UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

CENTRO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

Analysis of SOX2 expression in Potential Malignant Lesions and Oral Squamous Cell Carcinoma and the role of KLK6-SOX2 gene regulatory network during EMT

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ORIENTADOR: PROF. DR. MARCELO LAZZARON LAMERS

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Dissertação submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Mestre.

ORIENTADOR: PROF. Dr. MARCELO LAZZARON LAMERS

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A presente dissertação apresentada foi desenvolvida em conjunto com o Núcleo de Pesquisa Básica em Odontologia e no Laboratório de Migração Celular (LAMOC) localizados na Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, no Serviço de Otorrinolaringologia do Hospital de Clinicas de Porto Alegre (HCPA) e no Centro de Pesquisa Alemão em Câncer (DKFZ) em programa conjunto com a Seção Experimental e Translacional de Oncologia de Cabeça e Pescoço do Departamento de Otorrinolaringologia, Cirurgia de Cabeça e Pescoço do Hospital Universitário de Heidelberg. Todos participaram ativamente e financeiramente de todas as etapas do trabalho. Seguindo padrão internacional de colaboração a presente dissertação será apresentada em formato de dissertação e em inglês.

O presente trabalho foi submetido à apreciação do comitê de ética em pesquisa do Hospital de Clínicas da Porto Alegre e Universidade Federal do Rio Grande do Sul e teve parecer aprovado sob número: CAAE: 41110814.8.3001.5347.

The presented work was developed together with the Research Center Basic Dentistry and Cell Migration Laboratory (LAMOC) located at the Faculty of Dentistry, Federal University of Rio Grande do Sul, the Service of Otorhinolaryngology in Clinicas Hospital of Porto Alegre (HCPA) and the German Research Center Cancer (DKFZ) in a joint program with the Experimental Section and Translational Oncology Head and Neck Surgery, Department of Otolaryngology, Head and Neck Surgery, University Hospital of Heidelberg. All participates actively and financially in all stages of the work. Following international standards of cooperation this will be presented in dissertation format and in English.

The research proposal was submitted to the ethics committee in research of the Hospital Clínicas de Porto Alegre and Federal University of Rio Grande do Sul acceptance number: CAAE: 41110814.8.3001.5347.

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LIST OF ABBREVIATION

HNSCC - Head and Neck Squamous Cell Carcinoma

OSCC - Oral Squamous Cell Carcinoma

INCA - Brazilian National Cancer Institute

HPV - Human Papilloma Virus

PMOL - Potentially Malignant Oral Lesions

TNM – T: the tumor size, N: the presence or absence of metastasis to lymph nodes, M:distant metastasis.

ECM - Extracellular Matrix

EMT - Epithelial-Mesenchymal Transition

MMPs - Matrix Metalloproteinases

KLK6 - Kallikrein-Related Peptidase 6

IL-1β - Interleukin 1β

TGF-β3 - Transforming Growth Factor β3

IL-6 - Interleukin-6

TGF-β1 - Transforming Growth Factor β1

FISH- *hibridização in situ fluorescente*

p53 - tumor suppressorprotein

HIV - Human immunodeficiency virus

GDP - Guanosinediphosphate

GTP -Guanosine-5'-triphosphate

RhoA - induces the formation of actomyosin stress fibers

Rac1- stimulates polymerization of branched actin networks at the cell periphery

SOX2 – Transcriptional factor

TOPFLASH - TCF-dependent reporter

FOPFLASH - a mutant control plasmid

HCPA - Clinicas Hospital of Porto Alegre

TAE – tumor adjascentepitheluim

CT- Center of tumor

IZ – invasion zone

CEN 3 – centromere of chromosome 3

CEN – centromere

PBS – phosphate buffer saline

miRNA – micro RNA

FaDu - Human head and neck squamous cell lines

FaDu sh-KLK6 - Human head and neck squamous cell lines silenced for KLK6 gene

DAPI - 4',6-diamidino-2-phenylindole, is a fluorescent stain that binds strongly to A-T rich regions in DNA

HPR - The enzyme horseradish peroxidase

RESUMO

O fator de transcrição SOX2 regula a identidade celular em células embrionárias efacilita a reprogramação das células somáticas terminalmente diferenciadas para umestado de células-tronco pluripotente. A diminuição na expressão deSOX2 é um indicativo de pior prognóstico e baixa sobrevida de pacientes com câncer. Nossa hipótese é a de que, durante o processo de carcinogênese do carcinoma oral de células escamosas (OSCC) ocorre uma desregulação negativa na expressão de SOX2, contribuindo para o comportamento invasivo das células tumorais na matriz extracelular. Nós analisamos a expressão de SOX2 em biópsias humanas de carcinoma oral e lesões potencialmente malignas (PMOL). Foram utilizadas biópsias da mucosa oral (livre de lesão, n = 13), hiperplasia (n = 6) e displasias (n = 21), carcinomas orais com elevado (n = 12) ou baixo grau de malignidade (n = 12), os quais foram submetidos à análise de expressão de SOX2 por hibridização *in situ* fluorescente (FISH) e imuno-histoquímica para SOX2 e Vimentina, um marcador de células mesenquimais. Observou-se um aumento na expressão de SOX2 em regiões de proliferação em PMOLe no centro do tumor, mas nas zonas de invasão onde as células passam pelo processo de transição epitelio-mesênquima (EMT), SOX2 apresentou diminuição da expressão. Em seguida, analisamos o possível papel da SOX2 no EMT.Utilizou-se a linhagem celular FaDu, de tumor de cabeça e pescoço (HNSCC) e clones geneticamente modificados, nos quais a expressão da peptidase relacionados com calicreína 6 (FaDu-shKLK6) foi silenciada empregando *short hairpin*RNAs (shRNA). KLK6 pertence a uma família de 15 serino-proteases secretadas, que altera marcadores de EMT e um silenciamento de SOX2. Usando este sistema, os níveis de SOX2 foram restabelecidos e observou-se uma diminuição nos níveis de Vimentina, sugerindo um retorno a um fenótipo de célula epitelial. Utilizando-se de um sistema TOPFLASH-FOPFLASH, observou-se que a expressão de SOX2é inibida por β-catenina, o que sugere um papel da via da sinalização por Wnt para o processo de EMT SOX2-dependente. Os nossos resultados sugerem que, durante as patologias proliferativas, existe uma regulação positiva da expressão SOX2, mas para a indução de EMT é necessária uma regulação negativa de SOX2dependente de β-catenina.

Palavras chaves: SOX2, EMT, KLK6, OSCC, Wnt signaling, β-catenin.

ABSTRACT

The transcription factor SOX2 regulates cellular identity in embryonic stem cells and facilitates the reprogramming of terminally differentiated somatic cells back to a pluripotent stem cell state. The decrease in SOX2 expression is indicative of worse prognosis and poor survival of cancer patients. Our hypothesis is that during the Oral Squamous Cell Carcinoma (OSCC) carcinogenesis process, there is a dysregulation of SOX2 expression, contributing to the invasive behavior of tumor cells in the extracellular matrix. We analyzed the expression of SOX2 in human biopsies of Oral Squamous Cell Carcinoma (OSCC) and Potentially Malignant Oral Lesion (PMOL). Biopsies of oral mucosa (without lesions, n=13), hyperplasic (n=6) and dysplastic (n=21) lesions (PMOLs) and high (n=12) or low (n=12) differentiated HNSCC were used to analyses the SOX2 expression by fluorescent in situ hybridization (FISH). In addition, immunohistochemistry detection of SOX2 and the mesenchymalmarker Vimentin were conducted. It was observed an increase on SOX2 expression at proliferative regions. In PMOLS and center of the tumor but not, at the OSCC invasion zones where cells underwent epithelial-to-mesenchyme transition (EMT) process, SOX2 expression was reduced. Then, we analyzed the possible role of SOX2 in the EMT. We used the well-established OSCC tumor cell line FaDu and genetically modified clones in which the expression of the Kallikrein-related peptidase 6 (FaDu-shKLK6) was silenced employing shRNA. KLK6 belongs to a family of 15 secreted serine proteases, which depletion results in EMT an SOX2 silencing. Using this system, we restored SOX2 levels and observed a decrease in the Vimentin levels, suggesting a return to an epitheliallike phenotype. Using the TOPFLASH-FOPFLASH system, we observed that SOX2 expression is inhibited by β-catenin, which suggest a role for the Wnt signaling for the SOX2-dependent EMT process. Taken together, our results suggest that during proliferative disorders, there is an upregulation of SOX2 expression but it is necessary a β-catenin dependent downregulation of SOX2 for induction of EMT.

Key words: OSCC, Wnt signaling, β-catenin.

INTRODUCTION

The Head and Neck Squamous Cell Carcinoma (HNSCC) is one of the most common types of cancer, with an annual incidence of more than 500,000 cases worldwide(1, 2). HNSCC is a broad term that encompasses epithelial malignancies that arise in the paranasal sinuses, nasal cavity, pharynx, larynx and oral cavity (3). The Oral Squamous Cell Carcinoma (OSCC) is a well-known malignancy which accounts for 80% to 95% of cancers in the mouth (4). The OSCC is the eighth most common cancer in the United States and account for about 2.3% of cancer cases. In 2013, it was estimated to occur 41,380 new cases of oral cancer in US resulting in 8,000 deaths (1). According to the Brazilian National Cancer Institute (INCA), OSCC in 2014, it was estimated 11,280 new cases in men and 4,010 in women, which corresponds to an estimated risk of 11.54 per 100,000 men and 3.92 per 100,000 women (Brazilian MINISTRY OF HEALTH, 2014).

Standard treatment for OSCC consists of surgery and radiotherapy as a local approach and adjuvant chemo- or immunotherapy as a systemic approach for advanced tumors. The surgical process often results in mutilation, due to the infiltrative behavior of the tumor. The overall 5-year survival rate in OSCC has not significantly increased in the last few years (4). Only 40-50% of patients with an advanced disease will survive after five years from the primary treatment. This aggressively is mainly attributed to the heterogeneity and plasticity of cancer cells, which is a prerequisite for their clonal selection and expansion during therapy (5).

The etiology of oral cancer is multifactorial, consisting of endogenous factors such as genetic predisposition and environmental factors and exogenous behavioral, such as tobacco and alcohol consumption, exposure to solar radiation and carcinogenic chemicals (6, 7). The mechanisms by which tobacco and alcohol induce transformation and malignant progression of epithelial cells in the OSCC are not fully understood (7, 8). Alcohol acts as a permeabilization agent to the oral mucosa allowing the penetration of carcinogens contained in cigarette. Then, the carcinogenic agents present in tobacco can cause changes in the DNA resulting in changes in the structure and thus causes mutations. For example, loss of function of the p53 gene,

which encodes a tumor suppressor protein (p53) and interrupts the cell cycle, results in failure in cell cycle regulation and apoptosis impairment, leading to uncontrolled cell proliferation and perpetuation of new mutations (9, 10). However, despite most tobacco and alcohol users do not develop OSCC, the evidence of exposure to the common risk factors underscores the complexity of tumorigenesis and the important role of gene-environment interactions in the tumorigenic process.

Another recently risk factor particularly for oropharyngeal carcinoma is human papilloma virus (HPV) (8, 9), which is more prevalent in people who have a sexual life very active at a young age (11). HPV proteins such as E6 and E7 have a negative effect on cell proliferation, since they act in p53 ubiquitination or in pRB inactivation, and may mediate the anchorage independent growth by p63 degradation (12). Additionally, other factors may be considerate for the development with OSCC: deprivation of nutrients; defects in immunity, anyone who is chronically immunocompromised or HIV/AIDS; drugs and diseases (Fanconi anemia, dyskeratosiscongenita, xerodermapigmentosum, Li Fraumeni syndrome, lupus erythematosus, diabetes and scleroderma) (7, 8, 11).

Potentially Malignant Oral Lesions and OSCC histological features

The initiation and progression of head and neck cancer is a complex and multistep process that entails a progressive acquisition of morphological, genetic and epigenetic alterations (13). Many of these alterations occur early in tumorigenesis and result in proliferative disorders known as Potentially Malignant Oral Lesions (PMOL), which are identified clinically as a white (leukoplakia) or red (erythroplakia) plane that is not removable to scrap and might show as an increased volume nodule or as an ulcer that does not heal (14).

Histologically, PMOLs may have different levels of epithelial alterations that are classified as hyperkeratosis, hyperplasia and epithelial dysplasia and carcinoma *in situ*(15, 16). The hyperkeratosis and non-dysplastic epithelial hyperplasia are among the most common occurrences of histological diagnosis of leukoplakia, which is clinically subdivided in a homogeneous type (flat, thin,

uniform white in color) and a non-homogeneous type (white-and-red lesion, that may be either irregularly flat or nodular and was designated of “erythroleukoplakia” (14). Dysplasia are more likely to turn into an oral cancer and are characterized by a proliferation of epithelial layer followed by a subsequent degradation of the basal lamina (17). Due to altered proliferation rates, it is possible to observe microscopically changes in the epithelium such as: irregular epithelial stratification, basal layer hyperplasia, epithelial projections in form of drops, increased number of mitotic figures, loss of polarity of the basal layer cells, increased nuclear-cytoplasmic ratio, nuclear polymorphism, hyperchromatic nuclei, increase in the size of nucleoli, isolated cells or keratinization groups in the prickle cell layer and reduction of intercellular adhesion (14). The dysplasia degree is dependent of the structural and cytological changes. Mild dysplasias aredefined when the tissue architecture is limited to the lower third of the epithelium and have cytological atypia; moderate dysplasia, when two to four features are present and; severe dysplasia, when the architectural involves more than two thirds of the epithelium and five or more of the afore mentioned characteristics are present (14, 17). For *in situ* carcinoma, it is observed all of the architectural disturbance and cellular characteristics mentioned above, but without the disruption of basal lamina and the invasion of the underlying connective tissue – FIGURE 1(14, 18).

OSCC is graded histologically as well, moderately, or poorly differentiated carcinoma. According to the criteria established by Bryne(19) in 1992, the well-differentiated tumors contain orderly stratification and heavy keratinization in a pear formation; less than 20% of undifferentiated cells, which are cells that have not undergone the process of cell differentiation. Moderately differentiated tumors have less than 50% of undifferentiated cells, some stratification, and less keratinization. Poorly differentiated tumors are still recognizable as squamous cell carcinomas but manifest prominent nuclear pleomorphisms and atypical mitosis (13). The TNM classification system considered the size of the tumor (T) the presence or absence of metastasis to lymph nodes (N) and distant metastasis (M). From the combination of these parameters, the tumors are classified into stages where the first one presents a better prognosis, since tumors are smaller and without the presence of

metastasis, while stage IV has the worst prognosis due to the tumor size and/or the presence of metastasis (20).

