UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

RODRIGO FONSECA BUZO

Microscopic and immunohistochemical characterization of tumor development in immunocompromised mice xenografted with cancer stem cells of oral squamous cell carcinoma

Caracterização microscópica e imuno-histoquímica do desenvolvimento tumoral em camundongos imunodeficientes xenotransplantados com células-tronco de câncer de carcinoma epidermóide de boca

BAURU
2020

RODRIGO FONSECA BUZO

Microscopic and immunohistochemical characterization of tumor development in immunocompromised mice xenografted with cancer stem cells of oral squamous cell carcinoma

Caracterização microscópica e imuno-histoquímica do desenvolvimento tumoral em camundongos imunodeficientes xenotransplantados com células-tronco de câncer de carcinoma epidermóide de boca

Dissertação apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Biologia Oral.

Orientadora: Prof.ª Dr.ª Camila de Oliveira Rodini Pegoraro

BAURU
2020

Buzo, Rodrigo Fonseca

Microscopic and immunohistochemical characterization of tumor development in immunocompromised mice xenografted with cancer stem cells of oral squamous cell carcinoma / Rodrigo Fonseca Buzo – Bauru, 2020.

79p. : il. ; 31cm.

Dissertação (Mestrado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientador: Profa. Dra. Camila de Oliveira Rodini Pegoraro

Autorizo, exclusivamente para fins acadêmicos e científicos, a reprodução total ou parcial desta dissertação/tese, por processos fotocopiadores e outros meios eletrônicos.

Assinatura:

Data:

Comitê de Ética da FOB/USP
CEEPA-Proc. Nº 002/2017
Data: 05/05/2017

FOLHA DE APROVAÇÃO

DEDICATÓRIA

*Primeiramente, a **Deus**, pela sua infinita graça, amor e misericórdia que me alcançam todos os dias, me encorajando a realizar este sonho.*

*Aos meus pais **Fred** e **Isabela**, que nunca mediram esforços para me ajudar a chegar até aqui. Amo vocês. Agradeço por sempre estarem ao meu lado.*

AGRADECIMENTOS ESPECIAIS

Em especial agradeço,

*A minha orientadora **Profa. Dra. Camila de Oliveira Rodini**, por me orientar na elaboração deste trabalho. Obrigado pela confiança que depositou em mim. Saiba que você colaborou de forma imensurável para meu amadurecimento profissional.*

*À **Nádia Ghinelli Amôr**, por ser essa amiga e confidente que esteve ao meu lado desde o início, me mostrando sempre o bom que a vida tem para oferecer. Sou muito grato pela paciência em me corrigir e tranquilidade em me ensinar. Para mim, você é um exemplo de amor à pesquisa.*

*Ao **Rafael Carneiro Ortiz**, pela sua amizade e por cooperar grandemente em meu crescimento científico. Sou imensamente grato por todo sua dedicação com este projeto.*

*À **Luciana Mieli Saito** e **Nathália Martins Lopes** pelo apoio e contribuição como grupo de pesquisa. Sou grato por nossa amizade e bom convívio.*

*Às técnicas do laboratório de Histologia, **Danielle Ceolim e Patrícia Germínio**, pela inestimável ajuda prestada neste trabalho. Obrigado por sempre estarem ao me lado, me dando suporte e impulso nos momentos de dificuldade, e também me proporcionando momentos de bom-humor ao lado de vocês.*

*Às demais alunas do departamento de Histologia, **Angélica Fonseca**, **Jéssica Melchiades**, **Paula Sanches** e **Suelen Paini** pela convivência maravilhosa que temos no laboratório. Agradeço a colaboração e amizade.*

*A todos os demais **professores e funcionários da FOB-USP**, por estarem sempre dispostos a ajudar.*

Rodrigo Fonseca Buzo

AGRADECIMENTOS INSTITUCIONAIS

Ao Prof. Dr. Vahan Agopyan, *digníssimo reitor da Universidade de São Paulo;*

Ao Prof. Dr. **Pedro Vitoriano de Oliveira,** *digníssimo Secretário Geral da Universidade de São Paulo;*

Ao Prof. Dr. Carlos Ferreira dos Santos, *digníssimo Diretor da Faculdade de Odontologia de Bauru da Universidade de São Paulo;*

Ao Prof. Dr. Guilherme dos Reis Pereira Janson, *digníssimo Vice-diretor da Faculdade de Odontologia de Bauru da Universidade de São Paulo;*

Ao Prof. Dr. José Henrique Rubo, *digníssimo Prefeito do Campus da Faculdade de Odontologia de Bauru da Universidade de São Paulo;*

À Prof. Dra. Izabel Regina Fischer Rubira Bullen, *digníssima Presidente da Pós-Graduação da Faculdade de Odontologia de Bauru da Universidade de São Paulo;*

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES, *pela bolsa concedida no meu mestrado;*

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), *órgão de fomento deste trabalho (número de processo: 2013/07245-9).*

“ Todas as vitórias ocultam uma abdicação. ”

Simone de Beauvoir

RESUMO

Caracterização microscópica e imuno-histoquímica do desenvolvimento tumoral em camundongos imunodeficientes xenotransplantados com células-tronco de câncer de carcinoma epidermóide de boca

