, pH 9-9.3, 39 mM glycine, 20% methanol in semi-dry system Bio-Rad Laboratorioes Inc.). For mTOR analysis, samples were separated in gradient SDS-PAGE (8-20%) using the electrophoresis buffer (8.3 pH, 100 mM Tris, 190 mM glycine, 0.4% SDS) and transferred to PVDF membranes (Bio-Rad Laboratorioes Inc.) by using transfer buffer (25mM Tris,

pH 8-8.5, 192 mM glycine, 20% methanol in tank blotting system Bio-Rad Laboratorioes Inc.). The molecular weights of the protein bands were determined using as reference a standard molecular weight (1610374 - Bio-Rad Laboratorioes Inc). The bands were stained with Pounceau method. The membrane was blocked for 1 h (5% non fat milk) with TTBS buffer (100 ml TBS10x, 0.1% Tween 20, 800ml deionized water). Membranes were incubated with TTBS buffer, 5% non fat milk and antibodies. The following antibodies were used (Cell Signaling Technology): anti-Beclin 1 (3738S), anti-LC3b (2775S), anti-p62/SQSTM1 (5114S), anti-mTOR (2972S) and antiphospho-mTOR (2971S) – Cell Signaling Technology, which was titrated to 1:1000, overnight at 4°C. After, the membranes were incubated with conjugated secondary antibody goat anti-rabbit horseradish peroxidase IgG (1:1000). Glyceraldehyde-3-phosphate dehydrogenase was employed as housekeeping protein for further normalization using antibody anti-GAPDH (1:18000), incubated with conjugated secondary antibody goat anti-rabbit horseradish peroxidase IgG (1:18000) at room temperature. Chemiluminescent detection was performed using chemiluminescence reagent (ECL Advanced Western blotting kit Millipore Corporation) and exposed to a digital image acquisition system (L-Pix Chemi Molecular Imaging - Loccus biotecnologia, Cotia, São Paulo, Brazil). The membranes were quantified with public domain software ImageJ and results were expressed in percentage of arbitraty units (% AU).

Quantitative real time PCR (qRT-PCR)

RNA extraction, reverse transcription quantitative real-time PCR (RT-qPCR) – Total RNA was isolated from 30 mg of LV using MiRNeasy Mini kit (Qiagen, USA) according to the manufacturer's instructions. Concentration of RNA samples was assessed using the NanoDrop™ 1000 Spectrophotometer (260/280nm ratio – Thermo Fisher Scientific, USA). First-strand cDNA samples were synthesized from total RNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions. RT-qPCRs were performed in 7500 Real-time PCR System, using Taqman gene expression assays (both for Applied Biosystems Inc, USA), following the manufacturer's instructions. Gene expression was normalized for glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH); assay number 4352339E. Gene expression was measured for *Lc3, P62, Beclin-1, mTOR* and *Mstn* (Life Technologies assays numbers Mm00782868_sH, Mm01265461_m1, Mm00500417_m1, Mm00444968_m, Mm01254559_m1 respectively). The primers of these genes were tested for their efficiency in RT-qPCR reaction. The efficiencies were close to 100%, therefore the 2–ΔΔCt formula was considered for calculating [45] the relative gene expression of *Lc3, P62, Beclin-1, mTOR* and *Mstn*.

Statistics

Results are expressed as means ± SEM. Student T-test was used for two-group comparisons. Statistical analyses were performed using SPSS version 18 for Windows and a probability level of $p<0.05$ was considered statistically significant.

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AUTOR'S CONTRIBUTIONS

GHP was involved in conception and design of the study, data collection, data analysis and interpretation, as well as drafting and editing the final document for publication. MA was equally involved in data analysis, interpretation and reviewing the final document for publication. CC was envolved in data collection (sample preparing, molecular analysis), reviewing and writing paper. NM was involved in conception and design of the study, reviewing and writing paper, data analysis and reviewing final document. SATL was involved in interpretation and reviewing the final document for publication AB, LER and NOC was involved in conception and design of the study, data analysis, interpretation, reviewing and writing all parts of the final document for publication. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Figure legends

Figure 1. (A) Analysis of the Cardiac Hypertrophy by Left ventricular weight/ tibial length ratio (mg/mm). (B) Morphometry by cardiomyocytes diameter (µm). S7: sedentary at day 7; n = 5. T7: trained at day 7; n = 5. S28: sedentary at day 28; n = 12. T28: trained at day 28; n = 15. $^{*}p<0.01$; $^{**}p<0.001$ compared to sedentary group. Data are presented as means ± SEM (Student –t-Test).

Figure 2. Real-time PCR of myostatin gene expression. S7: sedentary at day 7; T7: trained at day 7; S28: sedentary at day 28; T28: trained at day 28. $^{*}p<0.05$ compared to sedentary group. Data were normalized to GAPDH gene and are expressed as mean ± SEM (Student t-Test).

