UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE
PIRACICABA

RENATA LUCENA MARKMAN

INTERFERÊNCIA FARMACOLÓGICA NOS LEITORES EPIGENÉTICOS BROMODOMÍNIO EM CARCINOMA MUCOEPIDERMÓIDE DE GLÂNDULA SALIVAR

INTERFERING WITH THE BROMODOMAIN EPIGENOME READERS IN MUCOEPIDERMOID CARCINOMA OF SALIVARY GLANDS

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Estomatopatologia, na Área de Estomatologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Pathology, in the Oral Medicine area.

Orientador: Prof. Dr. Márcio Ajudarte Lopes

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELA ALUNA RENATA LUCENA MARKMAN, E ORIENTADA PELO PROF. DR. MÁRCIO AJUDARTE LOPES

Piracicaba
2018

Agência(s) de fomento e nº(s) de processo(s): CNPq, 152660/2015-1; CAPES

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

L963i

Lucena Markman, Renata, 1990-
Interferência farmacológica nos leitores epigenéticos bromodomínio em carcinoma mucoepidermóide de glândula salivar / Renata Lucena Markman. – Piracicaba, SP : [s.n.], 2018.

Orientador: Márcio Ajudarte Lopes.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Neoplasias das glândulas salivares. 2. Epigenética. 3. Células-tronco neoplásicas. I. Lopes, Márcio Ajudarte, 1967-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Interfering with the bromodomain epigenome readers in mucoepidermoid carcinoma of salivary glands
Palavras-chave em inglês:
Salivary gland neoplasms
Epigenetics
Neoplastic stem cells
Área de concentração: Estomatologia
Titulação: Doutora em Estomatopatologia
Banca examinadora:
Márcio Ajudarte Lopes [Orientador]
Luiz Alcino Monteiro Gueiros
Rogério Moraes de Castilho
Alan Roger dos Santos Silva
Pablo Agustin Vargas
Data de defesa: 02-03-2018
Programa de Pós-Graduação: Estomatopatologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 02 de Março de 2018, considerou a candidata RENATA LUCENA MARKMAN aprovada.

PROF. DR. MÁRCIO AJUDARTE LOPES

PROF. DR. ROGÉRIO MORAES DE CASTILHO

PROF. DR. LUIZ ALCINO MONTEIRO GUEIROS

PROF. DR. ALAN ROGER DOS SANTOS SILVA

PROF. DR. PABLO AGUSTIN VARGAS

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATÓRIA

Aos meus pais, Brivaldo e Andrea, que souberam me mostrar com incansável carinho e amor o verdadeiro valor de uma família. Eles que nunca mediram esforços para dar a mim e às minhas irmãs, a melhor educação, a melhor experiência, o melhor de si. Fonte de toda a minha admiração, sou realmente privilegiada em ser sua filha.

Às minhas queridas irmãs, Deborah e Carolina, que dividiram comigo os melhores momentos da minha vida, que acreditam mais no meu potencial do que eu mesma e que sempre tiveram carinhosas palavras de incentivo.

Ao meu querido Leonardo, com quem cresci e amadureci imensamente nestes onze anos de relacionamento. A sua serenidade, amor e paciência inabaláveis foram imprescindíveis nas nossas conquistas.

AGRADECIMENTOS

À Faculdade de Odontologia de Piracicaba, na pessoa de seu Diretor, Professor Doutor Guilherme Elias Pessanha Henriques.

À Coordenadora dos cursos de pós-graduação da Faculdade de Odontologia de Piracicaba, Professora Doutora Cínthia Pereira Machado Tabchoury, e ao coordenador do Programa de pós-graduação em Estomatopatologia, Professor Doutor Márcio Ajudarte Lopes.

Ao Professor Márcio pela orientação, por ter me concedido a oportunidade de aprender com a sua imensurável experiência, pelo seu exemplo de profissional e sua admirável dedicação à família. Obrigada por todas as oportunidades que tive de crescer pessoalmente e profissionalmente.

À Coordenadora do Serviço de Odontologia Oncológica do Instituto do Câncer do Estado de São Paulo (ICESP) Dra. Thaís Bianca Brandão, pela atenção e oportunidades concedidas.

À Dra. Ana Carolina Prado Ribeiro por dividir toda a sua experiência no manejo odontológico de pacientes oncológicos, por sua amizade e contribuição com minha formação acadêmica.

Aos Professores Drs. Rogério Castilho e Cristiane Squarize com quem tive a oportunidade de aprender os encantos da vida laboratorial e do trabalho em equipe, além de poder ter uma incrível experiência de vida na Universidade de Michigan.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela bolsa de doutorado concedida (Processo: 156473/2013-5) pelos primeiros 20 meses de doutorado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa PDSE (Processo: 88881.132606/2016-01) e bolsa de doutorado.

Aos Professores Doutores das Áreas de Semiologia e Patologia da Faculdade de Odontologia de Piracicaba, Márcio Ajudarte Lopes, Alan Roger dos Santos Silva, Oslei Paes de Almeida, Pablo Agustín Vargas, Edgard Graner, Jacks Jorge Júnior, e Ricardo Della Coletta, pela maneira competente com que conduzem nosso grupo de trabalho.

Às minhas queridas amigas do ‘Sinto muito’, em especial Ana, Débora e Karina, com as quais criei imensa afinidade e foram essenciais durante esses anos de pós-

graduação. Obrigada pelos momentos de descontração e por vibrarem com minhas conquistas.

A Léo e Marisol, com quem pude compartilhar momentos memoráveis em Piracicaba e Ann Arbor, que me acolheram sempre e com quem posso contar em todos os momentos.

Aos amigos que fiz em Ann Arbor, em especial a Liana que me apoiou imensamente desde antes de pôr os pés lá. Estreitar nosso laço de amizade foi um dos grandes ganhos de 2017.

Aos amigos que tive a oportunidade de conhecer enquanto pós-graduandos em Estomatopatologia, Ana Camila, Bruno, Bel, Carol, Celeste, Diego, Ju, Mari, Nati, Pati, Vini e Wag, agradeço pelo convívio, por todas as risadas, por toda a ajuda recebida e por amenizarem um pouco as saudades de casa.

Aos profissionais do Orocentro e do Laboratório de Patologia, em especial ao Professor Alan e Rogério por compartilharem suas experiências, bem como Dona Cida, Dani e Fabi por estarem sempre prontas e dispostas a ajudar quando preciso.

Aos meus avós por todos os valores passados para nossa família. A nossa união é fruto de todo ensinamento que nos passaram. Obrigada por todo apoio e pelas melhores memórias. Aos meus tios e tias queridos que me receberam como filha - diversas vezes - em suas casas e em seus corações. Aos meus primos e primas com quem tenho o privilégio de dividir a nossa família.

Aos meus cunhados, Leonardo e João, por todo o carinho e por representarem os irmãos que nunca tive.

A tio Alexandre, tia Penha e Beca, que me acolheram com imenso carinho em sua família durante os últimos onze anos e que representam minha segunda família.

Às minhas inseparáveis amigas do Colégio Santa Maria, Dani, Duda, Gabi Acioli, Gabi Monteiro, Jú, Malu, Maga, Nati Loira, Nati Farias, Xande, Rafa e Thaís por todos os felizes momentos compartilhados durante a infância e adolescência.

Às minhas amigas de turma na UFPE, Lud, Mari e Rhay, por todas as horas de estudo compartilhadas, por todo apoio e conselhos oferecidos.

À minha família felina, Mel, Mia, Lia e Vida, companheiras incondicionais, que contribuem para minha felicidade diária.

A todos os demais profissionais e pessoas que de alguma forma contribuíram para a realização deste trabalho ou estiveram presentes em minha vida me fornecendo o suporte necessário para a conquista dos meus objetivos.

RESUMO

O carcinoma mucoepidermoide (CME) é a neoplasia maligna mais comum das glândulas salivares. O CME de alto grau possui uma evolução clínica imprevisível e é frequentemente associado a um pior prognóstico. Alguns estudos recentes, associam a progressão do câncer e a resposta inflamatória a uma classe de leitores epigenéticos do tipo bromodomínio. Bromodomínios estão envolvidos na transcrição gênica através de sua interação com resíduos de lisina na cauda das histonas. O BRD4, membro da família BET (*"Bromodomain and ExtraTerminal"*) de bromodomínios, tem sido associado a alguns oncogenes como o c-MYC e outros eventos inflamatórios. Neste estudo, demonstramos que CMEs apresentam uma maior expressão de BRD4 e a interferência farmacológica com estes bromodomínios foi capaz de diminuir a proliferação das células tumorais. Além disso, inibidores da família BET são moduladores eficientes da via do NFkB e potentes inibidores da população de células tronco tumorais. Em estudos prévios, foi demonstrado que CMEs apresentam altos níveis de NFkB que estão associados com sua resistência à radioterapia. Por fim, foi observado que a interferência da interação BRD4 e histona interrompe o ciclo celular e ativa senescência em células tumorais de CME. Em resumo, estes achados identificam bromodomínios como mecanismos epigenéticos que podem estar desregulados em CME e passíveis de interferência farmacológica como nova estratégia para tratamento deste tumor.