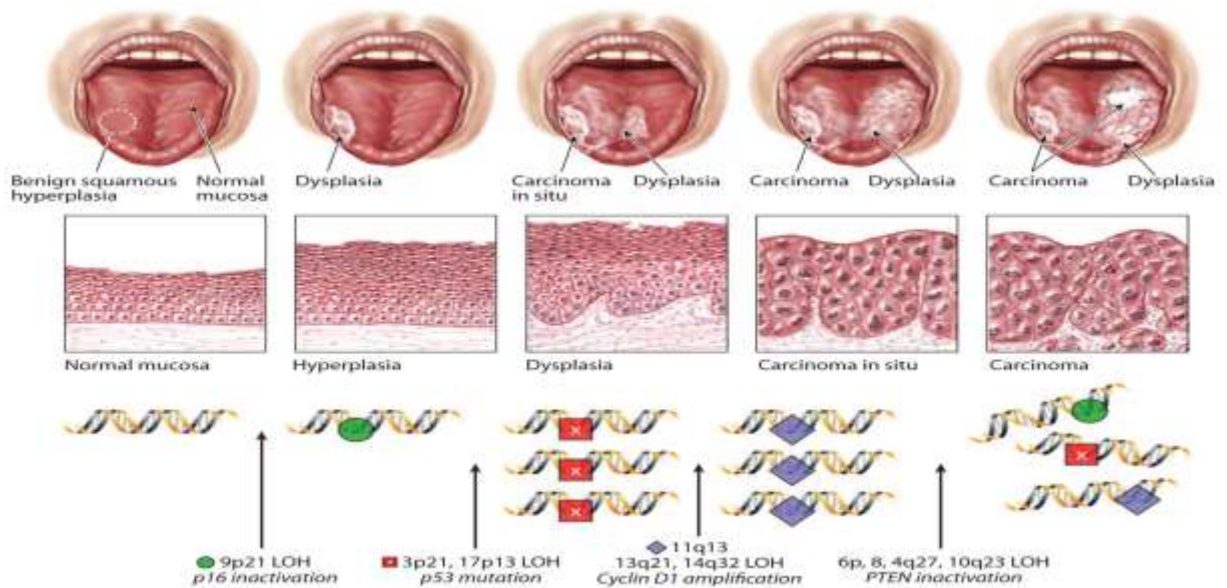

Figure 1: The multiple stages of carcinogenesis process in OSCC tumorigenesis. The first step starts from a PMOL and a stepwise acquisition of mutations and differentiation of cells, passing through epithelial hyperplasia, dysplasia stages until the formation of carcinoma *in situ* and more aggressive tumor stages. Figure from Pai, S. I. &Westra, W. H (21).

OSCC tumorigenesis and invasion

The origin of the precursor cell which gives rise to PMOLs and OSCC is uncertain but several studies hypothesize that stem cells can drive a start of the oral lesions. For instance, proliferative cells are present in the basal and parabasal layers of the oral epithelium contain one progenitor cell compartment. This progenitor cell compartment comprises two basic activities: a smaller population of tissue-specific stem cells and the larger population transiently in cell proliferation and differentiation (6, 22, 23). The stem cells contain the genomic information of the oral epithelium, are undifferentiated but have the ability to differentiate, divide and possess unlimited capacity for self-renewal (6). The maintenance of these stem cells is done through an asymmetric mitotic division that will give rise to two cells from which one will remain as stem cells in the basal layer, and other begins to differentiate into mature keratinocytes and continue the upper layers of the epithelium (6).

Another possibility for OSCC tumorigenesis is from a mature keratinocyte which has undergone cytogenetic alterations resulting in its dedifferentiation into

the analogue of an immature progenitor or stem cell which can express the deregulated intracellular pathways and transcription factors of a tissue-specific cancer stem cell phenotype (6). The keratinocytes genetically altered arise of similar or dissimilar clonal origin, make up a field of proliferative disorders in epithelium which clinically appears normal, but microscopically may show epithelial dysplasia or other proliferative lesion fields in the different anatomical site (6). PMOLs frequently develop from a proliferative disorder of epithelium which contain keratinocytes at different stages of transformation. These proliferative disorders fields, upon acquisition of additional genetic alterations, may become a carcinoma (6, 24).

The acquisition of an invasive and a metastatic behavior is a hallmark of cancer (25) and might be considered as one of the main lead of clinical failure. Invasion of OSCC involves a multistep process that in consequence of breaking of the basement membrane, cell migration through the extracellular matrix (ECM), extravasation into regional vasculature and extravasation at the metastatic site (26). Tumor cell motility might occur as individual or collective depending on intercellular signaling events. Cell migration is a coordinated phenomenon that requires a hierarchical activation of structural and signaling molecules. These molecules will determine a spatial asymmetry, generating a polarized morphology. The migration process takes place in a form of hierarchical and temporally defined switching between adhesion molecules (turnover), which are anchored in contractile bundles of actin-myosin II (stress fibers) and generate contractile forces, which facilitate movement of the body cell in a certain direction. The cycle ends with the release of adhesions in the posterior portion of the cell (27-32).

Most of cell migration events are regulated by low molecular weight GTPases belonging to the Rho family (Ras-homology). These proteins change from an inactive state (connected GDP) to an active state (bound to GTP). When activated, exert their effects through a vast amount of effector proteins. The most expressed GTPases is the Rac1 protein, which is mainly engaged in lammelipodium and focal complex formation (immature adhesions) while RhoA is involved in formation of stress fibers in the maturation of adhesions and cellular contractility (33, 34). The balance between these two GTPases determines cellular morphology and migratory behavior (35, 36). Activation of

Rho GTPase, RhoA and Rac1, particularly, are associated with increased invasiveness and migration of different tumor and high expression of these proteins has been associated with poorer prognosis of patients (37, 38). However, the molecular mechanisms that elicit the migratory behavior of tumor cells are still unknown.

All the events necessary for tumor invasion and metastasis rely not only in intrinsic properties of tumor cells, but also by cell-cell and cell-ECM elements, which are known as the tumor microenvironment (39, 40). The main cells that form the tumor microenvironment are fibroblasts; tumor associated fibroblasts; myofibroblasts and smooth muscle cells; endothelial cells; pericytes; macrophages and other cells of the immune system (17). The effects of tumor microenvironment on tumor biology are broad. For instance, studies have shown that inflammatory cells and their cytokines affect the migratory behavior of tumor cells indicating that there is a relationship between secretion of their products and early epithelial-mesenchymal transition (EMT) through activation of different transcription factors as the NF-kB, STAT-3 and Snail (41). Also, the action of matrix metalloproteinases (MMPs) from the tumor as well as from the microenvironment cells (42, 43) is necessary to basement membrane degradation(44). Since most of these factors are found at the invasive front, the interaction of tumor cells with elements of its microenvironment might pave pathways in the stroma for invasive tumor cells (21).

Our research group demonstrated that elements of the tumor microenvironment might elicit differential migratory performance according to the differentiation level of OSCC cells. For instance, the switch from a laminin (similar to normal epithelia) to a fibronectin (as observed in the connective tissue) rich environment increases the migration speed and directionality of low differentiated OSCC cells by modulating adhesion properties and Rac1 mediated signaling (45). Also, Stat3 activation by IL-6 stimuli also contributes to the migration performance in both low and highly differentiated OSCC cells (unpublished results). Since the OSCC is characterized by a heterogeneous population of cells, the understanding of the interaction of genetic reprogramming observed during EMT, elements of tumor microenvironment and the cellular events observed during OSCC invasion are a key step to search for biomarkers and new therapeutic strategies.

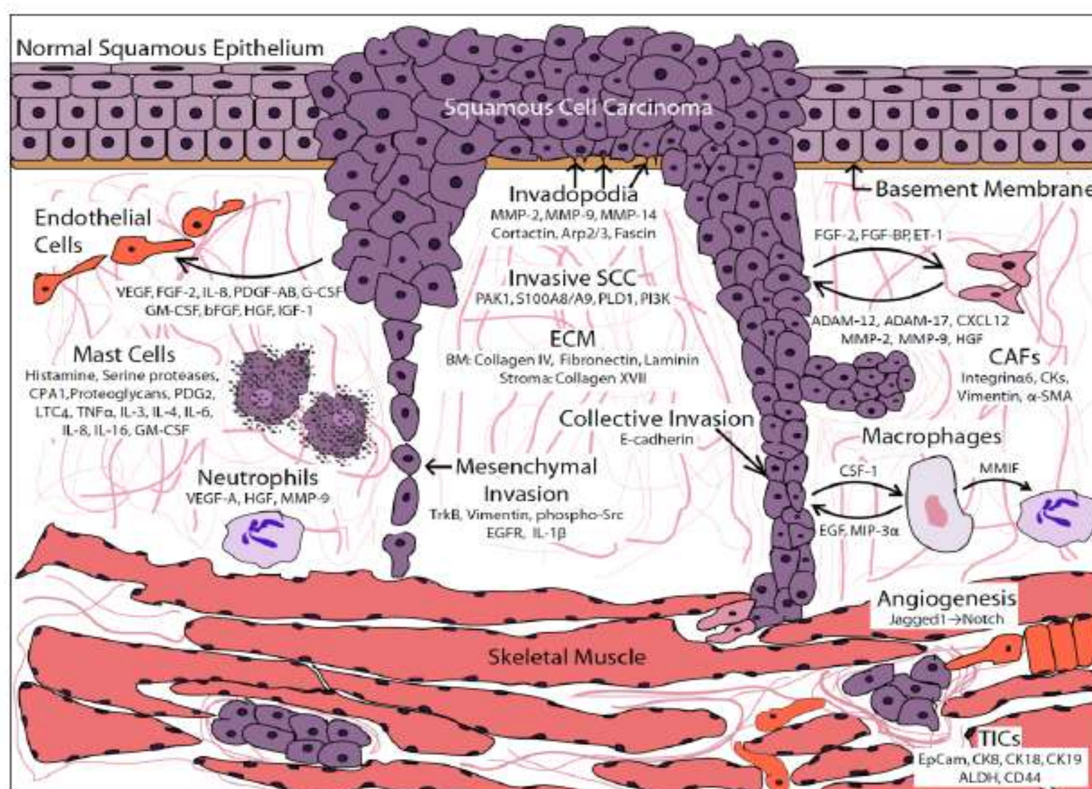

Figure 2: Diagram of tumor and stromal-based contributions that promote HNSCC invasion. Contribution of cells to the basement membrane degradation and tumor cell infiltration. Participation of tumor microenvironment with inflammatory cells, endoletilais cells, cytokines, integrins, interleukins, and other factors that contribute to the process of migration of tumor cells. Figure from Markwell SM & Weed SA(26).

Epithelial to mesenchymal transition

Under physiological conditions, epithelial cells establish close contacts with their neighbors and show a polarity in the apicobasal axis due to the sequential arrangement of adherent junctions that act as barriers during absorptive process. The epithelial cell layer maintains global communication through gap junction complexes, and it remains separated from adjacent tissues by a basal lamina. In order to invade the connective tissue, epithelial derived-tumor cells acquire a transient mesenchymal state, which is responsible by the capacity of cells to migrate to distant places, maintain stemness and initiates metastasis (46).

The EMT involves profound phenotypic changes that include the loss of cell-cell adhesion and cell polarity (47), due to changes on epithelial markers expression. E-cadherin, occludins, and cytokeratins are the most commonly used markers for the epithelial profile and N-cadherin and Vimentin for the

mesenchymal state. However, some malignant cells do not display specific epithelial nor mesenchymal phenotype during EMT (26). Recent data demonstrated there is two distinct subpopulations in HNSCC tumors, in which E-cadherin and Vimentin are inversely expressed. These two subpopulations demonstrate plasticity as well as both recovered cellular heterogeneity in culture and xenograft tumors derived from single subpopulations (26, 48-50).

Loss of E-cadherin is considered a fundamental step in the progression to invasive carcinoma cells and it is also a fundamental event in EMT (51), with the ability to influence the clinical course of the disease. For instance, tumors cells that are resistant to conventional chemotherapy are associated to EMT process (52, 53). Also, colon carcinoma epithelial cell lines that become resistant to Oxaliplatin exhibited a mesenchymal morphology and expressed several markers of EMT (54). The resistance of ovarian carcinoma epithelial cell lines to Paclitaxel is also associated with the acquisition of EMT markers and loss of the epithelial phenotype (55).

Cells need to undergo several molecular process in order to initiate an EMT process, for example: activation of transcription factors, expression of specific cell-surface proteins, reorganization of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs (46). Additionally, the switch in the expression of EMT markers might also contribute to modulate the behavior of tumor cells. For instance, loss of E-cadherin promotes modifications of chromatin structure and in the expression of many genes regulated at the transcriptional level, posttranslational modifications and control their nuclear localization or degradation. The loss of E-cadherin at cell-cell junctions enhances signaling resulting in increase of cytoplasmic β-catenin (56). The repress for E-cadherin transcription activate some of the direct repressors genes and also have multiple specific targets (47). Recent data showed the correlation between the transcription factor SOX2 and the Kallikrein-related peptidase 6 (KLK6) as one way to regulators of EMT in HNSCC (52, 57).

The EMT process might also be influenced by the tumor microenvironment. For instance, lung adenocarcinoma cells, after being stimulated by interleukin 1β (IL-1β) and transforming growth factor β3 (TGF-β3), significantly reduced the expression of E-cadherin and increased the migration

capability and invasion in different matrices (58). Studies with tumor cell lines of the biliary tract have shown that stimulation by interleukin-6 (IL-6) and transforming growth factor β1 (TGF-β1) increased the expression of cytokines (autocrine effect) and Vimentin and decreased the expression of E-cadherin, which resulted in increased migration and invasion capacity (59). IL-6 is also capable of inducing EMT in tumor cell lines from head and neck, also leading to increased expression of vimentin and decreased expression of E-cadherin (60).

The Kallikrein-related peptidase 6 (KLK6) is a member of a gene family mapping to chromosome 19q13.3 – 13.4, responsible for 15 secreted serine proteases with trypsin or chymotrypsin-like activity (52, 61). In normal physiology, these proteins also cleave a number of substrates including matrix metalloproteases (MMPs), insulin-like growth factor binding proteins (IGF2BPs), latent transforming growth factor β (TGF β), fibronectin and collagen(61). KLK6 up-regulation in human cutaneous skin cancer is associated with malignant progression. KLK6 expression in a mouse keratinocyte cell line promotes cell proliferation, migration, and invasion, most likely due to impaired E-cadherin–mediated cell-cell adhesion and beta-catenin accumulation in the nucleus (62). KLK6 is able to degrade components of the extracellular matrix *in vitro* and participates in physiological cellular processes (52, 61, 63, 64).

The research group Section Experimental and Translational Head and Neck Oncology at the University of Heidelberg and Molecular Mechanisms of Head and Neck Tumors at the German Cancer Research Center identified SOX2 and the KLK6 as novel key regulators of EMT in HNSCC (29, 36). Reduced expression of both proteins in established HNSCC cell lines was associated with a mesenchymal-like phenotype *in vitro* and *in vivo*, which is at least in part due to activation of Wnt/β-catenin signaling (29, 36) (unpublished data). Moreover, immunohistochemical staining of tissue microarrays with tumor specimens of a retrospective HNSCC patient cohort confirmed that low SOX2 or KLK6 expression are correlated with reduced progression-free and overall survival (29, 36).

