UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

LEONARDO AMARAL DOS REIS

O duplo papel da rapamicina no comportamento e no acúmulo de células-tronco tumorais em carcinomas mucoepidermóides.

The dual role of rapamycin on the behavior and cancer stem cell load of mucoepidermoid carcinomas.

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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 Doutor em Estomatopatologia, na Área de Patologia.

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 Pathology area.

Orientador: Prof. Dr. Jacks Jorge

Este exemplar corresponde a versão final da tese defendida pelo aluno Leonardo Amaral dos Reis e orientado pelo Prof. Dr. Jacks Jorge.

Piracicaba
2019

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

R277d

Reis, Leonardo Amaral dos, 1989-
O duplo papel da rapamicina no comportamento e no acúmulo de células-tronco tumorais em carcinomas mucoepidermóides / Leonardo Amaral dos Reis. – Piracicaba, SP : [s.n.], 2019.

Orientador: Jacks Jorge Junior.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Neoplasias das glândulas salivares. 2. Células-tronco neoplásicas. I. Jorge Junior, Jacks, 1962-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: The dual role of rapamycin on the behavior and cancer stem cell load of mucoepidermoid carcinomas
Palavras-chave em inglês:
Salivary gland neoplasms
Neoplastic stem cells
Área de concentração: Patologia
Titulação: Doutor em Estomatopatologia
Banca examinadora:
Pablo Agustin Vargas
Rogério Moraes de Castilho
Lívia Maris Ribeiro Paranaiba
Márcio Ajudarte Lopes
Ana Terezinha Marques Mesquita
Data de defesa: 02-08-2019
Programa de Pós-Graduação: Estomatopatologia

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: http://orcid.org/0000-0003-0701-1565
- Currículo Lattes do autor: http://lattes.cnpq.br/5715196126929124

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Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 02 de Agosto de 2019, considerou o candidato LEONARDO AMARAL DOS REIS aprovado.

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A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

DEDICATÓRIA

Aos meus pais, **Joaquim Domingos dos Reis** e **Maria dos Reis** que souberam me mostrar o verdadeiro sentido da palavra amor e valor de uma família. Eles que nunca mediram esforços, mesmo com todas as dificuldades, ofereceram-me a melhor educação e apoio. Sou grato por acreditarem no meu sonho.

Aos meus irmãos **Adilson, Renato e João Paulo**, que sempre estiveram do meu lado sempre me incentivando. A minha irmã gêmea **Lilian** com quem convivo desde o ventre e que compartilha tudo de melhor que a vida oferece, obrigado por ser minha melhor amiga e acreditar em todos meus projetos.

As minhas cunhadas **Marcela, Ana Rita** e **Fernanda** pela convivência harmoniosa e pelas manifestações de afeto. Aos meus sobrinhos **Rafaela, Isabela, Maria Laura e Caio**

Ao **Jonas** e toda sua família pelo apoio, pela ajuda e pelos bons momentos compartilhados. A sua serenidade e paciência foram imprescindíveis nesse ciclo que se encerra.

AGRADECIMENTOS

À Universidade Estadual de Campinas, na pessoa do Magnífico Reitor, **Prof**. **Dr. Marcelo Knobel.**

À Faculdade de Odontologia de Piracicaba, na pessoa de seu Diretor, **Prof. Dr. Francisco Haiter Neto.**

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) - Código de Financiamento 001

O presente trabalho foi realizado com apoio da Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), processo nº 88881.133023/2016-01

A Coordenadora dos cursos de pós-graduação da Faculdade de Odontologia de Piracicaba, **Profa Dra.Karina Ruiz**, e ao coordenador do Programa de pós-graduação em Estomatopatologia, **Prof. Dr. Márcio Ajudarte Lopes.**

Ao meu orientador, **Prof. Dr. Jacks Jorge** pelo incentivo e ensinamentos científicos, éticos e filosóficos, pelas conversas francas e pelas oportunidades concedidas.

Ao **Prof. Dr. Oslei Paes de Almeida** e **Prof. Dr. Pablo Agustin Vargas**, pelo exemplo de profissionalismo, pelo apoio e incentivo incondicional aos meus estudos. Agradeço pela oportunidade de fazer parte da rotina de histopatologia e aprender a cada dia. Minha admiração e gratidão.

Ao **Prof. Dr Marcio Ajudarte Lopes**, pela oportunidade de fazer parte da equipe do Orocentro, lugar este que tive a oportunidade de grande aprendizado no atendimento de pacientes com enfermidades bucais. Agradeço ainda a orientação em alguns trabalhos e as parcerias que fizemos. Pelo exemplo de professor e sempre reforçar a importância da família.

Ao **Prof. Dr. Rogério Castilho** e **Profa. Dra. Cristiane Squarize** com quem tive o imenso prazer de aprender Biologia molecular. Por ter passado seis meses de dedicação a este trabalho, pela organização e por ter aprendido o verdadeiro sentido de trabalho em equipe. Pela incrível experiência de morar em outro país e ao mesmo tempo sentir tão bem acolhido na Universidade de Michigan/USA.

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

À amiga **Fabiana Facco Casarotti,** pela amizade, pelo aprendizado durante o trabalho junto ao laborátório (histopatologia e Imunoístoquímica), pelos bons momentos compartilhados durante meu doutorado.

Aos funcionários do departamento de Diagnóstico Oral da FOP/UNICAMP **Emilio, Fábio,** pela ajuda e prontidão nas resoluções de problemas. Às secretárias do Orocentro **Dani** e **Eloisa** pela recepção carinhosa em todos os momentos.

Às minhas amigas de turma de Mestrado, que desde 2014 compartilham momentos de estudos, amizade e apoio. **Mariana Paglione** e **Florence Cuadra**, que nossa amizade permaneça por longos anos.

Aos meus irmãos de orientação, **Ciro Dantas** e **Bruno Mariz,** pelo apoio, pela grande amizade construída durante este tempo de convivência.

Aos amigos de Rotina de Histopatologia, velha gurada: **José Laurentino, Juliana, Marisol, Débora** e aos novos integrantes: **Gleyson, Amanda, Paola, Anna Luiza, Cinthia** que muito me ensinaram e que compartilharam todos os aprendizados que essa experiência proporciona.

Aos amigos do Orocentro **Raísa, Bruno, Patrícia, Isabel, Diego,**

Alexandro, Erika, Rogério, todas as quinta-feiras foram sem sombra de dúvida as melhores. Aqui não deixaria de destacar Pedro, grande amigo que nos deixou tão cedo, mas com quem sempre tive grande respeito.Os momentos de auxílio, confraternização, as risadas, os atendimentos.

Aos “Vipsons” **Luan, Iara, Raisa, João, Natália, Rodrigo, Raquel, Maurício** pelos momentos de descontração.

Aos demais amigos de pós-graduação: **Jéssica, Paola, Lígia, Erison, Ana Luiza, Felipe, Joab, Maria Eduarda, Renato, Thainá, Iasadora, Jamile, John Lennon, Reydson, Carla, Elisa,** por todos bons momentos que passamos juntos.

Aos meus queridos amigos egressos do programa**, José Laurentino, Priscilla Diniz, Celeste, Carolina Carneiro, Marcondes Sena, Vinicius Torregrossa.**

À minha querida e sempre amiga **Renata Lucena**, pelo companheirismo, pelo apoio incondicional em Michigan. Pela ajuda neste e em outros projetos. Você com certeza ganhou um lugar especial no meu coração.

À minha irmã Colombiana **Marisol Miranda**, que desde os primeiros dias compartilhamos a vida de pós-graduandos, sorrimos, choramos e aproveitamos cada dia juntos. Aqui não poderia deixar de falar sobre a **Florence**, que tem o coração maior que ela, o carinho que tenho por você transcende a amizade.

Aos queridos amigos de Diamantina, **Evandro, Max, Paulinho, Filipe,** que mesmo após a graduação continuam sempre mandando energias positivas.

Aos meus amigos e professores da UFVJM - Diamantina, em especial a **Professora Dra. Ana Terezinha Marques Mesquita**, pelo ensinamento, entusiasmo e dedicação a Estomatologia e a vida acadêmica, fonte de inspiração, contribuindo para meu crescimento pessoal e profissional.

À minha família, sempre na torcida e na manutenção da minha força nessa

caminhada distante. A minha **mãe**, minha eterna rainha, por me apoiar em todas as minhas escolhas, por sempre ter uma palavra amiga para acalmar nos momentos mais difíceis. Ao meu **pai,** meu exemplo de superação. À minha querida **vó Augusta**, por tudo que vivemos juntos, por todo carinho e cuidado. Meu objetivo é apenas ser motivo de orgulho para vocês. Obrigado por tudo.

RESUMO

O carcinoma mucoepidermoide (CME) é a segunda neoplasia mais comum de glândula salivar e a mais frequente dentre as malignas. O CME pode ser classificado histologicamente em três tipos (baixo, intermediário e de alto grau) de acordo com a quantidade de formação cística, proporção de cada tipo celular e grau de atipia celular. Apesar de todos os subtipos histológicos serem capazes de causar metástases, os de alto grau possuem uma evolução clínica imprevisível estando associados a um pior prognóstico. A sinalização disfuncional do ciclo circadiano é observada em uma variedade de condições patológicas. Alguns membros da família dos *clock genes* são regulados em células tumorais. Exploramos o resultado da inibição da via do mTOR, comumente encontrada e regulada positivamente em tumores sólidos, incluindo em CME, e seu efeito na população de células tronco tumorais (CTT). Avaliamos o potencial inibição da sinalização mTOR na expressão do membro da família dos *clock genes* BMAL1 e no comportamento do tumor usando o inibidor específico da mTOR - rapamicina. Foi observado que os CME são dotados de altos níveis de expressão de pS6 e BMAL1. A administração de rapamicina demonstra uma potente atividade antiproliferativa nas células de CME, juntamente com a capacidade de reduzir a invasão e migração tumoral. Descobrimos também que a rapamicina é um modulador eficaz dos clock genes BMAL1. Inesperadamente, a inibição da sinalização de mTOR resultou na proliferação de CTT em CME. Estas descobertas sugerem que a rapamicina tem efeitos diferentes em tumores sólidos. A administração de rapamicina foi eficiente no tratamento de CME, porém falhou em diminuiur a população de CSC.

Palavras-chave:Neoplasia maligna de glândula salivar, mTOR, pS6, BMAL1, células tronco tumorais, rapamicina.