Figure 3. Real-time PCR and western blot analysis of mTOR. S7: sedentary at day 7; T7: trained at day 7; S28: sedentary at day 28; T28: trained at day 28.$^{*}p<0.05$ compared to sedentary group. Data were normalized to GAPDH gene and protein phospho-mTOR was normalized to total and are expressed as mean ± SEM (Student t-Test).

Figure 4. Real-time PCR of autophagic-related genes. S7: sedentary at day 7; T7: trained at day 7; S28: sedentary at day 28; T28: trained at day 28. $^{*}P<0.05$ compared to sedentary group. Data were normalized to GAPDH gene and are expressed as mean ± SEM (Student t-Test).

Figure 5. Western blot analysis of protein expression. S7: sedentary at day 7; T7: trained at day 7; S28: sedentary at day 28; T28: trained at day 28. $^{*}p<0.05$ compared to sedentary group. Data were normalized to GAPDH protein and are expressed as mean ± SEM (Student t-Test).

Figure 1

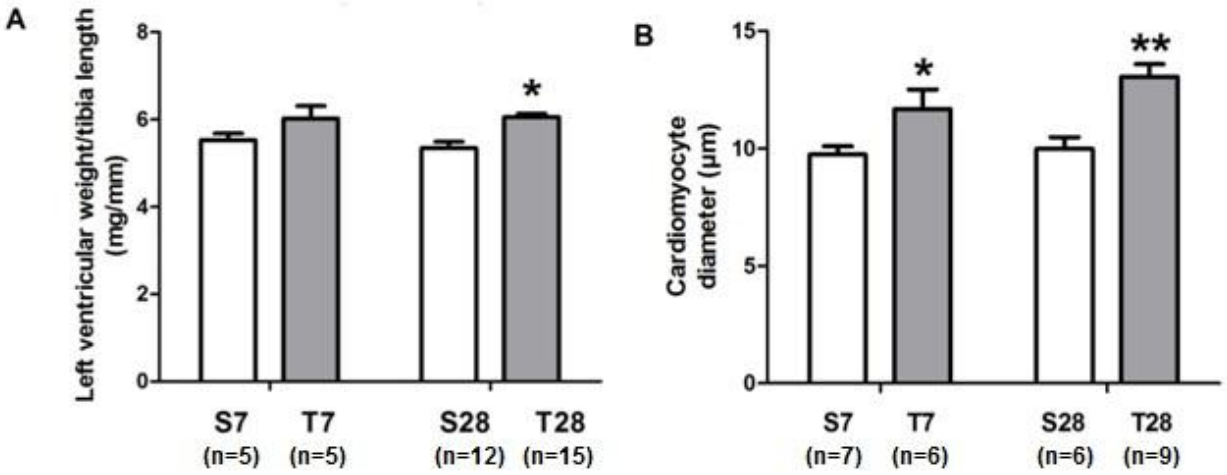

A
Left ventricular weight/tibia length (mg/mm)
8
6
4
2
0
*
S7 (n=5)
T7 (n=5)
S28 (n=12)
T28 (n=15)
B
Cardiomyocyte diameter (µm)
15
10
5
0
*
**
S7 (n=7)
T7 (n=6)
S28 (n=6)
T28 (n=9)

Figure 2

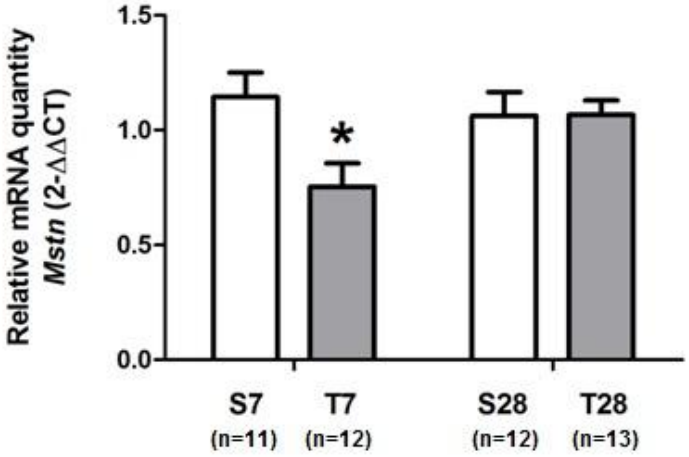

Relative mRNA quantity
Mstn (2-ΔΔCT)
1.5
1.0
0.5
0.0
*
S7
(n=11)
T7
(n=12)
S28
(n=12)
T28
(n=13)

