UNIVERSIDADE FEDERAL DE MINAS GERAIS

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM PATOLOGIA

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CARACTERIZAÇÃO DO MODELO GENETICAMENTE MODIFICADO C(3)1-TAG EM CAMUNDONGOS C57BL/6 E IDENTIFICAÇÃO DOS NERVOS SENSORIAIS NO CARCINOMA MAMÁRIO

Belo Horizonte

2020

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Dissertação apresentada ao Programa de Pós-Graduação em Patologia da Universidade Federal de Minas Gerais como requisito parcial para a obtenção do título de Mestre.

Orientador: Alexander Birbrair
Coorientadora: Leda Maria de Castro Coimbra Campos

Belo Horizonte

2020

043 Sena, Isadora Fernandes Gilson.
Caracterização do modelo geneticamente modificado C(3)1-TAG em camundongos C57BL/6 e identificação dos nervos sensoriais no tumor mamário [manuscrito] / Isadora Fernandes Gilson Sena. – 2020.
69 f. : il. ; 29,5 cm.

Orientador: Alexander Birbrair. Coorientadora: Leda Maria de Castro Coimbra Campos.
Dissertação (mestrado) – Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Programa de Pós-Graduação em Patologia.

1. Patologia. 2. Neoplasias da Mama. 3. Animais Geneticamente Modificados. 4. Neoplasias - Desenvolvimento. 5. Mama - Inervação. I. Birbrair, Alexander. II. Campos, Leda Maria de Castro Coimbra. III. Universidade Federal de Minas Gerais. Instituto de Ciências Biológicas. IV. Título.

CDU: 616

Ficha Catalográfica elaborada por Fabiane C. M. Reis – CRB: 6/2680

Programa de Pós-Graduação em Patologia

ATA DA DEFESA DA DISSERTAÇÃO DE MESTRADO N° 411 DE ISADORA FERNANDES GILSON SENA

Realizou-se, no dia 17 de fevereiro de 2020, às 9 horas, Sala Nello Rangel – Bloco K – 3º andar – sala 163 – ICB – da Universidade Federal de Minas Gerais, a defesa de Dissertação, intitulada ***"Caracterização do modelo geneticamente modificado C(3)1-TAg em camundongos C57Bl/6 e identificação dos nervos sensoriais no tumor mamário"***, apresentada por **Isadora Fernandes Gilson Sena**, número de registro 2018674794, graduada no curso de BIOMEDICINA, como requisito parcial para a obtenção do grau de Mestre em PATOLOGIA, à seguinte Comissão Examinadora: Prof. Alexander Birbrair – Orientador (UFMG), Profa. Leda Maria de Castro Coimbra Campos – Co-orientadora (UFMG), Profa. Ana Lucia Brunialti Godard (UFMG), Profa. Cristiana Buzelin Nunes (Faculdade de Medicina/UFMG).

A Comissão considerou a Dissertação:
(X) Aprovada
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Finalizados os trabalhos, lavrei a presente ata que, lida e aprovada, vai assinada por mim e pelos membros da Comissão.

Belo Horizonte, 17 de fevereiro de 2020.

Alexander Birbrair

Cristiana Nunes

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AGRADECIMENTOS

Agradeço a espiritualidade por ter me protegido e ajudado a chegar até aqui.

A minha mãe, Ana, e meu pai, Antônio, por serem meus exemplos de estudo e perseverança. Ela, doutora. Ele, mestre. Obrigada por terem me dado essa oportunidade de me mostrar desde criança o mundo acadêmico.

As minhas irmãs, Sarah e Clara, por deixarem ensinamentos sobre como equilibrar a vida estressada com uma meditação ou um filme.

Ao meu irmãozinho, Benício, por ser meu porto de risadas e felicidade.

Aos amigos do BirbrairLab. Todos aqueles que eu conheci desde 2016. Especialmente Bella, Lucas e Fernando, por sermos os quatro mosqueteiros que iniciaram esse laboratório. Ao Greg, que ajudou muito no início de tudo, nos orientando e nos fazendo rir de nervoso.

Aos fundadores da "base da pirâmide", Julia, Patrick, Gaby, Luiza e Bryan por serem os mais animados para fazer loucuras.

Aos sobreviventes, Gaby, Carol Leonel, Pedro, Carol Picoli, Alinne, Bia e Walison, por dividirem momentos inesquecíveis de risadas, persistência e trabalho.

A Lu, Vânia, Jaque e Naiara, por me auxiliarem com os blocos e cortes no micrótomo.

Ao professor Geovanni que auxiliou imensamente esse trabalho e a todos os integrantes do Laboratório de Patologia Comparada (LPC) que dividiram momentos de trabalho e risadas, em especial Ana Paula, Ana Emília, Diego e Gabi.