Palavras-chave: Tumor de glândula salivar, BET, BRD4, epigenética, células tronco tumorais, senescência, iBET762, epi-drug.

ABSTRACT

Mucoepidermoid carcinomas (MEC) are the most common malignancy of the salivary glands. High-grade MEC is particularly unpredictable and often associated with poor prognosis. Emerging evidence suggests the involvement of bromodomains as a conserved class of epigenome readers in cancer progression and inflammatory response. Bromodomains are directly associated with epigenetic modification of gene transcription through its interaction with the lysine residues of histone tails. The bromodomain family member BRD4 is particularly involved in the control of oncogenes including c-MYC, and in the maintenance of downstream inflammatory events and associated molecules. Here we showed that MECs are endowed with high expression levels of BRD4 and that targeting the acetyl-binding pockets of the bromodomains BET family demonstrated a potent anti-proliferative effect in MEC cells. Additionally, BET inhibitors are efficient modulators of the NFkB signaling pathway and capable of reducing the population of cancer stem cells. Notably, we have previously shown that MEC tumors present high levels of NFkB that are associated with its resistance to radiotherapy. Finally, we observed that targeting disruption of BRD4 histone interaction results in cell cycle arrest and activation of cellular senescence in MEC tumors cells. Altogether, our finding indicates that bromodomains constitute a new epigenetic mechanism found deregulated in MEC and that the use of BET inhibitors constitutes a feasible therapeutic strategy for managing MEC.

Keywords: Salivary gland tumor, BET, BRD4, epigenetic, cancer stem cells, senescence, iBET762, epi-drug.

SUMÁRIO

1 INTRODUÇÃO

1.1 NEOPLASIAS DE GLÂNDULA SALIVAR

As neoplasias de glândula salivar são incomuns e respondem por 0.4 a 13.5 casos a cada 100.000 indivíduos, anualmente. Já a frequência de neoplasias malignas é ainda menor, respondendo por apenas 0.4 a 2.6 casos a cada 100.000 indivíduos o que representa apenas 0.3% das doenças malignas nos Estados Unidos (Barnes et al., 2005). Este grupo de lesões é extremamente heterogêneo e é possível observar uma variação geográfica na distribuição dos tipos de tumores. A maioria dos estudos que envolve a América do Norte e América Latina demonstram uma maior predominância de tumores benignos ao passo que estudos realizados na África, Europa e Ásia, com exceção do Japão, descrevem predominantemente tumores malignos (Abrahão et al., 2016). Embora muitos estudos retrospectivos sobre a incidência de tumores de glândula salivar tenham sido descritos, a epidemiologia destas neoplasias não é bem estabelecida devido às limitações dos estudos disponíveis. A origem do espécime também pode se mostrar como um viés na determinação da prevalência de tumores por localização anatômica. Enquanto que hospitais recebem mais espécimes de glândulas salivares maiores, laboratórios de patologia oral e faculdades de odontologia tendem a receber uma maior quantidade de espécimes de glândulas salivares menores (Fonseca et al. 2012).

A maioria das neoplasias de glândula salivar ocorre na glândula parótida, seguido de glândulas salivares menores e da glândula submandibular. Nas glândulas salivares menores, neoplasias malignas são geralmente mais frequentes (Lopes et al., 1999; Vargas et al., 2002; Ito et al., 2005). A distribuição entre os gêneros é usualmente igualitária, apresentando uma pequena tendência para predileção pelo sexo feminino e as lesões usualmente são diagnosticadas entre a quarta e quinta décadas de vida (Fonseca et al., 2012; Vasconcelos et al., 2016).

A principal linha de tratamento para neoplasias malignas de glândula salivar é a ressecção cirúrgica. A remoção profilática dos linfonodos cervicais não é recomendada, exceto para casos específicos. A radioterapia é reservada para pacientes com tumores inoperáveis ou pode ser utilizada de forma adjuvante ao tratamento para pacientes com doença residual, presença extensiva de metástase nodal, ruptura de cápsula, casos indiferenciados ou de alto grau histológico, presença

de invasão perineural, doenças avançadas com envolvimento do nervo facial, casos com margens cirúrgicas comprometidas e invasão de vasos sanguíneos e/ou linfáticos. A quimioterapia é considerada paliativa e indicada apenas para casos com doença metastática avançada ou incuráveis (Guzzo et al., 2010).

1.1.1 CARCINOMA MUCOEPIDERMÓIDE

O carcinoma mucoepidermoide (CME) é a neoplasia maligna de glândula salivar mais comum, representando 30-60% dos tumores malignos (Lopes et al., 1998; Vargas et al., 2002; Cruz Perez et al., 2004; Pires et al., 2007; Fonseca et al., 2012; Saghravanian et al., 2013). A localização mais comum nas glândulas salivares maiores é a parótida; e o palato para tumores de glândulas salivares menores (Lopes et al., 1998; Fonseca et al., 2012). O comportamento clínico do CME é muito variado. Esta neoplasia geralmente se apresenta como uma massa de crescimento indolor e tumores superficiais demonstram coloração azulada semelhante a um mucocele ou tumor vascular (Figura 1). Dependendo da localização de origem do CME, a apresentação clínica, sintomatologia e tratamento podem variar (Coca-Pelaz et al., 2015).

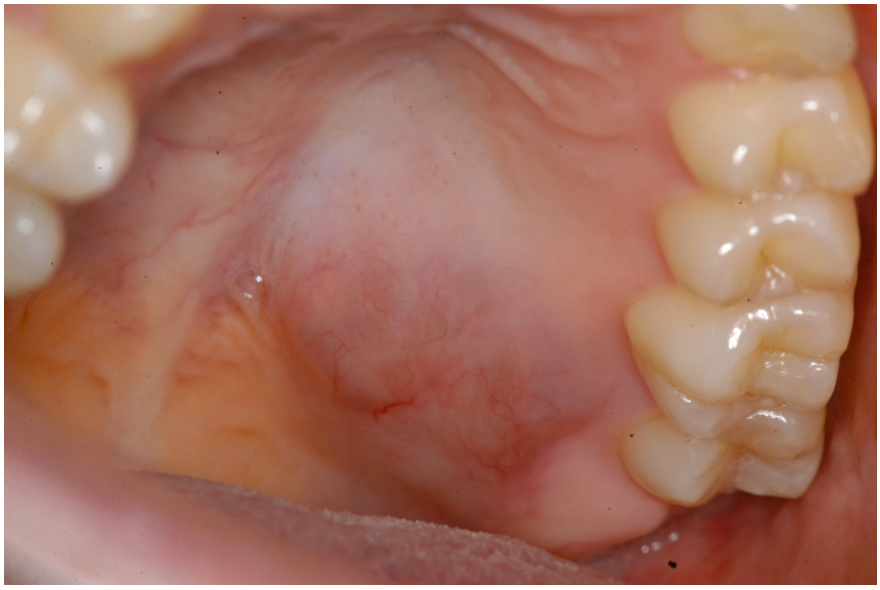

Figura 1. Imagem clínica de tumefação em palato duro, lado esquerdo. Após biópsia incisional, o diagnóstico foi de carcinoma mucoepidermóide. (Imagem cedida pelo prof. Márcio Ajudarte Lopes, acervo Orocentro).

Muitos fatores prognósticos têm sido estudados a fim de elucidar o comportamento altamente variável deste tumor. Características clínicas e

histopatológicas convencionais como idade, gênero, localização, estadio, classificação TNM e extravasamento capsular, terapia adjuvante e comprometimento de margens cirúrgicas, tem se mostrado importantes parâmetros preditores de sobrevivência, embora inconstantes (Granic et al., 2017). Dentre estes, a translocação t(11;19) que acarreta na fusão dos genes CRT1-MAML2, tem sido descrita em aproximadamente 40% dos casos e é associada com curso mais indolente do tumor (Okabe et al., 2006). No entanto, os fatores prognósticos de sobrevida mais relevantes, permanecem o grau do tumor e o estadio da doença (Granic et al., 2017).

A sobrevida livre de doença é geralmente favorável em casos específicos como em tumores de baixo grau, de pequenas dimensões e sem envolvimento linfonodal (Byrd et al., 2013). No entanto, tumores de alto grau e com estadio avançado tem pior prognóstico e sobrevida (Lopes et al., 2006; Birkeland et al., 2017).

O tratamento para CME segue a mesma linha das demais neoplasias malignas de glândula salivar, sendo a excisão cirúrgica com margens de segurança, a terapia mais indicada (Lopes et al., 2006). Radioterapia adjuvante é reservada para casos com margens cirúrgicas comprometidas ou tumores de alto grau com grande risco de recorrência. A quimioterapia é pouco utilizada em CME, pois estes tumores são pouco quimiossensíveis. Esta modalidade terapêutica tem sido utilizada no contexto paliativo para pacientes com doença incurável, embora apresente baixo impacto no prognóstico (Gilbert et al., 2006; Coca-Pelaz et al., 2015).