O carcinoma epidermóide de boca (CEB) é uma das neoplasias mais comuns da região de cabeça e pescoço, com sobrevida global inferior a 5 anos. O pior prognóstico da doença está associado à presença de metástases linfonodais, que tem participação de uma subpopulação de células-tronco presente nos tumores, conhecidas por células-tronco de câncer (CSC, do inglês *cancer stem cells*). Essa subpopulação sofre transição epitélio-mesenquimal (EMT, do inglês *epitelial-mesenchymal transition*), processo no qual as células epiteliais adquirem um fenótipo mesenquimal tornando-se migratórias e invasivas. CSC podem ser identificadas por meio de biomarcadores, sendo a proteína CD44 a mais utilizada em CEB. Vale ressaltar que na pesquisa do câncer bucal, os modelos animais têm sido amplamente utilizados como estratégia para entender a carcinogênese, bem como para testar novos agentes antineoplásicos e desenvolver novas abordagens terapêuticas. O objetivo deste estudo foi avaliar e comparar microscopicamente tumores murinos induzidos por xenotransplante de duas subpopulações de CSC, $CD44^{High}ESA^{High}$ (epitelial) e $CD44^{High}ESA^{Low}$ (mensenquimal), presentes em linhagens de CEB humano LUC4. Após isolamento por meio de citometria de fluxo (BD FACSAria™ Fusion), foi realizado o xenotransplante das duas subpopulações com $5x10^3$ células inoculadas na língua, em dois grupos com 12 camundongos machos NOD/SCID cada. Após 49 dias, os tumores formados foram medidos, coletados e submetidos ao processamento histotécnico para caracterização microscópica e imuno-histoquímica. A subpopulação $CD44^{High}ESA^{High}$ apresentou maior potencial tumorigênico, formando tumores (média da área: 4,22 mm²) em todos os animais inoculados. Em contrapartida, apenas seis animais (50%) xenotransplantados com células $CD44^{High}ESA^{Low}$ desenvolveram tumores microscopicamente visíveis (média da área: 0,20 mm²). Foram observadas alterações estruturais e celulares semelhantes ao CEB de humanos em ambos os grupos. Além disso, os animais do grupo $CD44^{High}ESA^{High}$ apresentaram maior perda de peso comparado ao grupo $CD44^{High}ESA^{Low}$ ($p= 0,0217$). A correlação de subpopulações de CSC com seus tumores correspondentes *in vivo* representa uma abordagem confiável para futuras pesquisas sobre câncer bucal, destacando o papel de diferentes fenótipos de CSC no desenvolvimento e progressão de CEB. Estudos adicionais devem ser realizados

nesse campo, por exemplo, para entender como eles respondem na terapêutica comumente usada e desenvolver técnicas para superar os mecanismos de resistência.

Palavras-chave: Carcinoma Epidermóide. Células-tronco Neoplásicas. Transição Epitelial-Mesenquimal. Modelo Animal.

ABSTRACT

Microscopic and immunohistochemical characterization of tumor development in immunocompromised mice xenografted with cancer stem cells of oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is one of the most common neoplasms of the head and neck region, with overall survival <5 years. The worst prognosis of the disease is lymph node metastasis associated with a subpopulation of stem cells in tumors, known as cancer stem cells (CSC). Studies have shown that this subpopulation undergoes epithelial to mesenchymal transition (EMT), a process which epithelial cells acquire a mesenchymal phenotype. In OSCC, CSC can be identified by biomarkers, CD44 transmembrane protein being the most *commonly* used. In oral cancer research, animal models have been widely used as a strategy to understand carcinogenesis as well as to test new antineoplastic agents and to develop new therapeutic approaches. We aimed to evaluate and compare microscopically murine tumors induced by CSC xenotransplantation. Two subpopulations $CD44^{High}ESA^{High}$ (epithelial) and $CD44^{High}ESA^{Low}$ (mensenchymal) were isolated from OSCC cell line LUC4 by flow cytometry (BD FACSAria ™ Fusion). Xenotransplantation was performed with $5x10^3$ cells injected in the tongue into two groups with twelve NOD/SCID mice each one. Forty-nine days post-injection, tumors were measured, collected and submitted to histotechnical processing for microscopic and immunohistochemical analyses. $CD44^{High}ESA^{High}$ cells showed great tumorigenic potential, being able to originate larger tumors in twelve animals (average tumor area: 4.22 mm²). In contrast, only six animals (50%) xenografted with $CD44^{high}ESA^{low}$ cells developed microscopically visible tumors (average tumor area: 0.20 mm²). Structural and cellular changes similar to the human OSCC were observed in both groups. In addition, animals xenografted with $CD44^{High}ESA^{High}$ cells showed greater weight loss compared to the $CD44^{High}ESA^{Low}$ group ($p = 0.0217$). The correlation of CSC subpopulations with their corresponding tumors *in vivo* represents a reliable approach for future research in oral cancer, highlighting the role of different CSC phenotypes in OSCC development and progression. Further studies should be conduct in this field, for example, in understanding how they respond in commonly used therapeutics and develop techniques to overcome their resistance mechanisms.

Keywords: Squamous Cell Carcinoma. Neoplastic Stem Cells. Epithelial-Mesenchymal Transition. Animal Model.