A querida professora Leda que chegou para salvar o mundo. Não sei o que seria esse trabalho sem o seu norteamento. Agradeço imensamente por todo o auxílio e ensinamentos para a vida profissional e pessoal.

Ao meu orientador, Alexander Birbrair, pela oportunidade de trabalho e aprendizado.

E todos aqueles que passaram por mim nesses anos e me ajudaram de alguma forma, em especial Flávia, Pâmela, Mari, Luiz, Nina, Mayra, Samantha, Helen, Vitor e Ricardo.

Médicos e enfermeiros andavam, muito ocupados, de quarto em quarto, conferindo tabelas, redigindo ordens e distribuindo remédios. Mas o laboratório de Farber era apático e vazio, uma cova desnuda de produtos químicos e jarros de vidro ligada ao hospital por uma série de gélidos corredores. O forte cheiro de formalina usada para embalsamar flutuava pelo ar. Não havia pacientes nos quartos, apenas corpos e tecidos de pacientes trazidos através de túneis para autópsias e exames. Farber era patologista.

O Imperador de Todos os Males – Siddhartha Mukherjee

RESUMO

O câncer de mama é a doença maligna que mais atinge as mulheres em todo o mundo e, no Brasil, é a principal causa de morte por câncer nessa população. Vários tipos de modelos animais são usados para entender melhor o desenvolvimento do câncer de mama e seu microambiente. Um desses modelos é o C(3)1-TAg, um animal geneticamente modificado que desenvolve o câncer de mama endogenamente. Porém, a literatura científica não possui trabalhos sobre esse modelo na linhagem C57BL/6. Por isso, realizamos o estudo da progressão tumoral desse animal, assim como analisamos o microambiente tumoral focando nosso estudo na inervação sensorial. Nossos resultados mostraram que o animal C3(1)-TAg na linhagem C57BL/6 possui tumores sólidos, aumento da fibrose, área diminuída de adipócitos e possui expressão de receptor de estrógeno e progesterona, mas não de HER2. Isso o torna um modelo vantajoso para estudar o desenvolvimento de câncer de mama do tipo sólido basalóide do carcinoma adenóide cístico, permitindo realizar análises desde o início do desenvolvimento do tumor e em estágios importantes do câncer de mama. Nesse trabalho, também identificamos que animais com câncer de mama possuem uma maior densidade de inervação sensorial nas mamas e, por sua vez, esses nervos estão mais próximos de vasos sanguíneos. Esses dados sugerem a possibilidade de uma relação entre nervo sensorial e o desenvolvimento do câncer de mama, que será melhor investigada em futuros estudos.

Palavras-Chave: Tumor mamário; animal transgênico; câncer de mama; desenvolvimento tumoral; inervação sensorial

ABSTRACT

Breast cancer is the malignant disease that most affects women worldwide and, in Brazil, it is the main cause of death in this population. Various types of animal models are used to better understand the development of breast cancer and its microenvironment. One of these models is C(3)1-TAg, a genetically engineered modified mouse that develops breast cancer endogenously. However, the scientific literature does not have studies on this model in the C57BL/6 strain. Therefore, we conducted the study of the tumor progression of this animal, as well as analyzed the tumor microenvironment focusing our study on sensory innervation. Our results showed that the animal C3(1)-TAg in the C57BL/6 strain has solid tumors, increased fibrosis, decreased area of adipocytes and has expression of estrogen and progesterone receptor, but not of HER2. This makes it an advantageous model for studying the development solid-basaloid adenoid cystic carcinoma, allowing analysis to be carried out from the beginning of tumor development and at important stages of breast cancer. In this work, we also identified that animals with mammary cancer have a higher density of sensory innervation in the mammary tissue and, in turn, these nerves are closer to blood vessels. These data suggest the possibility of a relationship between sensory nerve and the development of breast cancer, which will be further investigated in future studies.

Keywords: Mammary tumor; transgenic animal; breast cancer; tumor development; sensory innervation

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LISTA DE ABREVIATURAS

BDNF - *Brain-Derived Neurotrophic Fator*/Fator Neurotrófico Derivado Do Cérebro

BRCA1 – *Breast Cancer Gene 1*/Gene 1 do Câncer de Mama

BRCA2 - *Breast Cancer Gene 2/*Gene 2 do Câncer de Mama

C3(1) - *Regulatory Control Of The Rat Prostatic Steroid Binding Protein*/Controle Regulador Da Proteína De Ligação De Esteroide Prostático De Rato

CGH - *Comparative Genomic Hybridization*/Hibridização Genômica Comparativa

CGRP - *Calcitonin Gene-Related Peptide/*Peptídeo Relacionado ao Gene da Calcitonina