Considerando que os CME avançados apresentam taxas de sobrevida extremamente baixas, observa-se a necessidade de desenvolvimento de novas estratégias terapêuticas mais eficazes para estes casos. Além disso, terapias sistêmicas eficazes podem ser utilizadas para pacientes com tumores mais brandos visando evitar o procedimento cirúrgico – muitas vezes mutilador - e melhorar substancialmente a qualidade de vida (Wagner, 2016). Desta forma, a descoberta de novos alvos terapêuticos para tratamento do CME é importante a fim de melhorar o manejo dos pacientes acometidos por esta neoplasia.

1.2 LEITORES EPIGENÉTICOS BROMODOMÍNIO

Todas as células do organismo contem a mesma sequência de DNA, mas são capazes de se diferenciar e manter fenótipos distintos que expressam funções biológicas diferentes. Este processo é possível devido à epigenética (Pérez-Salvia e

Esteller, 2017). O termo “epigenética” foi primeiramente citado por C.H. Waddington em 1939 (Waddington, 1939) e posteriormente definido como mudanças hereditárias na expressão gênica sem alterar a sequência de pares de bases do DNA (Waddington, 2012). Aberrações na regulação epigenética são frequentes no câncer e o evento epigenético mais conhecido é a metilação do DNA. Em termos gerais, células cancerosas sofrem uma hipometilação generalizada do seu DNA, e hipermetilação selecionada de alguns genes promoters, especificamente genes supressores de tumor (Pérez-Salvia e Esteller, 2017; Castilho et al., 2017).

Além da metilação do DNA, modificações nas histonas também são um importante evento epigenético. Histonas podem sofrer vários tipos de modificações, as mais comuns sendo fosforilação, acetilação, metilação, ubiquitinação e sumoilação. A alteração dos padrões normais de modificação das histonas é comum no câncer, assim como mutações e desregulação de enzimas responsáveis por adicionar, remover ou reconhecer essas marcas epigenéticas (Pérez-Salvia e Esteller, 2017).

A acetilação de histonas é uma das principais modificações que ocorrem nas caudas destas proteínas e tem sido extensamente estudada no contexto do código das histonas. Acetilação da cromatina tem geralmente sido associada com seu estado “aberto” e ativação da transcrição (Castilho et al., 2017), embora alguns estudos tenham achado algumas marcas de acetilação como responsáveis pela compactação da cromatina (Shogren-Knaak et al., 2006), estabilização de proteínas e regulação da interação proteína-proteína (Kouzarides, 2007).

A acetilação dos resíduos de lisina na porção amino-terminal da cauda das histonas é regulada pelas enzimas histona acetiltransferases (HATs) - que adiciona grupamentos acetil - e histonas deacetilases (HDACs) – que remove estes grupamentos. Muitas vezes, estas enzimas possuem expressão aberrante no câncer, sofrendo mutações e estando sujeitas a outros mecanismos de desregulação (Castilho et al., 2017).

Modificações epigenéticas também contribuem para a plasticidade celular durante a progressão do câncer e na formação das células tronco tumorais (CTT) – uma pequena população de células tumorais envolvidas na resistência terapêutica, recorrência e recidivas dos tumores. Desta forma, a caracterização dos eventos epigenéticos envolvidos em carcinogênese, e a identificação de marcadores

epigenéticos associados às CTT se mostram promissores no desenvolvimento de novas estratégias para tratamento dos tumores (Castilho et al., 2017).

Os bromodomínios (BRDs) são pequenos módulos de aproximadamente 110 aminoácidos que possuem um importante papel na regulação da transcrição e remodelamento da cromatina (Filippakopoulos et al., 2012). Estas proteínas parecem ser alvos epigenéticos com potencial de interferência farmacológica o que encorajou o desenvolvimento de inúmeras moléculas inibidoras nos últimos anos (Pérez-Salvia e Esteller, 2017).

Um total de 61 bromodomínios foram encontrados em 46 diferentes proteínas do proteoma humano. Eles são classificados em subfamílias de acordo com sua estrutura. Os bromodomínios adotam uma estrutura de 4 α-hélices, cada uma separada por regiões conectoras flexíveis de diferentes tamanhos, formando bolsões hidrofóbicos capazes de reconhecer lisinas acetiladas (Filippakopoulos et al., 2012).

Uma família de bromodomínios em especial, a BET (*"Bromodomain and ExtraTerminal"*) tem sido extremamente investigada. A família BET é composta por BRD2, BRD3, BRD4, e BRDT, que são expressos de forma ubíqua, exceto pelo BRDT, expresso somente nos testículos. A característica principal da BET são dois bromodomínios em sequência (BD1 e BD2) na extremidade N-terminal e um domínio extraterminal (ET) na extremidade C-terminal (Figura 2) (Pérez-Salvia e Esteller, 2017).

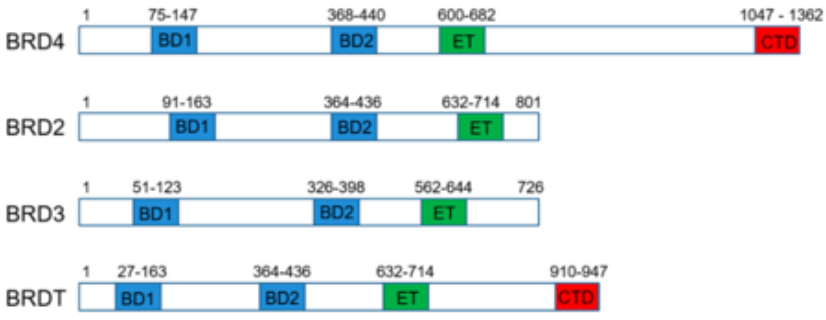

Figura 2. Organização esquemática dos membros da família BET de bromodomínios (Adaptado de Hajmirza et al., 2018).

1.2.1 BRD4

BRD4 foi primeiramente associado ao carcinoma de linha média por identificação de uma translocação t(15; 19)(q13;p13.1) que ocasionou uma interrupção na sequência codificadora do BRD4 e sua fusão ao gente NUT. O envolvimento do BRD4 e provavelmente do NUT na regulação da cromatina sugere que a fusão BRD4-NUT possa prevenir a expressão de genes necessários para a diferenciação epitelial (French, 2001). Desde sua primeira associação a um tumor maligno, estudos *in vitro* e *in vivo* sobre o BRD4 revelaram que esta proteína desempenha um significante papel na regulação do ciclo e da proliferação celular, e também de genes dependentes de NFkB, sendo importante em cânceres desencadeados pelo NFkB (Houzelstein et al., 2002; Mochizuki et al., 2008).

BRD4 é um regulador universal da transcrição gênica, desta forma, era de se esperar que sua inibição causasse *downregulation* de toda atividade gênica. No entanto, a inibição de BRD4 *downregulates* apenas poucas centenas de genes, a maioria dos quais envolvidos no processo de tumorigênese. Em vista disto, BRD4 se mostrou um alvo farmacológico passível de interferência para tratamento do câncer e possui inúmeros inibidores atualmente em ensaios clínicos (Pérez-Salvia e Esteller, 2017).

O iBET762 é um composto sintético que foi desenvolvido para inibir a família BET de bromodomínios (Chung et al., 2011). Atualmente, esta droga está em ensaio clínico para inúmeras malignidades como doenças hematológicas (NCT01943851), carcinoma tipo NUT de linha média (NCT01587703), câncer de mama receptor hormonal positivo (NCT02964507) e câncer de próstata resistente à castração (NCT03150056) (Clinical trials from https://clinicaltrials.gov/ - acessado em 24/10/2017).

Tendo em vista estes dados previamente descritos, o objetivo geral desta tese foi avaliar o efeito *in vitro* de novas alternativas terapêuticas para o CME através do uso de uma droga que atua diretamente em vias possivelmente envolvidas com os processos de tumorigênese. Além disso, o efeito desta terapia sobre a população de CTT foi avaliado uma vez que estas estão diretamente relacionadas com a resistência a terapias convencionais e são as principais responsáveis pelo desenvolvimento de metástases e recidivas após o tratamento.

2 Interfering with the Bromodomain Epigenome Readers in Salivary Gland Tumors

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Word count manuscript: 3,149
Figures: 5

Key words: Salivary gland tumor, BET, BRD4, epigenetic, cancer stem cells, senescence, iBET762, epi-drug.

Abstract

Mucoepidermoid carcinomas (MEC) are the most common malignancy of the salivary glands. High-grade MEC is particularly unpredictable and often associated with poor prognosis. Emerging evidence suggests the involvement of bromodomains as a conserved class of epigenome readers in cancer progression and inflammatory response. Bromodomains are directly associated with epigenetic modification of gene transcription through its interaction with the lysine residues of histone tails. The bromodomain family member BRD4 is particularly involved in the control of oncogenes including c-MYC, and in the maintenance of downstream inflammatory events and associated molecules. Here we showed that MECs are endowed with high expression levels of BRD4 and that targeting the acetyl-binding pockets of the bromodomains BET family demonstrated a potent anti-proliferative effect in MEC cells. Additionally, BET inhibitors are efficient modulators of the NFkB signaling pathway and capable of reducing the population of cancer stem cells. Notably, we have previously shown that MEC tumors present high levels of NFkB that are associated with its resistance to radiotherapy. Finally, we observed that targeting disruption of BRD4 histone interaction results in cell cycle arrest and activation of cellular senescence in MEC tumors cells. Altogether, our finding indicates that bromodomains constitute a new epigenetic mechanism found deregulated in MEC and that the use of BET inhibitors constitutes a feasible therapeutic strategy for managing MEC.