ABSTRACT

Mucoepidermoid carcinomas (MEC) are the most common malignancy of the salivary glands. The CME can be classified histologically into three types (low, intermediate and high grade) according to the amount of cystic formation, proportion of each cell type and degree of cellular atypia. High-grade MEC is particularly unpredictable and often associated with poor prognosis. Dysfunctional clock signaling is observed in a variety of pathological conditions. Many members of the clock gene family are upregulated in tumor cells. Here, we explored the role of the disruptedmTOR signaling pathway, and its population of cancer stem cells (CSC). We also explored the potential implication on the disruption of the mTOR signaling on the expression of the clock gene family member BMAL1 and in tumor behavior using the specific mTOR inhibitor Rapamycin. We showed that MEC and HNSCC are endowed with high expression levels of mTOR and BMAL1. Administration of Rapamycin, demonstrate a potent anti-proliferative activity in MEC cells, along with the ability to reduce tumor invasion and migration. We also found that Rapamycin is an effective modulator of the core clock gene BMAL1. Unexpectedly, inhibition of mTOR signaling resulted in the proliferation of CSC MEC. These findings suggest that Rapamycin have different effects on CSC of different solid tumors. While administration of Rapamycin was efficient in treating MEC tumors and depleting its CSC, Rapamycin failed in disrupting the population of CSC.

Keywords: Salivary gland tumor, mTOR, pS6, BMAL1, cancer stem cells, rapamycin

SUMÁRIO

1 INTRODUÇÃO

1.1 NEOPLASIAS DE GLÂNDULA SALIVAR

As neoplasias de glândulas salivares correspondem a um grupo de lesões extremamente heterogêneas com alta variação de incidência de acordo com a distribuição geográfica. 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). Segundo dados da Organização Mundial da Saúde (OMS, 2017) os casos de tumores de glândulas salivares variam de 0,4 a 13,5 casos por 100 mil habitantes a cada ano (Araya *et al.,* 2015; Fonseca *et al.,* 2012; Poetsch, Lorenz e Kleist 2002).

A partir de levantamentos dos dados da literatura, a OMS mostrou que os tumores de glândula salivar afetam mais frequentemente a parótida, representando de 64% a 80% de todos os casos, seguido por glândulas salivares menores com 9% a 23%, glândula submandibular variando de 7% a 11% e, por fim, glândula sublingual que é afetada por menos de 1% dos tumores (Fonseca *et al.,* 2016). Além disso, a literatura mostra que o percentual de tumores benignos pode variar de 54% a 79%, enquanto os tumores malignos acometem de 21% a 46% dos casos (Fonseca *et al.,* 2012; 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). Além disso, as neoplasias de glândulas salivares possuem diversos subtipos histológicos e os mais comumente encontrados nas grandes séries de casos incluem o adenoma pleomórfico e o tumor de Warthin como tumores benignos e o carcinoma mucoepidermoide e o carcinoma adenoide cístico como tumores malignos (Araya *et al.,* 2015; Fonseca *et al.,* 2016; Ito *et al.,* 2005).

A principal forma de tratamento para neoplasias malignas de glândula salivar é a ressecção cirúrgica sendo que a remoção profilática dos linfonodos cervicais não é recomendada, exceto para casos específicos (Fonseca *et al.,* 2012, 2016; Ito *et al.,* 2005). A radioterapia é reservada para pacientes com tumores irressecáveis ou como 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 uma opção terapêutica paliativa e indicada apenas para casos com doença metastática avançada ou incurável (Guzzo *et al.,* 2010).

1.2 CARCINOMA MUCOEPIDERMÓIDE

O carcinoma mucoepidermoide (CME) é a neoplasia maligna de glândula salivar mais comum, representando 30-60% dos tumores malignos (Da Cruz Perez *et al.,* 2004). A localização mais comum nas glândulas salivares maiores é a parótida; e o palato para tumores de glândulas salivares menores (Fonseca *et al.,* 2012; Lopes, *et al.,* 1998). O comportamento clínico do CME é muito variado. Esta neoplasia geralmente se apresenta como uma massa de crescimento indolor e tumores superficiais. Em regiões de mucosa superficial, pode-se observar algumas vezes uma coloração azul-avermelhada que pode levar o clínico a suspeitar de lesão vascular ou até mesmo mucocele, uma lesão benigna bastante comum (Seethala e Stenman, 2017).

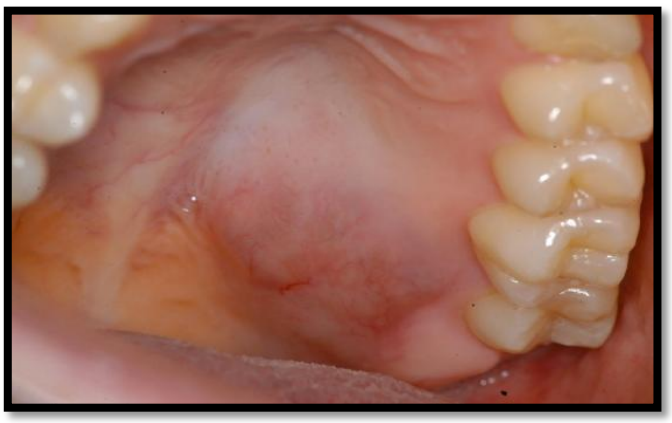

Figura1: Imagem clínica de tumefação em palato duro, lado esquerdo. (Imagem cedida pelo prof. Dr. Márcio Ajudarte Lopes, acervo Orocentro).

Histologicamente, o CME se origina em células do epitélio ductal de glândulas e é caracterizado pela presença de células epiteliais, intermediárias e mucosas, podendo apresentar ainda células colunares e claras(Bai *et al.*, 2013; Ito *et al.,* 2005; Vargas *et al.,* 2008). A distribuição desses tipos celulares e o seu

arranjo leva a uma classificação em gradações de baixo, intermediário e alto grau que leva em consideração a proporção de células mucosas, intermediárias e epidermoides, componente cístico, a presença de invasão neural, presença de necrose, quantidade de mitoses encontradas e anaplasia (Seethala e Stenman, 2017).

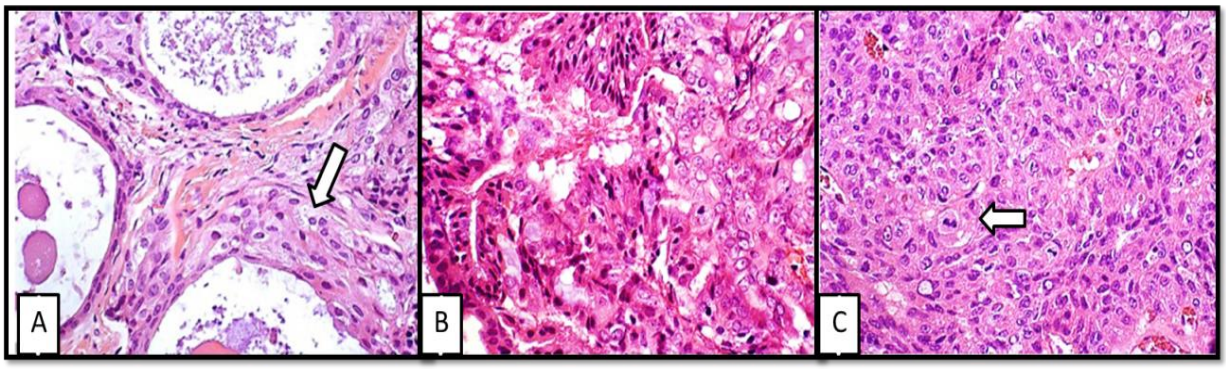

Figura 2: Fotomicrografias em H&E (200X) representando a gradação histológica dos carcinomas mucoepidermóides: **(A)** Tumor de baixo grau mostrando áreas císticas e células mucosas (indicadas pela seta branca). **(B)** Tumor de grau intermediário com perda de espaços císticos e elevado número de células intermediárias. **(C)** tumor de alto grau mostrando padrão sólido com células atípicas (seta branca).

Fatores prognósticos têm sido estudados a fim de elucidar o comportamento altamente variável deste tumor. 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 (Noda *et al.,* 2013). Em termos de prognóstico, o CME apresenta-se como uma neoplasia com boa taxa de resposta ao tratamento, apresentando uma taxa de sobrevida de 5 anos que pode variar de 57% a 92% (Pires *et al.*, 2004; Okumura *et al.*, 2011; Bai *et al.*, 2013; Shigeishi *et al.*, 2014). Um estudo realizado no Brasil com 173 casos de CME mostrou que 12,7% dos pacientes apresentaram recorrência local do tumor, 9,8% tiveram metástase regional e 9,2% tiveram 15 metástase à distância(Pires *et al.,* 2004). Além disso, outros estudos mostram que graus histológicos mais altos da neoplasia estão relacionados com pior prognóstico(Lopes *et al.,* 1998; Lopes *et al.,* 2006; Pires *et al.,* 2004; Riaz *et al.,* 2016).

O tratamento para o CME segue a mesma linha das demais neoplasias

malignas de glândula salivar, sendo a remoção 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 (Coca-Pelaz *et al.,* 2015; Gilbert *et al.,* 2006).

Alguns casos de CME apresentam comportamentos mais agressivos, mesmo apresentando estágios clínicos menos avançados. Assim, a busca por marcadores moleculares têm sido amplamente discutida na literatura uma vez que estes marcadores podem estar relacionados com a biologia e com o prognóstico de alguns CME, trazendo benefícios para a escolha do tratamento adequado. Terapias sistêmicas eficazes podem ser utilizadas para pacientes com tumores menos agressivos visando evitar o procedimento cirúrgico e melhorar substancialmente a qualidade de vida (Markman *et al.,* 2019; Wagner *et al.,* 2018). 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.

1.3 Inibição da via mTOR pela rapamicina

mTOR é uma serina/treonina quinase que foi descoberta há cerca de 16 anos através de investigações sobre o mecanismo de ação do seu inibidor, a rapamicina, um macrolídeo com potente imunossupressor e ações antineoplásicas em células de mamíferos (Kuss-Duerkop et al. 2017; Mita, Mita e Rowinsky, 2003). A via mTOR é composta por dois complexos proteicos diferentes: mTORC1, que diretamente fosforila S6 quinase (S6K), proteína de ligação 4E1 (4EBP1), e subsequentemente ativa a proteína ribossómica alvo S6 a jusante (S6RP); e mTORC2, que fosforila AKT (Laplante e Sabatini 2012; Strimpakos *et al.,* 2009). A ativação aberrante do mTOR desempenha papéis críticos na patogênese de processos pró-inflamatórios e algumas doenças como, aterosclerose, doenças neurodegenerativas e câncer (Marques *et al.,* 2016; Pritchard *et al.,* 2018).