DCIS - *Ductal Carcinoma In Situ*/Carcinoma Ductal In Situ

DRG – *Dorsal Root Ganglion*/Gânglio Da Raiz Dorsal

EGFR - *Epidermal Growth Factor Receptor*/Receptor De Fator De Crescimento Epidérmico

GEMM – *Genetically Engineered Mouse Model*/Modelo de Camundongo Geneticamente Modificado

HER2 - *Human Epidermal Growth Factor Receptor-Type 2*/Receptor de Fator de Crescimento Epidérmico Humano 2

IARC - *International Agency for Research on Cancer*/Agência Internacional de Pesquisa em Câncer

INCA – Instituto Nacional de Câncer

LTR - *Long Terminal Repeat*/Repetição Terminal Longa

MIN – *Mammary Intraepithelial Neoplasia*/Neoplasia Intra-Epitelial Mamária

MMTV - *Mouse Mammary Tumor Vírus*/Vírus Do Tumor Mamário De Camundongo

NF200 - Neurofilamento 200

NGF – *Nerve Growth Factor*/Fator De Crescimento Neural

NIH – *National Institute of Health*/Instituto Nacional de Saúde dos Estados Unidos

OMS - Organização Mundial da Saúde

PGP 9.5 - *Protein Gene Product 9.5/*Produto Proteico do Gene 9.5

PTEN - *Phosphatase and tensin homolog*/homólogo de fosfatase e tensina

RE - Receptor de Estrógeno

RP - Receptor de Progesterona

TAg – *T Antigen*/Antígeno T

TDLU - *Terminal Ductal Lobular Unit*/Unidade Terminal Ducto-Lobular

TEB – *Terminal End Buds/*Brotos Terminais

TGF-α – *Tumor Growth Factor*/Fator De Crescimento Tumoral-α

TP53 – *Tumor Protein 53/*Proteína Tumoral 53

VEGF-A - *Vascular Endothelial Growth Factor-A*/Fator de Crescimento Endotelial Vascular-A

WAP – *Whey Acidic Protein*/Proteína Àcida do Soro de Leite

SUMÁRIO

1. INTRODUÇÃO

O câncer de mama é a principal causa de morte por câncer, exceto pele, em mulheres no Brasil e no mundo (BRAY et al., 2018; MARCELI DE OLIVEIRA, 2018). Desse modo, inúmeros estudos tentam desvendar o câncer analisando, caracterizando e classificando os tumores para identificar melhores tipos de tratamento e prevenção contra essa doença (LONNING, 2007; HARBECK *et al.*, 2019). Nas pesquisas científicas esses estudos podem ser realizados em modelos *in vitro* ou modelos *in vivo* (WAGNER, 2004; TAUBENBERGER, 2014).

Uma boa ferramenta investigativa *in vivo* são os modelos de camundongos geneticamente modificados, pois eles: permitem a avaliação da evolução do tumor, desde os estágios iniciais; não possuem o sistema imune deprimido; desenvolvem o tumor no microambiente natural; e apresentam alterações genéticas mais semelhantes a dos seres humanos (HOLEN et al., 2017; PARK et al., 2018; SWIATNICKI & ANDRECHEK, 2019).

O C(3)1-TAg, é um modelo de camundongo geneticamente modificado que permite analisar o desenvolvimento do câncer de mama. Porém, a literatura científica, possui somente análises do desenvolvimento tumoral na linhagem de camundongos FVB (MAROULAKOU *et al.*, 1994; LIU *et al.*, 1998; FAN *et al.*, 2014). Em contrapartida, já é descrito que linhagens diferentes podem gerar respostas imunofisiológicas variadas em diversas doenças, inclusive no câncer (WOODWORTH *et al.*, 2004; SZADE *et al.*, 2015). Além disso, recentes pesquisas tem se dedicado a desvendar a complexidade do microambiente tumoral, destacando a interação das células neoplásicas com outros elementos celulares, como por exemplo, componentes do sistema nervoso (BALKWILL *et al.*, 2012; PUNDAVELA *et al.*, 2015; WANG *et al.*, 2017). Deste modo, este trabalho teve como objetivo estudar o modelo C(3)1-TAg na linhagem C57BL/6 e a presença da inervação sensorial no modelo Nav1.8-Cre/TdTomato/C(3)1-TAg.