Introduction

Mucoepidermoid carcinoma (MEC) represents the most common primary malignant salivary gland tumor (de Oliveira et al. 2009; Fonseca et al. 2012; Vargas et al. 2002). Overall, salivary gland tumors are rare and comprised of a heterogeneous group of lesions accountable for less than 1% of all tumors and between 3-5% of all head and neck cancers (Saghravanian et al. 2013). The annual incidence of salivary gland tumors ranges between 0.4 - 13.5 cases per 100,000 population (Barnes et al. 2007).

Although uncommon, salivary gland tumors are difficult to manage. While small and early stage tumors can be well managed with surgery, advanced cases and high-grade tumors respond poorly to available therapy. Current treatment of choice for MEC is complete surgical resection with radio and chemotherapy frequently reserved for positive surgical margins, regional metastasis, recurrent or inoperable cases resulting in common functional and aesthetic complications (Cerda et al. 2014; Coca-Pelaz et al. 2015; Grisanti et al. 2008; Posner et al. 1982).

Over the years, cancer research efforts have focused mostly on the genetic basis of tumor development and progression. However, different from genetic mutations, epigenetic alterations cause changes in gene expression independent from genetic alterations in the underlying DNA sequences. In fact, epigenetic modifications have been associated with critical changes on gene expression during initiation and progression of human cancers. Remarkably, epigenetic alterations are potentially reversible by small molecules known as Epi-drugs; therefore, targeting epigenetics as cancer therapeutic strategy is gaining growing interest (Castilho et al. 2017; Cerda et al. 2014).

A highly conserved class of epigenome readers, the bromodomains (BRDs) target chromatin-modifying enzymes and other protein machinery to specific sites in the chromatin, which results in strong gene transcription regulation (Perez-Salvia and Esteller 2017; Segura et al. 2013). The bromodomain and extra-terminal domain (BET) family of proteins is made up by BRD2, BRD3, BRD4, and BRDT (bromodomain testis-associated), which are ubiquitously expressed. The exception to this rule is BRDT, which is only found in the testis. Their main features are two bromodomains in tandem (called BD1 and BD2) present in the N-terminal and a C-terminal extra-terminal (ET) domain (Perez-Salvia and Esteller 2017).

Notably, BRD4 was first associated with aggressive midline carcinoma by identification of a translocation that caused an interruption in BRD4 coding sequence gene and BRD4 fusion also displays an oncogenic function (French et al. 2001). Ever since *in vitro* and *in vivo* studies of BRD4 have revealed a critical role in the regulation of cell cycle progression and cellular proliferation (Houzelstein et al. 2002; Mochizuki et al. 2008). Therefore, BRD4 appears to be a potential druggable epigenetic target for cancer treatment, which has encouraged the discovery and development of several small-molecule inhibitors in recent years (Perez-Salvia and Esteller 2017). Of these, iBET762 is currently in clinical trial for NUT-midline carcinoma, refractory hematologic malignancies, estrogen receptor-positive breast cancer, solid tumors and castrate-resistant prostate cancer (Clinical trials from https://clinicaltrials.gov/ - accessed on October 24, 2017).

In this study, we assessed the effect of pharmacologically displacing the BET family of proteins in MEC cell lines. Altogether, our results support a role for epigenetic readers in MEC maintenance and the target of bromodomains as a novel therapeutic intervention against this disease.

Materials and Methods

Human tissue specimens and Immunofluorescence

Human MEC tissue specimens (diagnosed between January 2011 and February 2017) were retrieved from archives of the Oral Pathology Laboratory of State University of Campinas, Piracicaba, São Paulo, Brazil (Human Research Ethics Committee approval: 60264016.5.0000.5418). The original hematoxylin-eosin stained slides were reviewed by 2 independent pathologists) to confirm the diagnosis. Patient information and tumor grading are presented in Figure 1C. Paraffin blocks were sectioned into 3-μm sections, deparaffinized in xylene and hydrated in descending grades of ethanol. Tissues were blocked in 0.5% (v/v) Triton X-100 in PBS and 3% (w/v) bovine serum albumin (BSA) and then incubated with anti-BRD4 (Cell Signaling Technology). Cells were then washed three times, incubated with Alexa 488 - conjugated secondary antibody and stained with Hoechst 33342 for visualization of DNA content. Images were taken using a QImaging ExiAqua monochrome digital camera attached to a Nikon Eclipse 80i Microscope (Nikon Melville, NY, USA) and visualized with QCapturePro software.

Cell lines

MEC cell lines UM-HMC3A, UM-HMC3B and UM-HMC5, were established at the University of Michigan School of Dentistry. All tumor cell lines authenticity is routinely verified by short tandem repeat (STR) profiling. Cells were maintained in a 5% CO_2 humified incubator at 37°C and cultured in DMEM – High glucose (Hyclone Laboratories Inc, Logan, UT USA), supplemented with 10% Fetal Bovine Serum (Thermo Scientific, Waltham, MA USA), 1% antibiotic (Invitrogen, Carlsbad, CA, USA), 1% L-glutamine (Invitrogen), 20 ng/ml epidermal growth factor (PeproTech, Rocky Hill, NJ, USA), 400 ng/ml hydrocortisone (Sigma-Aldrich) and 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO USA). Where indicated, UM-HMC3A, UM-HMC3B, and UM-HMC5 were treated with 2 μM of iBET762 (Sigma-Aldrich).

Colony forming assay

For the colony forming assay, cells were plated into 6-well culture plates at a concentration previously determined by plating efficiency. After overnight incubation, cells were treated with iBET762 every 12 hours with a 2μM concentration for 8 days. Following, cells were stained with 0.1% crystal violet. Colonies with more than 50 cells were counted as surviving colonies and normalized with the colony number observed in control (vehicle) cells.

Flow cytometry

MEC cancer stem cell-like cells were resuspended and counted following the assay for aldehyde dehydrogenase (ALDH) activity and CD44 stain (BD Biosciences, Mountain View, CA, USA) expression using flow cytometry. The Aldefluor kit (StemCell Technologies, Durham, NC, USA) was used according to the manufacturer's instructions to identify cells with high ALDH enzymatic activity. Positive control and negative controls were included. In addition, cells with or without pretreatment, as indicated in individual experiments, were suspended with activated Aldefluor substrate (BODIPY amino acetate) or negative control (dimethylamino benzaldehyde, a specific ALDH inhibitor) for 45 minutes at 37°C. Then, cells were washed and suspended with CD44 – APC conjugated and incubated for 25 min in shaking rotor at 4°C. All samples were analyzed using a flow cytometer Accuri C6 (BD Biosciences, USA) equipped with two excitation lasers: a solid blue state (488 nm) and a diode red (640 nm) providing

up to 6 simultaneous detection parameters, including 4 fluorescent colors plus FSC and SSC.

Cell Cycle

Cell cycle distribution was accessed by propidium iodide staining. After treatment with iBET762, cells were harvested and fixed with 70% ethanol on ice for 2 hours. The cell pellet was resuspended in 0.5mL PBS containing 0.25% Triton X-100 for permeabilization and incubated for 15 minutes on ice. Cells were then incubated with PBS containing propidium iodide (Sigma-Aldrich; 20 μg/mL) and RNase solution (Sigma-Aldrich; 10 μg/mL) for 30 min at room temperature. The relative number of cells in different phases of the cell cycle was accessed by flow cytometry, and the percentages of cells in G1, S, and G2 were calculated.

Immunohistochemistry

Cells were plated on glass coverslips in 6-well plates. After the indicated treatment, cells were fixed with 3 % paraformaldehyde for 20 minutes. The avidin-biotin blocking kit was used to block nonspecific binding (Kit Vector Laboratories, Burlingame, CA, USA). Slides were incubated overnight with anti-$p16^{ink4}$ (BD Biosciences, Mountain View, CA, USA) and then incubated with diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, St. Louis, MO, USA) and counterstained with Mayer's hematoxylin.

Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Statistical tests used were a one-way analysis of variance (ANOVA) and Student's *t*-test. Asterisks denote statistical significance (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; and NS $P > 0.05$).