Genetic markers of EMT

The number of acquired genetic alterations progressively increases from squamous hyperplasia through graded dysplasia to invasive carcinoma. The

progressive accumulation of genetic alterations may affect critical components of key genetic pathways regulating cell growth, motility, and stromal interaction and this genetic damage often precedes microscopic changes (21, 65). The accumulation of multiple genetic and epigenetic alterations is a multistep process that involves mutations, deletion and *de novo* promoter methylation gene of tumor suppressor regions, and amplification or overexpression of oncogenes. Several studies have focused on molecular alterations and chromosomal disorders such as primary target (66-69). Although it is not understood what factors are involved in the regulation of oncogenes and tumor suppressor genes, the genetic polymorphisms, inflammation and angiogenesis mechanisms may be associated with the development and progress of HNSCC (70-72).

The abnormal expression of transcription factors also contributes to the EMT process. For instance, Snail and Zeb factors induce the expression of metalloproteases that can degrade the basement membrane, thereby favoring invasion (47). More recently, it was seen that genetic imbalances on the transcription factor SOX2 (3q26.3-q27) is related to several types of cancer. The SOX gene family is involved in the biology of stem cells during embryonic development by encoding a major transcription factor involved in the functions of stabilizing embryonic stem cells into pluripotent state and reprogram somatic cells. SOX2 is critical for differentiation of tongue, esophagus, stomach, lung, retina and brain (73). It has been proposed that SOX2 may mark the tumor stem cell population in cancers and it is probable related to the tumor relapse and to regrowth of tumor at the metastatic site(74). Up regulated SOX2 expression in several types of cancer is correlated with poor survival outcome of patients (71). Paradoxically, low SOX2 expression was shown to be correlated with poor outcome of HNSCC patients and demonstrate higher risk for treatment failure due to more aggressive and invasive behavior of cancer cells (57).

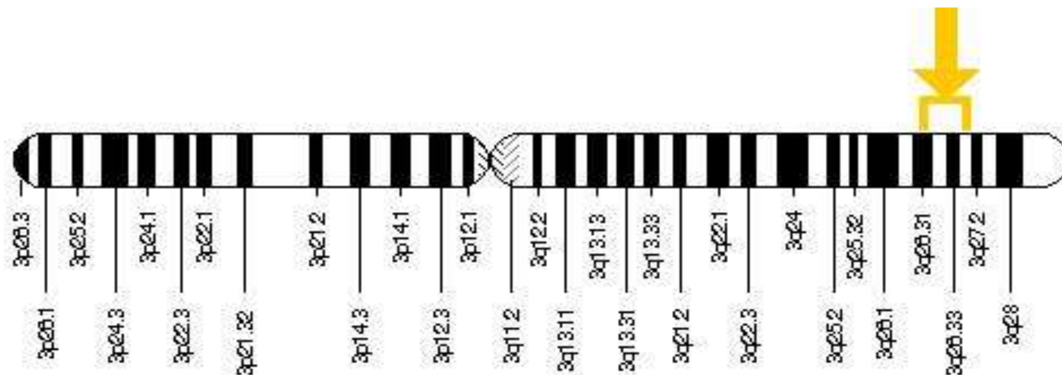

Figure 3: Cytogenetics Location SOX2 in the regionSRY: 3q26.3-q27. Adapted: Genetics Home Reference.

However, it is important to note that for the cell to migrate and form new tumor focus, it needs to somehow lose the characteristic of embryonic cell and invade the tissue to promote a new invasive focus. It was demonstrated that when carcinoma cells are muted for SOX2 they show a greater migration profile (57). Since the EMT is a plastic process (53), it is possible that the deletion or downregulation of SOX2 might be a determinant of a mesenchymal-like state, and this feature might help to explain the failure on cancer treatment due to cellular heterogeneity.

Since the OSCC presents a heterogeneous cell population that responds differently to stimuli the tumor microenvironment, it is possible that the profile of gene transcription factor expression is differentially expressed during the process of tumorigenesis and in the tumor, itself with the potential of influence invasiveness. The understanding of this genetic relation to the behavior of cells can lead to the development of possible strategies for tracking and improving recurrences and even pursue a strategy of personalized treatment.

HYPOTHESIS

SOX2 expression is deregulated during oral carcinogenesis process, which might contribute to the proliferative behavior of premalignant lesions and/or tumor cells.

AIM:

To analyze the SOX2 expression levels in Oral Squamous Cell Carcinoma and Potentially MalignantOral Lesions and address the main players involved in SOX2 regulation.

SPECIFIC AIMS:

1. To analyze SOX2 expression levels in normal mucosa, hyperplastic and dysplastic epithelia (PMOLs) and low invasive and highly invasive OSCC;

2. To correlate the SOX2 expression with the differentiation level of the lesions;

3. To analyze the role of KLK6, SOX2 and β-catenin during the EMT process.

CHAPTER 1

This chapter is based on results data from patient samples. The work was conducted in Laboratoryof Cell Migration (LAMOC), in the Dentistry Faculty of Federal University of Rio Grande do Sul, and it was performed under supervision of Dr. Marcelo Lazzaron Lamers. This paper would be revised and submitted at a high impact factor after complete the review.

SOX2 expression is deregulated during Oral Squamous Cell Carcinoma progression

SOX2 as a potential regulatory gene of invasion process in the recurrence and metastasis of Head and Neck Squamous Cell Carcinoma

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Unraveling the underlying molecular principles of SOX2 will not only help to develop innovative strategies for more efficient and less toxic therapy of HNSCC patients, but could also pave the way for biomarker-driven clinical trials in the future. The scientific and translational impact of the expected data could also be valuable for other human malignancies with comparable etiology, risk factors or treatment modalities. The results of this study will open a new perspective in achieving better prognosis and less toxicity therapy.

ABSTRACT

Oral Squamous Cell Carcinoma (OSCC) is a highly malignant tumor, showing an aggressive phenotype with high recurrence. It is one of the most common types of oral cancer and can develop from Premalignant Lesions (PMOL) of epithelial origin, such as hyperplastic and dysplastic lesions. Despite improvements in treatment, clinical prognosis of these patients remains low primarily due to the migratory and invasive behavior of tumor cells. The SOX2 transcription factor is a potential factor controlling the development of OSCC, due to its role in cell fate definition of embryonic stem cells in oral epithelia. The aim of this study was to analyze SOX2 expression in PMOL and OSCC samples and its role in tumor invasion. To that end, we used biopsies samples for immunohistochemistry and in situ hybridization fluorescence. Data indicate that there is an inhibition of gene expression during tissue invasion and after establishment of new tumor foci, followed by relief and re-establishment of protein levels similar to those found in healthy epithelium. This behavior can be regulated by environmental and genetic factors, as well as by tumor microenvironment itself.

KEY WORDS: OSCC, Potential Malignant Oral Lesions, EMT, FISH.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) corresponds to 95% of oral tumors and it is one of the most common and lethal human malignancies worldwide [1,2]. The etiology of oral cancer is multifactorial, consisting of endogenous factors such as genetic predisposition, environmental and exogenous behavioral factors, such as tobacco and alcohol consumption, exposure to solar radiation and HPV[3,4]. Some lesions, known as potentially malignant oral lesions (PMOL), may precede oral cancer, although not all necessarily progress to cancer. PMOLs are characterized by an initial proliferative disorder of epithelial cells that is accompanied by increasing levels of morphological changes due to accumulation of mutations [5]. Then, cells develop an infiltrative and migratory behavior which results in invasion, loco-regional or distant metastasis characteristic of OSCC [2,6-8]. Treatment failure in OSCC patients is mainly attributed to the heterogeneity and plasticity of cancer cells, which is a prerequisite for their clonal selection and expansion during therapy [9]. The survival rate for OSCC changed only marginally in the last decades, despite major improvement in the treatment of other types of human malignancies [10,11].

The epithelial to mesenchymal transition (EMT) is characterized by a set of well-established morphological and molecular features that drives the invasive behavior of epithelial-derived cancer cells [12]. The functional hallmark of the physiological EMT program is to allow stationary epithelial cells to acquire the ability to migrate and invade during developmental morphogenesis [13-15]. However, tumor cells are able to oscillate not only between the full epithelial and full mesenchymal states, but rather, they move through a spectrum of

intermediary phases [16]. This cell plasticity observed during the EMT process is known as a driver of epithelial-derived tumor malignancies [17,18]. For instance, the enhancement of mesenchymal-like features may epigenetically reprogram epithelial cancer cells to better adapt to new microenvironments and thus may contribute to local or distant recurrence [19,20].

During the past few years it has been shown that genetic imbalances on the transcription factor SOX2 are related to several types of cancer. SOX2 is involved in many physiological actions including normal development and pathological processes that have an important impact on epithelial cell behavior [7,21]. It is also involved in the proliferative activity [22], anchorage-dependent growth[23], drug resistance [20], and metastasis[24]in several cancer cell types. Up-regulated SOX2 expression is correlated with low survival rates of patients with many types of cancer [20]. Paradoxically, low SOX2 expression was shown to be correlated with poor outcome of Head and Neck Squamous Cell Carcinoma (HNSCC) patients, displaying higher risk for treatment failure due to a more aggressive and invasive behavior of cancer cells [25]. These data indicate a heterogeneity among tumor cells in which individual cell behavior relies in pleiotropic signaling factors that regulate the expression of specific transcription factors and consequently influence cell responses such as proliferation or migration [11,16].

The molecular mechanisms that lead to a potentially malignant lesion to evolve and become a carcinoma with invasive/infiltrative profile are still poorly understood. It is possible that the association of extrinsic factors, such as components of tumor microenvironment, with intrinsic tumor changes, such as regulation of oncogenes and genetic polymorphism, might result in the invasive

phenotype. However, the detection of potential biomarkers for this switch in tumor cell behavior is still unknown. It has already been shown that SOX2 is not only involved in embryonic self-renewal but also in cancer stem cells [26]. Our hypothesis is that SOX2 regulation varies according to the degree of aggressiveness of oral lesions and its deregulated expression may contribute to the invasive behavior of tumor cells.

Herein we analyzed the expression profile of the transcription factor SOX2 in OSCC, potentially malignant, and normal oral mucosa biopsies. It was observed that proliferative disorders show increased SOX2 expression. However, SOX2 expression is severely lost in cells at the invasion front of the invasion indicating a possible role of SOX2 as a negative regulator of tumor invasiveness.

MATERIALS AND METHODS

Patient Samples

All protocols were approved by the ethics committee from UFRGS and Hospital de Clínicas de Porto Alegre (HCPA) (CAAE: 41110814.8.3001.5347) and samples were obtained from the biorepository at the Basic Research Nucleus of the Dentistry School at UFRGS. Cases of oral mucosa (n=13), potential malignant lesions and oral squamous cell carcinoma were selected (Table1). The cases of PMOL were histopathologically separated into hyperplasia (n=6) or dysplastic lesions (n=21). The histopathology grading of OSCC cases was performed according Barnes, 2005[29]and Kujan, 2006[28]. The following morphological characteristics were evaluated: degree of keratinization, pleomorphic nuclei, invasion pattern and lymphocytic infiltration. Each feature

received a score of 1 to 4 and according to the total sum of the scores. Each OSCC case was sorted into the following grades of malignancy: OSCCI (sum=4-12) or OSCCII (sum=13-16). For analysis of immunohistochemistry labeling, both OSCC groups were analyzed at three specific regions: tumor adjacent epithelia (TAE), center of the tumor (CT) and invasion zone (IZ), where the IZ was considered as the last six tumor cell layers nearest connective tissue[27].The following morphological characteristics were evaluated: degree of keratinization, pleomorphic nuclei, invasion pattern and lymphocytic infiltration. Each feature received a score of 1 to 4 and according to the total sum of the scores. Each OSCC case was sorted into the following grades of malignancy: OSCCI (sum=4-12) or OSCCII (sum=13-16). For analysis of immunohistochemistry labeling, both OSCC groups were analyzed at three specific regions: tumor adjacent epithelia (TAE), center of the tumor (CT) and invasion zone (IZ), where the IZ was considered as the last six tumor cell layers nearest connective tissue[27]. All paraffin blocks were submitted to sequential cut in microtome (5µm), cleared in xylene, hydrated in decreasing ethanol concentration and phosphate-buffered saline (PBS) solution. Then, slices were submitted to hematoxylin/eosin staining, Fluorescence In Situ Hybridization or immunostaining for SOX2 or Vimentin.

***Table 1:* Distribution of samples and general characteristics of the lesions.**

Biopsy	Region	Gender	Age Average	Ethnicity
Mucosa (n=13)	Gingiva / alveolar ridge (9) Lip (3) Unknown (1)	F(6) M (7)	40	Afro-descendant (4) Caucasian (9)
Hyperplasia (n=6)	Tongue (1) Gingiva / alveolar ridge (3) Unknown (2)	F (5)	45	Afro-descendant (1) Caucasian (4) Unknown (2)
Dysplasia (n=21)	Tongue (9) Gingiva / alveolar ridge (5) Lip (1) Palate (2) Jugal mucosa (2) Unknown (2)	F(10) M (9) Unknown (2)	50	Afro-descendant (2) Caucasian (13) Unknown (6)
OSCCI (n=12)	Tongue (5) Gingiva / alveolar ridge (5) Floor of the mouth (4) Palate (3)	F(4) M (8)	61	Afro-descendant (2) Caucasian (9) Unknown (1)
OSCCII (n=12)	Tongue (5) Gingiva / alveolar ridge (4) Palate (2) Unknown (1)	F(2) M(10)	57	Afro-descendant (1) Caucasian (8) Unknown (3)

Fluorescence *in situ* Hybridization (FISH) analysis

Unstained slices were previously analyzed in microscope and the most representative area was selected and marked with a diamond pen. Pretreatment of slides, hybridization, post hybridization processing, and signal detection were carried out as per instructions of Zyto*Light*® SPEC SOX2/CEN 3 Dual Color Probe, which was designed for the detection of SOX2 gene (ZytoVision GmbH, Bremerhaven, Germany). The SPEC SOX2/CEN 3 Dual Color Probe is a mixture of an orange fluorochrome direct labeled probe specific for the alpha satellite centromeric region of chromosome 3 (CEN 3) and a green fluorochrome direct labeled SPEC SOX2 probe specific for the SOX2 gene at 3q26. Briefly, sections were digested with protease K (0.5 mg/mL) at 37°C for 30 minutes. Slices were denatured at 95°C for 5 minutes and dehydrated in ethanol. The probes were denatured for 15 minutes at 75°C before

hybridization. Slides were hybridized overnight at 37°C, washed and the nuclei were counterstained with DAPI. All slices were analyzed using a fluorescence microscope (Olympus BX51) at a 100x magnification objective and equipped with 405 nm (DAPI), 503nm (SOX2) and 547nm (CEN3) filters. For semi-quantitative analysis we followed previous protocols for SOX2 quantitation[29]. The number of centromere signals (CEN3 – orange fluorochrome) and the number of copies of individual genetic locus (SOX2 – green fluorochrome) were counted individually in well-defined nuclei that have no signs of overlap. Tumor signals were scored as amplified when 10% of cells showed eight or more signals or tight clusters of signals of the gene probe (SOX2). Less than eight signals per cell were considered as low-level gains and not scored as gene amplification[30,31]. The signals of the centromere probes were used to control adequate hybridization and to exclude artifacts. Only morphologically abnormal nuclei with FISH signals for both colors were counted.