Figure 3

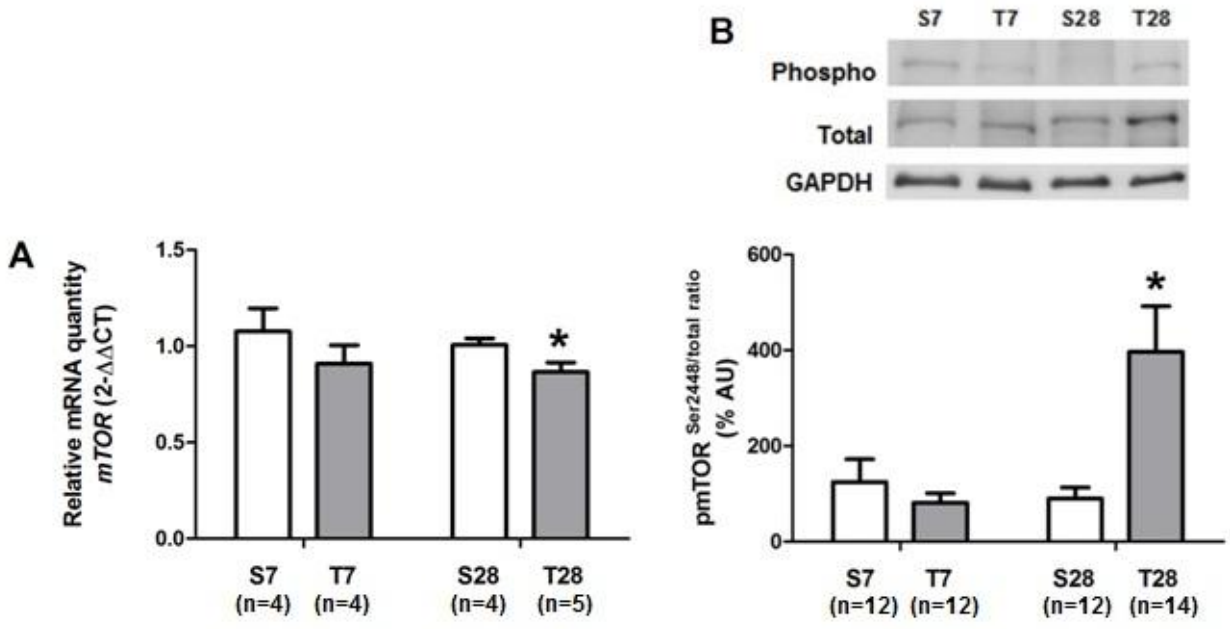

B
S7
T7
S28
T28
Phospho
Total
GAPDH
A
Relative mRNA quantity
mTOR (2-ΔΔCT)
1.5
1.0
0.5
0.0
*
S7 (n=4)
T7 (n=4)
S28 (n=4)
T28 (n=5)
pmTOR Ser2448/total ratio (% AU)
600
400
200
0
*
S7 (n=12)
T7 (n=12)
S28 (n=12)
T28 (n=14)