Results

Mucoepidermoid carcinomas express high levels of BRD4

The activation of epigenome readers leads to the recruitment of chromatin-modifying enzymes, which results in an important regulator of gene transcription. Normal salivary gland (NGS) tissues presented low levels of the epigenome reader

BRD4 in the nucleus, which were considered as basal levels (Fig. 1A_BRD4-red). Interestingly, we found that MEC tumor tissue samples present a high number of BRD4 positive cells compared to normal salivary gland (NSG) (Fig. 1B) (*** P<0.001). All samples were from MEC tumors diagnosed as low and intermediate grade (Fig. 1C). We also found that MEC cell lines also present most of the tumor cells expressing nuclear BRD4 (Fig. 1D), aligned with our findings in paraffin sections of human MEC tumors, in which all cell lines contained nuclear BRD4. Although no clinical association could be established in our samples, the increase in BRD4 expression suggests that this pathway plays a role in MEC behavior.

Interfering with BRD4-histone interaction

The post-translation modification driven by the acetylation of lysines can result in changes in gene transcription and activation of signaling pathways; and these processes were observed in normal physiology and tumors, including those from the head and neck (Castilho et al. 2017; Martins et al. 2016; Wagner et al. 2016). The main readers of N-acetyl lysine marks are bromodomains that interact with histone acetylation through a hydrophobic acetyl-lysine binding site. Here, we administered the compound iBET762 (GSK525762A) that competitively and selectively bind to the acetyl-lysine binding motifs and displace BRD4 protein from the chromatin (Mirguet et al. 2013; Nicodeme et al. 2010). As expected, we confirmed that also in MEC cells, BRD4 protein levels did not change upon administration of iBET762 (Fig. 2A, ns P>0.05). Although this was not shown in MECs before, our findings are aligned with iBET762 pharmacokinetics, which displaces BRD4 from the chromatin; therefore, blocking its function (but does not induce its degradation). Interestingly, we found that iBET762 was able to reduce the protein levels of NFkB, a key regulator of cytokine and chemokine production, in all MEC cell lines (Fig. 2B) (** P<0.01, *** P<0.001) indicating that NFkB is a novel molecular cue for BRD4 activity in MEC.

iBET762 disrupts the colony forming ability of MEC cell lines

Here, we explored the effects of BET selective inhibition on the colony forming ability of human MEC cell. Administration of iBET762 result in a dramatic reduction in the ability of tumor cells to form colonies (Fig. 3A). BRD4 displacement caused a significant decrease in colony formation from UM-HMC5 (* P<0.05), while UM-HMC3A *** P<0.001) and UM-HMC3B (** P<0.001) could not form colonies larger than 50 cells.

Indeed, when exploring the effects of iBET762, we observed that tumor cells were not undergoing cell death, but rather tumor cells were stationary or quiescent, compared to control cells receiving vehicle alone (Fig. 3A – inserts) (* P<0.05, ** P<0.001, *** P<0.001).

Bromodomain proteins are required for the maintenance of the population of MEC cancer stem cells

The combination of ALDH activity and CD44 expression enables the identification of a unique population of tumor cells characterized by increased tumorigenic potential, ability to self-renew and to differentiate into cells that establish the tumor mass (Adams et al. 2015). These tumor cells are known as cancer stem cells (CSCs) and are also implicated in tumor recurrence and resistance to chemo and radiotherapy (Guimaraes et al. 2016; Wagner et al. 2016). Here we showed that by targeting and displacing BRD4 with the use of iBET762, we disrupted colony forming properties of MEC tumor cells. Using a similar approach, we administered iBET762 for 48 hours following the identification of ALDH/CD44 double positive cells using fluorescence-activated cell sorting (FACS). Surprisingly, we found that iBET762 significantly reduces the population of CSCs in UM-HMC3B, UM-HMC3B and UM-HMC5 when compared to vehicle-treated cells (Fig. 3B and C) (* P<0.05, ** P<0.001). Our findings demonstrate that disruption of the BET family of bromodomains in MEC tumors is an effective therapeutic strategy to target CSCs.

iBET762 induces G1-cell cycle arrest and boosts activation of cellular senescence

Our results showed induction of G1 cell cycle arrest of MEC cells upon administration of iBET762 (Fig. 4A). To further explore the effects of iBET762 on tumor cells we decided to investigate the molecular mechanism associated with the reduced colony forming and cell cycle arrest. Recently, Wang et al. found that BRD4 inhibitors blocked telomere elongation in mouse cells overexpressing telomerase and long treatment with these drugs proved effective in telomere shortening in both mouse and human cells (Wang et al. 2017). Here we found that all three MEC cell lines receiving iBET762 undergo cellular senescence (Fig. 4B). Indeed, the expression levels of the senescence marker $p16^{ink4}$ were elevated in iBET762-treated cells compared to control (vehicle) (** P<0.001, *** P<0.001), suggesting that BRD4 plays a role in telomere

maintenance, activation of senescence and cell cycle arrest in MEC cell lines (Fig. 5A and B).

Discussion

Although MEC is the most common malignant salivary gland tumor, only a few studies have addressed the role of systemic therapy in the adjuvant and palliative protocols. While the combination of high-dose chemotherapy and radiation (compared to radiation alone) has led to significant improvements in survival of patients with head and neck squamous cell carcinoma, the efficacy of chemotherapy seems to be limited for high-grade MEC in both the curative and palliative settings (Granic et al. 2017). The discovery of new druggable pathways is critical to the development of new therapeutic strategies to better manage MEC. This is particularly true for patients afflicted with recurrent tumors or demonstrating resistance to available therapy. Although MEC tumors present common oncogenic gene fusions (CRTC1/3-MAML2)(Birkeland et al. 2017), recent evidence suggests that epigenetic alterations are also implicated in the early stages of carcinogenesis. Of interest to this study, BET plays a vital role in early tumor progression by interaction with histones and modifying transcription (Devaiah et al. 2016).

Over the last decade, the development of new inhibitors targeting the acetyl-binding pockets of the bromodomains BET family has shown a potent anti-proliferative effect in several malignancies (Segura et al. 2013; Wu et al. 2017; Zuber et al. 2011). These proteins activate transcription through their ability to bind to acetyl-modified lysine residues of histone tails and also recruit complexes of RNA polymerase II, thus ensuring transcriptional initiation and elongation. Two classes of BET inhibitors, benzodiazepines, and quinolines have significant antiproliferative activity against a variety of hematologic and solid tumors (Chaidos et al. 2014). In 2010, a diazepine-based compound, called iBET762 was shown to be an inflammatory suppressor (Nicodeme et al. 2010). This drug displaced a tetra-acetylated H4 peptide with a pre-bound to tandem bromodomains of BET and had a high affinity for BRD4. The BRD4 protein constitutes part of the machinery that activates transcription and is key for multiple gene activation events, particularly cell proliferation, cell division, metabolic adaptation and cell survival that are controlled by the oncogene c-MYC (Baylin and Jones 2016; Delmore et al. 2011). BRD4 also regulates NFkB-dependent genes and

prevents the degradation of Rel A, which maintains NFkB activity and thus it plays an important role in NFkB-driven cancers (Perez-Salvia and Esteller 2017).

Recently, we described a high nuclear expression of NFkB (active form) in MEC human samples and cell lines indicating that NFkB accumulation leads to the development of resistance to ionizing radiation in salivary gland tumors (Wagner et al. 2016). In this context of therapy resistance, the tumor cells are responsive to conventional chemotherapy whereas CSCs are resistant to therapy and ultimately re-establish the tumor at the local or distant sites (Castilho et al. 2017). The presence of CSCs was also recently shown in several MEC cell lines (Guimaraes et al. 2016). Therefore, the prospective ability of BET inhibitor to disrupt NFkB signaling and deplete CSCs is exciting and noteworthy as a single agent or as an adjuvant therapy for MEC.

Here we found that MEC tumors expressed higher levels of nuclear BRD4 compared to normal salivary gland tissues. Similar to our findings, a higher expression of BRD4 is found in melanoma compared to nevi and in renal cell carcinoma compared to normal adjacent tissue (Segura et al. 2013; Wu et al. 2017). In addition to the higher amounts of nuclear BRD4 in human MEC samples, we also found it in human MEC cell lines. As expected, MEC cells receiving iBET762 did not modify the levels of BRD4. These results are similar to the results using another BET inhibitor (JQ1) in breast cancer cells (Perez-Salvia and Esteller 2017). Furthermore, these results were consistent with multiple analysis such as immunofluorescence, western blot and real-time PCR (qRT-PCR).

Aligned with our results showing an inhibitory effect in CSC, Yokoyama, and collaborators (Yokoyama et al. 2016) also found that BET inhibitors significantly suppressed ALDH activity by abrogating BRD4-mediated ALDH1A1 expression in ovarian cancer cells. BRD4 plays a key role in CSCs by selectively regulating ALDH1A1 super-enhancer, and its inhibition suppresses the expression of stem-related genes by reducing BRD4 association with their promoters (Yokoyama et al. 2016). When using ALDH activity and CD44 expression to identify CSCs in MEC cell lines, we found a significant decrease of ALDH/CD44 positive cells after treatment with iBET-762. Given the role of CSCs in tumor resistance and recurrence, these results suggest that patients with MEC might benefit from the targeted ablation of this population of cancer cells as seen in iBET762 treatment.