Immunohistochemistry

After hydration, slices were submitted to the blockage of endogenous peroxidase activity (3% H_2O_2 in methanol, 10min, room temperature) and antigen retrieval was performed at steamer (90° C, 25 min). Slices were then incubated overnight (1:100, 4° C) with monoclonal antibodies against SOX2 (Cell Signaling Technology, Inc., Danvers, USA) or monoclonal antibody against the mesenchymal marker Vimentin (Dako, California, USA). For the detection of primary antibody, it was used the EnVision™+ Dual Link System-HRP (Dako, California, USA) and labeling was visualized with 3,3'-diaminobenzidine (Dako, California, USA). Last, tissues were washed in PBS, dehydrated in ethanol, xylene and cover slipped using Permount, (Fisher, Massachusetts, USA). The

whole stained slice was scanned using a microscope Imager M 2.0 (Zeiss, Germany) with a 20 x magnification objective and immunolabeling was evaluated microscopically by two pathologists. The semi-quantitative analysis for SOX2 was performed according to the perception of stained cells while the qualitative analysis of vimentin staining considered the intensity of the labeling and cell type. The pathologist used the Kappa test 2 weeks after the analysis and the concordance value obtained was 75%.

Statistical Analysis

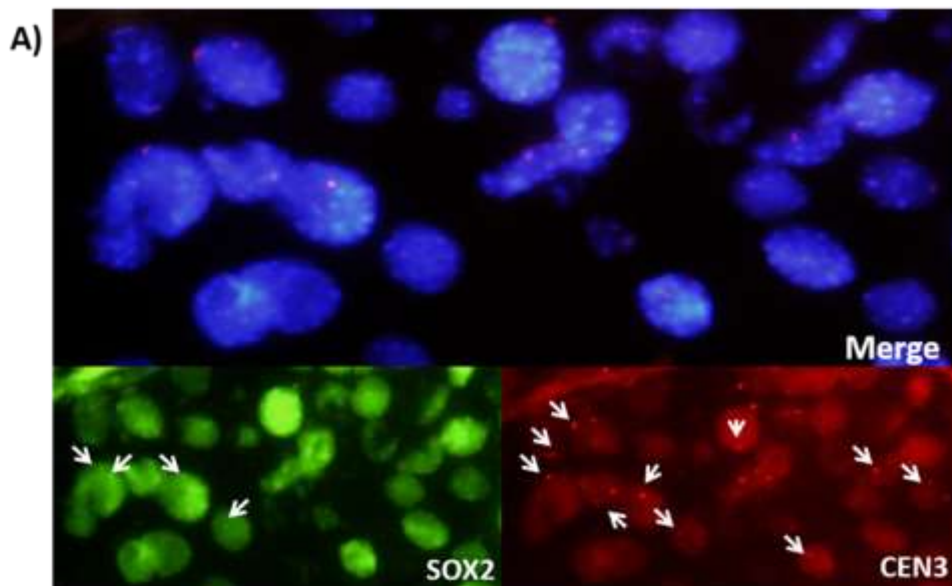
The relationship between independent variables was evaluated using two-way ANOVA followed by Tukey test. A P-value $\leqq$ 0.05 was considered statistically significant. Analyzes were performed with Prism GraphPad 6 (San Diego, CA, U.S.A.).

RESULTS

Sox2 transcription levels are diminished according to lesion aggressiveness:

Since the epithelial-to-mesenchyme transition involves genetic reprogramming, we analyzed the expression level of the transcription factors SOX2 in samples of normal oral mucosa, hyperplasia and dysplasia biopsies as well as low (OSCCI) and high (OSCCII) aggressive oral squamous cell carcinoma samples. Each biopsy sample was submitted to detection of SOX2 and centromere (CEN) signal by FISH. In each sample 20 nuclei were analyzed and the number of signals of CEN and SOX2 were quantified. For each cell, the number of signals for CEN was divided by the total number of signals for SOX2, yielding the current transcription activity for the gene. We found different signaling patterns and a heterogeneous distribution of gene copy numbers. For hyperplasia, 66.1% (±8.48, n=6) of the cases showed presence of

the SOX2 gene, while dysplasia samples had 44.2% (±31.99, n=12) of the cases. Both, OSCCI 26.47% (±15.20, n=6) and OSCCII 40.2% (±14.37, n=9) showed a decrease in the percentage of cells with the presence of SOX2 in chromosome 3q.26. All results demonstrate a negative correlation of SOX2 expression level and the oral lesion aggressiveness. Since centromeres were monitored, it was possible to observe the presence of chromosome 3 associated with lower SOX2 expression, suggesting that loss of SOX2 is regulated by gene expression itself and not deletion of the chromosome. Data also suggest that a decrease in SOX2 is necessaryfor increased invasiveness and migratory ability of tumor cells, as observed during embryogenesis (Fig. 1).



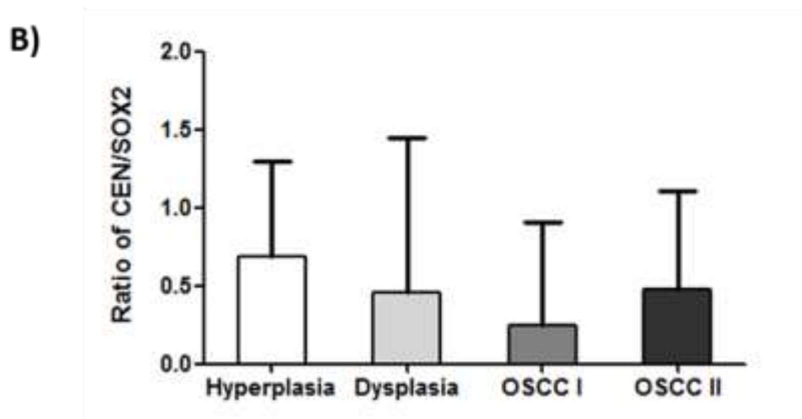


Fig. 1.: SOX2 signal decreases as lesion dedifferentiation level increases: A) The number of centromere signals (CEN3 – orange fluorochrome) and the number of copies of individual genetic loci (SOX2 – green fluorochrome) were counted individually in well-defined nuclei (Blue – DAPI) that had no signs of overlap. Images were obtained at high magnification (100x) using triple filter. Tumor signals were scored as amplified when 10% of cells showed eight or more signals or tight clusters of signals of the gene probe (SOX2). B) Biopsies of PMOLs (hyperplasia and dysplasia, n= 6 and 12 respectively) and low (OSCCI, n=6) or highly (OSCCII, n=9) aggressive OSCC were submitted to dual color FISH assay for detection of SOX2 and CEN3 signal. Data presented as mean +/- SD. Tukey test, P-value ≤ 0.05.Did not present statistical difference.

SOX2 protein levels are decreased at the tumor invasion area.

In order to confirm the transcript results of SOX2, we performed a quantitative analyzes of the SOX2 protein levels in different regions of the biopsy by immunohistochemistry. Also, in order to correlate SOX2 with a

possible EMT process, we performed a qualitative analysis for the mesenchymal marker vimentin in the subsequent sequential cut slice from the biopsy.

For normal mucosa (Figure 2 and 3A-C), SOX2 staining was visible in ~25% (±29.1%, n=13) of the cells at the basal layer of epithelia, while vimentin showed a weak signal in cells present in the connective tissue surrounding the epithelia. In hyperplasia regions (Figure 2 and 3D-F), SOX2 was present in ~20% (±18.7%, n=6) of the cells at the basal layer, but it was also observed in all extracts of the epithelia, while Vimentin labeling was observed in blood vessels, fibroblasts and macrophages. In dysplastic samples (Figure 2 and 3G-I), SOX2 was present in ~48% (±26.32%, n= 21) of the cells at the basal layer as well as in all tracts of the epithelia, while Vimentin was present in blood vessels, inflammatory cells and fibroblasts. These data combined with those of FISH indicate that during the evolution of aggressiveness in potential lesions, where cells show mainly proliferative activity, there is an increase in SOX2 protein level, probably due to decreased protein turnover.

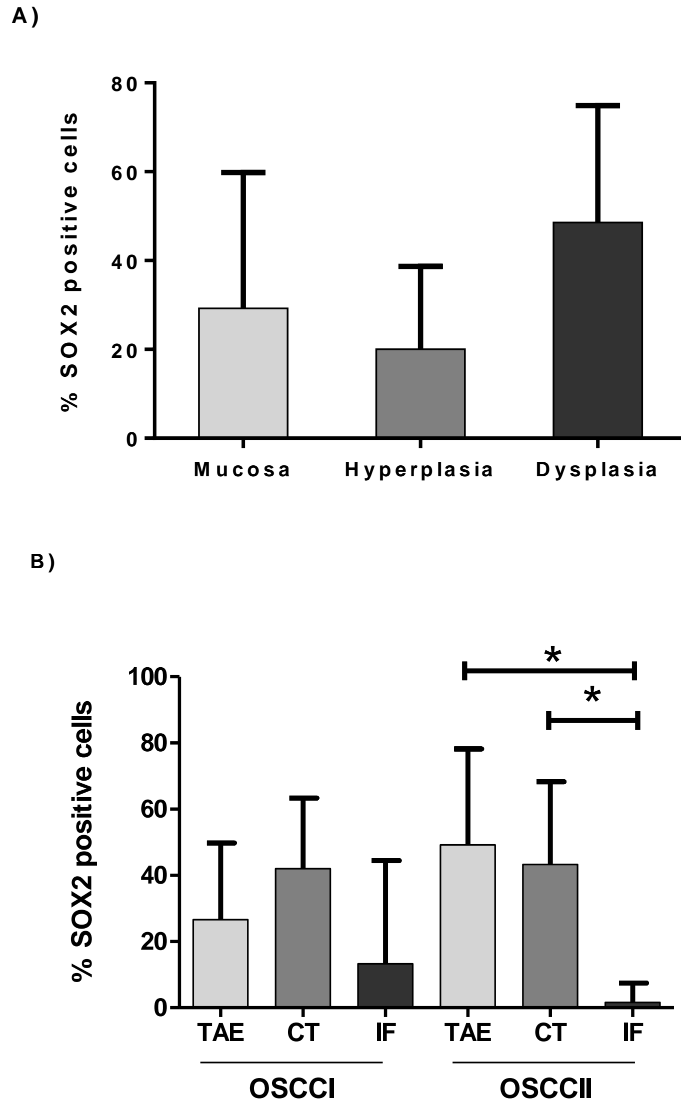


FIG 2.: Immunohistochemistry analyzes in PML and HNSCC. The quantification of SOX2 positive cells: A) Normal oral mucosa (n=13) vs PMOLs (hyperplasia n=6, dysplasia n=21). B) Different regions of low (OSCCI, n=12) vs highly (OSCCII, n=12) aggressive OSCC biopsies.Tukey test, P-value ≤ 0.05.

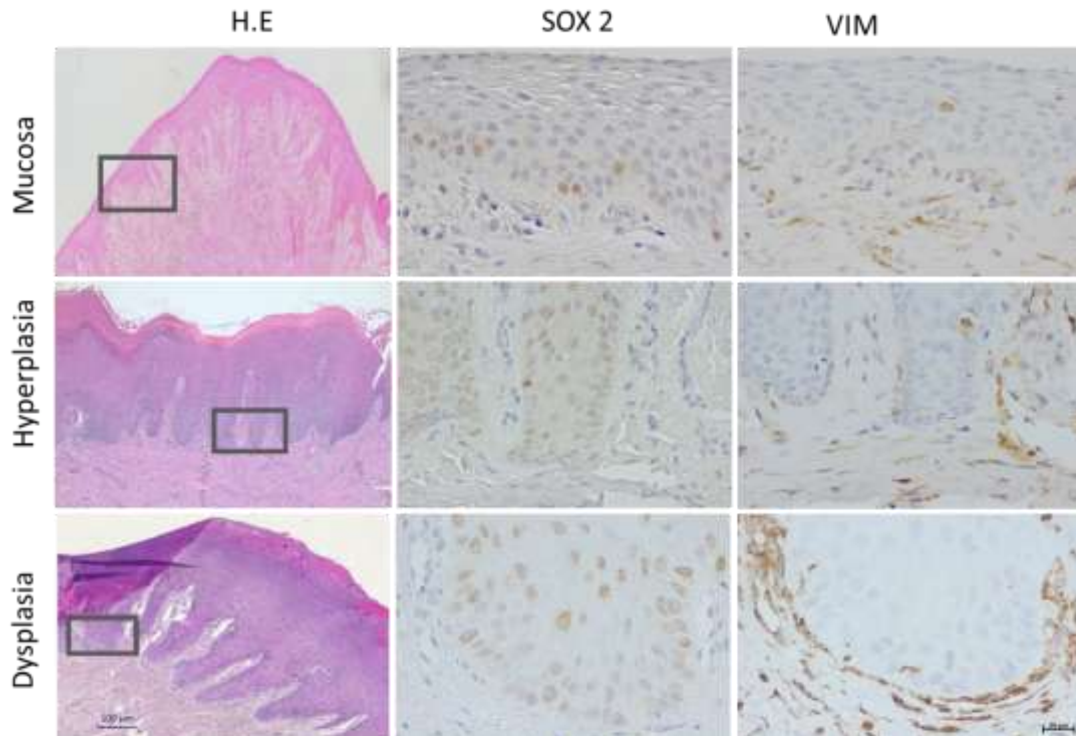


FIG 3.: SOX2 and Vimentin expression in normal mucosa and Potential Malignant Lesions. Samples of oral mucosa (upper line), hyperplasia (center line) or dysplasia (lower line) were stained for HE (left column), SOX2 (center column) or Vimentin (right column). HE images were obtained at low magnification (10x) and black box represent the area where images were taken at higher magnification (63x) from sequential series slices submitted to SOX2 or Vimentin immunostaining. **B-** Quantification of SOX2 positive cells in biopsies of oral mucosa (n=13), hyperplasia (n=6) and dysplasia (n=21). Analysis was performed by two pathologists.

In OSCC samples, we performed a segmented analyzes according to tumor region. It was observed the tumor adjacent epithelia (TAE), center of the tumor (CT) and the invasion front of the tumor (IF), which was defined as the last six tumor cell layer nearest to the connective tissue[27]. In high differentiated/low invasive carcinoma (OSCCI, n=12, Figure 2 and 4; Supplementary Figure 1) the expression of SOX2 was observed in 29.16% (±21.51%) of cells in TAE, 38.65% (±17.59%) of cells at CT and 13.33% (±31.13%) of positive cells at IF. In low differentiated/highly invasive carcinoma (OSCCII, n=12, Figure 2 and 5; Supplementary Figure 2) the expression for SOX2 was observed in 49.16% (±29.16%) of cells at TAE, 43.33% (±24.98%) in CT and only in 4.33% (±14.39%) of cells at IF of positive cells. Interestingly, for

both grades of OSCC, Vimentin staining was observed only in cells at the connective tissue at TAE and CT regions, but in IF zones, Vimentin labeling was also observed in tumor cells (Figure 4H and 5H). Taken together, our data indicate that in proliferative lesions and/or highly differentiated regions, such as dysplasia, tumor adjacent epithelia and center of the tumor, SOX2 protein levels are elevated, while at invasion regions with low differentiated cells SOX2 protein levels are silenced.