LIST OF FIGURES

DISSERTATION

ARTICLE

LIST OF ABREVIATIONS

OSCC	Oral Squamous Cell Carcinoma
ITF	Invasive Tumor Front
CSC	Cancer Stem Cells
OCT-4	Octamer-binding Transcription Factor 4
SOX-2	Sex Determing Region Y-box 2
SC	Stem Cells
NOD/SCID	Non-obese Diabetic/severe Combined immunodeficient
ALDH1	Aldehyde Dehydrogenase Enzyme 1
BMI1	B-lymphoma Moloney murine leukemia virus insertion 1
HNSCC	Head and Neck Squamous Cell Carcinoma
TSSC	Tongue Squamous Cell Carcinoma
DMBA	9, 10-dimethyl-1, 2-benzanthracene
4NQO	4-nitroquinoline 1-oxide
DNA	Deoxyribonucleic acid
CEB	From Portuguese "*Carcinoma epidermóide de boca*"
CD44	Principal cell surface receptor for hyaluronate

SUMMARY

1 INTRODUCTION

1 INTRODUCTION

1.1 Oral squamous cell carcinoma

Oral squamous cell carcinoma (OSCC) is one of the most common malignant neoplasms of the head and neck region. It involves inner mucosa of the lips, tongue, floor of mouth, gingiva, palate, and buccal mucosa. The major risk factors for OSCC include the frequent use of tobacco and alcohol, as well as papilloma virus infection, associated with genetic susceptibility (PETRICK; WYSS; BUTLER; CUMMINGS *et al.*, 2014). The prognostic factor with the most significant impact is the presence of metastasis in cervical lymph nodes, which occurs in 25 to 65% of the cases (HANNEN; VAN DER LAAK; MANNI; PAHLPLATZ *et al.*, 2001; JÄRVINEN; AUTIO; KILPINEN; SAARELA *et al.*, 2008; KALLURI; WEINBERG, 2009; KOSUNEN; PIRINEN; ROPPONEN; PUKKILA *et al.*, 2007; SZANISZLO; FENNEWALD; QIU; KANTARA *et al.*, 2014; VERED; YAROM; DAYAN, 2005)

OSCC is microscopically characterized by islets and cords of tumor epithelial cells with cellular atypia (nuclear hyperchromasia, pleomorphism, altered nuclear-*cytoplasmic* ratio, frequent and abnormal mitoses, dyskeratosis and keratin pearls) and architectural changes (loss of stratification and basement membrane). The tumors are graded into well-diferentiated areas that resemble closely to normal squamous epithelium, moderately-differentiated areas exhibiting usually less keratinization, and poorly-differentiated (or undifferentiated) areas with predominance of immature cells and minimal keratinization (WHO, 2005). In addition, perivascular, perimuscular and perineural invasion by OSCC cells is often present in the invasive tumor front (ITF) that corresponds to three to six cell layers or detached tumor cell groups at the advancing edge (PIFFKÒ; BÀNKFALVI; OFNER; BRYNE *et al.*, 1997).

1.2 Cancer stem cells

Two hypotheses currently predominate to explain how cancer develops. In the stochastic model (Fig. 1A), all tumor cells are proliferative and display unlimited tumor-initiating capacities (WACLAW; BOZIC; PITTMAN; HRUBAN *et al.*, 2015). In contrast, the cancer

stem cells (CSC) theory (Fig. 1B) suggests that only one subpopulation among all tumor cells is highly proliferative and initiate a new tumor growth (CLEVERS, 2011; KOREN; FUCHS, 2016; REYA; MORRISON; CLARKE; WEISSMAN, 2001). However, the tumor hierarchy is not a one-way route, but can be reversible, which differentiated cells can dedifferentiate and obtain stem-like properties under specific conditions, such as OCT-4, Nanog and SOX-2 overexpression (HERREROS-VILLANUEVA; ZHANG; KOENIG; ABEL *et al.*, 2013; MEACHAM; MORRISON, 2013).