In search for the biological effects of iBET762 administration, we found that MEC cells undergo cellular senescence. Activation of senescence is a cellular

response to stress that is triggered by many mechanisms, such as telomere shortening, and culminates in an irreversible cell cycle arrest program (Castilho et al. 2009; Collado et al. 2007; Liu et al. 2007; Tasdemir et al. 2016). In fact, activation of cellular senescence is characterized by the inability of cells to progress through the cell cycle, typically arrested in G1. Nonetheless, senescent cells remain metabolically active (Campisi and d'Adda di Fagagna 2007). Notably, iBET762 treatment induced G1-cell cycle arrest in the MEC cell lines resulting in impaired colony formation. These findings are consistent with the BRD4 knockdown in melanoma cell lines which caused impaired cell cycle progression into S phase and reduced overall proliferation by increasing levels of G1 arrest (Mochizuki et al. 2008; Segura et al. 2013). Additionally, BRD4 was recently identified as a novel positive regulator of telomere length (Wang et al. 2017). Telomere maintenance is required for long-term division of cells and interesting, is evident in virtually all types of malignant cells contributing to limitless replicative potential, a known hallmarks of cancer (Hanahan and Weinberg 2000). Relatedly, three BRD4 inhibitors were shown to block telomere elongation induced by telomerase overexpression in a dose-dependent manner (Wang et al. 2017), which might be the mechanism that triggered senescence observed in our study by the accumulation of $p16^{ink4}$ when MEC cells were treated with iBET762.

Finally, our findings reveal BRD4 with prooncogenic functions in MEC as a regulator of multiple processes such as cell-cycle progression, CSCs, proliferation, and senescence. In this regard, and knowing that BET inhibitors are already progressing through clinical trials, our experimental results support the possibility of using iBET-762 as a novel treatment to MEC, allowing for strong regulation of the epigenetic and transcriptional machinery in control of gene expression in this tumor.

Acknowledgment

This work was conducted during a visiting scholar period at University of Michigan, sponsored by the Capes Foundation within the Ministry of Education, Brazil (grant n. BEX / 88881.132606/2016-01 PDSE). This grant was funded by the University of Michigan School of Dentistry faculty grant, and the Cancer Center Support Grant (P30 CA046592).

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Author contributions

RLM performed most of the cell culture based assays, IHC, IF, and participated in the organization of the figures. CHVNF executed the flow cytometry, LAR helped with IHC and IF, LPW helped with the immunofluorescence and quantifications. PAV, MAL, CHS, and RMC contributed to the conception, design, data organization, and writing of the manuscript. Authors gave final approval and agreed to be accountable for all aspects of the work.

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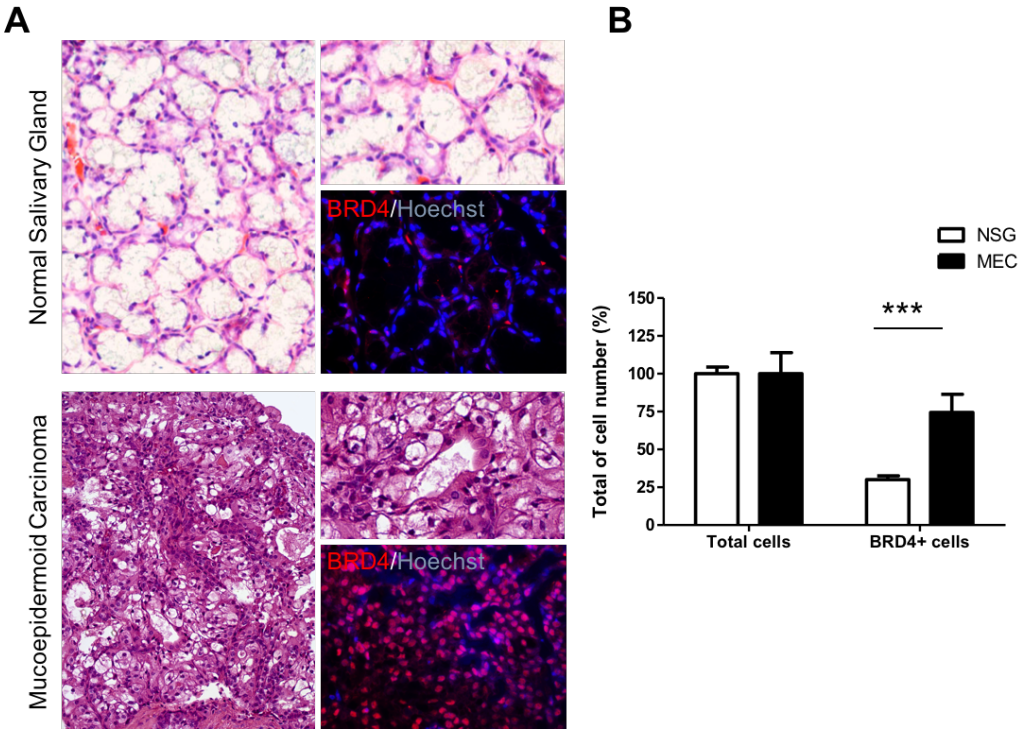
Figure 1



C

Histologic grade	Gender	Age	Location
low grade	male	13 yo	hard palate
intermediate	female	52 yo	buccal mucosa
low grade	female	38 yo	buccal mucosa
intermediate	female	42 yo	hard palate
NA	female	70 yo	retromolar trigone

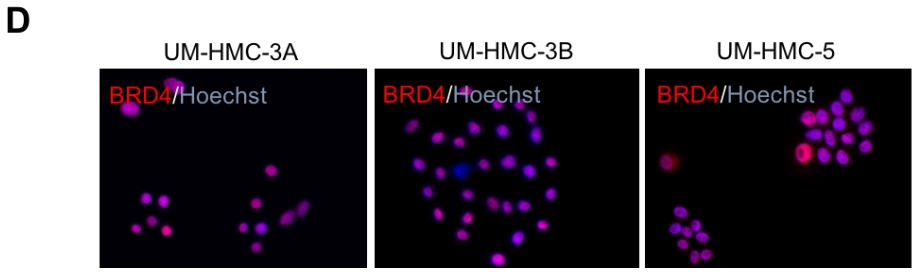


Figure 1: BRD4 is expressed in MEC tissue tumors and MEC cell lines. (A) Representative histological sample of a normal salivary gland and Mucoepidermoid Carcinoma. Upper right insert demonstrates histological magnification of representative histological sections. Lower right depict immunofluorescence for BRD4 (red) and DNA staining using Hoechst (blue). (B) Quantification of the total number of positive cells for BRD4 from normal salivary glands (30% SEM 2.58) and MEC tissue samples (74.4% SEM 12.02). Results present % of positive cells baseline corrected for the total number of cells stained for Hoechst (*** P<0.001). (C) Histological grading and location of MEC tissue samples. (D) MEC cell lines (UM-HMC-3A, UM-HMC-3B, UM- HMC-5) presenting nuclear expression of BRD4 (red) and stained for Hoechst (blue).