Figure 4

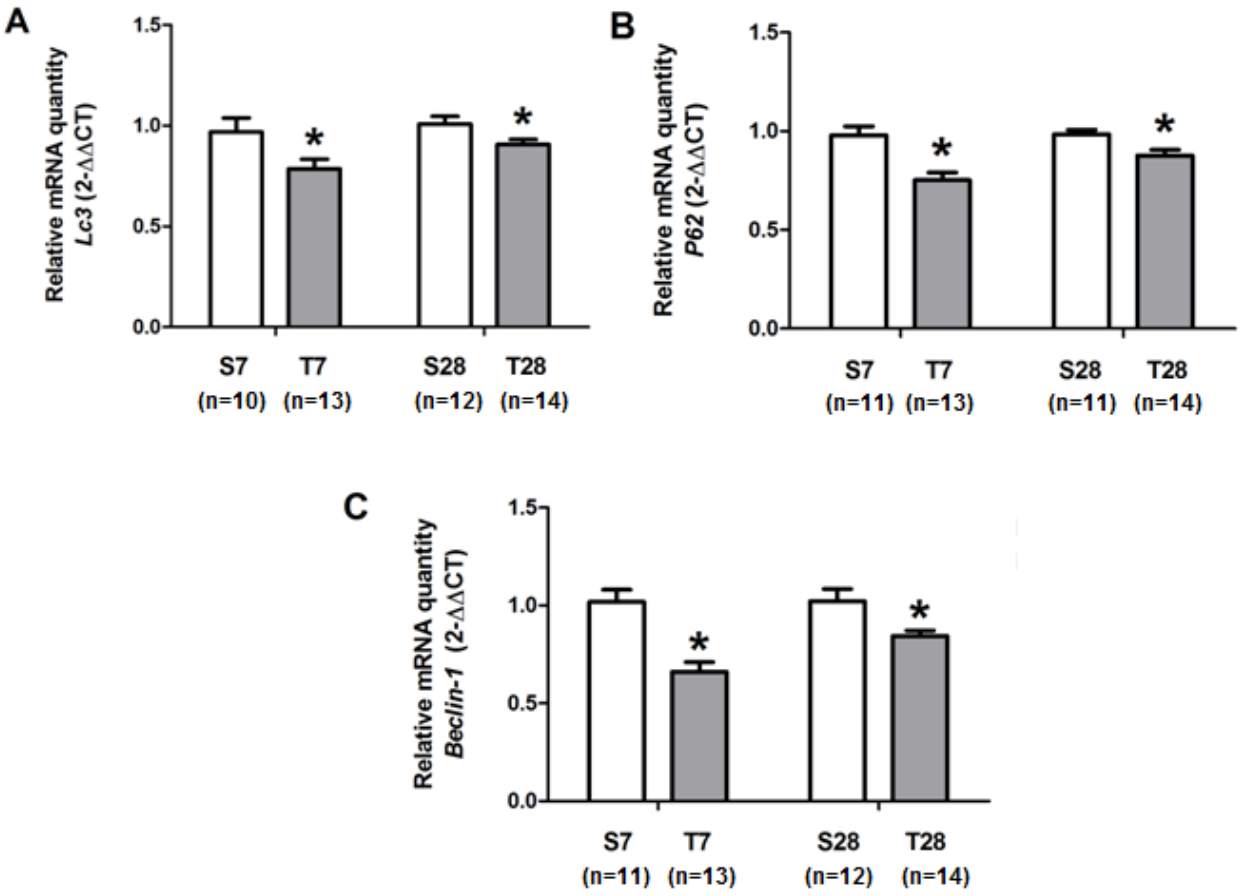

A
Relative mRNA quantity
Lc3 (2-ΔΔCT)
1.5
1.0
0.5
0.0
*
*
S7
(n=10)
T7
(n=13)
S28
(n=12)
T28
(n=14)
B
Relative mRNA quantity
P62 (2-ΔΔCT)
1.5
1.0
0.5
0.0
*
*
S7
(n=11)
T7
(n=13)
S28
(n=11)
T28
(n=14)
C
Relative mRNA quantity
Beclin-1 (2-ΔΔCT)
1.5
1.0
0.5
0.0
*
*
S7
(n=11)
T7
(n=13)
S28
(n=12)
T28
(n=14)