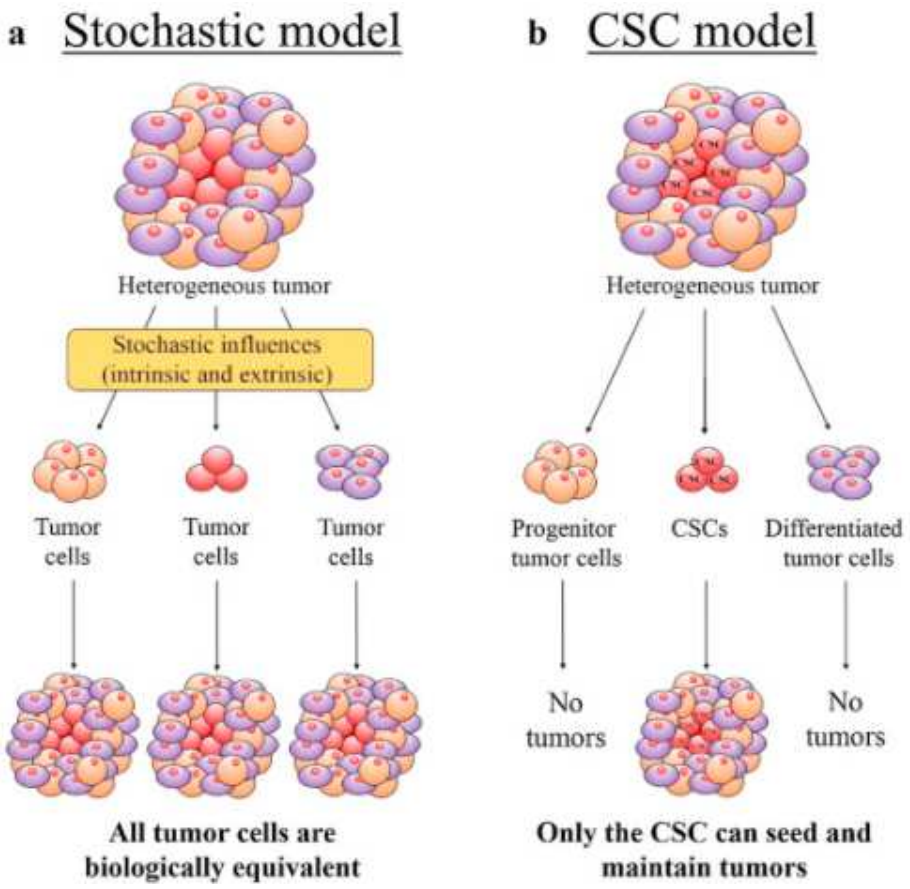

Figure 1. Models of cellular heterogeneity in solid tumors. Tumors are composed of a heterogeneous population of cells with different phenotypic characteristics. Different cell types are shown in different colors; (A) stochastic model: all tumor cells are biologically equivalent and have equal tumorigenic capacities. The behavioral variation is the result of intrinsic and extrinsic stochastic influences; (B) Cancer stem cell hypothesis: only a subpopulation of tumor cells (CSC, in red) is able to self-renew and originate new tumors (KOREN; FUCHS, 2016).

In normal tissues, stem cells (SC) are defined as immature and non-specialized cells that display a unique ability to self-renew and differentiate into specialized cells in an attempt to maintain tissue integrity (EGUSA; SONOYAMA; NISHIMURA; ATSUTA *et al.*, 2012; LEMISCHKA, 2005; VALENT; BONNET; DE MARIA; LAPIDOT *et al.*, 2012). CSC have similar properties to SC and they were identified as an essential source of tumor cells in

different malignancies, such as in oral cancer (ERAMO; LOTTI; SETTE; PILOZZI *et al.*, 2008; O'BRIEN; POLLETT; GALLINGER; DICK, 2007; RODINI; LOPES; LARA; MACKENZIE, 2017). Importantly, these cells are chemoresistant and capable to propagate and support tumorigenesis (DAWOOD; AUSTIN; CRISTOFANILLI, 2014; SHIGDAR; LI; BHATTACHARYA; O'CONNOR *et al.*, 2014). According to Singh *et al*. (2003), the ability to initiate tumors and originate heterogeneous cell populations found in the original tumor are exclusive properties of CSC. In view of that, it is important to develop specific therapies targeting CSC for cancer treatment.

The first evidence of CSC in tumor progression was demonstrated when human leukemic tumor cells were xenografted in non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, and only $CD34^{+}/CD38^{-}$ cells were capable to initiate tumor growth (BONNET; DICK, 1997). Thereafter, CSC were identified by specific markers in solid brain tumors ($CD133^{+}$ cells) (SINGH; CLARKE; TERASAKI; BONN *et al.*, 2003), breast tumors ($CD44^{high}/CD24^{low}/ESA^{+}$) (AL-HAJJ; WICHA; BENITO-HERNANDEZ; MORRISON *et al.*, 2003), prostate tumors ($CD44^{+}$) (PATRAWALA; CALHOUN; SCHNEIDER-BROUSSARD; LI *et al.*, 2006) and oral tumors ($CD44^{+/high}$) (LOCKE; HEYWOOD; FAWELL; MACKENZIE, 2005; PRINCE; SIVANANDAN; KACZOROWSKI; WOLF *et al.*, 2007). Researchers also identified CSC by the high activity of the aldehyde dehydrogenase enzyme (ALDH1) in breast and oral cancers (CLAY; TABOR; OWEN; CAREY *et al.*, 2010; GINESTIER; HUR; CHARAFE-JAUFFRET; MONVILLE *et al.*, 2007).

The subsequent studies on CSC biomarkers revealed that this subpopulation is significantly involved with tumor behavior. BMI1 (also known as B-lymphoma Moloney murine leukemia virus insertion region 1) is involved in the self-renewal, differentiation and initiation of brain and prostate tumors (ABDOUH; FACCHINO; CHATOO; BALASINGAM *et al.*, 2009; LUKACS; GOLDSTEIN; LAWSON; CHENG *et al.*, 2010). Overexpression of BMI1 in an $ALDH1^{+}$ subpopulation was correlated with increased tumor formation, cell migration, local invasion, and metastasis in head and neck squamous cell carcinoma (HNSCC) (YU; LO; CHEN; HUANG *et al.*, 2011). This suggests that presence of BMI1 can be used as a predictive marker of CSC in addition to CD44 and ALDH1.

Research of our group is investigating the role of CSC and tumor microenvironment in OSCC development and progression (Young Investigator Grant, FAPESP # 2013/07245-9). Recently, we found that CD44 immunoexpression was associated with lymph node metastasis,

while high expression of ALDH1 was associated with angiolymphatic invasion (ORTIZ; LOPES; AMÔR; PONCE *et al.*, 2018).

1.3 Epithelial-mesenchymal transition

The epithelial to mesenchymal transition (EMT) is a biological process involved in early stages of embryonic development. Epithelial cells loss apical-basal polarity and intercellular junctions, acquiring a mesenchymal phenotype that enables them to migrate beyond the primary site to integrate into surrounding or distant tissues (PARSANA; AMEND; HERNANDEZ; PIENTA *et al.*, 2017). Importantly, studies have demonstrated that carcinoma cells undergo EMT, acquiring migratory and- invasive properties, which are a critical step during tumor metastasis events (FEDELE; CERCHIA; CHIAPPETTA, 2017; THIERAUF; VEIT; HESS, 2017).