Várias mutações foram identificadas na via da fosfoinositida 3-quinase

(*PI3K*)/alvo de rapamicina em mamíferos (*mTOR*), incluindo *PIK3CA* (8–10%), *TSC1/2* (5-8%) e *PTEN* (5-10%) todos resultando na ativação da via PI3K/AKT/mTOR(Stransky et al. 2011). O gene PTEN codifica uma proteína de 403 aminoácidos, também conhecida como *MMAC (Mutated in Multiple Advanced Cancers)*, capaz de hidrolisar o grupamento fosfato da posição 3 em *PIP3* (Fosfatidilinositol 3,4,5-Trifosfato) para formação de *PIP2* (phosphatidylinositol (4,5)-bisphosphate), e regular negativamente a via de sinalização *PIP3* (Maehama Dixon, 1998). Quando fosforilado, *PIP3* ativa a proteína *Akt* que controla a proliferação e apoptose celular (Matsumoto *et al*., 2016) (figura 3). O *Akt* hiperfosforilado tem sido relatado em diferentes tipos de neoplasias de glândula salivar, incluindo o carcinoma mucoepidermóide (Marques *et al.,* 2008)**.**

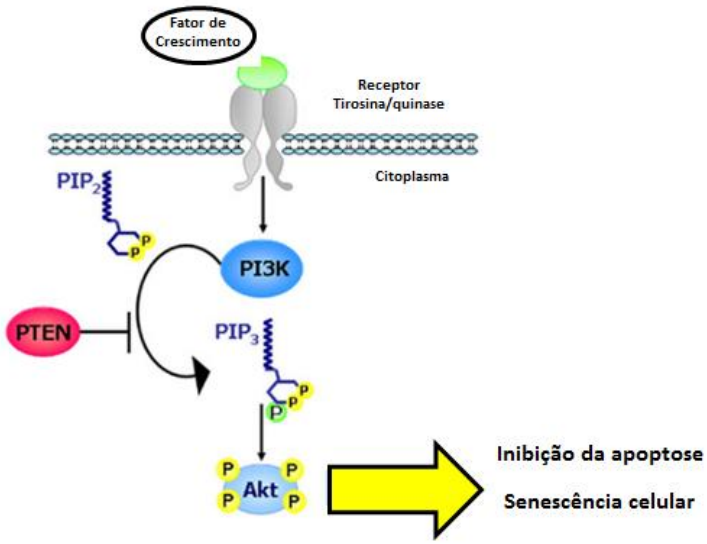

Figura 3: Organização esquemática da via PI3K-PTEN-Akt. (adaptado de Molinarie Frattini M 2014).

A rapamicina, um inibidor seletivo da via mTOR, é uma droga aprovada pela FDA e que tem sido usada na clínica desde 1999. Análogos de rapamicina de segunda geração (rapalogs), incluindo aqueles que estão disponíveis por via oral, também são aprovados pela FDA e estão sendo testados em ensaios clínicos (Acevedo-Gadea *et al.,* 2015; Guba *et al.,* 2002; Li *et al.,* 2013; Zhou, Luo e Huang, 2012) (https://clinicaltrials.gov/). Guba *et al.,* publicaram em 2012 um estudo mostrando um potente efeito da rapamicina na oncogênese induzida em ratos, apresentando efeito anti-angiogênico e antiproliferativo.

A via mTOR está sob controle do relógio circadiano. Os relógios circadianos

são as oscilações diárias nos processos comportamentais, fisiológicos e metabólicos. Em células eucarióticas, estes ritmos são gerados por um relógio molecular endógeno (King e Takahashi 2002). Pesquisadores descobriram que 43% de todos os genes mostraram oscilações circadianas na transcrição em algum lugar do corpo (tecidos periféricos como retina, coração, pulmão e fígado) grande parte dessas oscilações são específicas de cada órgão (Yamazaki *et al.,* 2002). Além disso, sabe-se que a funcionalidade do relógio circadiano é atenuada ou interrompida com a idade (James *et al.*, 2017; Kondratova e Kondratov, 2012). Como consequência, o rompimento do relógio circadiano tem sido associado a uma variedade de estados fisiopatológicos, variando de distúrbio metabólico ao câncer (Korkmaz *et al.,* 2018).

Dados epidemiológicos sugerem que indivíduos com perturbação do relógio circadiano ocupacional têm um risco de cancer (Stevens, 2009). Usando a proteína ribossômica S6 fosforilada (pS6) como um marcador da atividade de *mTOR*, eles mostraram a íntima relação desta proteína com a via *mTOR* (Cao *et al.,* 2011). A atividade de *mTOR* e a oscilação circadiana de pS6 é similar e/ou paralela à expressão da proteína *BMAL1(Brain and muscle Arnt-like protein-1)*. Sabe-se que atualmente um conjunto de proteínas tem sido envolvidas na expressão da ritmicidade do relógio central de mamíferos e que a primeira alça de retroalimentação negativa é composta por CLOCK (CLK) e *BMAL1* (Gekakis, 1998).

Alterações nas vias de sinalização mTOR também contribuem para a plasticidade celular durante a progressão do câncer e na formação das células tronco tumorais (CTT). Desta forma, a caracterização dessas alterações envolvidos em carcinogênese, e a identificação de marcadores da via mTOR e CTT se mostram promissores no desenvolvimento de novas estratégias para tratamento dos tumores.

1.4 Célula tronco-tumoral

Células-tronco tumorais (CTT) formam um conjunto de células neoplásicas malignas que apresentam uma capacidade de proliferação indefinidamente e de formar tumores e/ou metástases em modelos animais(Clarke *et al.,* 2006). As neoplasias malignas do sistema hematopoiético como as leucemias, favorecem as evidências de que células-tronco normais sofrem mutação e se transformam em

CTT com consequente proliferação de células malignas neoplásicas. De fato, a leucemia e o mieloma múltiplo proporcionaram as primeiras evidências da existência deste tipo celular, sendo descritas inicialmente como “células-tronco leucêmicas” (Reya *et al.,* 2001).

A capacidade de autorrenovação e de diferenciação são as principais características das células-tronco. Para que as CTT sejam caracterizadas, estas capacidades das células-tronco devem ser identificadas e confirmadas. A caracterização dessas células são baseadas na identificação e isolamento, como ensaios funcionais (capacidade de formação de esferóides e a atividade de enzimas como aldeído desidrogenase-ALDH), (Krishnamurthy e Nör 2012; Mertins, 2015).

A correlação entre a perda de expressão de PTEN e ativação da sinalização mTOR está bem documentado pelo nosso grupo e por outros (Squarize *et al.,* 2010; Squarize, Castilho e Gutkind 2008); no entanto, a inativação simultânea da proteína PTEN e a regulação positiva de genes do ciclo circadiano como o BMAL1 e pS6, tem sido uma recente descoberta do nosso grupo de pesquisa. 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. 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. ARTIGO

Rapamycin differentially impacts the population of cancer stem cells from salivary mucoepidermoid carcinomas and head and neck squamous cell carcinomas

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Word count manuscript: 4632 Figures: 7

Keywords: Salivary gland tumor, mTOR, Rapamycin, cancer stem cells, clock gene, oral squamous cell carcinoma.

Abstract

Mucoepidermoid carcinoma (MEC) is the most common malignancy of the salivary glands. High-grade MEC is particularly challenging to manage as presents an unpredictable clinical progression being often associated with poor prognosis. Here, we explored the role of the mTOR pathway in salivary MEC tumors. mTOR signaling is commonly found deregulated in solid tumors such as prostate cancer, breast cancer, and head and neck squamous cell carcinoma. We have previously shown that dysfunctional mTOR drives the aberrant expression of clock genes in head and neck squamous cell carcinomas and conditional knockout mice for Pten, a major regulator of PI3K/mTOR signaling. Here we explored the potential disruption of the mTOR signaling in MEC tumors and further impact over the expression levels of BMAL1. We have also explored the therapeutic efficacy of the mTOR inhibitor Rapamycin on the behavior of MEC tumors and its cancer stem cells (CSC). We observed that MEC express high levels of mTOR and BMAL1. Interfering with mTOR signaling using Rapamycin resulted in a potent anti-proliferative effect over MEC cells, along with reduced tumor invasive and migratory abilities. We also observed that Rapamycin could efficiently downregulate BMAL1 in MEC tumors. Unexpectedly, inhibition of mTOR signaling resulted in the accumulation of CSC in all analyzed MEC cell lines. Interestingly, we did not observe a similar effect of Rapamycin on Head and Neck Squamous Cell Carcinomas (HNSCC). Our results suggest that the mechanisms controlling CSC from salivary gland tumors significantly differs from HNSCC.

INTRODUCTION

Mucoepidermoid carcinoma (MEC) is the most commonly reported malignant salivary gland tumour[1–4], and the second most common tumor from the salivary glands, being second only to the pleomorphic adenoma[4]. This tumor affects men and women in a wide age group that can vary from 5 to 96 years, usually presenting its peak of incidence between the fourth and sixth decades of life [5,6]. The most common location for MEC in major salivary glands is the parotid; and the palate for minor salivary glands[3]. Management of MEC includes surgical excision with safety margins[7], and the use of radio- or chemotherapy is mostly reserved to cases presenting compromised surgical margins, the presence of local metastases, recurrent disease, and inoperable tumors [8,9].

Several solid tumors including HNSCC, glioblastomas, breast cancer, endometrial, and prostate cancer are endowed with dysfunctional PTEN/PI3K signaling resulting in upregulation of the mammalian target of Rapamycin (mTOR)[10–15]. Deregulation of the tumor suppressor gene PTEN also results in the accumulation of the clock gene BMAL1. We have previously shown that PTEN-driven upregulation of BMAL1 is mTOR-driven in normal epithelial cells presenting loss of PTEN (KO) and in HNSCC[11]. However, the correlation between PTEN/PI3K/mTOR signaling and BMAL1 expression is salivary gland tumors remains unknown. Targeted therapy to the PTEN/PI3K/mTOR signaling may constitute an attractive approach to manage salivary gland carcinomas[16]. Indeed, Rapamycin is a well-known specific inhibitor of mTOR[17] that is currently in clinical trials for HNSCC, bladder carcinoma, renal cell carcinoma, breast cancer, classical Hodgkin lymphoma, and hepatocellular carcinoma, (https://clinicaltrials.gov/).

Here, we assessed the effect of pharmacological inhibition of mTOR using Rapamycin in MEC cells. Our results demonstrate that the inhibition of mTOR interferes with the expression of the clock gene BMAL1 in MEC tumors. We also show that Rapamycin impacts the migration and invasion ability of MEC; however, tumor cell lines respond to therapy by accumulating CSC. Puzzled with these results, we also accessed the effects of Rapamycin over HNSCC and found that mTOR inhibition result in a reduction on CSC number from HNSCC. Our data suggest different regulatory roles of mTOR in CSC from MEC and HNSC.

Materials and Methods

Human tissue specimens

Human MEC tissue samples (n = 20) were retrieved from the archives of the Oral Pathology Laboratory of the State University of Campinas, Piracicaba, São Paulo, Brazil (Human Research Ethics Committee approval: 60025316.6.0000.5418. All MEC tissue samples were stained with hematoxylin-eosin and reviewed by two independent pathologists to confirm the diagnosis. Patient information and tumor grades are listed in Table 1.