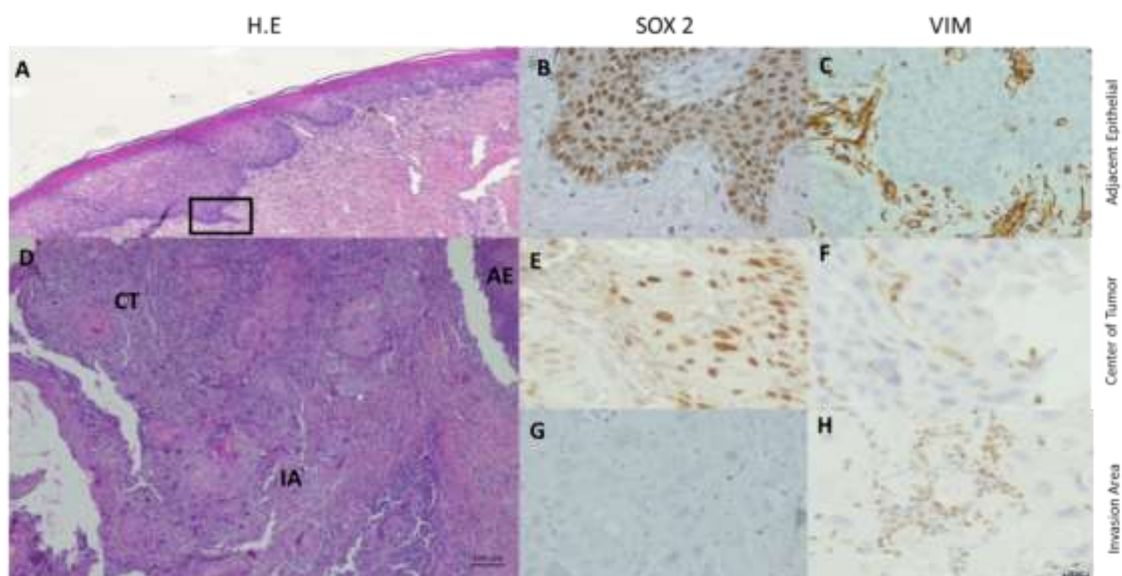


FIG 4.: SOX2 and Vimentin expression in biopsies of high differentiated Oral Squamous Cell Carcinoma (OSCCI). A- Regions of the biopsies corresponding to the tumor adjacent epithelia (B-C) and the tumor (E-H) werestained for HE (A, D), SOX2 (B, E, G) or Vimentin (C, F, H). HE images were obtained at low magnification (10x) and black box represent the area where images were taken at higher magnification (63x) from sequential series slices submitted to SOX2 or Vimentin immunostaining. At the tumor region, analysis was divided into cells from the center of the tumor (E, F) and cells at the invasion zone (G, H). Representative images from n=12.

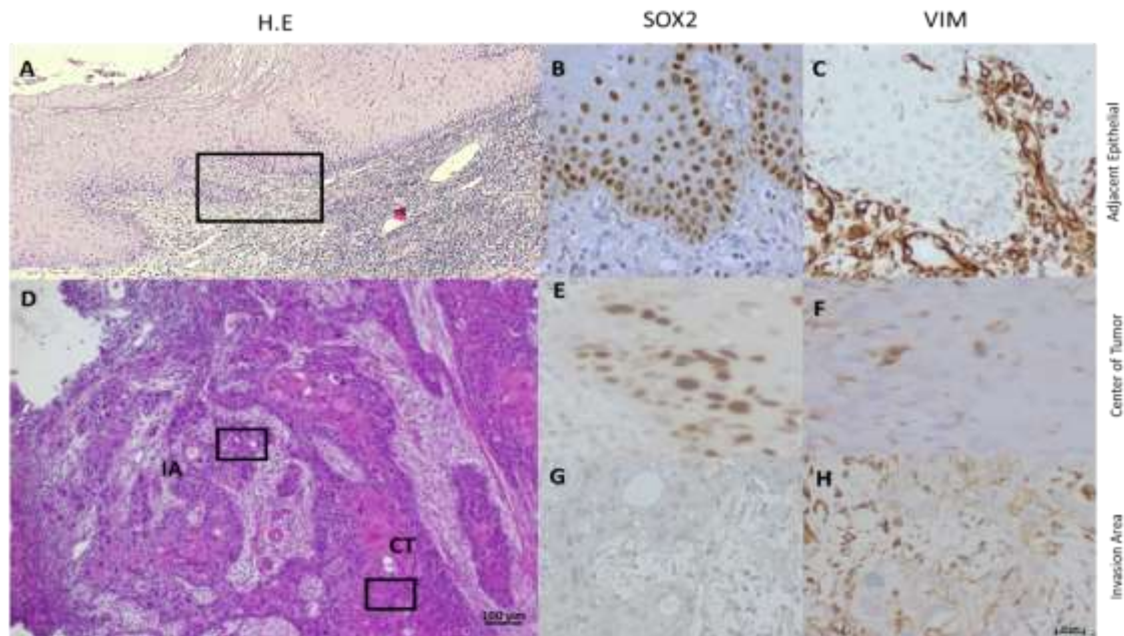


FIG 5.: SOX2 and Vimentin expression in biopsies of low differentiated Oral Squamous Cell Carcinoma (OSCCII). A- Regions of the biopsies corresponding to the tumor adjacent epithelia (B-C) and the tumor (E-H) werestained for HE (A, D), SOX2 (B, E, G) or Vimentin (C, F, H). HE images were obtained at low magnification (10x) and black box represent the area where images were taken at higher magnification (63x) from sequential series slices submitted to SOX2 or Vimentin immunostaining. At the tumor region, analysis was divided into cells from the center of the tumor (E, F) and cells at the invasion front (G, H). Representative images from n=12.

DISCUSSION

About one third of HNSCC patients are under 55 years and the 5 years survival rate is 55% [3,6,11, 32]. Standard treatment consists of surgery and radiotherapy as a local approach and adjuvant chemo- or immunotherapy as a systemic approach for advanced tumors.Treatment failure in HNSCC is mainly attributed to the plasticity and aggressive nature of cancer cells allowing invasiveness, dissemination and as a consequence the ability to develop locoregional relapse and/or metastasis. Although it is still not completely understood how oncogenes and tumor suppressor genes are regulated, genetic polymorphisms and tumor microenvironment-related mechanisms may influence the development and progress of HNSCC [20, 26, 33].

Oral Squamous Cell Carcinoma are the most incident subgroup of HNSCC and might evolve from initially proliferative disorders named potentially malignant lesions that due to the accumulation of mutations might acquire an invasive phenotype[5]. We identified in PMOLs an increase in SOX2 protein levels, which was also observed in regions of the center of the tumor and tumor adjacent epithelia. Interestingly, the amplification signal of SOX2 is decreased according to the region degree of aggressiveness. This indicates that during the proliferative stage, there is an accumulation of SOX2 probably by regulation of the protein turnover, and not by increase in gene transcription. In fact, blockage of SOX2 was able to reduce tumor growth in a xenograft mouse model for gastric tumors where the expression of SOX2 was related to higher proliferation behavior while inhibition led to cell cycle arrest in G2/M phase [34], which was also observed in prostate cancer cells [35]. SOX2 has been reported to be essential for proliferation of glioma and breast cancer cells [36, 37], whereas downregulation of SOX2 inhibited proliferation and induced apoptosis in human lung cancer cells [38-40]. Studies suggest that upregulation of SOX2 induces proliferation and anchorage independent growth in epithelial cells [25, 41, 42]. Since amplification and expression of SOX2 may also occur in the Squamous Cell Carcinoma in esophagus [43], SOX2 might have similar effects on other oncogenic factors, such as, *LEF1*, *MAPK1*, *TGFBR2*, and *CCNB1, which* are candidate genes acting downstream of SOX2 [43-45]. Amplification of SOX2 levels indicates histological differentiation degree, whereas a homogeneous expression in different regions of the same tumor is a potential indicative of improved survival of patients [33].

In our results we found that tumor cells at invading areas, where the cells are migrating and establishing new metastatic foci, shows a decrease on SOX2 expression but exhibit high expression of EMT markers. It has been proposed that SOX2 may mark the tumor stem cell population in cancers [41, 47]. These distinct subpopulation of cells persists in tumors and are responsible to cause relapse and metastasis by giving rise to new tumors [47]. It is not clear if the mesenchymal-like cells, cancer stem cell (CSC) and circulating tumor cells (CTC) are the same subpopulations for OSCC, but all show tumor initiating capacity that can be utilized to form metastases [48]. However, it is important to note that for cells to migrate and form a new tumor focus, it needs to increase the characteristic of embryonic cell and invade the tissue to promote a new invasive focus. It was already demonstrated that when SOX2 is mutated, with a consequent decrease in the protein level, carcinoma cells show a greater migration profile [28]. SOX2 negative cells have a more invasive and migratory behavior and showed a poor prognosis for HNSCC [28]. Several genetic alterations have been reported in HNSCC which include activation of proto-oncogenes and inactivation of tumor suppressor genes [49, 50]. The genetic alterations driving the malignant remain to be fully elucidated [51].

The undifferentiated mesenchymal cells are characterized by a switch from E- to N-cadherin expression, the expression of Snail factors, vimentin, and metalloproteases. The normal stem cells and cancer stem cells may share a mesenchymal phenotype that enhances their ability to preserve stemness, to retain migratory properties, and to respond to different stimuli during expansion and differentiation [52].

Cell-cell junctions are important to maintain cell and tissue polarity and integrity[18]. E-cadherin is a transmembrane protein involved in the maintenance of cell-cell adhesion but is also involved in other regulatory mechanism. The extracellular region of E-cadherin extends from the cell surface and bind to another E-cadherin present on adjacent cells. The intracellular region contains binding sites to interact with catenin and other regulatory proteins. The *α*-catenin and *β*-catenin proteins connect E-cadherin to actin bundles leading to stabilization of cell-cell adhesion and preserving the adherent junctions [53-56]. *β*-catenin is a key regulator in the canonical Wnt signaling, where cytoplasmic *β*-catenin translocate to the nucleus and function as an activator of transcription factors involved in cell adhesion, tissue morphogenesis, and tumor development [57-59].

During carcinogenesis, it is proposed that there is a loosening of E-cadherin-mediated cell-cell contact and activation of Wnt/β-actin leading to changes in the SOX2 expression levels. Corroborating this data, we found higher SOX2 protein levels at tumor regions where there are high amounts of E-cadherin mediated cell-cell adhesion [56], while SOX2 was abolished at regions characterized by dedifferentiated tumor cells. This suggests that variation in SOX2 levels can be a final step in the process of tissue invasion and might be triggered by a decrease in cell-cell adhesion signaling.

Another possible mechanism of SOX2 regulation in tumor behavior is the modulation by microRNAs. Frequently miRNA expression patterns correlate with disease progression and patient survival. Thus, a small change in miRNA level can rapidly switch gene expression pattern in a cell and tissue-dependent manner. It is interesting to note that some miRNAs may have dual functions as

both tumor suppressors and oncogenes [60]. It was already observed that miR-302 is regulated by SOX2 to promote cancer stemness in OSCC [61]. However, microRNA profile is not yet elucidated for HNSCC. Epigenetic modifications, transcriptional control, alternative splicing, protein stability, and subcellular localization may be other mechanisms playing roles in regulation by SOX2. Despite the efforts to identify common regulatory networks in normal development and disease it is not clearif some pathways work isolated. Unraveling the underlying molecular principles of SOX2 will not only help to develop innovative strategies for more efficient and less toxic therapy of HNSCC patients, but could also pave the way for biomarker-driven clinical trials in the future.

CONCLUSION

An inverse correlation between SOX2 expression and aggressiveness of oral lesions was observed, suggesting that loss of SOX2 is a common feature of malignant progression that drives motility and invasive ability of tumor cells.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST:

The authors declare that they have no competing interests.

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CHAPTER 2

This chapter is based on results that are still in progress and will be correlated with data from patient samples. The work was conducted over a 4-month internship during the year 2015 in The Section of Experimental and Translational Head and Neck Oncology at the Department of Otolaryngology, Head and Neck Surgery of the University Hospital Heidelberg in Germany, and it was performed under supervision of Dra. Adriana Jou and the principal investigator Dr. Jochen Hess who kindly received me in the laboratory and allowed great exchange of experiences of my knowledge in Molecular Biology of Cancer.

The aim of this topic is to dissect the molecular mechanisms by which SOX2 is involved in EMT process. The German group, under supervision of Dra. Adriana Jou and the principal investigator Dr. Jochen Hess, have a preliminary data that demonstrated that established HNSCC cell lines silenced for the cysteine protease Kallikrein 6 (KLK6) also showed a decrease on SOX2 expression and it correlated to a mesenchymal-like phenotype *in vitro* and *in vivo*(52, 57). Using this system, we restored SOX2 expression levels and observed its effect in several players of the EMT process. Briefly, we noted that overexpression of SOX2 decreased the amounts of Vimentin, a mesenchymal marker. Also, SOX2 expression can be regulated by the accumulation of β-catenin in the nucleus, avoiding their transcription.The series of data strongly support the assumption that SOX2 and KLK6act in a common signaling and generegulatory network.

Materials and Methods:

Cellular Model

Human head and neck squamous cell lines (FaDu) were obtained from ATCC and cultivated with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, 2 mM L-Glutamine and 50 µg/ml Penicillin-Streptomycin in a humidified atmosphere of 6-8 % CO_2 at 37 °C. The transfection of FaDu cells were established and cultured as previously described (52). In summary, FaDu cells were transfected with a control pRS vector encoding a non-effective Hush 29-mer scrambled shRNA cassette (TR30012, OriGene Technologies, USA) or a pRS-shKLK6 plasmid (TR316673, OriGene Technologies, USA) using FuGene HD Transfection Reagent (Promega, Germany) according to the manufacturer's instruction. After, the selection of the clones expressing the plasmid was done with 1.5 µg/ml Puromycin (Gibco life technologies, Germany) for one week and stable clones were isolated and KLK6 silencing was confirmed on RNA level(52). With this approach it was obtained the two clones used in this study: one with normal expression for KLK6 (FaDu Mock) and a second cell line silenced for KLK6 (FaDu sh- KLK6).