Notably, CSC undergo EMT mediated by cell transcription factors (SLUG, SNAIL and TWIST) activated in response to WNT signaling pathways, leading to intracellular changes such as hyperregulation of vimentin expression, inhibition of E-cadherin expression and nuclear translocation of β-catenin (THIERY, 2003). In OSCC, Biddle *et al*. identified two distinct phenotypes of CSC: one described as proliferative with epithelial characteristics, $CD44^{High}ESA^{High}$; and another with migratory traits, $CD44^{High}ESA^{Low}$, which exhibits mesenchymal properties. Consequently, it is worth to emphasize the role of biomarkers to identify these phenotypes as a target in further cancer therapies (BIDDLE; LIANG; GAMMON; FAZIL *et al.*, 2011).

Liu *et al*. investigated the immunoexpression of EMT-related proteins in tongue squamous cell carcinoma (TSSC). SNAIL, E-cadherin, N-cadherin, and Vimentin were associated with tumorigenesis and pathological outcomes. Vimentin was described as a potential prognostic factor for TSCC patients (LIU; KANG; WU; SUN *et al.*, 2017). In a different study, SNAIL expression in HNSCC was correlated with metastasis and poor prognosis (YANG; CHANG; CHIOU; LIU *et al.*, 2007). Reduced E-cadherin expression at the invasive tumor front suggest that this transmembrane glycoprotein is a noteworthy EMT marker in OSCC (COSTA; LEITE; CARDOSO; LOYOLA *et al.*, 2015). Moreover, high expression of N-cadherin has been correlated to enhanced tumor cell invasion and migration, leading to tumor

growth in carcinomas (SMITH; TEKNOS; PAN, 2013). Thus, SNAIL, E-cadherin, N-cadherin, and Vimentin expression is well recognized and established as EMT markers.

1.4 Experimental models of tumorigenesis *in vivo*

The most used protocol for experimental models of tumorigenesis *in vivo* involves the use of chemical agents as carcinogens. DMBA (9, 10-dimethyl-1, 2-benzanthracene) and 4NQO (4-nitroquinoline 1-oxide) are the most used in oral experimental chemical carcinogenesis. DMBA causes DNA adduct formation, failure in the repair of which leads to the development of cancer (MAAYAH; GHEBEH; ALHAIDER; EL-KADI *et al.*, 2015). DMBA also promotes local irritation, resulting in inflammatory response and necrosis. Thus, early epithelial lesions become difficult to analyze (KANOJIA; VAIDYA, 2006; RAJU; IBRAHIM, 2011). On the other hand, 4NQO is a water-soluble agent, which induces tumors in the oral cavity with histological and molecular changes similar to oral carcinogenesis in humans (VERED; YAROM; DAYAN, 2005). This model often results in multiple lesions, which enables to study all stages of tumorigenesis, including dysplasia, invasive tumor at the primary site and metastasis. However, this model includes a long time for tumor development (at least 48 weeks), the size and the number of tumors are hardly reproducible among replicates, and the mice display low rate of lymphatic metastasis (SZANISZLO; FENNEWALD; QIU; KANTARA *et al.*, 2014).

The gold-standard method applied in the study of CSC phenotype is the xenoimplatation of human tumor cells into immunocompromised animals (SCHATTON; FRANK, 2010). This experimental model generates lesions that displays similarities to the cellular and molecular changes which occur during the development and progression of OSCC in humans (RAJU; IBRAHIM, 2011). In addition, animal models can be used for the development of biological markers for diagnosis and prognosis of the disease (KANOJIA; VAIDYA, 2006).

Immunoexpression of CD44, ALDH1 and BMI1 have been reported as potential prognostic markers in OSCC. In view of the mutual relationship between CSC and EMT in tumor progression, EMT-related proteins (E-cadherin, SNAIL and Vimentin) might be relevant biomarkers in the study of CSC phenotypes. Therefore, we aimed to induce tumorigenesis by xenoimplantation of the CSC subpopulations ($CD44^{High}ESA^{High}$ and $CD44^{High}ESA^{Low}$ into the

tongue of NOD/SCID mice. Further, formed tumors were characterized microscopically by H&E and submitted to immunohistochemistry for the above-mentioned markers.

4 CONCLUSION

4 CONCLUSION

In conclusion, using the surface markers CD44 and ESA we isolated two different phenotypes of CSC from OSCC. Epi-CSC displayed high tumorigenic potential to form heterogeneous tumors into NOD/SCID mice 49 days-post inoculation. Moreover, these tumors reproduced histopathological and immunolabeling pattern similar to human OSCC suggesting that xenografting Epi-CSC into immunocompromised mice represent a trustworthy approach for future oral cancer research.

REFERENCES

REFERENCES

ABDOUH, M.; FACCHINO, S.; CHATOO, W.; BALASINGAM, V. *et al.* BMI1 sustains human glioblastoma multiforme stem cell renewal. **J Neurosci**, 29, n. 28, p. 8884-8896, Jul 2009.

AHMED, S.; JAYAN, L.; DINESHKUMAR, T.; RAMAN, S. Oral squamous cell carcinoma under microscopic vision: A review of histological variants and its prognostic indicators. **SRM Journal of Research in Dental Sciences**, 10, n. 2, p. 90-97, April 1, 2019 2019. Review Article.

AL-HAJJ, M.; WICHA, M. S.; BENITO-HERNANDEZ, A.; MORRISON, S. J. *et al.* Prospective identification of tumorigenic breast cancer cells. **Proc Natl Acad Sci U S A**, 100, n. 7, p. 3983-3988, Apr 2003.