Immunofluorescence

Immunofluorescence was performed on paraffin-embedded tissue sections of MEC and cell lines. Briefly, paraffin blocks were sectioned into 3-um sections, deparaffinized in xylene and hydrated in descending grades of ethanol. Cell lines were cultured over coverslips and fixed with 3% paraformaldehyde. Tissues and cells were blocked in 0.5% (v/v) Triton X-100 in PBS and 3% (w/v) bovine serum albumin (BSA) and then incubated with pS6 (S235/236, Cell Signaling Technology, Danvers, MA), anti-BMAL1 (NB100-2288, Novus Biological, Littleton, CO), and ALDH1A1 (Aldehyde dehydrogenase – Abcam, Cambridge, MA). After incubation (overnight), slides were washed with PBS and incubated with a secondary antibody conjugated with either Alexa Fluor 488 or 568 (ThermoFisher Scientific/Invitrogen, Grand Island, NY). Tissue sections and cells were further washed and incubated with Hoechst 33342 for genomic content (ThermoFisher Scientific/Invitrogen, Grand Island, NY), and mounted using an aqueous mounting medium (Fluoroshield, Sigma-Aldrich, St. Louis, MO, USA). All samples were washed three times with PBS between each step. 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 and ten fields of each slide were counted.

Cell lines and administration of Rapamycin

Mucoepidermoid Carcinoma cell lines UM-HMC-3A, UM-HMC-3B, and UM-HMC-5 and was established at the University of Michigan School of Dentistry[18]. Head and Neck Squamous Cell carcinoma cell lines WSU-HN6, WSU-HN12, and WSU-

HN13 were established at Wayne State University and derived from the base of the tongue, lymph node metastasis, and tongue respectively[19]. Identity-based DNA genotyping of all cell lines by short tandem repeat (STR) profiling was performed by Biosynthesis Inc. (Lewisville, TX, USA) using 15 autosomal STR loci and one gender identity locus (amelogenin). Cells were maintained in a 5% CO_2 humidified incubator at 37ºC 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). Mucoepidermoid cell lines were further supplemented with 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, MEC and HNSCC cell lines were treated with 50nM of Rapamycin for 24 hrs (LC Laboratories Sigma–Aldrich).

Flow cytometry

MEC cells were resuspended and counted using a Countess II FL automatic cell counter (Invitrogen, Carlsbad, CA, USA). MEC cancer stem cell-like were identified by its Aldehyde dehydrogenase (ALDH) activity using an Aldefluor kit (StemCell Technologies, Durham, NC, USA) combined with CD44 antibody combined with APC (BD Biosciences, clone G44-26) using flow cytometry. The Aldefluor kit was used according to the manufacturer's instructions. Briefly, cells receiving treatment or vehicle were suspended with activated Aldefluor substrate (BODIPY amino acetate) or negative control (dimethylamino benzaldehyde, a specific ALDH inhibitor) for 45 min at 37°C followed by the incubation of the CD44 antibody for 25 min using a shaking rotor at 4°C. All samples were analyzed using an Accuri C6 Flow Cytometer (BD Accuri™ C6 Plus). All assays were performed in triplicate.

Tumorsphere formation assay

The sphere formation assay was performed as previously described[20]. Briefly, MEC cells were plated on ultra-low attachment 6-well plates (Corning, New York, USA) and allowed to grow for 5 days. The assay was performed in triplicate with and

without pretreatment. Spheres growing in suspension were collected on day 5 and transferred to a glass slide by centrifugation (4 °C) at 1500 rpm for 10 min using a cytospin system. Spheres were stained with hematoxylin and eosin and mounted in aqueous mounting media (Sigma).

In vitro scratch wound assay

In vitro scratch-induced wound model was chosen to assess the migratory ability of the MEC and HNSCC cells upon treatment with Rapamycin. Briefly, MEC cells were plated at high confluence, followed by a mechanical scratch using a 200-µl pipette tip across the diameter of each well. The migration speed of tumor cells into the wound area was recorded at 0, 6, 12, 24, 36, 48, 60, 72, 84, 96, and 108 hrs under a phase contrast microscope. The percentage of wound closure at each time point was calculated using a computerized image analyzer system (AxioVision 4.8.1, Carl Zeiss, Thornwood, New York). All scratch assays were performed in triplicate.

Cell Invasion Assay

MEC cell lines and HNSCC cell lines were used for this assay. Cells were seeded over a thin homogeneous layer of fibronectin (BD Biosciences, Bedford, MA, USA) in Millicell Cell Culture Inserts (Millipore, Billerica, MA, USA) containing a polycarbonate filter membrane with 8 µm-diameter pores in 24-well plates. DMEM supplemented with 10% FBS, 1% antibiotics, was placed in the lower chamber. Cells were incubated for 24hrs at 37ºC in a 5% CO_2-humidified incubator. Invasive cells in the lower chamber were stained with hematoxylin and eosin (H&E). Images were taken using a QImaging ExiAqua monochrome digital camera attached to a Nikon Eclipse 80i Microscope (Nikon, Melville, NY, USA) and visualized using QCapturePro software.

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

mTOR and the CSC marker ALDH1A1 are expressed in different subsets of tumor cells.

The activation of the mTOR signaling pathway is identified by the expression of phosphorylated S6 ribosomal protein (hereafter referred to as pS6). In normal conditions, pS6 is exclusively expressed in most of the differentiated cells from the oral mucosa (Fig. 1A_red) and epidermis (data not shown). The proliferative basal layer of the oral mucosa is entirely negative for pS6 (Fig. 1A_insert). Interestingly, the expression levels of pS6 in MEC tumors vary depending on the histological grade (Fig. 1B). While low and intermediate grades of MEC tumors express a mean of 41,8% and 40,56% of positive cells, respectively, high-grade tumors express a mean of 27,37% of positive cells for pS6. We further accessed the expression levels of MEC cancer stem cells (CSC) as determined by the positivity to ALDH1A1 marker (Fig. 1C). It is interesting to note that there is minimal overlap of tumors cells expressing ALDH1A1 and pS6 (Fig. 1C_arrow) and that the ratio between ALDH and pS6 positive cells change depending on the histological grade (Fig. 1D). The vast majority of tumor cells expressing ALDH1A1 do not express pS6. The expression levels of ALDH1A1 also varies accordingly with the grading of MEC tumors (Fig. 1E, * $p<0.05$; **** $p<0.0001$). Intermediate and high-grade MEC also express reduced levels of pS6, when compared with low-grade MEC (Fig. 1F, ns $p>0.05$; * $p<0.05$; *** $p<0.001$). Our data suggest that pS6 and ALDH1A1 identify distinct populations of cancer cells.

We have previously shown that activation of the mTOR signaling in solid tumors is accompanied by the expression of the core clock gene BMAL1. Here we show that constitutive activation of the mTOR signaling observed in MEC cell lines (Fig. 2A) is accompanied by the expression of BMAL1 (Fig. 2B). Similar to the expression of pS6 in normal oral mucosa and MEC tissue samples, BMAL1 is also expressed at the differentiated layers of the oral mucosa (Fig. 2C), and ALDH1A1 positive cells also do not co-localize with the expression of BMAL1 (Fig. 2D).

Rapamycin interferes with the expression of pS6 and BMAL1 levels in MEC cell lines and reduces aggressive tumor behavior.

The accumulation of pS6 and BMAL1 in HNSCC with compromised PTEN function suggests crosstalk between mTOR and clock signaling. We have previously shown that disruption of the PTEN/PI3K pathway results in activation of the circadian clock gene BMAL1 in an mTOR-dependent signaling[13]. Here we shown that administration of Rapamycin disrupts the accumulation of pS6 (Fig. 3A, ****p<0.0001) and the accumulation of the core clock protein BMAL1 from MEC cells (Fig. 3B, **** p<0.0001). Our results suggest that BMAL1 has the potential to be used as a marker for mTOR activity in MEC tumors similar to our previous observations on normal and head and neck cancer cells[11]. This data also suggests that MEC present deregulated circadian rhythm.

We further decided to explore the effects of administration of Rapamycin to the behavior of MEC cells. Using scratch assay, we observed that Rapamycin reduced tumor migration on all analyzed MEC cell lines (Fig. 4A and B, *p<0.05, **p<0.001, ***p<0.001). We also assessed the ability of Rapamycin to disrupt the invasion of cancer cells. Using a Millicell Cell Culture Insert system coated with fibronectin, we observed that Rapamycin efficiently disrupted the ability of tumor cells to invade the substrate from all our MEC cell lines (Fig. 4C, **** p<0.0001).

Rapamycin induces the accumulation of CSC in MEC tumors.

The cancer stem cells (CSC) model implicates that each tumor consists of a heterogeneous population of tumor cells and a small subpopulation of cancer cells endowed with stem cell properties and the ability to generate new tumors and metastasize[21]. The presence of CSC in solid tumors has been implicated in tumor recurrence and the acquisition of tumor resistance to chemo and radiotherapy ([22–24]). Here we evaluated the effects of Rapamycin on CSC from MEC. Tumor cells received 50nM/ml of Rapamycin for 24 hrs following by the identification of $ALDH^{bright}$ $CD44^{+}$ cells using flow cytometry. Surprisingly, we found that Rapamycin did not reduce the population of CSC as previously shown in solid tumors[25–27]. Rater, Rapamycin-induced a significant accumulation of $ALDH^{bright}$ $CD44^{+}$ cells (Fig. 5A, * p<0.05). In order to verify our results, we decided to run a sphere forming assay

using all our MEC cell lines receiving vehicle or Rapamycin. Supporting our flow cytometry data, we observed that administration of Rapamycin resulted in a significant accumulation of tumorspheres in all MEC cell lines (Fig. 5B, * $p<0.05$).

Rapamycin differentially modulates CSC from squamous cell carcinomas and MEC

Following our unexpected results on the Rapamycin-induced accumulation of MEC CSC, we decided to explore the effects on Rapamycin over the behavior and population of CSC from HNSCC. Towards this goal, we used 3 HNSCC cell lines (HN-6, HN-12, and HN-13) that represent head and neck tumors from the base of the tongue, lymph node metastasis, and tongue, respectively. HNSCC cell lines present similar expression levels of pS6 and BMAL1 when compared with MEC cells (Suppl. Fig. 1A) and respond well to the administration of Rapamycin leading to the downregulation of pS6 and BMAL1 protein levels (****$p<0.0001$). We observed that administration of Rapamycin also disrupts the ability of HNSCC to invade as we have observed in MEC cells (Fig. 6A, **** $p<0.0001$). Further, we assessed the ability of Rapamycin in disrupting the population of CSC from HNSCC following the same protocol used for MEC cells. Interesting, we found unlike MEC cells, Rapamycin was able to reduce the overall number of CSC from all the analyzed HNSCC cell lines within 24 hours of treatment (Fig. 6B, * $p<0.05$).

Altogether our results suggest that CSC from MEC tumors are not sensitive to the inhibition of the PI3K/mTOR signaling when compared with HNSCC CSC. Our data also suggests that Rapamycin may be employed to reduce migration and invasive ability of MEC, however, a neoadjuvant therapy to control CSC may be required.