Luciferase reporter assay

For analysis of gene expression, $3x10^4$ FaDu-shKLK6 cells were seeded in 24-well plates and transfected with either 0.5 µg TOPFLASH or 0.5 µg FOPFLASH (upstate biotechnology,USA) using FuGene HD Transfection Reagent (Promega, Germany) according to the manufacturer's instruction. As reference for transfection efficiency all cells were co-transfected with 0.5 µg pTK-RL (Promega, Germany). Firefly and Renilla luciferase activity was quantified with a Sirius Luminometer (Berthold Detection Systems, Germany) using the Dual-Luciferase Reporter Assay System (Promega, Germany) according to the manufacturer instruction. Briefly, these measures are able to analyses the activation of Wnt/β-catenin signaling and associated with the phenotype after silencing or overexpression.The independent FaDu-shKLK6 clones were transfected with a TCF-dependent reporter (TOPFLASH) or a

mutant control plasmid (FOPFLASH). The graph represents the relative luciferase activity as mean value + SD of three independent experiments. The value of one FOPFLASH-transfected mock control was set to one.

Immunofluorescence staining

For analysis of SOX2 and Vimentin protein distribution, $3x10^4$ cells were seeded on glass coverslips in a 12-well plate. After seeding (48h), cells were fixed with 4% paraformaldehyde (PFA) and washed with PBS. Permeabilization of cells was done in X-buffer (0.5% Triton X-100 in PBS) for 30min at room temperature (RT). After washing with PBS, cells were blocked with T-buffer (1% BSA/0.2% Tween20 in PBS) for 30min at RT. Primary antibody for anti-SOX2 (rabbit; 1:300; Cell Signaling) or Anti-Vimentin (mouse; 1:100; Progen)was diluted in T- buffer and incubated for 1-2h. After washing with PBS, cells were incubated withsecondary antibody was applied and incubated for 1h in the dark. Antibodies Cy3 (goat anti-rabbit; 1:200; Invitrogen) and Alexa 488 (goat anti-mouse;1:100; Invitrogen) were used. The nuclei were visualized by incubation of cells with DAPI. Finally, coverslips were mounted with Mowiol.

Immunoblotting

Cell lysates (20 µg protein per sample) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% acrylamide gels and proteins transferred to a PDVF membrane. For immunoblotting, membranes were blocked with non-fat milk (4%) and then incubated with primary antibody forAnti-SOX2 monoclonal antibody (Cell Signaling Technology, USA, 1:1000), Anti-Vimentin monoclonal antibody (Progen Biotecnik, Germany, 1:1000) and Anti-β-actin monoclonal antibody (Sigma Aldrich, USA, 1:10000). For secondary immunodetection, anti-rabbit IgG - HRP or anti-mouse IgG - HRP (Santa Cruz, Germany) were used at a dilution of 1:10000. Antibody binding was detected using Pierce ECL Western Blotting Substrate (Thermo Scientific).

Results:

Silencing of KLK6 blocks SOX2 expression in OSCC cells line

In order to confirm the preliminary data from the German group regarding the correlation between KLK6 and SOX2 expression, we analyze the effects of KLK6 knock down on SOX2 levels and on the EMT marker Vimentin. FaDu cells were separated in two groups: FaDu – Mock, with normal levels for KLK6, and FaDu-shKLK6, which was silenced for KLK6. The SOX2 and Vimentin levels were measured by western blotting and immunofluorescence. It was observed that silencing of KLK6 induced a severe decrease on SOX2 levels, which correlated with an increase on the mesenchymal marker Vimentin. Then, both clones were transfected for overexpression of SOX2 and, after 48 hours, cells were submitted to immunofluorescence for both, SOX2 and Vimentin. It was observed that FaDu Mock cells overexpressing SOX2 show almost no signal for Vimentin. However, FaDu shKLK6 cells that overexpressed SOX2 showed a decrease in Vimentin levels when compared to untransfected cells. Taken together, these data indicate that depletion of SOX2 might be necessaryfor the EMT process induced by the silencing of KLK6 model.

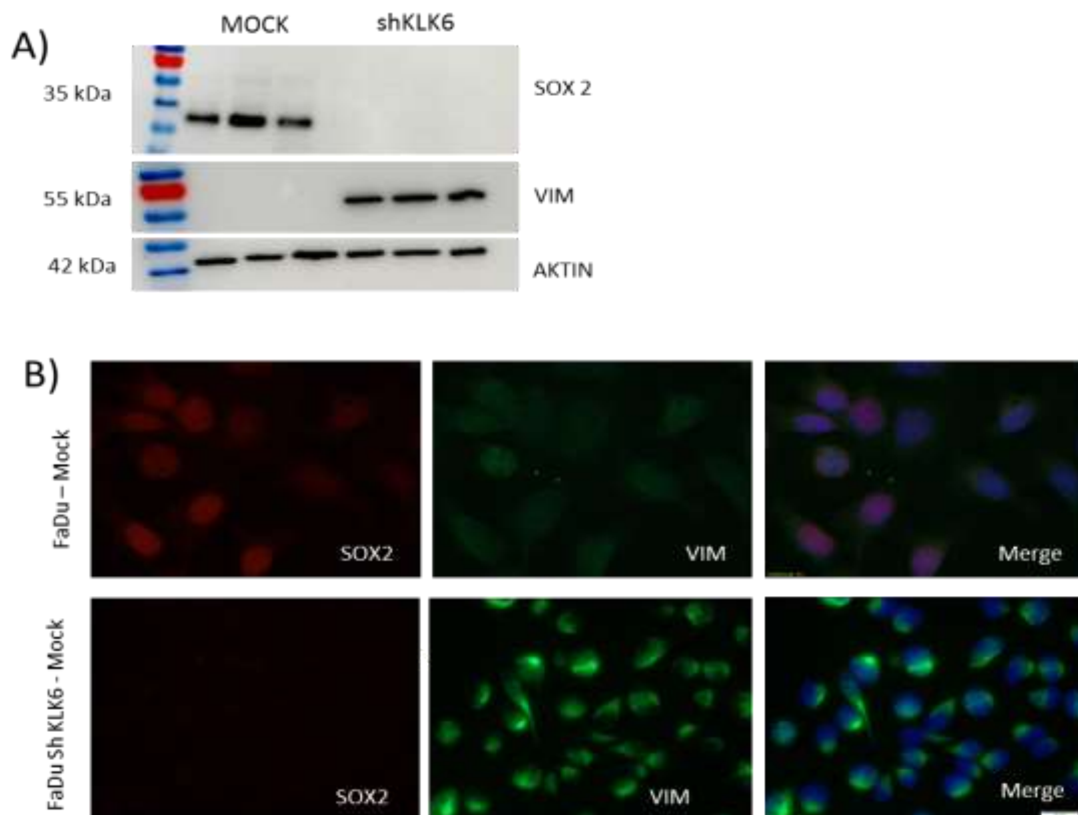


Figure 1: Knockdown of KLK6 decreases SOX2 levels. Analysis of SOX2 and Vimentin protein levels (A) and intracellular distribution (B) in mock control (M) or stable silencing for KLK6 (sh-KLK6). Nuclei were stained with DAPI. Representative images of an n=3 clones. Scale bar = 20µm.

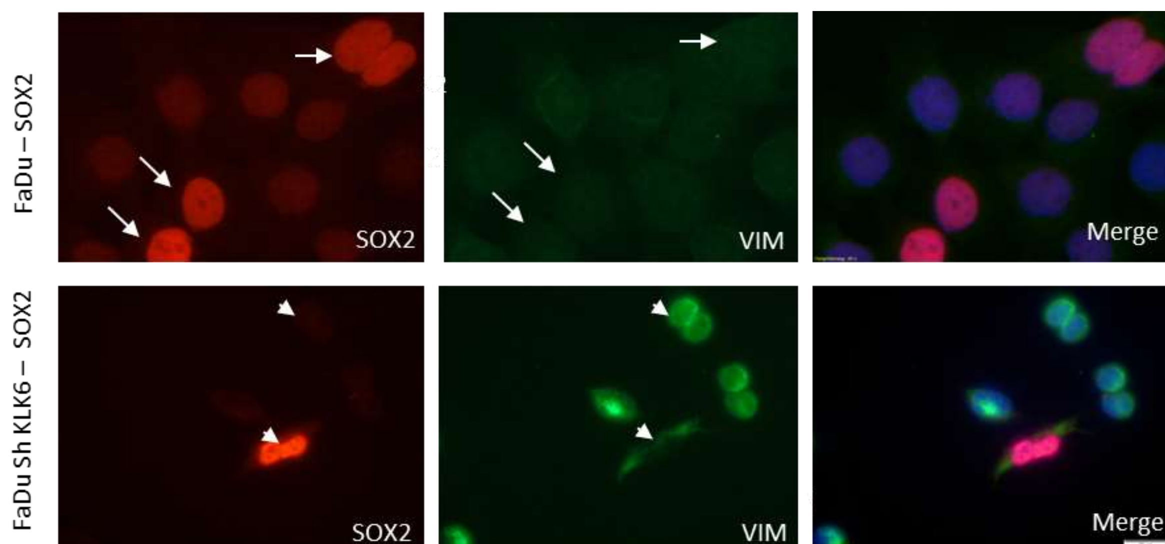


Figure 2: Overexpression of SOX2 in KLK6 knockdown cells decreases Vimentin levels. Control FaDu Cells FaDu Mock) or stable knock down for KLK6 (FaDu-shKLK6) were transfected for overexpression SOX2 and stained for SOX2 and Vimentin. Nuclei were stained with DAPI. Representative images of an n=3 clones.Scale bar = 20µm.

KLK6 affects SOX2 expression through β-catenin-mediated signaling

A possible mechanism by which KLK6 affects SOX2 expression is through β-catenin activation due to changes in the Wnt signaling (52, 75-77). In order to analyze this hypothesis, we performed a TOP/FOP assay, which consists in the use of a luciferase reporter plasmid for β-catenin (FOP) and reporters (TOP) for a promoter of a constitutively transcripted gene (p-DEST), KLK6 (p-DEST-KLK6) or SOX2 (p-DEST-SOX2). The ratio between the desired gene (TOP) and the levels of β-catenin (FOP) indicates the transcription level of the gene. It was observed that when cells were overexpressed for KLK6, FaDu sh-KLK6 showed an increase on KLK6 reporter (mean ±2,4 SD ±1,2) when compared to housekeeping gene (mean ±1,73, SD ±0,45), indicating and increase on KLK6 gene transcription. However, FaDu sh-KLK6 transfected with the reporter for SOX2, the signal was decreased (mean ± 0,81 SD ±0,02), indicating that β-catenin driven Wnt-transcriptional is involved in the regulation of SOX2 expression.

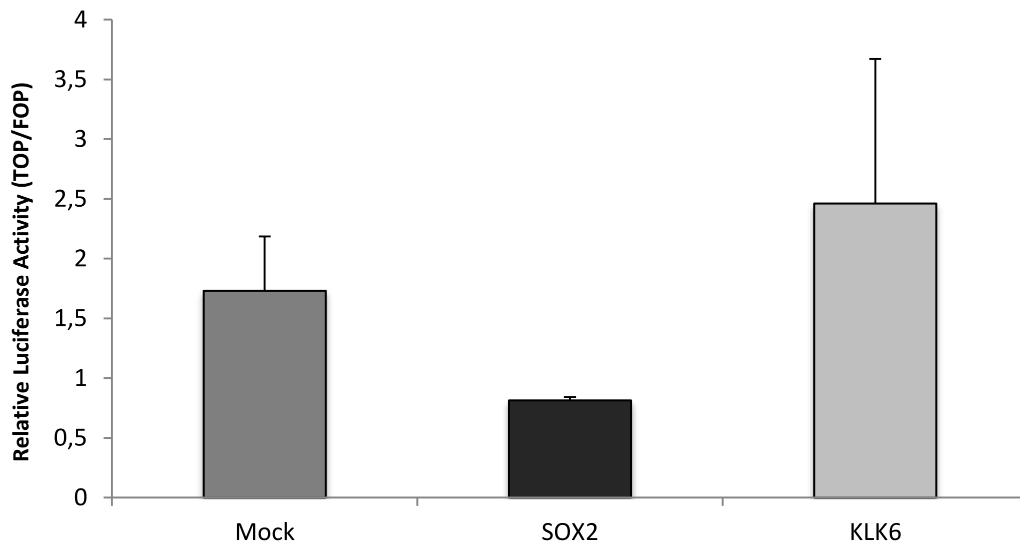


Figure 3:SOX2 expression correlates with β-catenin signaling. FaDushKLK6 cells were transiently co-transfected with TOPflash/FOPflash reporter plasmid system. Forcontrol gene (MOCK), pDest – SOX2 (SOX2) and pDest KLK6 (KLK6) plasmid was used and the expression was measure with Luciferase system. Each assay was performed in triplicate and the reporter activity was expressed as mean ±SD, n=3 clones. Student T Test, P-value ≤ 0.05.

In order to confirm these results, we performed and immunofluorescence to analyze the cellular distribution and the amount of β-catenin and SOX2 in FaDu Mock and in FaDu shKLK6 cells. It was observed that FaDu Mock cells, which have normal levels of SOX2 in the nuclei, β-catenin was more evidently at cell-cell contacts. When cells were silenced of KLK6, it was observed a decrease in SOX2 levels in the nuclei, which correlated with a decrease of β-catenin in the cytoplasm. To confirm if the KLK6-mediated change on β-catenin distribution was dependent of SOX2, FaDu Mock and FaDu shKLK6 were submitted to overexpression of SOX2 and stained for SOX2 and β-catenin. It was observed that FaDu Mock cells overexpressing SOX2, β-catenin remained at the cell-cell contact regions. However, FaDu shKLK6 overexpressing SOX2 showed the same nuclei distribution for β-catenin then cells with low levels of SOX2. Taken together, the luciferase assay and the immunofluorescence images indicate that β-catenin is upstream to SOX2 and downstream to KLK6.

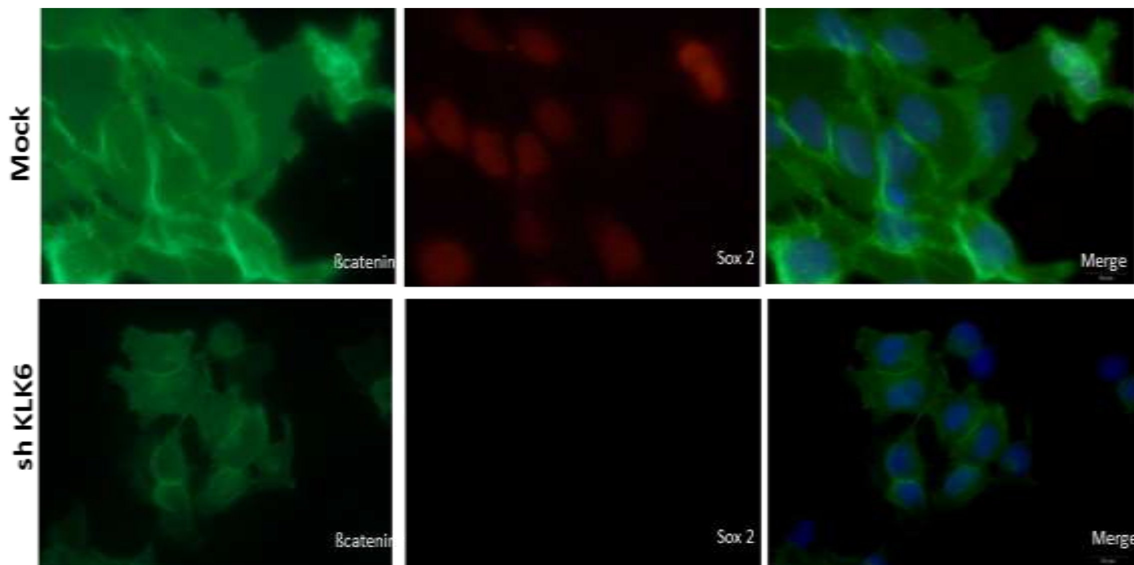


Figure 4:Distribution of β-catenin in FaDu cells. Control FaDu Cells (FaDu Mock) or stable knock down for KLK6 (FaDu-shKLK6) were stained for SOX2 and β-catenin. Nuclei were stained with DAPI. Representative images of an n=6 clones and images were done in magnificance 44x with a fluorescence microscope.Scale bar = 20μm.