BANSAL, N.; BARTUCCI, M.; YUSUFF, S.; DAVIS, S. *et al.* BMI-1 Targeting Interferes with Patient-Derived Tumor-Initiating Cell Survival and Tumor Growth in Prostate Cancer. **Clin Cancer Res**, 22, n. 24, p. 6176-6191, Dec 2016.

BIDDLE, A.; LIANG, X.; GAMMON, L.; FAZIL, B. *et al.* Cancer stem cells in squamous cell carcinoma switch between two distinct phenotypes that are preferentially migratory or proliferative. **Cancer Res**, 71, n. 15, p. 5317-5326, Aug 2011.

BONNET, D.; DICK, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. **Nat Med**, 3, n. 7, p. 730-737, Jul 1997.

CEKANOVA, M.; RATHORE, K. Animal models and therapeutic molecular targets of cancer: utility and limitations. **Drug Des Devel Ther**, 8, p. 1911-1921, 2014.

CHINN, S. B.; DARR, O. A.; OWEN, J. H.; BELLILE, E. *et al.* Cancer stem cells: mediators of tumorigenesis and metastasis in head and neck squamous cell carcinoma. **Head Neck**, 37, n. 3, p. 317-326, Mar 2015.

CLAY, M. R.; TABOR, M.; OWEN, J. H.; CAREY, T. E. *et al.* Single-marker identification of head and neck squamous cell carcinoma cancer stem cells with aldehyde dehydrogenase. **Head Neck**, 32, n. 9, p. 1195-1201, Sep 2010.

CLEVERS, H. The cancer stem cell: premises, promises and challenges. **Nat Med**, 17, n. 3, p. 313-319, Mar 2011.

COSTA, L. C.; LEITE, C. F.; CARDOSO, S. V.; LOYOLA, A. M. *et al.* Expression of epithelial-mesenchymal transition markers at the invasive front of oral squamous cell carcinoma. **J Appl Oral Sci**, 23, n. 2, p. 169-178, 2015 Mar-Apr 2015.

DANIELSSON, F.; PETERSON, M. K.; CALDEIRA ARAÚJO, H.; LAUTENSCHLÄGER, F. *et al.* Vimentin Diversity in Health and Disease. **Cells**, 7, n. 10, Sep 2018.

DAWOOD, S.; AUSTIN, L.; CRISTOFANILLI, M. Cancer stem cells: implications for cancer therapy. **Oncology (Williston Park)**, 28, n. 12, p. 1101-1107, 1110, Dec 2014.

DE ANDRADE, N. P.; RODRIGUES, M. F.; RODINI, C. O.; NUNES, F. D. Cancer stem cell, cytokeratins and epithelial to mesenchymal transition markers expression in oral squamous cell carcinoma derived from ortothopic xenoimplantation of CD44. **Pathol Res Pract**, 213, n. 3, p. 235-244, Mar 2017.

EGUSA, H.; SONOYAMA, W.; NISHIMURA, M.; ATSUTA, I. *et al.* Stem cells in dentistry--part I: stem cell sources. **J Prosthodont Res**, 56, n. 3, p. 151-165, Jul 2012.

ERAMO, A.; LOTTI, F.; SETTE, G.; PILOZZI, E. *et al.* Identification and expansion of the tumorigenic lung cancer stem cell population. **Cell Death Differ**, 15, n. 3, p. 504-514, Mar 2008.

FEDELE, M.; CERCHIA, L.; CHIAPPETTA, G. The Epithelial-to-Mesenchymal Transition in Breast Cancer: Focus on Basal-Like Carcinomas. **Cancers (Basel)**, 9, n. 10, Sep 2017.

GINESTIER, C.; HUR, M. H.; CHARAFE-JAUFFRET, E.; MONVILLE, F. *et al.* ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. **Cell Stem Cell**, 1, n. 5, p. 555-567, Nov 2007.

GÖTZ, C.; BISSINGER, O.; NOBIS, C.; WOLFF, K. D. *et al.* ALDH1 as a prognostic marker for lymph node metastasis in OSCC. **Biomed Rep**, 9, n. 4, p. 284-290, Oct 2018.

HANNEN, E. J.; VAN DER LAAK, J. A.; MANNI, J. J.; PAHLPLATZ, M. M. *et al.* Improved prediction of metastasis in tongue carcinomas, combining vascular and nuclear tumor parameters. **Cancer**, 92, n. 7, p. 1881-1887, Oct 2001.

HERREROS-VILLANUEVA, M.; ZHANG, J. S.; KOENIG, A.; ABEL, E. V. *et al.* SOX2 promotes dedifferentiation and imparts stem cell-like features to pancreatic cancer cells. **Oncogenesis**, 2, p. e61, Aug 2013.

HU, J.; MIRSHAHIDI, S.; SIMENTAL, A.; LEE, S. C. *et al.* Cancer stem cell self-renewal as a therapeutic target in human oral cancer. **Oncogene**, 38, n. 27, p. 5440-5456, 07 2019.

IRANI, S.; JAFARI, B. Expression of vimentin and CD44 in mucoepidermoid carcinoma: A role in tumor growth. **Indian J Dent Res**, 29, n. 3, p. 333-340, 2018 May-Jun 2018.

JÄRVINEN, A. K.; AUTIO, R.; KILPINEN, S.; SAARELA, M. *et al.* High-resolution copy number and gene expression microarray analyses of head and neck squamous cell carcinoma cell lines of tongue and larynx. **Genes Chromosomes Cancer**, 47, n. 6, p. 500-509, Jun 2008.