Discussion

The combination of therapies including high-doses of chemotherapy and radiation (compared to radiation alone) has led to improvements in the survival of patients with HNSCC; however, similar results are not observed in MEC, in special high grade-tumors[24]. MEC can be presented in a wide variety of morphological characteristics that suggest a heterogeneous disease[28,29]. From a therapy standpoint, few studies have addressed the use of systemic chemotherapy in MEC

from the salivary glands, and the results are disappointing[30]. Much of the poor outcomes observed after therapy advent from our limited understanding of the disease. Besides the known oncogenic gene fusion CRTC1/3-MAML2 observed in MEC tumors, other pathways are also found upregulated affecting tumor progression[31]. For example, CRTC1 is known to activate the EGFR ligand AREG[32], whereas EGFR alone is observed upregulated in high-grade tumors, even in tumors negative for CRTC1-MAML2 fusion[33]. TP53 is the most commonly mutated gene in intermediate and high-grade MEC[34]. Other pathways, including the c-MET, are also observed active in MEC[35]. Several oncogenic pathways are also associate with the development of a resistance phenotype of solid tumors. This is the case of the NFκB signaling pathway that is found overexpressed in MEC and associated with a resistant phenotype to chemotherapy[36]. We have shown that interference with the IKKβ/IκBα/NFκB axis using the NFκB inhibitor Emetine resulted in the sensitization of MEC tumors to radiotherapy. Similar to the NFκB signaling, upregulation of the bromodomains in MEC tumors is also a common event and interfering with BRD4 using BET inhibitors leads to G1 cell cycle arrest and activation of tumor senescence[37]. Aligned with the inhibition of bromodomains, the administration of histone deacetylase inhibitors (HDACi) leads to the depletion of CSC and sensitization of MEC tumors to Cisplatin[22].

The presence of an active PI3K/AKT/mTOR signaling is a common finding in solid tumors. Salivary gland tumors like salivary duct carcinoma and the mucoepidermoid carcinoma express higher levels of AKT and mTOR[38,39], similar to the oral squamous cell carcinoma[40]. We have previously shown that disruption of PTEN, a master regulator of the PI3K signaling leads to the accumulation of mTOR and the core clock gene BMAL1 in normal and malignant epithelial cells[11]. Here, we demonstrate that MEC tumors also present a direct correlation between the activation of the mTOR signaling and BMAL1. Similar to the upregulation of mTOR, circadian dysrhythmia is also associated with an increased risk for cancer development. This is particularly true for night-shift workers that undergo constant sleep disruption resulting in higher chances for males to develop colon cancer and females to develop breast cancer[41–44]. Overexpression of BMAL1 is also related to the reduced overall survival of colorectal cancer patients and found activated in HNSCC[11,45]. Although our data do not aim at correlating high levels of BMAL1 with

poor prognosis or tumor behavior, it becomes clear that BMAL1 is dependent on the levels of mTOR as suggested by the chemical inhibition of mTOR using Rapamycin in MEC cells.

Rapamycin has been shown to efficiently re-sensitize HNSCC to cisplatin/radiation therapy[46], along with reducing the lymphatic vascular density of HNSCC tumors[47]. Rapamycin has also successfully lead to an increased antitumor activity of Cetuximab in HNSCC expressing PIK3CA and RAS[48]. However, little is known on the effects of Rapamycin administration over the population of CSC in MEC or HNSCC. Here we show that Rapamycin has an antitumoral effect by reducing the invasive and migratory abilities of MEC. Nonetheless, Rapamycin unexpectedly induced the accumulation of CSC and the formation of tumor spheres. However, HNSCC cells exposed to the same protocol of Rapamycin administration responded by reducing its invasive abilities and reduced the overall number of CSC. The discrepancy on the CSC response to Rapamycin by MEC and HNSCC cells suggest differences in the inherent regulation of CSC by the mTOR signaling. While mTOR activity seems to control the number of CSC in MEC tumors, and Rapamycin set CSC free to accumulate, CSC from HNSCC depend on the mTOR signaling to accumulate.

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 (88881.133023/2016-01). This grant was funded by the University of Michigan School of Dentistry faculty grant, and the Cancer Center Support Grant.

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

Author contributions

LAR performed most of the cell culture based assays, IHC, IF, and participated in the organization of the figures. RLM executed the flow cytometry, LPW helped with IHC and IF and helped with the immunofluorescence and quantifications. PAV, JJ, 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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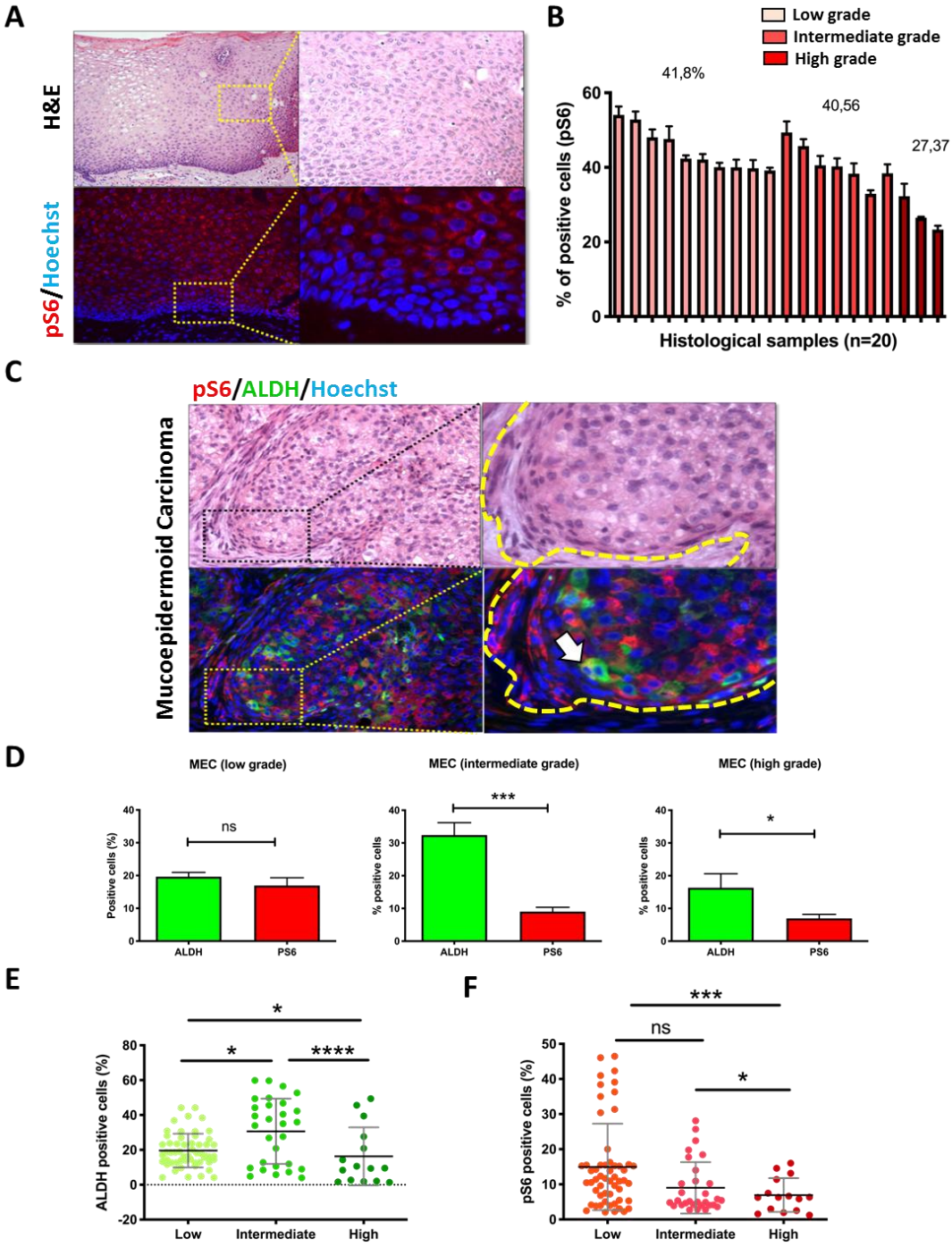
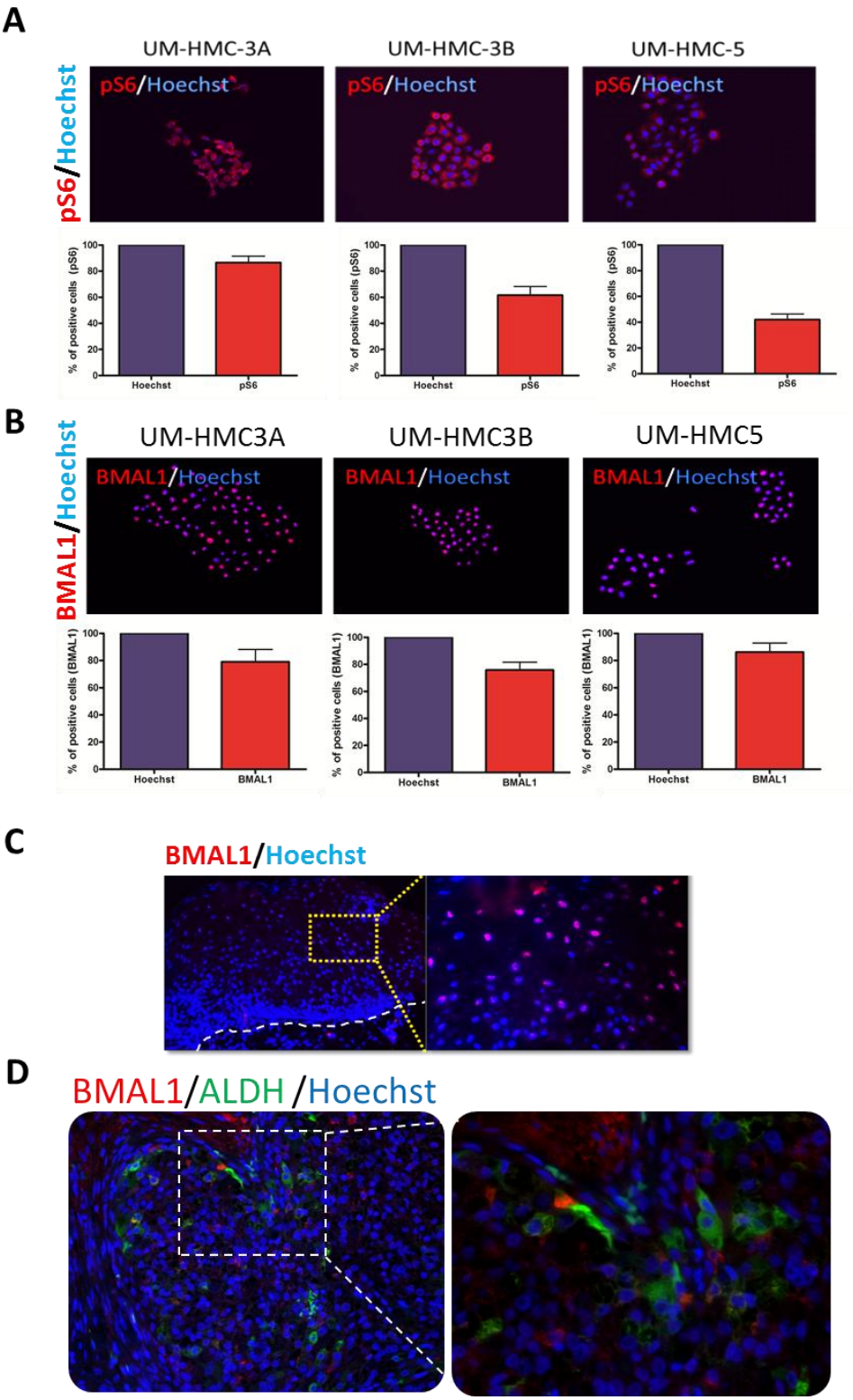


Figure 1: pS6 is expressed in normal tissues and overexpressed in MEC tumor and cell lines. **(A)** Representative histological section of a normal oral mucosa stained for H&E and pS6. Note the presence of pS6 positive cells exclusively at differentiated epithelial layers. Insert image demonstrate the basal layer of the oral mucosa negative for pS6. **(B)** Percentage of positive cells for pS6 in 20 cases of MEC tumors distributed by its histological grade as low (41,8%), intermediate (40,56%), and high grade (27,37%). **(C)** Representative H&E and immunofluorescence sections of MEC tumors demonstrate combined expression of pS6 (red) and ALDH (green) positive cells. Note that a minority of tumor cells present double positivity to pS6 and ALDH (white arrow). **(D)** The ratio of ALDH and pS6 positive cells change according to the tumor histological grade. **(E and F)** Quantification of total number of ALDH or pS6 positive cells in MEC tumors accordingly with its histological grade (ns $p>0.05$; * $p<0.05$; *** $p<0.001$; **** $p<0.0001$).