Figure 5

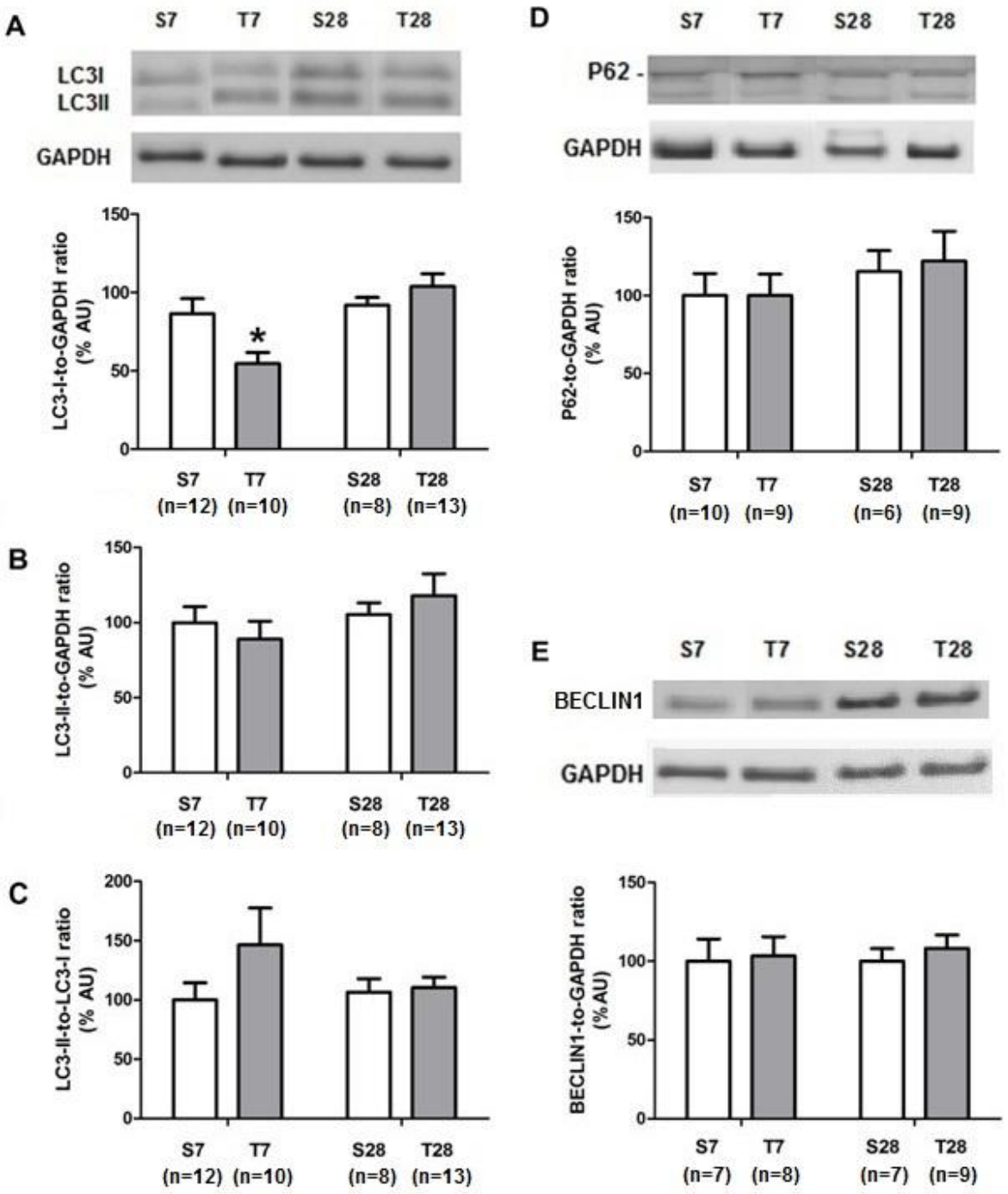

A
S7
T7
S28
T28
LC3I
LC3II
GAPDH
LC3-I-to-GAPDH ratio (% AU)
150
100
50
0
*
S7 (n=12)
T7 (n=10)
S28 (n=8)
T28 (n=13)
B
LC3-II-to-GAPDH ratio (% AU)
C
LC3-II-to-LC3-I ratio (% AU)
200
D
P62
P62-to-GAPDH ratio (% AU)
S7 (n=10)
T7 (n=9)
S28 (n=6)
T28 (n=9)
E
BECLIN1
BECLIN1-to-GAPDH ratio (% AU)
S7 (n=7)
T7 (n=8)
S28 (n=7)
T28 (n=9)

Table:

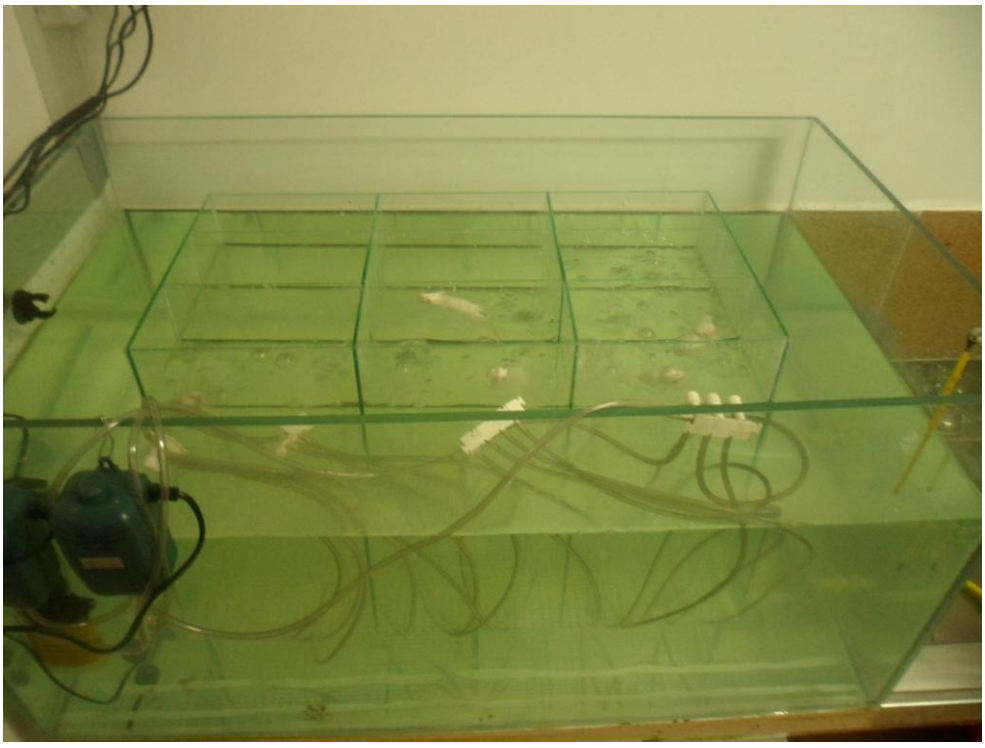
Table1. Morphometric analysis of sedentary and trained animals