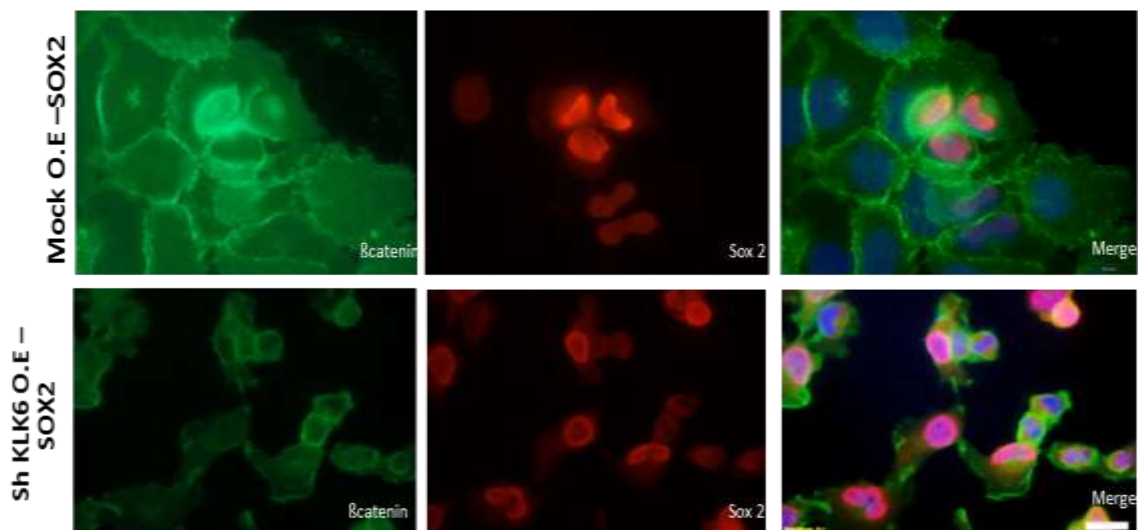


Figure 5: Overexpression of SOX2 in KLK6 knockdown cells decreases β-catenin levels and Co-localization in the nuclei. Control FaDu Cells (FaDu Mock) or stable knock down for KLK6 (FaDu-shKLK6) were transfected for overexpression SOX2 and stained for SOX2 and β-catenin. Nuclei were stained with DAPI. Representative images of an n= 6 clones and images were done in magnificance 44x with a fluorescence microscope.Scale bar = 20μm.

CONCLUSION

The preliminary data demonstrate that loss of SOX2 expression in tumor cells silenced for KLK6 is necessary for the induction of mesenchymal-like phenotype in OSCC cells, and the mechanism for downregulation of SOX2 might be influenced by the accumulation ofβ-catenin in nucleus.

DISCUSSION

SOX2 is a transcription factor that plays a critical role in the maintenance of embryonic stem cells, adult stem cells and it is involved in the maintenance of multiple tissues(72, 78-80). It was already demonstrated that SOX2 is predominantly expressed in progenitor cells at the basal layer of theepithelium(80), which has the ability to maintain proliferation of epithelial cells, but the overexpress for SOX2 is ably to increase the proliferation and inhibits the differentiation in progenitor cells in the stratified epithelium (80).However, SOX2 gene amplification and protein overexpression are observed in the development of Squamous Cell Carcinoma (SCC) from several human tissues, including lung, oral cavity and esophagus (67, 70, 78, 80).Amplification of SOX2 levels indicates histological differentiation grade and a homogeneous expression at different regions of the same tumor is an indicative towards improved survival of patients (72).

We identified in PMOLs an increase on SOX2 protein levels, which was also observed in regions of the center of the tumor and tumor adjacent epithelia, but not at invading areas.Studies suggest that up-regulation of SOX2 induces proliferation and anchorage independent growth in epithelial cells (81-83).We observed a decrease on gene expression but it was accompanied by an accumulation of SOX2 in the cells. This indicates that during the proliferative state, there is an accumulation of SOX2 probably by regulation of the protein turnover, and not by increase in gene transcription (17, 80, 84).

At invading areas, where the cells are migrating and establishing new metastatic foci, SOX2 expression was lower,whileVimentin expression exhibited high levels, characterizing a predominant SOX2 negative and Vimentinpositive cell population. This distinct subpopulation of SOX2 negative cells in tumors might beassociated to tumor relapse and metastasis by giving rise to new tumors (17, 74). It is not clear if the cancer stem cell (CSC) or/and circulating tumor cells (CTC) are the same subpopulations for OSCC, but both show tumor initiating capacity that can be utilized to form metastases (26). However, it is important to note that for cells to migrate and form new tumor focus, it is necessary to decrease the characteristic of embryonic cell and invade the tissue to promote a new invasive focus. It was already demonstrated that when SOX2

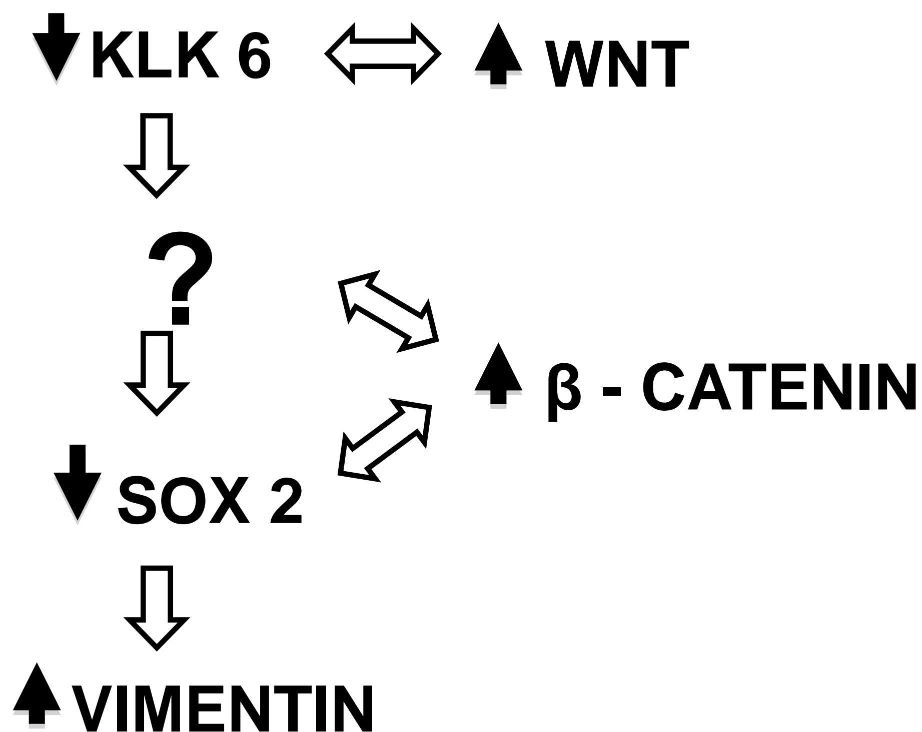
is mutated, with a consequent decrease in the protein level, carcinoma cells show a greater migration profile (57). SOX2 negative cells have a more invasive and migratory behavior and demonstrated a poor prognosis for HNSCC (57). Several genetic alterations have been reported in HNSCC, which include activation of proto-oncogenes and inactivation of tumor suppressor genes (65, 85). But it is not completely understood which genetic alterations drive the malignant behavior (86).

In FaDu cell line, we demonstrated that the induction of EMT in the KLK6- cells relies in SOX2 downreguation. The KLK6 is one member of proteinases family that degrades major components of the basal membrane and participated in EMTprocess (62).Our data showsthat KLK6would be upstream to SOX2 and thatVimentin levels arelikely to be regulated by these SOX2 (Scheme 1).

SOX2 overexpression results in increase of β-catenin levels in the nuclei of KLK6 knockdown cells. The presence of nuclear β-catenin was observed after a strong induction of TCF-dependent transcription determined by a transient transfection of two independent (FaDu-Mock and FaDu-shKLK6) clones, with the TOP/FOPFLASH reporter plasmid. In this case, β-catenin migrated to cytoplasm and accumulates in the nuclei. This indicates that SOX2 may be regulated by the accumulation of β-catenin in the nucleus, preventing their transcription. Several genes were related to Wnt pathway and formation of cancer process. In this way, it has already shown that SOX2 antagonizes TCF/β-catenin activity, repressing the Wnt and promotion the negative regulation in the transcription process (75). These data indicates that the proposed KLK6-SOX2-Vimentin axis is apparently influenced by Wnt signaling, since possibly by accumulation of β-catenin affected SOX2 expression (Scheme 1).

In summary, ours results demonstrated the involvement of SOX2 in the carcinogenesis process, since SOX2 participate in the proliferation process, it was also demonstrated that the loss of SOX2 expression occurs in invasive regions which can be correlated with the modulation of EMT that interfere in the Wnt signaling.It was not completely elucidated whether SOX2 alone is able to induce new tumor foci or if it would be the last step of the many transformations necessary for mutations occurring cancer. Other regulatoryprocesses should be

investigated, such as: epigenetic modifications, transcriptional control, alternative splicing, protein stability, and subcellular localization of SOX2. Despite the efforts to identify common regulatory networks in normal development and disease it is not clear that some pathways work isolated. Unraveling the underlying molecular principles of SOX2 and KLK6 will not only help to develop innovative strategies for more efficient and less toxic therapy of HNSCC patients, but could also pave the way for biomarker-driven clinical trials in the future.



Scheme 1: Schematic of the proposed effects of SOX2 during the EMT process.

CONCLUSION

The SOX2 protein content is increased in oral human biopsies of proliferative epithelial disorders, such as hyperplasia, dysplasia, tumor adjacent epithelia and center of the tumor. Nevertheless,SOX2 expression is lost at the invasion zones of the tumor.

In cell culture, SOX2 is involved in the induction of mesenchymal-like phenotype in the KLK6 knock down probably by Wnt signaling. This via might be a player on SOX2 expression by modulating the β-catenin levels, which is a negative regulator of SOX2 expression.

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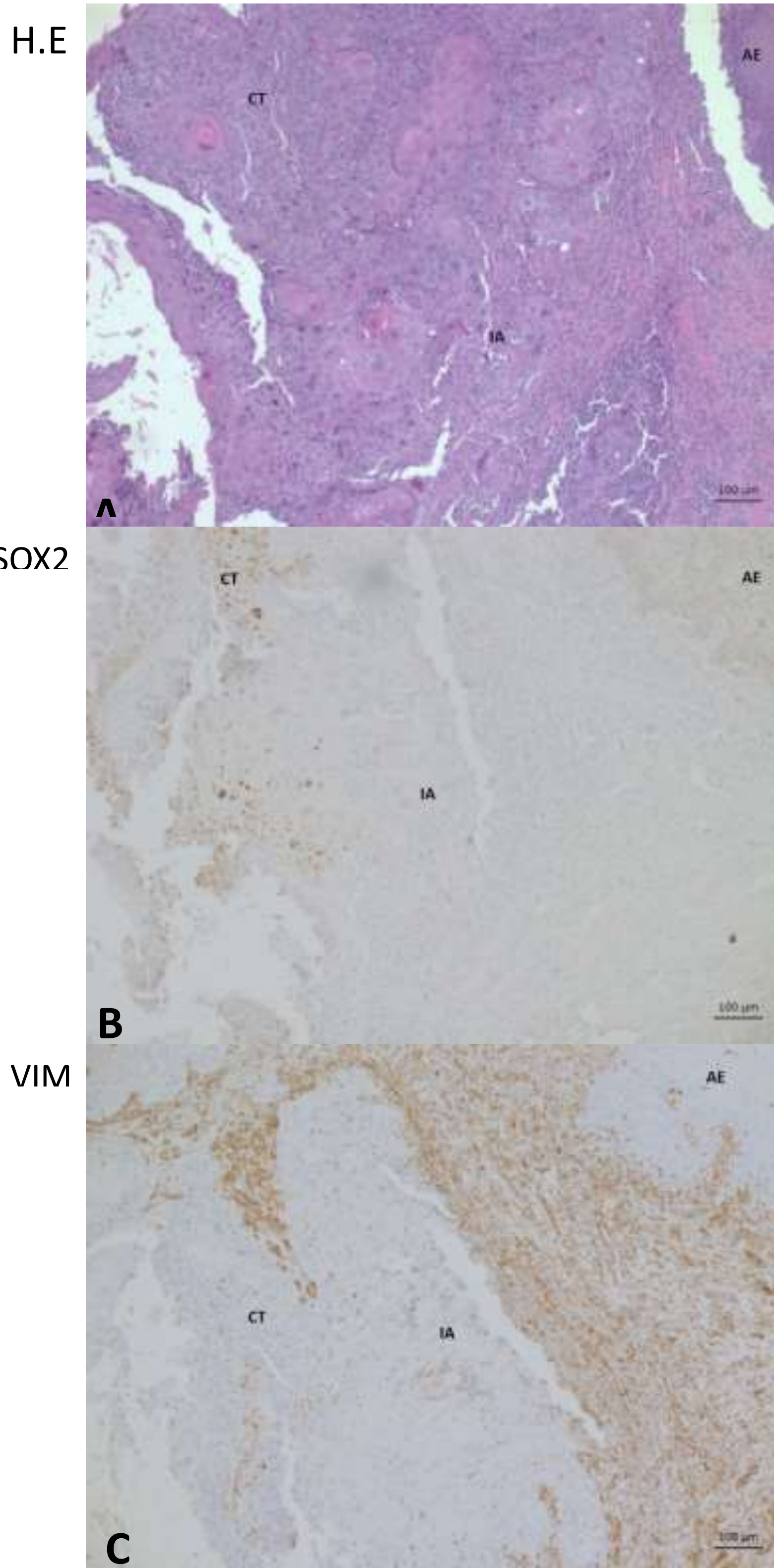
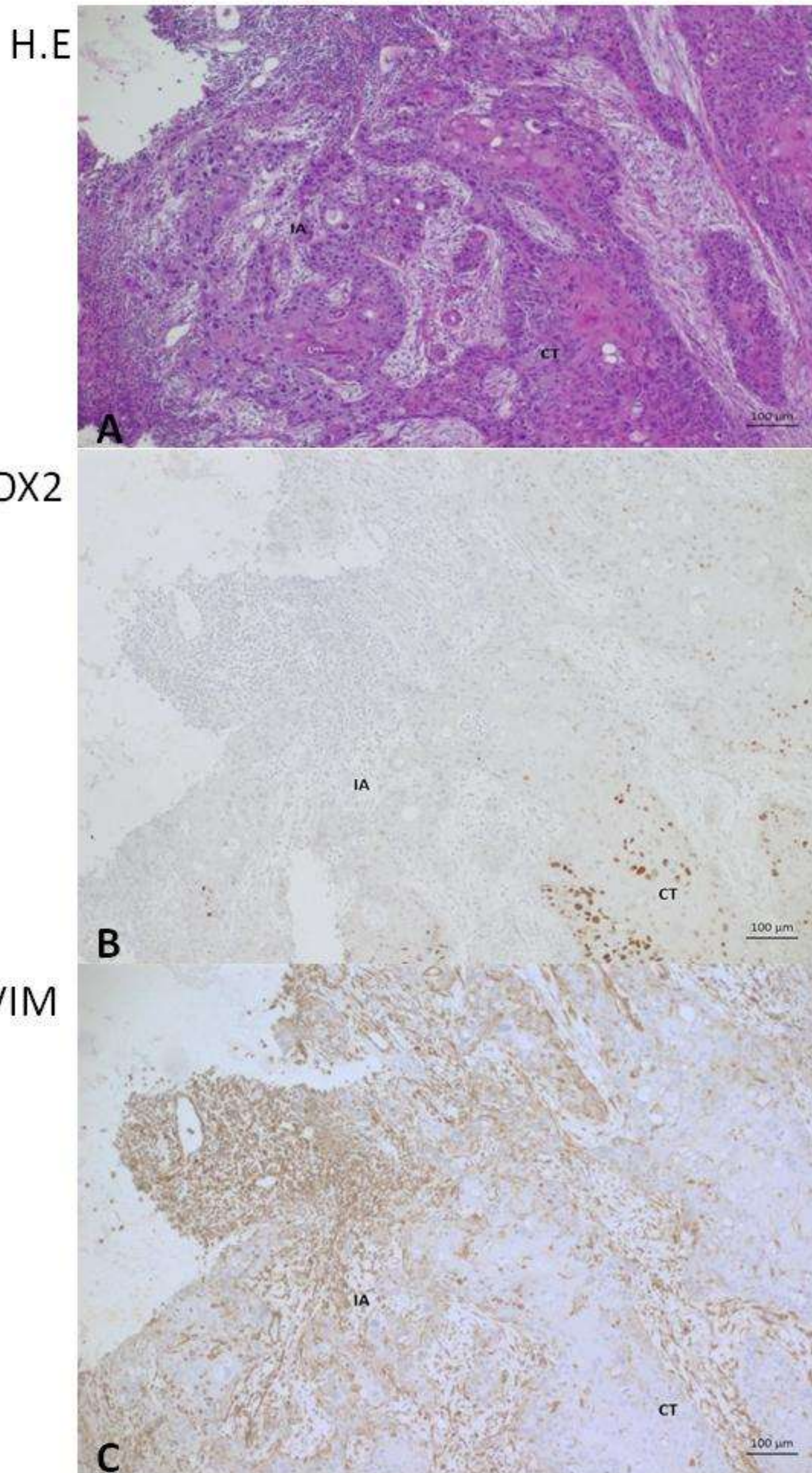
APPENDIX