KALLURI, R.; WEINBERG, R. A. The basics of epithelial-mesenchymal transition. **J Clin Invest**, 119, n. 6, p. 1420-1428, Jun 2009.

KANOJIA, D.; VAIDYA, M. M. 4-nitroquinoline-1-oxide induced experimental oral carcinogenesis. **Oral Oncol**, 42, n. 7, p. 655-667, Aug 2006.

KOREN, E.; FUCHS, Y. The bad seed: Cancer stem cells in tumor development and resistance. **Drug Resist Updat**, 28, p. 1-12, 09 2016.

KOSUNEN, A.; PIRINEN, R.; ROPPONEN, K.; PUKKILA, M. *et al.* CD44 expression and its relationship with MMP-9, clinicopathological factors and survival in oral squamous cell carcinoma. **Oral Oncol**, 43, n. 1, p. 51-59, Jan 2007.

LEMISCHKA, I. R. Stem cell biology: a view toward the future. **Ann N Y Acad Sci**, 1044, p. 132-138, Jun 2005.

LIU, P. F.; KANG, B. H.; WU, Y. M.; SUN, J. H. *et al.* Vimentin is a potential prognostic factor for tongue squamous cell carcinoma among five epithelial-mesenchymal transition-related proteins. **PLoS One**, 12, n. 6, p. e0178581, 2017.

LOCKE, M.; HEYWOOD, M.; FAWELL, S.; MACKENZIE, I. C. Retention of intrinsic stem cell hierarchies in carcinoma-derived cell lines. **Cancer Res**, 65, n. 19, p. 8944-8950, Oct 2005.

LUKACS, R. U.; GOLDSTEIN, A. S.; LAWSON, D. A.; CHENG, D. *et al.* Isolation, cultivation and characterization of adult murine prostate stem cells. **Nat Protoc**, 5, n. 4, p. 702-713, Apr 2010.

MAAYAH, Z. H.; GHEBEH, H.; ALHAIDER, A. A.; EL-KADI, A. O. *et al.* Metformin inhibits 7,12-dimethylbenz[a]anthracene-induced breast carcinogenesis and adduct formation in human breast cells by inhibiting the cytochrome P4501A1/aryl hydrocarbon receptor signaling pathway. **Toxicol Appl Pharmacol**, 284, n. 2, p. 217-226, Apr 2015.

MEACHAM, C. E.; MORRISON, S. J. Tumour heterogeneity and cancer cell plasticity. **Nature**, 501, n. 7467, p. 328-337, Sep 2013.

MOGNETTI, B.; DI CARLO, F.; BERTA, G. N. Animal models in oral cancer research. **Oral Oncol**, 42, n. 5, p. 448-460, May 2006.

O'BRIEN, C. A.; POLLETT, A.; GALLINGER, S.; DICK, J. E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. **Nature**, 445, n. 7123, p. 106-110, Jan 2007.

ORTIZ, R. C.; LOPES, N. M.; AMÔR, N. G.; PONCE, J. B. *et al.* CD44 and ALDH1 immunoexpression as prognostic indicators of invasion and metastasis in oral squamous cell carcinoma. **J Oral Pathol Med**, 47, n. 8, p. 740-747, Sep 2018.

PARSANA, P.; AMEND, S. R.; HERNANDEZ, J.; PIENTA, K. J. *et al.* Identifying global expression patterns and key regulators in epithelial to mesenchymal transition through multi-study integration. **BMC Cancer**, 17, n. 1, p. 447, Jun 2017.

PATRAWALA, L.; CALHOUN, T.; SCHNEIDER-BROUSSARD, R.; LI, H. *et al.* Highly purified CD44+ prostate cancer cells from xenograft human tumors are enriched in tumorigenic and metastatic progenitor cells. **Oncogene**, 25, n. 12, p. 1696-1708, Mar 2006.

PETRICK, J. L.; WYSS, A. B.; BUTLER, A. M.; CUMMINGS, C. *et al.* Prevalence of human papillomavirus among oesophageal squamous cell carcinoma cases: systematic review and meta-analysis. **Br J Cancer**, 110, n. 9, p. 2369-2377, Apr 2014.

PIFFKÒ, J.; BÀNKFALVI, A.; OFNER, D.; BRYNE, M. *et al.* Prognostic value of histobiological factors (malignancy grading and AgNOR content) assessed at the invasive tumour front of oral squamous cell carcinomas. **Br J Cancer**, 75, n. 10, p. 1543-1546, 1997.

PRINCE, M. E.; SIVANANDAN, R.; KACZOROWSKI, A.; WOLF, G. T. *et al.* Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. **Proc Natl Acad Sci U S A**, 104, n. 3, p. 973-978, Jan 2007.

RAJU, B.; IBRAHIM, S. O. Pathophysiology of oral cancer in experimental animal models: a review with focus on the role of sympathetic nerves. **J Oral Pathol Med**, 40, n. 1, p. 1-9, Jan 2011.

REYA, T.; MORRISON, S. J.; CLARKE, M. F.; WEISSMAN, I. L. Stem cells, cancer, and cancer stem cells. **Nature**, 414, n. 6859, p. 105-111, Nov 2001.

SALUJA, T. S.; ALI, M.; MISHRA, P.; KUMAR, V. *et al.* Prognostic Value of Cancer Stem Cell Markers in Potentially Malignant Disorders of Oral Mucosa: A Meta-analysis. **Cancer Epidemiol Biomarkers Prev**, 28, n. 1, p. 144-153, 01 2019.