	S7 N=11	T7 N=13	S28 N=12	T28 N=15
Body weight (g)	27.0 ± 0.6	26.7 ± 0.3	28.6 ± 0.7	26.0 ± 0.5*
Left ventricular weight (mg)	87.7 ± 2.7	96.3 ± 2.6*	88.4 ± 2.7	100.0 ± 1.6*
Left ventricular weight/Body weight (mg/g)	3.3 ± 0.05	3.6 ± 0.08*	3.1 ± 0.08	3.8 ± 0.3*

Data are presented as means ± SEM (Student t-Test). S7: sedentary at day 7; T7: trained at day 7; S28: sedentary at day 28; T28: trained at day 28; $^{*}p<0.05$ compared to sedentary group.

Supplementary data

Supplementary figure: Swimming apparatus for mouse physical training adapted from Evangelista et al [3]. The system is composed of a glass tank measuring 10 mm thick, 60 cm wide, 100 cm long, and 50 cm in height. The inner tank is made up of glass with 4mm divided into 9 groups with surface area of 30x15 per lane with 35cm depth to allow individual training. To avoid the fluctuation of the animal during swimming, a bubbles system in water pipe connected to an air pump which was coupled to the lower base of the inner tank was used. An automated heating system maintained the temperature between 30 and 32°C with the filter capacity of 420 L/h for cleaning the swimming apparatus.



Artigo Submetido para Revista Autophagy: normas

Manuscript Preparation

Types of Papers

Reviews

The editor will solicit reviews on all topics related to autophagy. In general, the preferred type of review article will be written by multiple investigators from several labs, providing a more thorough, in-depth overview of the literature. Nonsolicited review articles are also welcome. All review articles, including solicited reviews, will be subject to peer review, and publication is not guaranteed.

Research Papers/Reports

Research Papers or Reports should include the following sections in the following order:

- Abstract: A single paragraph of fewer than 250 words. The primary goal of the abstract should be to make the general significance and conceptual advance of the work clearly accessible to a broad readership. References should not be cited in the abstract.
- Keywords: Include 5–10 for indexing purposes.
- Introduction.
- Results: Present results in a logical sequence in tables and illustrations. In the text, explain, emphasize or summarize the most important observations. Units of measurement should be expressed in accordance with Systeme International d'Unites (SI Units).
- Discussion: Do not repeat in detail the data given in the Results section. Emphasize the new and important aspects of the study. Relate observations to other relevant published studies. On the basis of your findings (and others'), discuss possible implications/conclusions for the area an field.

When stating a new hypothesis, clearly label it as such. State the strengths and weaknesses/limitations of the study.

- Patients and Methods/Materials and Methods: Describe the selection of patients or experimental animals, including controls. Do not use patients' names or hospital numbers. Identify methods, apparatus (manufacturer's name and address) and procedures in sufficient detail to allow other workers to reproduce the results. Provide references and brief descriptions of methods that have been published. When using new methods, evaluate their advantages and limitations. Identify drugs and chemicals, including generic name, dosage and route(s) of administration.
- Indicate whether the procedures were approved by the Ethics Committee of Human Experimentation in your country, or are in accordance with the Helsinki Declaration of 1975.
- For reagents listed in the Materials and Methods section, the company that supplied the reagent and the catalog number should be listed in parentheses; do not list the company location.
- References: No more than 125.
- Figure legends must be included.
- Tables: Tables should be numbered consecutively with Arabic numerals and include descriptive titles and legends.

Brief Reports

Brief Reports should constitute unusually interesting data combined with a discussion of what the data might mean, or an explanation of why the data contradicts current paradigms. The primary goal of the abstract should be to make the general significance and conceptual advance of the work clearly accessible to a broad readership. Please include the following:

- Abstract (one paragraph of fewer than 150 words)
- 5–10 key words for indexing purposes

Toolbox

This category is for papers that follow the general guidelines for Research Papers or Brief Reports but have a focus on methodology. The number of references should be commensurate with the length of the article. The

difference between Toolbox and Protocol papers is that the former should include new data in the form of an experiment(s) demonstrating the utility of the approach.

Resource

The Resource category is for papers that provide useful information for the community, but that may not be complete stories, or may not yet have mechanistic information. Examples of appropriate papers include large-scale screens. Resource papers should be written in the style of Research Papers.

Protocol

These are papers that provide detailed descriptions of procedures that may be of use to researchers in the autophagy field. The methodology should be described in sufficient detail, including all of the necessary reagents (along with source and catalog number), to allow the procedure to be repeated by another lab without the need for extensive reference to other publications. That is, the protocol should provide an essentially self-sufficient set of directions, with the exception of standard procedures such as transformation/transfection, etc. However, with regard to determining what is “standard,” keep in mind that a researcher from outside your own field/system may wish to use the protocol. In contrast to Toolbox papers, Protocols do not need to provide new data. The Protocols should be written using a hierarchical numbering system, which makes it easy to refer to specific sections.