SUPPLEMENTARY FIGURE 1.:Histological Analysis of Squamous Cell Carcinoma (SCC) Grade I. Staining for Hematoxylin and Eosin (A), SOX2 (B) or Vimentin (C) from three regions of SCC biopsies: Tumor adjacent Epithelia (AE), Center of Tumor (CT) and Invasion Front (IA). Images were obtained at low magnification (10x) It is observed a loss of signal for SOX2 at the invasion front of tumor when compared to center of tumor and adjacent epithelia.



SUPPLEMENTARY FIGURE 2: Histological Analysis of Squamous Cell Carcinoma (SCC) Grade II. Staining for Hematoxylin and Eosin (A), SOX2 (B) or Vimentin (C) from three regions of SCC biopsies: Tumor adjacent Epithelia (AE), Center of Tumor (CT) and Invasion Front (IF). Images were obtained at low magnification (10x) It is observed a lost of signal for SOX2 at the invasion area of tumor when compared to center of tumor and adjacent epithelia.

CURRICULUM VITAE

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EDUCATION

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A. Positions:

MasterResearch: Cell Migration Laboratory, Federal University of Rio Grande do Sul.

Thesis: Analysis of the expression of SOX2 during development of Potential Malignant Lesions and Oral Squamous Cell Carcinoma
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Scientific Initiation (Undergraduate Research):

2008.01 - Volunteer Internship in Production and Characterization of Monoclonal Antibodies. Biotechnology Center, UFRGS – Supervisor: Prof. Dra.Sandra Estrazulas Farias.

2008.02 -Scholarship of Scientific Initiation Poly and Monoclonal Antibodies. Biotechnology Center, Supervisor: Prof. Dra.Aoi Masuda.

Work position:

2009 – at moment: Biotechnology Laboratory Technician, Department ofMorphological Sciences, Histophysiology Laboratory Compared.

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B. Activities in the Scientific Community

2007 - Extension Course in Health and Safety in the Workplace. UFRGS.

2010 - II Neuroscience Course - UFRGS. Summer Course of Graduate Program in Neurosciences – ICBS/UFRGS.

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Group of Migration, Adhesion and Cell Signaling – Leader Group: Prof. PhD Marcelo Lazzaron Lamers

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2009 – 2013: EFFECTS OF PHYSICAL EXERCISE ON THE ACTIVITY AND CELLULAR NEUROTROPHIC SUPPORT THE ROOT GANGLION CELLS DORSAL L5 AND DORSAL HORN OF SPINAL CORD OF ADULT MALE RATS SUBMITTED TO DIABETIC NEUROPATHY. SUPERVISOR: MATILDE ACHAVAL ELENA.

2009-2011: ASSOCIATION OF EXPOSURE TO LIPOPOLYSACCHARIDE, ANOXIA RESTRICTION AND SENSORY-MOTOR: A NEW ANIMAL MODEL FOR CEREBRAL PALSY. SUPERVISOR: MATILDE ACHAVAL ELENA.

2009-2013: EFFECTS OF CHRONIC ADMINISTRATION OF ACID FOLIC ON BEHAVIORAL PARAMETERS, BIOCHEMICAL AND MORPHOLOGICAL IN RATS SUBMITTED TO HYPOXIA-ISCHEMIA NEONATAL. SUPERVISOR: LENIR ORLANDI PERREIRA SILVA.

2010-2012: INFLUENCE OF ENVIRONMENTAL ENRICHMENT ON HIPPOCAMPAL NEUROGENESIS AND MEMORY DEFICIT RATS IN DIABETIC STREPTOZOTOCIN-INDUCED. SUPERVISOR: SIMONE MARCUZZO.

2010 – 2014: BEHAVIORAL ANALYSIS AND MORPHOLOGICAL OF THE PRENATAL EXPOSURE OF ASSOCIATION AND CHOKING LIPOPOLYSACCHARIDE INTRAUTERINE. SUPERVISOR: SIMONE MARCUZZO.

2011-2013: EFFECTS OF ENVIRONMENTAL ENRICHMENT IN RATS SUBMITTED TO HYPOXIA ISCHEMIA NEWBORN: BEHAVIORAL APPROACH AND MORPHOLOGICAL DEVELOPMENT OF LONG. SUPERVISOR: LENIR ORLANDI PERREIRA SILVA.

2012-2015: NEUROTROPHIN-3 IN SPINAL CORD (L4-L6) IN RATS SUBMITTED TO PERIPHERAL NERVE INJURY AND BALANCING EXERCISE AND COORDINATION OR EXERCISE AERÓBICOE. SUPERVISOR: MARIA CRISTINA FACCIONI HEUSER.

2012-2014: EFFECT BEHAVIORAL AND ENVIRONMENTAL MORPHOLOGICAL EARLY ENRICHMENT IN A CEREBRAL PALSY MODEL IN RATS. SUPERVISOR: SIMONE MARCUZZO.

2012-2014: CHARACTERISATION DEATH CELL AND EFFECTS ON DEVELOPMENT AFTER TREATMENT FOLIC ACID ANIMAL SUBJECT TO HYPOXIA-ISCHEMIA NEONATAL. SUPERVISOR: LENIR ORLANDI PERREIRA SILVA.

2012-2015: EVALUATION OF THE ENVIRONMENT THROUGH EARLY STIMULATION RICH IN BRAIN INJURY OF HYPOXIA-ISCHEMIA TYPE: EXPERIMENTAL MODEL A CLINIC. SUPERVISOR: LENIR ORLANDI PERREIRA SILVA.

2012-2016: ADMINISTRATION FOLIC ACID, AS STRATEGY NEUROPROTECTIVE IN RATS SUBMITTED TO HYPOXIA-ISCHEMIA NEONATAL. SUPERVISOR: LENIR ORLANDI PERREIRA SILVA.

2012-2015: NEUROTROPHIN-3 IN SPINAL CORD (L4-L6) IN RATS SUBMITTED TO NERVE INJURY AND PERIPHERAL BALANCE EXERCISE AND COORDINATION OR EXERCISE AEROBICOE. SUPERVISOR: MARIA CRISTINA FACCIONI HEUSER.

2015-2016: PHYSICAL EXERCISE IN MATURITY AND PREGNANCY: EFFECTS ON PHYSICAL DEVELOPMENT AND COGNITION OFFSPRING OF PARENTS AND FERTILITY. SUPERVISOR: SIMONE MARCUZZO.

E. Additional training:

2007 – Course of Histotechnical Processes. (Hours: 150).Federal University of Rio Grande do Sul.

2010 - Experimental Models Of Neurological Diseases. (Hours: 3).Brazilian Society of Neuroscience and Behavior, SBNeC, Brazil.

2010- DEPRESSION: neurochemistry, ANIMAL MODELS AND FARMACO. (Hours: 3).Brazilian Society of Neuroscience and Behavior, SBNeC, Brazil.

2012- Training on classification, collection and labeling of laboratory chemical waste.(Hours: 2). Federal University of Rio Grande do Sul.

2014 - Waste Management Course in Laboratory Environment and Domestic. (Hours: 40). Federal University of Rio Grande do Sul.

2014 - Epigenetics principles in Neuroscience. (Hours: 3).Brazilian Society of Neuroscience and Behavior, SBNeC, Brazil.

F. Publication List:

1. NASCIMENTO, PATRÍCIA S. DO ; LOVATEL, GISELE A. ; **BARBOSA, SÍLVIA** ; Ilha, Jocemar ; CENTENARO, LÍGIA A. ; Malysz, Tais ; XAVIER, LÉDER L. ; SCHAAN, BEATRIZ D. ;Achaval, Matilde . Treadmill training improves motor skills and increases tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta in diabetic rats. Brain Research, v. 1382, p. 173-180, 2011.

2. BAGATINI, PAMELA BRAMBILLA ; XAVIER, LÉDER LEAL ; NEVES, LAURA TARTARI ; SAUR, LISIANI ; **BARBOSA, SÍLVIA** ; BAPTISTA, PEDRO PORTO ALEGRE ; AUGUSTIN,

OTÁVIO AMÉRICO ; DE SENNA, PRISCYLLA NUNES ; MESTRINER, RÉGIS GEMERASCA ; SOUTO, ANDRÉ ARIGONY ; Achaval, Matilde . Resveratrol prevents akinesia and restores neuronal tyrosine hydroxylase immunoreactivity in the substantia nigra pars compacta of diabetic rats. Brain Research, v. 1592, p. 101-112, 2014.

3. MARQUES, MARÍLIA ROSSATO ; STIGGER, FELIPE ; SEGABINAZI, ETHIANE ; AUGUSTIN, OTÁVIO AMÉRICO ; **BARBOSA, SÍLVIA** ; PIAZZA, FRANCELE VALENTE ; Achaval, Matilde ;MARCUZZO, SIMONE . Beneficial effects of early environmental enrichment on motor development and spinal cord plasticity in a rat model of cerebral palsy. Behavioural Brain Research, v. 263, p. 149-157, 2014.

4. BONETTI, LEANDRO VIÇOSA ; SCHNEIDER, ANA PAULA KRAUTHEIN ; **BARBOSA, SILVIA** ; Ilha, Jocemar ; FACCIONI-HEUSER, MARIA CRISTINA . Balance and coordination training and endurance training after nerve injury. Muscle & Nerve (Print), v. 51, p. 83-91, 2014.

5. BONETTI, LEANDRO VIÇOSA ; Ilha, Jocemar ; SCHNEIDER, ANA PAULA KRAUTHEIN ; **BARBOSA, SILVIA** ; FACCIONI-HEUSER, MARIA CRISTINA . Balance and coordination training, but not endurance training, enhances synaptophysin and neurotrophin-3 immunoreactivity in the lumbar spinal cord after sciatic nerve crush. Muscle & Nerve (Print), v. 1, p. n/a-n/a, 2015.

6. TREVISAN, P. ; **Silvia Barbosa** ; SPEROTTO, G. ; COSTI, C. ; OMENA FILHO, R. L. ; SILVA, A. P. ; VARELLA-GARCIA, M. ; FIEGENBAUM, M. ; ROSA, R. F. M. ; ZEN, P. R. G. Macrothrombocytopenia as diagnosis predictor of 22q11 deletion syndrome among patients with congenital heart defects. American Journal of Medical Genetics. Part A, p. n/a-n/a, 2015.

7. LUCÉIA F. SOUZAA,∗, CARLOS HENRIQUE PAGNOA, NIARA DA S. MEDEIROS B, SÍLVIA BARBOSAC, PAULA C.P. DOS SANTOS B, ALESSANDRO RIOS A, MATILDE ACHAVAL C, ERNA V. DE JONGA. The effect of the carotenoid bixin and annatto seeds on hematological markers and nephrotoxicity in rats subjected to chronic treatment with cisplatin. Revista Brasileira de Farmacognosia,2016. Accepted 7 March 2016, Available online 7 April 2016. doi:10.1016/j.bjp.2016.03.005

8. DE CASTRO L., ALEXANDRE; BONETTO P., JÉSSICA HELLEN; ORTIZ D., VANESSA; MÜLLER D., DALVANA; CARRARO C., CRISTINA; BARBOSA, SÍLVIA; NEVES T., LAURA ; XAVIER L., LÉDER; SCHENKEL C., PAULO; SINGAL, PAWAN; KHAPER, NEELAM; ARAUJO R., ALEX SANDER; BELLÓ-KLEIN, ADRIANE. Sulforaphane effects on post-infarction cardiac remodeling in rats: modulation of redox-sensitive pro-survival and pro-apoptotic proteins. Manuscript (JNB-16-140R1) was accepted for publication in the Journal of Nutritional Biochemistry (www.elsevier.com/locate/jnutbio/). May 11, 2016.