SCHATTON, T.; FRANK, M. H. The in vitro spheroid melanoma cell culture assay: cues on tumor initiation? **J Invest Dermatol**, 130, n. 7, p. 1769-1771, Jul 2010.

SEN, S.; CARNELIO, S. Expression of epithelial cell adhesion molecule (EpCAM) in oral squamous cell carcinoma. **Histopathology**, 68, n. 6, p. 897-904, May 2016.

SHIGDAR, S.; LI, Y.; BHATTACHARYA, S.; O'CONNOR, M. *et al.* Inflammation and cancer stem cells. **Cancer Lett**, 345, n. 2, p. 271-278, Apr 2014.

SINGH, S. K.; CLARKE, I. D.; TERASAKI, M.; BONN, V. E. *et al.* Identification of a cancer stem cell in human brain tumors. **Cancer Res**, 63, n. 18, p. 5821-5828, Sep 2003.

SMITH, A.; TEKNOS, T. N.; PAN, Q. Epithelial to mesenchymal transition in head and neck squamous cell carcinoma. **Oral Oncol**, 49, n. 4, p. 287-292, Apr 2013.

SZANISZLO, P.; FENNEWALD, S. M.; QIU, S.; KANTARA, C. *et al.* Temporal characterization of lymphatic metastasis in an orthotopic mouse model of oral cancer. **Head Neck**, 36, n. 11, p. 1638-1647, Nov 2014.

THIERAUF, J.; VEIT, J. A.; HESS, J. Epithelial-to-Mesenchymal Transition in the Pathogenesis and Therapy of Head and Neck Cancer. **Cancers (Basel)**, 9, n. 7, Jul 2017.

THIERY, J. P. Epithelial-mesenchymal transitions in development and pathologies. **Curr Opin Cell Biol**, 15, n. 6, p. 740-746, Dec 2003.

THOMPSON, L. D. R. Squamous cell carcinoma variants of the head and neck. MINI-SYMPOSIUM:HEADANDNECK PATHOLOGY. Current Diagnostic Pathology. 9**:** 384-396 p. 2003.

VALENT, P.; BONNET, D.; DE MARIA, R.; LAPIDOT, T. *et al.* Cancer stem cell definitions and terminology: the devil is in the details. **Nat Rev Cancer**, 12, n. 11, p. 767-775, 11 2012.

VERED, M.; YAROM, N.; DAYAN, D. 4NQO oral carcinogenesis: animal models, molecular markers and future expectations. **Oral Oncol**, 41, n. 4, p. 337-339, Apr 2005.

WACLAW, B.; BOZIC, I.; PITTMAN, M. E.; HRUBAN, R. H. *et al.* A spatial model predicts that dispersal and cell turnover limit intratumour heterogeneity. **Nature**, 525, n. 7568, p. 261-264, Sep 2015.

YAMASHITA, N.; TOKUNAGA, E.; INOUE, Y.; TANAKA, K. *et al.* Clinical significance of co-expression of E-cadherin and vimentin in invasive breast cancer. **Journal of Clinical Oncology**, 33, n. 15_suppl, p. e22013-e22013, 2015.

YANG, M. H.; CHANG, S. Y.; CHIOU, S. H.; LIU, C. J. *et al.* Overexpression of NBS1 induces epithelial-mesenchymal transition and co-expression of NBS1 and Snail predicts metastasis of head and neck cancer. **Oncogene**, 26, n. 10, p. 1459-1467, Mar 2007.

YU, C. C.; LO, W. L.; CHEN, Y. W.; HUANG, P. I. *et al.* Bmi-1 Regulates Snail Expression and Promotes Metastasis Ability in Head and Neck Squamous Cancer-Derived ALDH1 Positive Cells. **J Oncol**, 2011, 2011.

ZHOU, J.; TAO, D.; XU, Q.; GAO, Z. *et al.* Expression of E-cadherin and vimentin in oral squamous cell carcinoma. **Int J Clin Exp Pathol**, 8, n. 3, p. 3150-3154, 2015.

ANNEX

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

CEEPA-Proc. Nº 002/2017.

Bauru, 5 de maio de 2017.

Senhora Professora,

Informamos que Projeto de Pesquisa denominado ***"Caracterização da Relação Funcional entre Macrófagos, Fenótipo Célula-Tronco de Câncer e Fenômeno de Transição Epitélio-Mesenquimal no Carcinoma Epidermóide de Boca"*** tendo Vossa Senhoria como Pesquisador Responsável, que envolve a utilização de animais (roedores), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi analisado e considerado APROVADO em reunião ordinária da Comissão de Ética no Ensino e Pesquisa em Animais (CEEPA), realizada nesta data.

Vigência do projeto:	*Maio/2017 a Maio/2020*
Espécie/Linhagem:	*Camundongos imunodeficientes NOD/SCID*
Nº de animais:	*300*
Peso/Idade	*20g/6-8 semanas*
Sexo:	*Machos e fêmeas*
Origem:	*Centro de Bioterismo da Faculdade de Medicina da USP*

Esta CEEPA solicita que ao final da pesquisa seja enviado um Relatório com os resultados obtidos para análise ética e emissão de parecer final, o qual poderá ser utilizado para fins de publicação científica.

Atenciosamente,

Profª Drª Ana Paula Campanelli

Presidente da Comissão de Ética no Ensino e Pesquisa em Animais

Profa. Dra. Camila de Oliveira Rodini Pegoraro
Docente do Departamento de Ciências Biológicas

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
e-mail: ceepa@fob.usp.br – Fone/FAX (0xx14) 3235-8356
http://www.fob.usp.br