Letters to the Editor

Letters to the Editor are aimed at publishing short, but important, breakthrough data not embedded within a complex story. This can also be what is considered a Small Publishable Unit. In other words, data that is sufficient in itself to be

published, but not a part of a larger story that would comprise an entire research article.

Letters to the Editor can also be mini-reviews with an small addition of novel data. The abstract should not be longer than 120 words. The paper should be structured as a research paper (see above), but without the headings and subheadings. No more than 50 references and no more than 4,500 words altogether.

Commentaries and Views

Commentaries and Views may be short and focused opinion articles, commentaries on papers recently published in *Autophagy* or elsewhere, or commentaries on significant conceptual changes, important trends or new directions in the field. These may include figures and up to 30 references. Please include an abstract of 15–200 words and 5–10 key words for indexing purposes.

Puncta

Puncta are essentially auto-commentary. The Editorial Board will solicit authors of the most significant recent and forthcoming papers, published elsewhere, to provide a short summary with additional insights, new interpretations or speculation on the relevant topic; currently, puncta are only being accepted for papers published in journals with an impact factor higher than that of *Autophagy*. The puncta should not include data, but model figures are acceptable; if you wish to include unpublished data, please use the Brief Report format. The first paragraph will constitute the abstract and the text is limited to 1,000 words (not including figure legends, which should be a maximum of 150 words). Puncta will have no references, but the original paper will still be noted on the first page.

Meeting Reports

Meeting Reports are summaries of presentations from recent meetings in the field. Authors are encouraged to contact the Editor-in-Chief with proposals for meeting reports. Also, please contact the meeting organizers to verify that reports will be permitted. Please include an abstract of 150–200 words and 5–10 key words for indexing purposes.

Organization

All manuscripts should be in English. Please ensure that manuscripts are clear, concise and grammatically correct.

Text should be prepared in MS Word, double-spaced, with page numbers throughout and line numbering turned on. Click here for directions on adding line numbers.

Organize manuscripts in the following manner:

- Title page, including titles, author's names (first, MI, last) and affiliations
- 5-10 keywords (for indexing purposes)
- A list of abbreviations and acronyms used throughout the text
- An abstract (please see Type of Paper for word limit), the primary goal of which is to make the general significance and conceptual advance of the work clearly accessible to a broad readership. (References should not be cited in the abstract.)
- Text (length and organization depends upon type of paper)
- Acknowledgments
- References
- Figure legends
- Tables (with descriptive titles and legends)

There are no word limits for papers published, however, accepted manuscripts are published with the understanding that page and color charges will be assessed. Please see the section, Page and Color Charges below.

If your paper is to be published in a journal indexed by PubMed/Medline, the citation of your article will be sent to PubMed within one week of acceptance; therefore, please ensure that all information is correct.

Text Files/Tables

Please save text and table files as MS Word documents. Figure legends should be at the end of the manuscript following references. Tables will be reformatted during production and therefore should only be minimally formatted in your text file and follow the figure legends.

Figure Preparation

Figures should be as small and simple as clarity permits. Unnecessary figures and panels in figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Avoid unnecessary complexity, coloring and excessive detail. Figures should not contain more than one panel unless the parts are logically connected. Where possible, text, including keys to symbols, should be provided in the text of the figure legend rather than on the figure itself. Any image processing should be explained clearly in the Materials and Methods section of your manuscript.

To aid in the processing and turnaround of issues, we ask that authors please adhere to the following figure guidelines. Authors will be asked to revise details and images if they do not adhere to the figure protocols.

Guidelines for Figure Preparation

<u>Image presentation</u>

(These guidelines for image presentation are adapted from the “Instructions for Authors” that are posted on the *Journal of Cell Biology* web site, and are included here with permission).

As you prepare your figures, please adhere to the following guidelines to accurately present your data:

1. No specific feature within an image may be enhanced, obscured, moved, removed, or introduced.
2. The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (i.e., using dividing lines) and in the text of the figure legend.
3. Adjustments of brightness, contrast, or color balance are acceptable if they are applied to the whole image and as long as they do not obscure, eliminate, or misrepresent any information present in the original, including the background. Non-linear adjustments must be disclosed in the figure legend.

A more detailed discussion of image presentation can be found at the following URL:http://jcb.rupress.org/content/166/1/11.full (Rossner and Yamada, J. Cell Biol. 166:11–15)

Resolution

All submitted images must be of high quality and have resolutions of 300 dpi ready for print.