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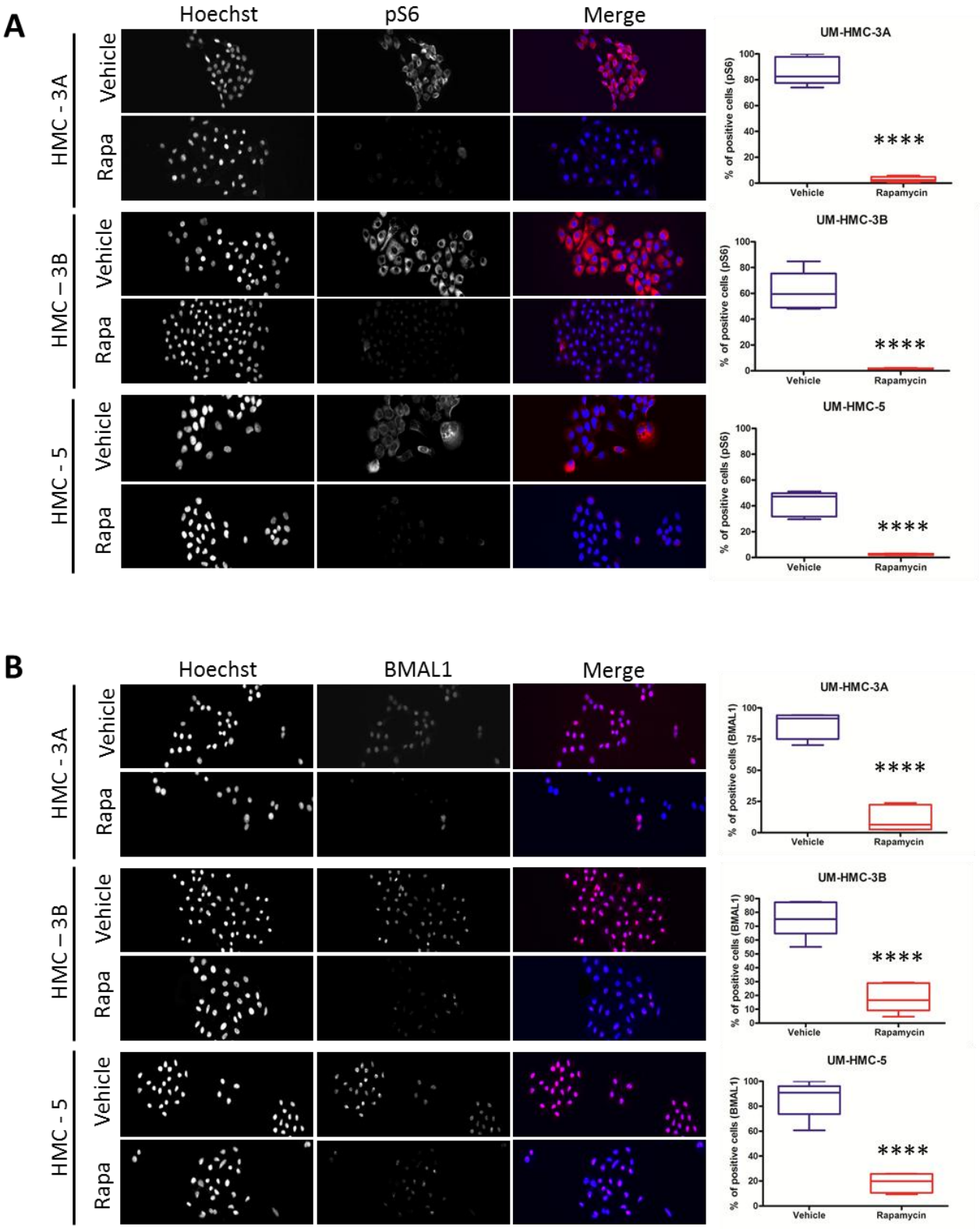
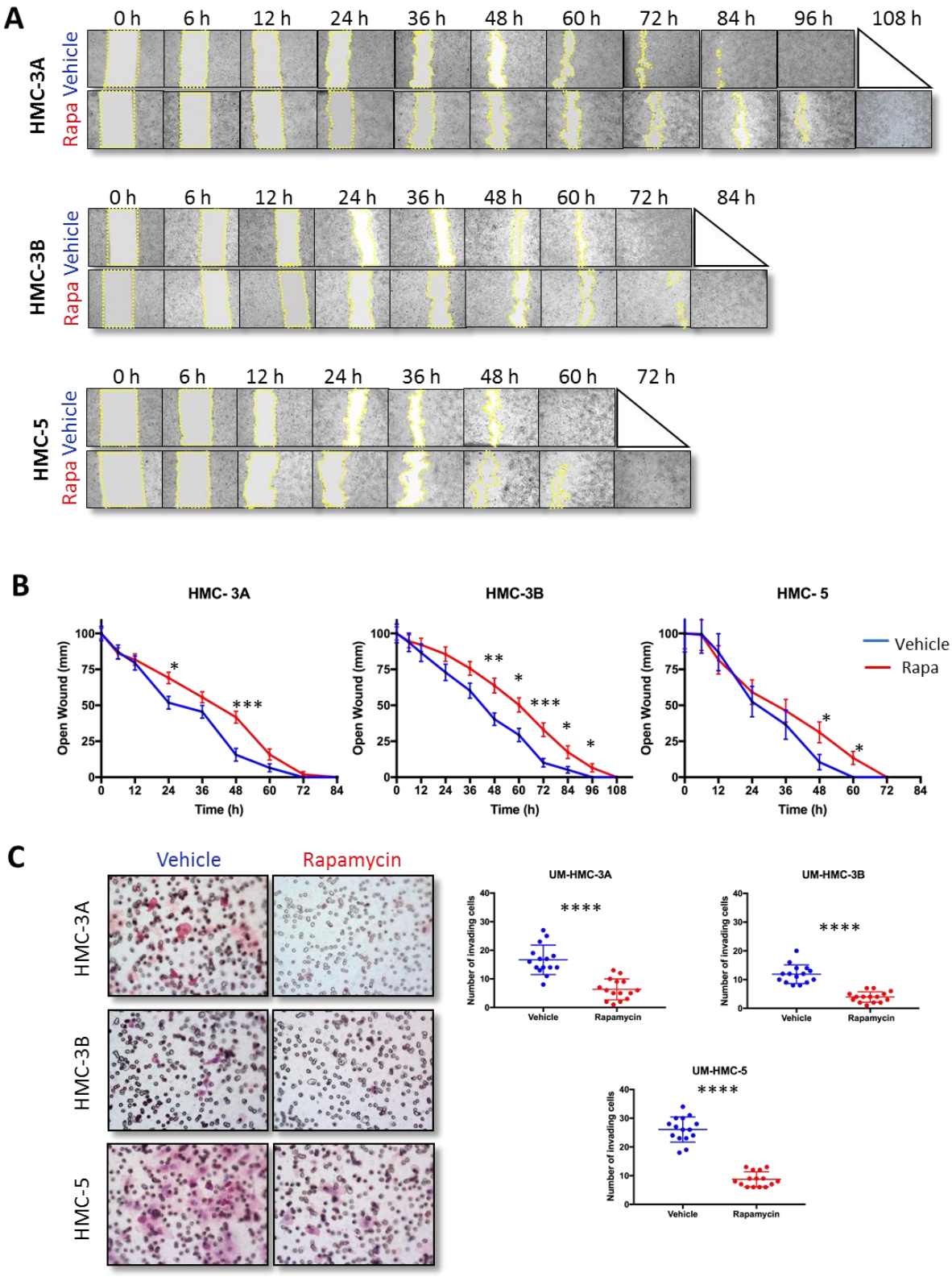


Figure 3: Inhibition of mTOR signaling in MEC cell lines. Administration of Rapamycin for 24 hrs to HMC-3A, HMC-3B, and HMC-5 cell lines resulted in abrupt downregulation of pS6 **(A)** and BMAL1 **(B)** from MEC cell lines (****p<0.0001). Results are represented as a % of total cells with baseline correction for the total number of cells stained for Hoechst.