Figure 2

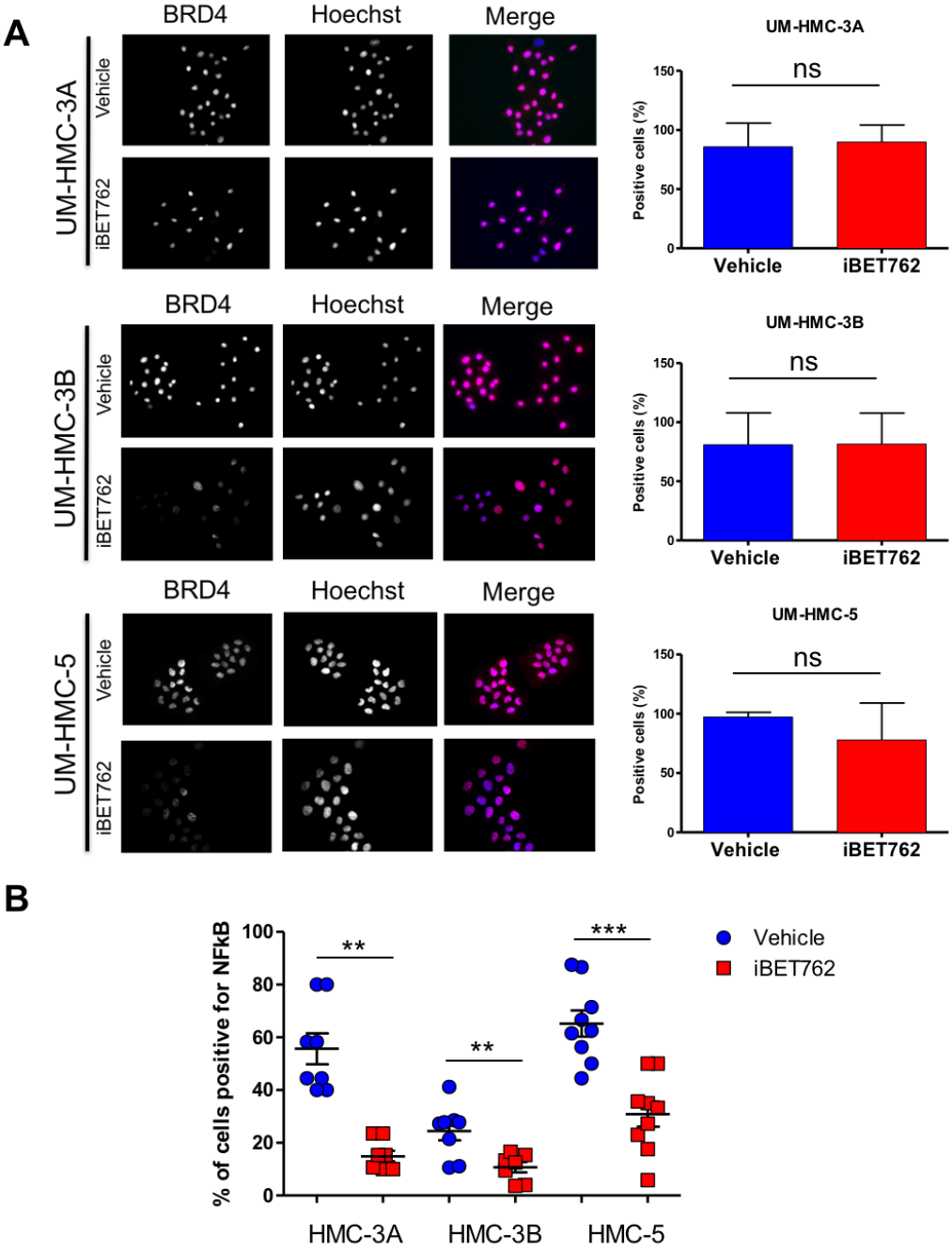


Figure 2: BRD4 and NFkB expression after administration of iBET762. (A) Protein levels of BRD4 were not disrupted by iBET762 in all MEC cell lines as observed by immunofluorescence. iBET762 was administered for 12 hours. Results are plotted in graphs. (ns, P>0.05). (B) NFkB expression levels in MEC cell lines upon administration of iBET762 for 12 hours. (** P<0.01, *** P<0.001).

Figure 3

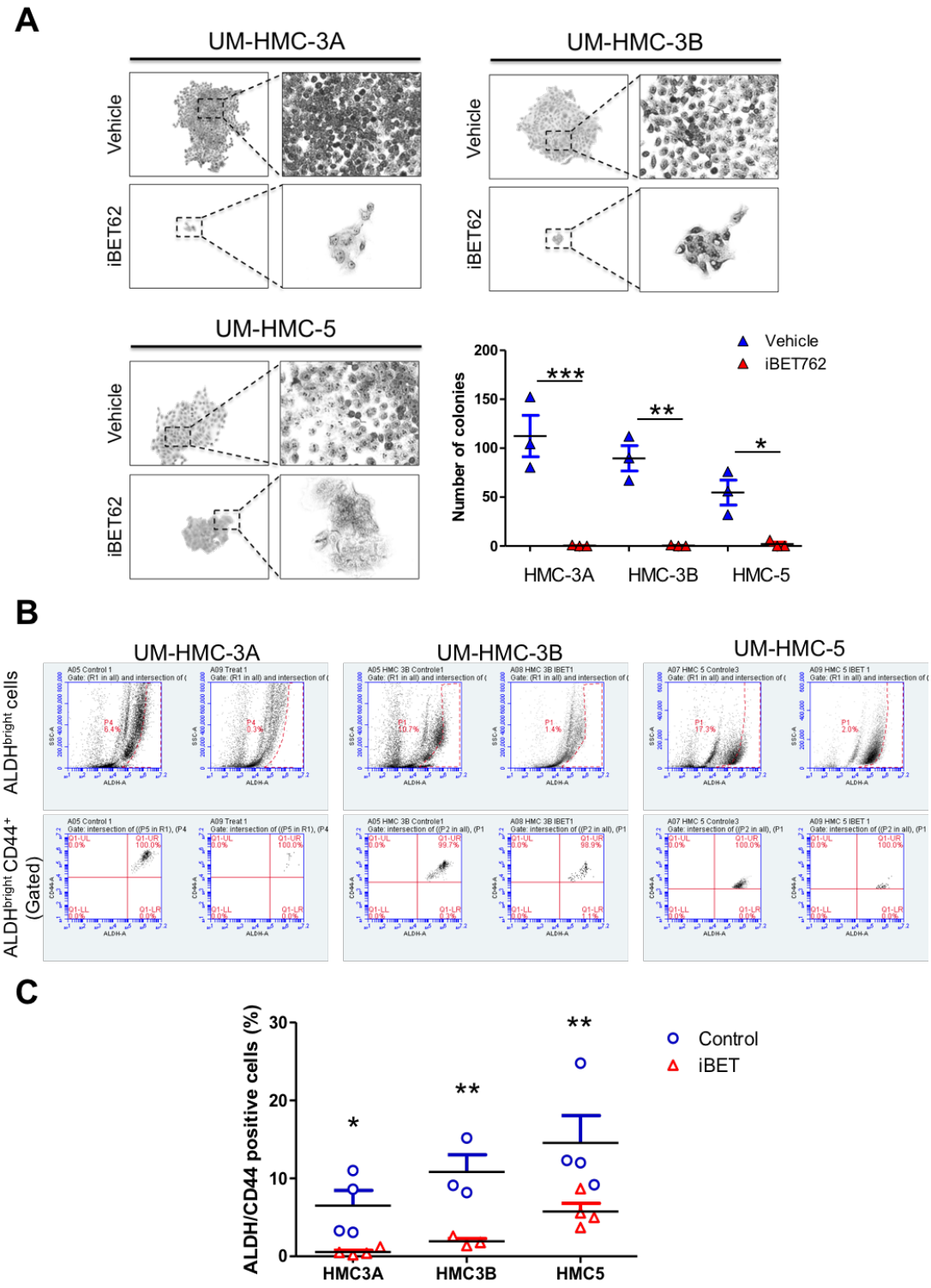


Figure 3: BET inhibitor disrupts the ability of tumor-forming colonies and depletes CSCs. (A) Colony forming assay of MEC tumor cells cultured for 8 days. Note the difference in colony size when comparing vehicle and iBTE762-treated MEC cells. Quantification of the total number of colonies formed by at least 50 cells each is depicted in the graphic. (* P<0.05, ** P<0.01, *** P<0.001).
Cells receiving iBET762 every 12 hours for 48 hours were collected and processed for ALDH enzymatic activity (ALDH bright) and CD44+ using fluorescence-activated cell

sorting (FACS). (B) Representative samples of ALDHbright cells are shown in the first row; Gated ALDHbright/CD44+ cells are represented in the second row. (C) Quantification of BRD4 displacement leads to depletion of CSCs in MEC cell lines compared to vehicle alone represented in the upper right (UR) quadrant of the Gated ALDHbright/CD44+ cells. (* $P<0.05$, ** $P<0.01$).