Formats

We require figures in electronic format. Please do not send PowerPoint, MS Word, presentation or paint files as they are inadequate for the creation of high quality images. Much of the information contained in PowerPoint or other file types is lost or skewed in the conversion of images. Figures should be provided as TIF, Photoshop, EPS or high resolution PDF files. Compatible graphic art programs are Adobe Illustrator and Adobe Photoshop.

Figure size

Figures should be submitted at the size they are to be published. Maximum width = 7.1 in. Maximum height = 9.5 in.

For multi-panel figures (such as figure 1a, 1b, 1c, etc.), each panel should be assembled into one image file. Do not include separate panels on multiple pages, i.e. A, B, C and D should all fit on one page. Each panel should be sized so that the figure as a whole can be reduced by the same amount and

reproduced on the printed page at the smallest size at which essential details, including type, are visible and readable.

Color mode

Save all color figures in CMYK mode at 8 bits/channel. Layering type directly over shaded or textured areas and using reversed type (white lettering on a colored background) should be avoided.

Type

Please be sure to embed all fonts. Use a sans serif font such as Helvetica. The font size should be no greater than 9 pt. and no smaller than 6 pt; however, panel labels (A, B, C) should be 15 pt. uppercase (not bold). Lettering in figures (labeling of axes and so on) should be in lowercase type, with the first letter capitalized and no full stop. Please keep font size relatively the same throughout the figures so as to avoid scaling issues. Also note that readability suffers if type is layered over a pattern or color other than white or black.

Units

Units should have a single space between the number and the unit, and follow SI nomenclature or the nomenclature common to a particular field. Thousands should be separated by commas (1,000). Unusual units or abbreviations should be defined in the legend. Please use the proper microsymbol (denoting a factor of one millionth) rather than a lower case u.

References

Include in the reference list only those articles that have been published or have been accepted for publication. All references to unpublished data or personal communications must be cited within the text.

Use the citation-sequence system: The list of references should be numbered consecutively according to the sequence of first appearance within the article text. For in-text references, use only the number assigned to the reference:

Correct: according to Jones.[1]

Incorrect: according to Jones[1].

Correct: noted by Smith et al.[1]

Incorrect: Smith et al (1).

When referring the reader to specific references as part of a sentence please use the following format:

Correct: For a review see refs. 20-25.

Incorrect: For a review see 20-25

Journal References

- The reference format is the same for all of our journals. You may download the output style for *Cell Cycle*from Endnotes, or view it in the CSL Style Repository.
- List, at minimum, ten author names before using “et al.”
- Abbreviate journal names according to the style used in Index Medicus or a comparable source and omit punctuation after journal titles. Spell out foreign or less commonly known journal names.

Standard format:

[Author's last name] [Author's initials], [First ten author's last names followed by their initials]. [Title of article with only the first word capitalized]. [Journal's standard abbreviated name] [Year]; [Volume]:[Inclusive pages].

For Example:

Haegel H, Thioudellet C, Hallet R, Geist M, Menguy T, Le Pogam F, Marchand J, Toh M, Duong V, Calcei A, et al. A unique anti-CD115 monoclonal antibody which inhibits osteolysis and skews human monocyte differentiation from M2-polarized macrophages toward dendritic cells. mAbs 2013; 5: 243–56.

Other Types of References

For all other types of reference styling formats, please refer to the National Library of Medicine Style Guide for Authors, Editors and Publishers which is available here: http://www.ncbi.nlm.nih.gov/books/NBK7256

Supplementary Files

The following fees apply for any supplementary material posted with a manuscript. A $100 fee is assessed for all text, figures and/or tables. Supplementary movies carry a rate of $150 for the first five movie files and $50 for each subsequent movie file. Fees will be outlined on the publication charge form authors receive with galley proofs.

Please provide supplementary material in the following formats:

- **Text**: MS Word file
- **Table/Data**: MS Word file or Excel file
- **Figures**: Please provide figures in a MS Word file or in a PPT file, clearly labeled with figure legends below them.
- **Video Files**: Video submissions for viewing online should be Audio Video Interleave (.avi), MPEG (.mpg, .mp4), or Quick Time (.qt, .mov).
    - AVI files can be displayed via Windows Media Player; MPEG files can be displayed via Windows Media Player; Quick Time files require Quick Time software (free) from Apple
    - Videos should be brief whenever possible (<2-5 minutes). Longer videos will require longer download times and may have difficulty playing online. Videos should be restricted to the most critical aspects of your research. A longer procedure can be restructured as several shorter videos and submitted in that form.
    - It is advisable to compress files to use as little bandwidth as possible and to avoid overly long download times. Video files should be no larger than 5 megabytes. This is a suggested maximum. If files are larger, please contact the Managing Editor.

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- If your paper is accepted for publication you may wish to supply the editorial office with several different resolutions of your video files. This will allow viewers with slower connections to download a lower resolution version of your video.

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