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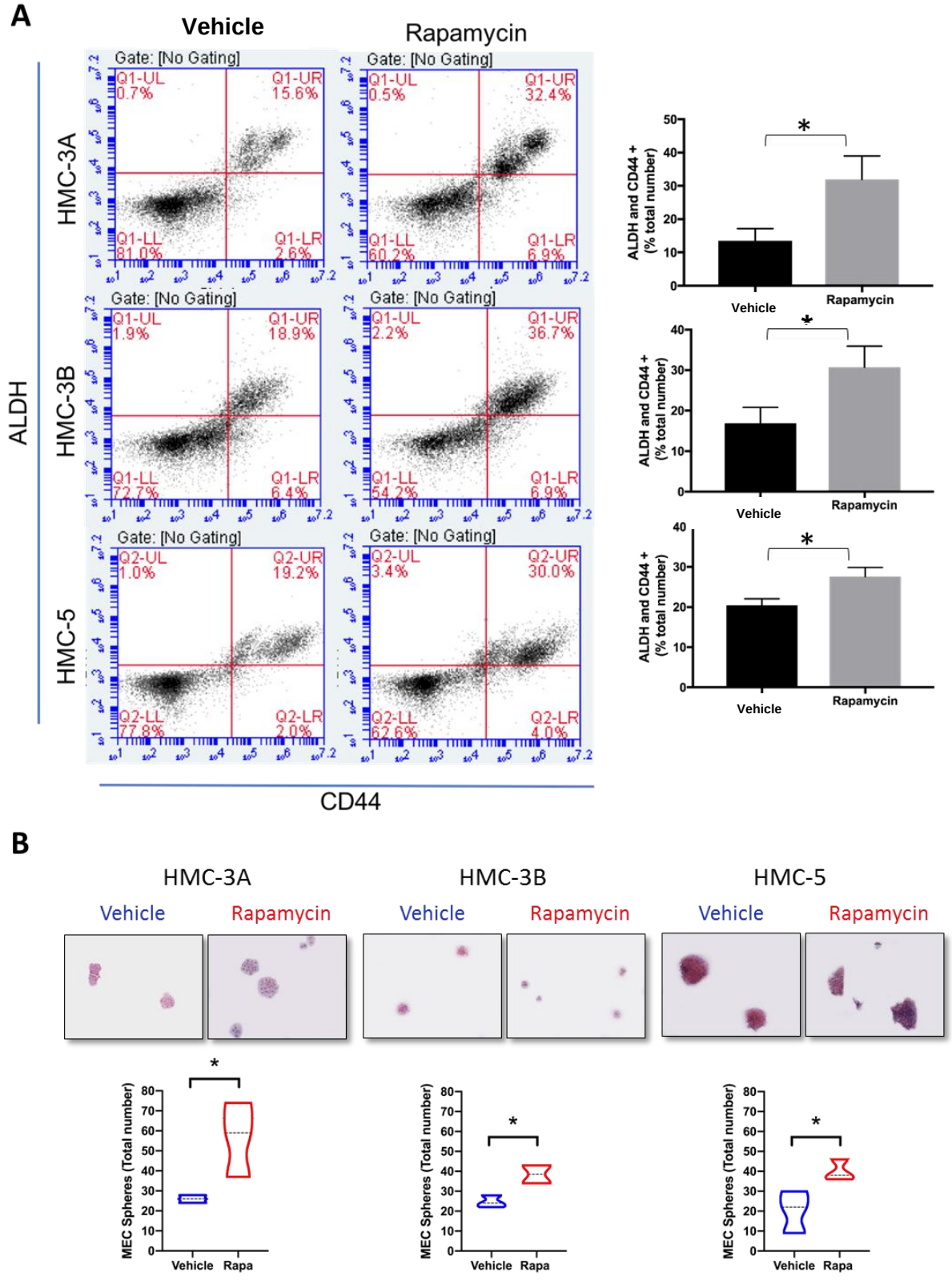
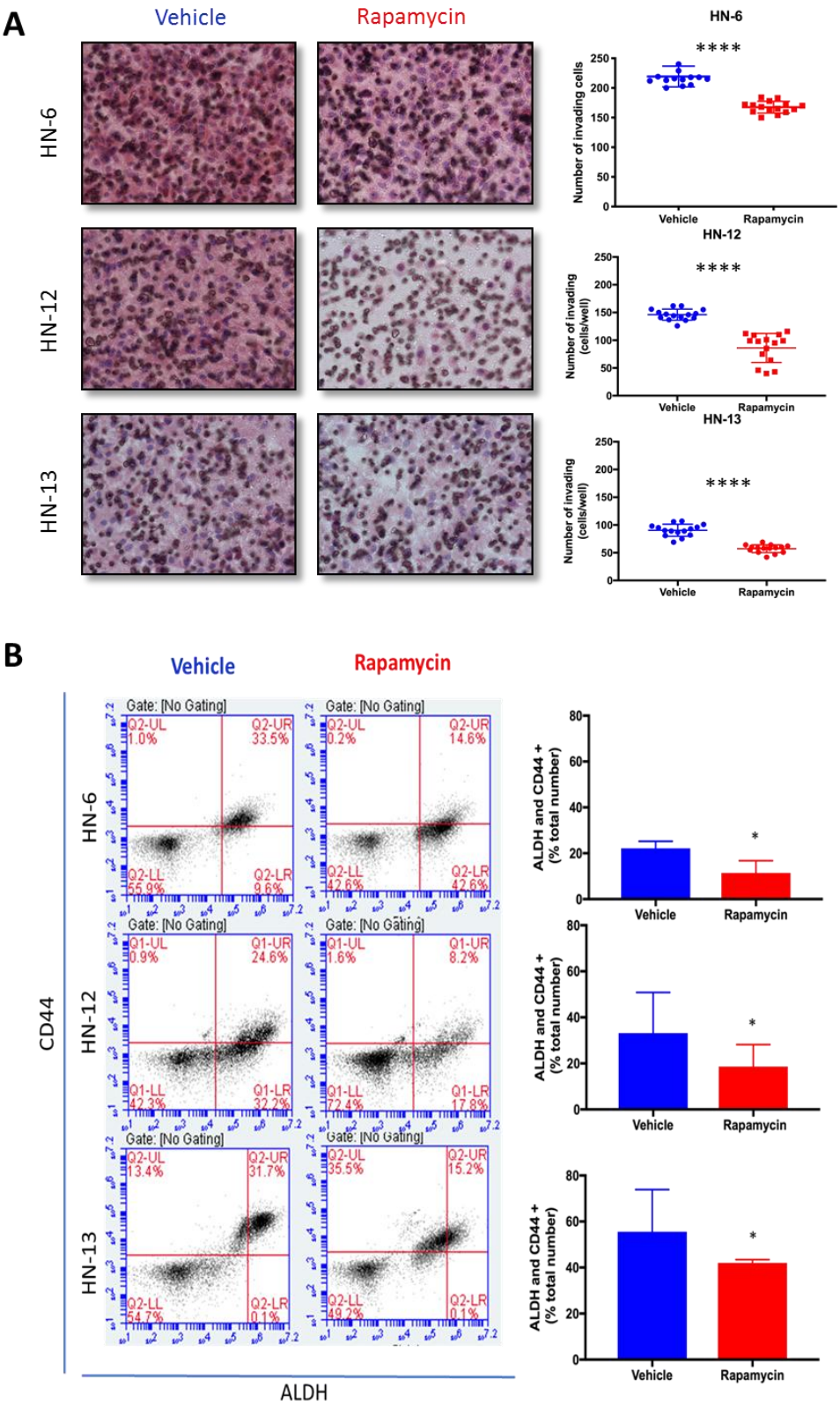


Figure 5: Rapamycin induces the accumulation of cancer stem cells. (A) Representative image of ALDH enzymatic activity combined with the presence of CD44 positive cells in all 3 MEC cell lines during the administration of Rapamycin (24 hrs, 50nM). The percentage of $ALDH^{bright}/CD44^{+}$ is represented in column bar (*$p < 0.05$). (B) Tumorsphere assays demonstrate an increased number of spheres in after 5 days of ultra-low adhesion culture conditions. Violin graphic representation of tumorspheres for all MEC cell lines (*$p < 0.05$).



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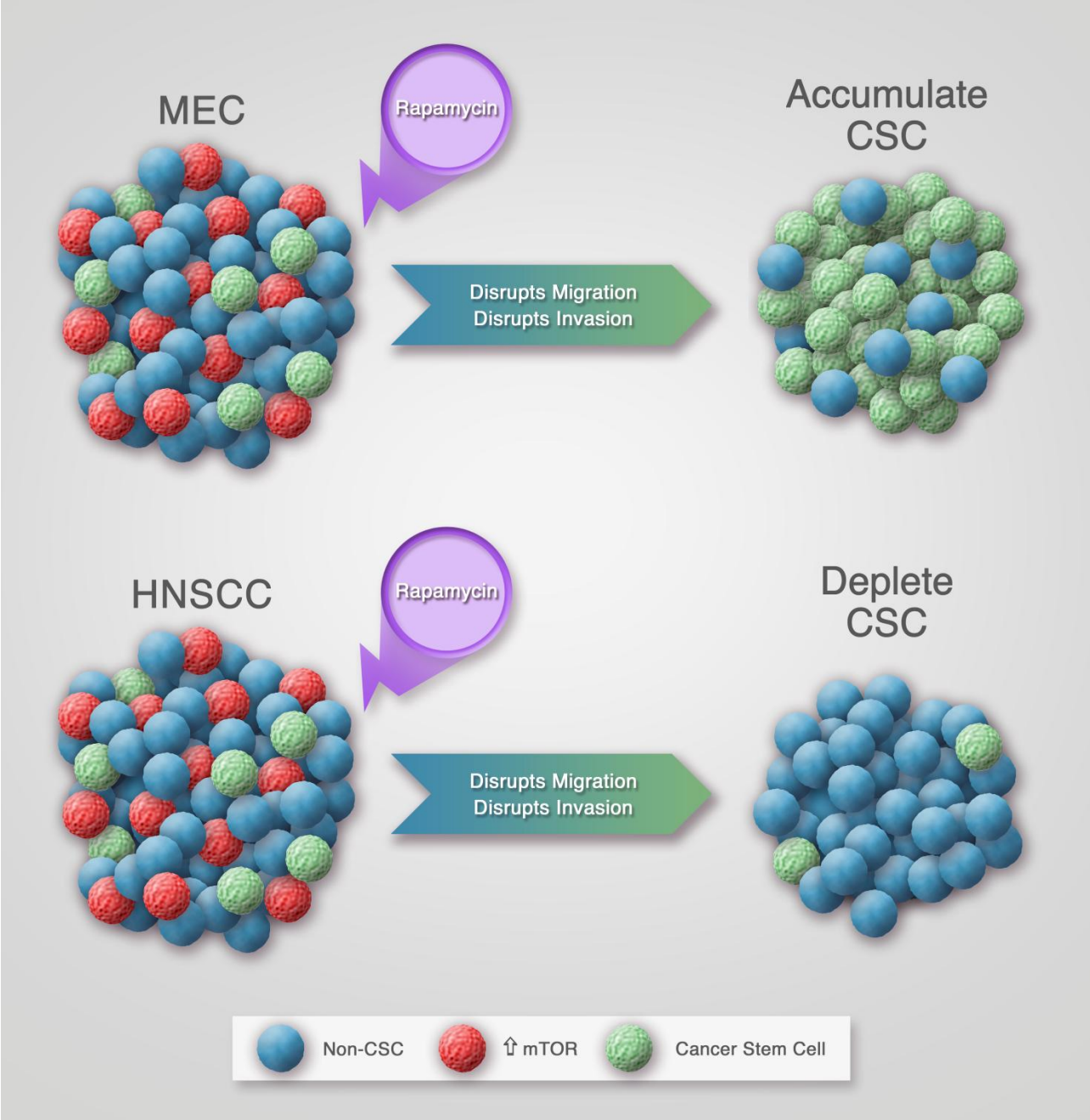
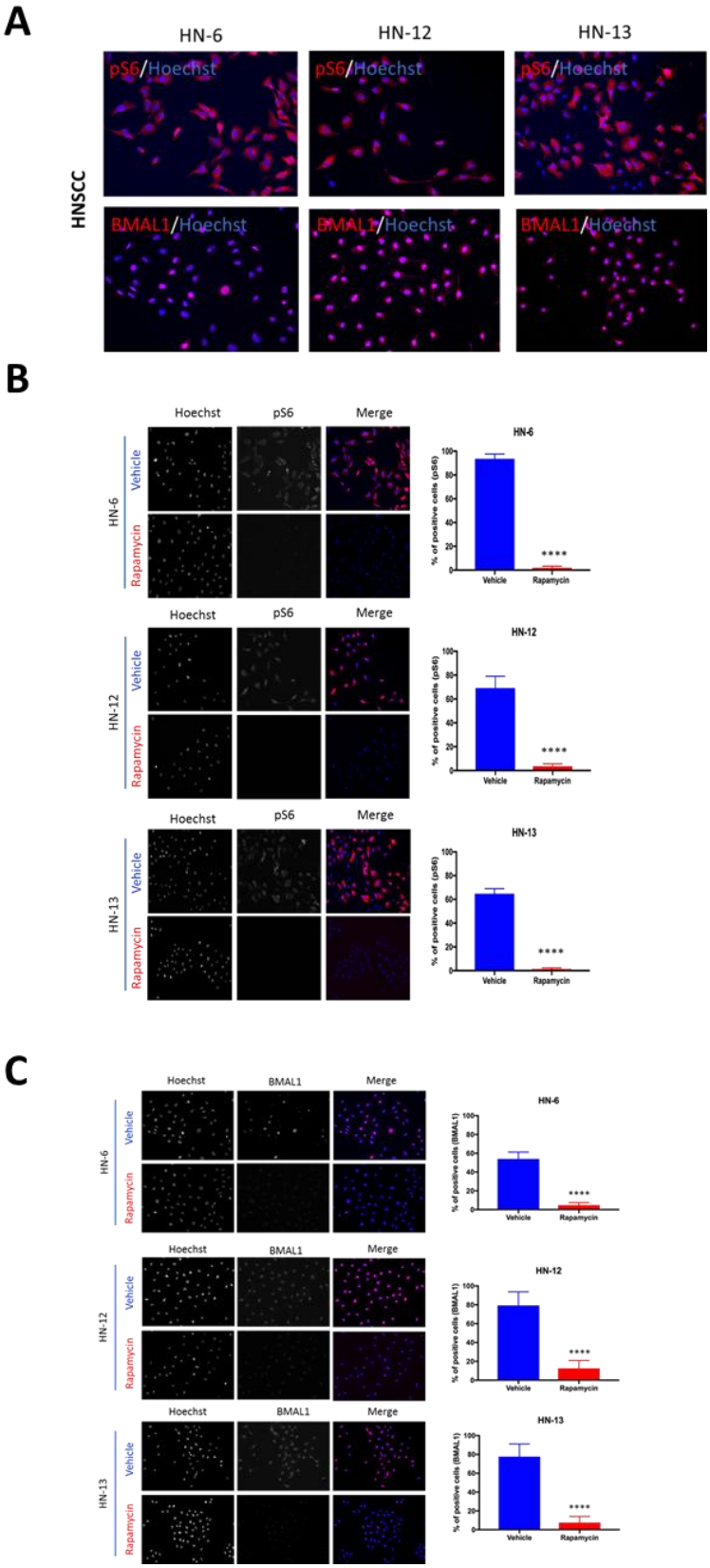