Figure 4

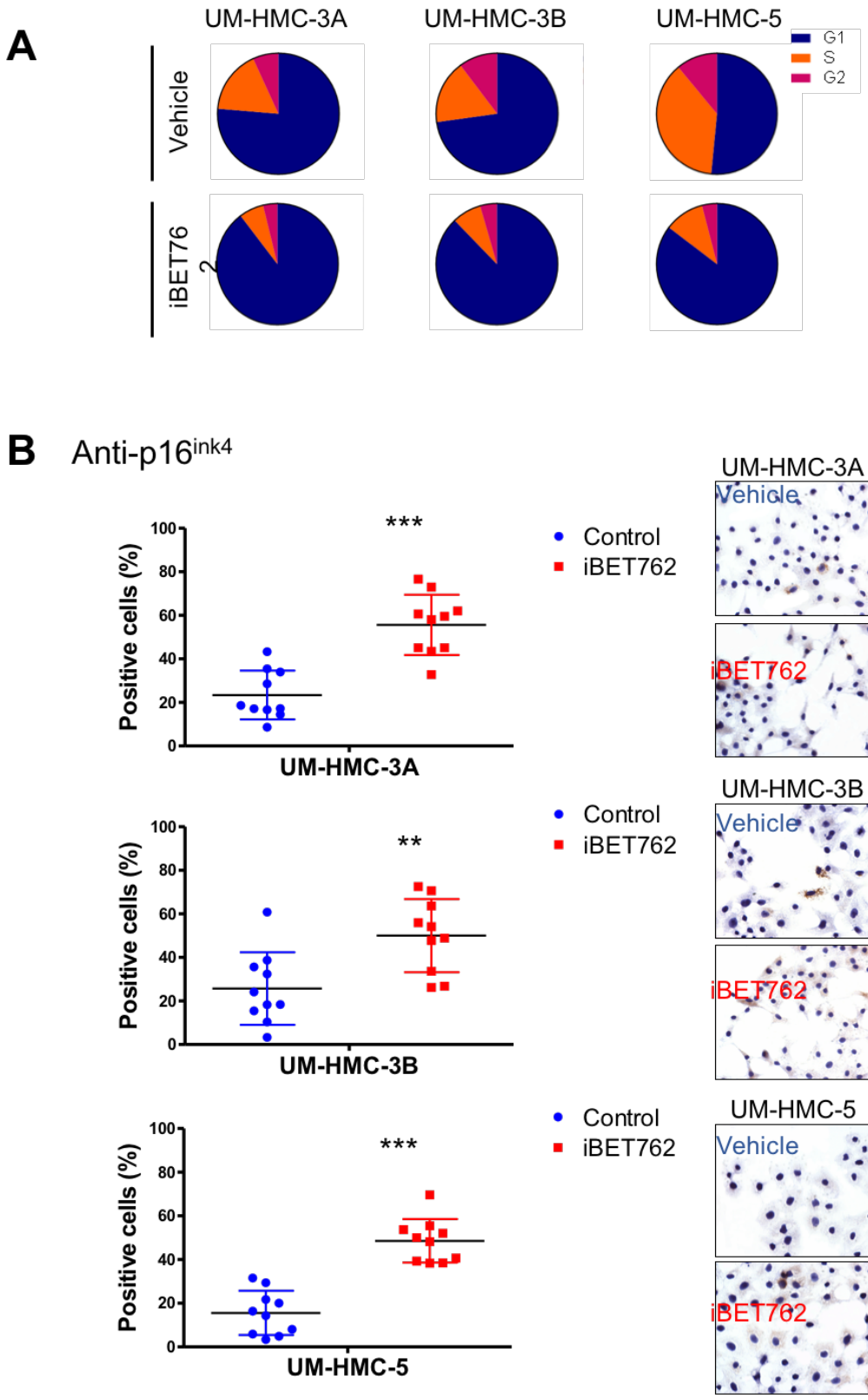


Figure 4: iBET762 induces G1-cell cycle arrest and boosts activation of cellular senescence. (A) Cells were treated with iBET762 every 12 hours for 48 hours. iBET762 causes G1-cell cycle arrest in all cell lines. (B) Accumulation of p16INK4 protein was detected using immunocytochemistry after administration of iBET762 every 6 hours for 24 hours. Positive cells in ten fields were quantified using ImageJ 1.50i. Results are plotted in graphs (** P<0.01, *** P<0.001).

Figure 5

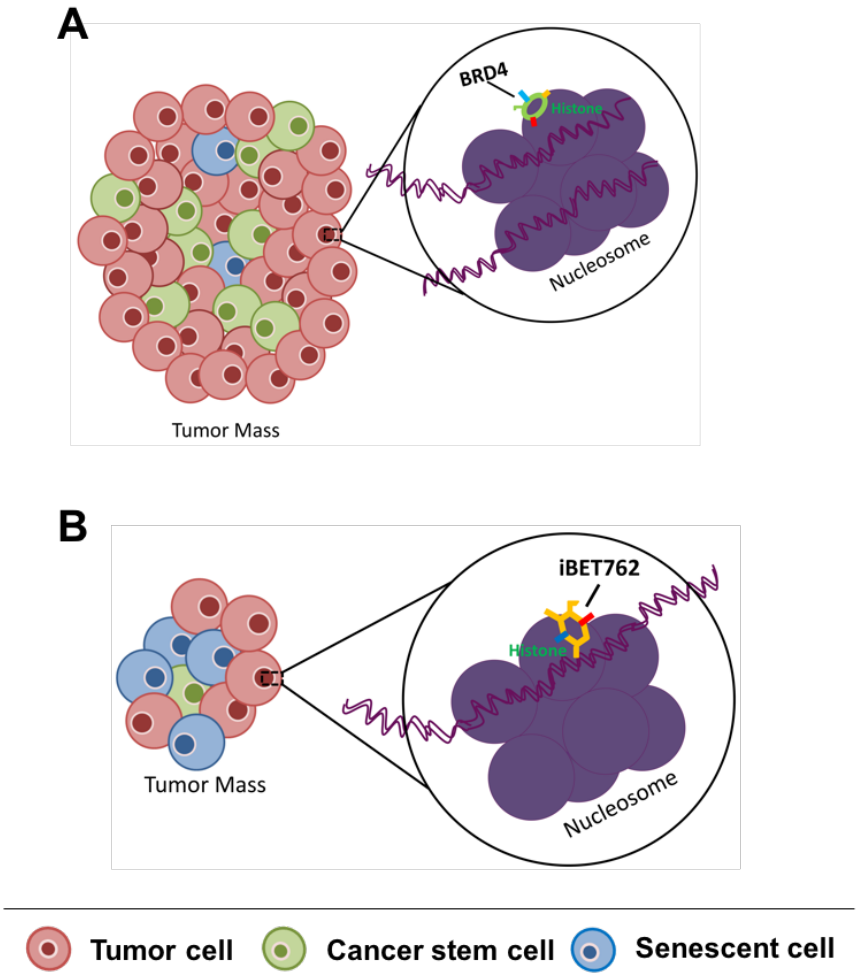


Figure 5: Diagram of important findings demonstrate (A) heterogeneous population of cancer cells are composed of tumor cells and CSCs upon iBET762 displacement of histone H4; (B) upon administration of iBET762, the total number of tumor cells and CSCs are reduced while remain cells undergo cellular senescence.

3 CONCLUSÃO

O leitor epigenético bromodomínio BRD4:

- estava mais expresso em CME do que em glândulas salivares normais,
- demonstrou apresentar funções pró-oncogênicas em CME,
- foi capaz de interferir na progressão do ciclo celular em células tumorais,
- apresentou um importante papel na proliferação celular e formação de colônias em linhagens de CME,
- estava envolvido na manutenção da população de células tronco tumorais,
- é um importante regulador epigenético passível de interferência farmacológica para tratamento do CME.

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Alegre: Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul; 2016.

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ANEXOS

Anexo 1

Certificado do Comitê de Ética em Pesquisa da Faculdade de Odontologia de Piracicaba – UNICAMP

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Caracterização da via PI3K em carcinomas mucoepidermoides**", CAAE **60264016.5.0000.5418**, dos pesquisadores **Renata Lucena Markman** e **Marcio Ajudarte Lopes**, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em 08/11/2016.

The Research Ethics Committee of the School of Dentistry of Piracicaba of the University of Campinas (FOP-UNICAMP) certifies that research project "**Characterization of the PI3K pathway in mucoepidermoid carcinomas**", CAAE **60264016.5.0000.5418**, of the researcher's **Renata Lucena Markman e Marcio Ajudarte Lopes**, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on the Eighth of November of 2016.

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.

Anexo 2

Comprovante de submissão de artigo científico realizada em 15 de Novembro de 2017.

Journal of Dental Research

Interfering with the Bromodomain Epigenome Readers in Salivary Gland Tumors

Journal:	*Journal of Dental Research*
Manuscript ID	JDR-17-1228
Manuscript Type:	Research Reports
Date Submitted by the Author:	15-Nov-2017
Complete List of Authors:	Markman, Renata; Univerity of Michigan, Department of Periodontics and Oral Medicine ; Department of Oral Diagnosis (Pathology), Piracicaba Dental School, State University of Campinas, Brazil. Nascimento Jr, Carlos; Univerity of Michigan, Department of Periodontics and Oral Medicine ; Universidade Estadual Paulista Julio de Mesquita Filho - Campus de Sao Jose do Rio Preto Reis, Leonardo; Univerity of Michigan, Department of Periodontics and Oral Medicine ; Department of Oral Diagnosis (Pathology), Piracicaba Dental School, State University of Campinas, Brazil. Webber, Liana; Univerity of Michigan, Department of Periodontics and Oral Medicine Vargas, Pablo Agustin ; Department of Oral Diagnosis (Pathology), Piracicaba Dental School, State University of Campinas, Brazil. Lopes, Marcio; FOP-UNICAMP, Oral Diagnosis Squarize, Cristiane; University of Michigan - School of Dentistry, POM Castilho, Rogerio; Univerity of Michigan, Department of Periodontics and Oral Medicine
Keywords:	Tumor biology, Epigenetic(s), Stem cell(s)
Abstract:	Mucoepidermoid carcinomas (MEC) are the most common malignancy of the salivary glands. High-grade MEC is particularly unpredictable and often associated with poor prognosis. Emerging evidence suggests the involvement of bromodomains as a conserved class of epigenome readers in cancer progression and inflammatory response. Bromodomains are directly associated with epigenetic modification of gene transcription through its interaction with the lysine residues of histone tails. The bromodomain family member BRD4 is particularly involved in the control of oncogenes including c-MYC, and in the maintenance of downstream inflammatory events and associated molecules. Here we showed that MECs are endowed with high expression levels of BRD4 and that targeting the acetyl-binding pockets of the bromodomains BET family demonstrated a potent anti-proliferative effect in MEC cells. Additionally, BET inhibitors are efficient modulators of the NFkB signaling pathway and capable of reducing the population of cancer stem cells. Notably, we have previously shown that MEC tumors present high levels of NFkB that are associated with its resistance to radiotherapy. Finally, we observed that targeting disruption of BRD4 histone interaction results in cell cycle arrest and activation of