Figure 7: Schematic representation of the effects of Rapamycin over MEC and HNSCC tumors. Administration of Rapamycin results in the disruption of cancer migration and invasion of MEC and HNSCC tumors. Rapamycin induces accumulation of MEC cancer stem cells, while HNSCC responds to Rapamycin by reducing the number of CSC.

Table 1 Clinico-pathological information of all MEC cases, including histological grade, gender, age and anatomical location of the tumors.

Case	Histologic grade	Gender	Age (years)	Location
1	Low	Female	38	Buccal mucosa
2	low	Female	38	Hard Palate
3	low	Female	48	Buccal mucosa
4	low	Female	50	Soft palate
5	low	Female	42	Hard palate
6	Low	Male	31	Hard palate
7	Low	Female	77	Hard palate
8	Low	Male	40	lower lip
9	Low	Male	46	Hard palate
10	Low	Male	62	Hard palate
11	low	Male	61	Hard palate
12	Intermediate	Female	70	Retromolar area
13	Intermediate	Female	52	Buccal mucosa
14	Intermediate	Female	58	Hard palate
15	Intermediate	Female	45	Retromolar area
16	Intermediate	Female	38	Buccal mucosa
17	Intermediate	Female	46	Hard palate
18	High	Female	80	Upper Lip
19	High	Male	52	Buccal mucosa
20	High	Male	16	Hard palate



Supplementary Figure 1: (A) Immunofluorescence staining of HNSCC expressing high levels of pS6 and BMAL1. (B) Administration of Rapamycin to HNSCC result in a reduction of pS6 levels in all cell lines (****p<0.0001). (C) Immunofluorescence of HNSCC for BMAL1 demonstrate a reduced number of positive cells upon treatment with Rapamycin (****p<0.0001).

Figure legends

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3 CONCLUSÃO

Os resultados apresentados permitem concluir que a via mTOR foi mais expressa em carcinoma mucoepidermóide do que em tecidos normais, e que a inibição dessa via pela rapamicina apresentou funções anti-oncogênicas, como a diminuição de invasão e migração cellular. A rapamicina levou a manutenção e indução da população de células-tronco tumorais. O uso da rapamicina no tratamento de CME deve ser associado a um inibidor de células-tronco tumorais.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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ANEXO

Anexo 1 – Certificado do Comitê de Ética em Pesquisa

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 "Correlação da expressão dos *"clock genes"* com proteínas supressoras tumorais – pten e suas vias em carcinoma mucoepidermóide salivar", CAAE 60025316.6.0000.5418, dos pesquisadores Leonardo Amaral dos Reis e Jacks Jorge Junior, 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 27/09/2016.

The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that research project "Correlation of the expression "clock genes" with suppressor protein - pten and routes in salivary mucoepidermoid carcinoma", CAAE 60025316.6.0000.5418, of the researcher's Leonardo Amaral dos Reis and Jacks Jorge Junior, 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 September, 27 2016.

Fernanda Miori Pascon

Profa. Fernanda Miori Pascon

Vice 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 do Artigo

Comprovante de submissão do Artigo – periódico Cellular Oncology

Re: "Rapamycin differentially impacts the population of cancer stem cells from salivary mucoepidermoid carcinomas and head and neck squamous cell carcinomas"
Full author list: Leonardo Amaral Reis, DDS, PhD; Renata L. Markman, DDS, PhD; Liana P. Webber, DDS, PhD; Manoela Martins Domingues, DDS, Ph.D.; Pablo A. Vargas, DDS, PhD; Jacks Jorge, DDS, PhD; Cristiane H. Squarize, DDS, PhD; Rogerio M. Castilho, DDS, PhD

Dear Dr Leonardo Reis,

We have received the submission entitled: "Rapamycin differentially impacts the population of cancer stem cells from salivary mucoepidermoid carcinomas and head and neck squamous cell carcinomas" for possible publication in Cellular Oncology, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dr. Rogerio M. Castilho who will be able to track the status of the paper through his/her login.

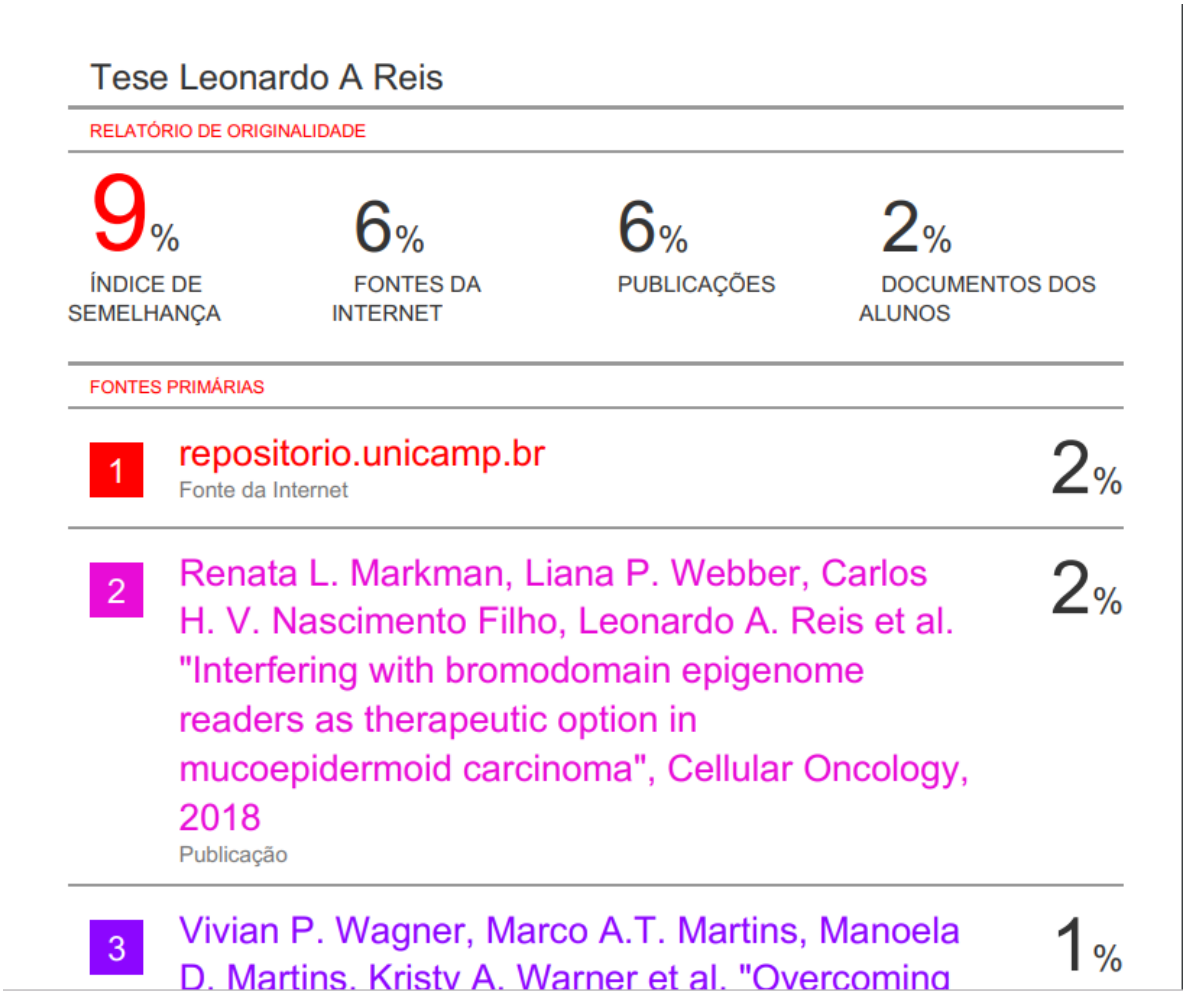
If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office
Cellular Oncology

Anexo 3 - Verificação de originalidade e prevenção de plágio



Tese Leonardo A Reis

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