2. REVISÃO BIBLIOGRÁFICA

2.1. Câncer de Mama

Em todo o mundo o câncer de mama, exceto pele, é o que mais afeta as mulheres e é um dos cânceres mais agressivos para essa população (FUSELER *et al.*, 2014; HARBECK *et al.*, 2019). De acordo com a Agência Internacional de Pesquisa em Câncer (IARC), em 2018, o número de novos casos de câncer de mama ultrapassou dois milhões e o número de mortes por esta doença ultrapassou 600.000 casos. Isso faz do câncer de mama o segundo câncer mais comum na população mundial (BRAY *et al.*, 2018).

No Brasil, o câncer é a segunda causa de morte em pessoas com menos de 70 anos e a principal causa de morte em mulheres. O Instituto Nacional do Câncer (INCA) estimou para o biênio 2018-2019 cerca de 60 mil novos casos de câncer de mama. A IARC estimou que, em 2018, mais de 18.000 mulheres morreram no Brasil devido a essa doença (Fig. 1) (BRAY *et al.*, 2018; MARCELI DE OLIVEIRA, 2018).

Fatores genéticos, hormonais, ambientais e comportamentais aumentam o risco do desenvolvimento do câncer de mama. Menarca precoce, falta de aleitamento materno, menopausa tardia, gravidez em idade avançada, uso de álcool e falta de exercício físico são alguns fatores que aumentam o risco de desenvolvimento da doença (HARBECK *et al.*, 2019). Mutações em genes como TP53, BRCA1, BRCA2 e PTEN também influenciam a predisposição para o desenvolvimento do câncer de mama (SHIOVITZ & KORDE, 2015).

O diagnóstico e prognóstico do câncer de mama são baseados pelo estadiamento da doença identificado por meio de técnicas histopatológicas e de biologia molecular. Assim os tumores podem ser classificados de acordo com a expressão de receptores hormonais, oncogenes, genes supressores de tumor e alterações celulares (LONNING, 2007). Segundo a Organização Mundial da Saúde (OMS) os tumores mamários podem ser classificados histologicamente em mais de quinze subtipos, sendo o tumor ductal o mais incidente na população (BOMBONATI & SGROI, 2011; SINN & KREIPE, 2013). Harbeck e colaboradores (2019) também discutem que uma análise patológica precisa conter detalhes histológicos do tumor bem como características hormonais. Os tipos de receptores hormonais presentes no tumor são reconhecidos como fatores norteadores para tomada de decisão no tratamento oncológico, destacando-se o receptor de estrógeno (RE), receptor de progesterona (RP) e o receptor de fator de crescimento epidérmico humano 2

(HER2) (HARBECK *et al.*, 2019). A partir da análise do perfil proteico, por meio de imuno-histoquímica desses marcadores, e do perfil de expressão gênica os tumores podem ser agrupados como tipo basal, HER2 enriquecido, Luminal B- HER2+, Luminal B-HER2– e Luminal A (PEROU *et al.*, 2000; PARK *et al.*, 2018).

Os tumores luminais A e B são os subtipos mais encontrados na população (VODUC *et al.*, 2010; BOMBONATI & SGROI, 2011). O tumor luminal A apresenta alta expressão de RE e RP, baixa expressão de HER2 e baixo índice de proliferação celular. Enquanto que o tumor luminal B apresenta uma expressão menor de RE e RP, podendo ser HER2 positivo ou negativo, e possui um alto índice de proliferação celular. Os tumores HER2 enriquecido não exibem a expressão de RE e RP, mas superexpressam HER2 e possuem um alto índice de proliferação celular. Já os tumores basais não possuem a expressão de RE, RP e HER2, possuem um alto índice de proliferação celular e são caracterizados por possuírem um pior prognóstico devido à dificuldade de resposta à terapia (PEROU *et al.*, 2000; BOMBONATI & SGROI, 2011; HARBECK *et al.*, 2019).

Análises histopatológicas são importantes para determinar o estadiamento do tumor e, consequentemente, o melhor tratamento para o paciente (PARK *et al.*, 2018). Os primeiros estágios do câncer de mama podem ser representados pela proliferação epitelial com atipia, hiperplasia ductal atípica e carcinoma ductal in situ (DCIS) (BOMBONATI & SGROI, 2011). O DCIS apresenta a característica de glândulas mamárias com a membrana basal intacta, impedindo as células tumorais de invadir os tecidos adjacentes (POSNER & WOLMARK, 1992). Essa fase é de extrema importância para o estabelecimento de uma estratégia terapêutica, de modo que a doença não avance para o estágio invasivo. O carcinoma invasivo (onde as células tumorais invadem outros tecidos) é o estágio final–do desenvolvimento do câncer de mama, que possui uma alta heterogeneidade celular, baixa taxa de sobrevivência e alta probabilidade de apresentar metástase (MAUGHAN *et al.*, 2010; AKRAM *et al.*, 2017; HARBECK *et al.*, 2019).

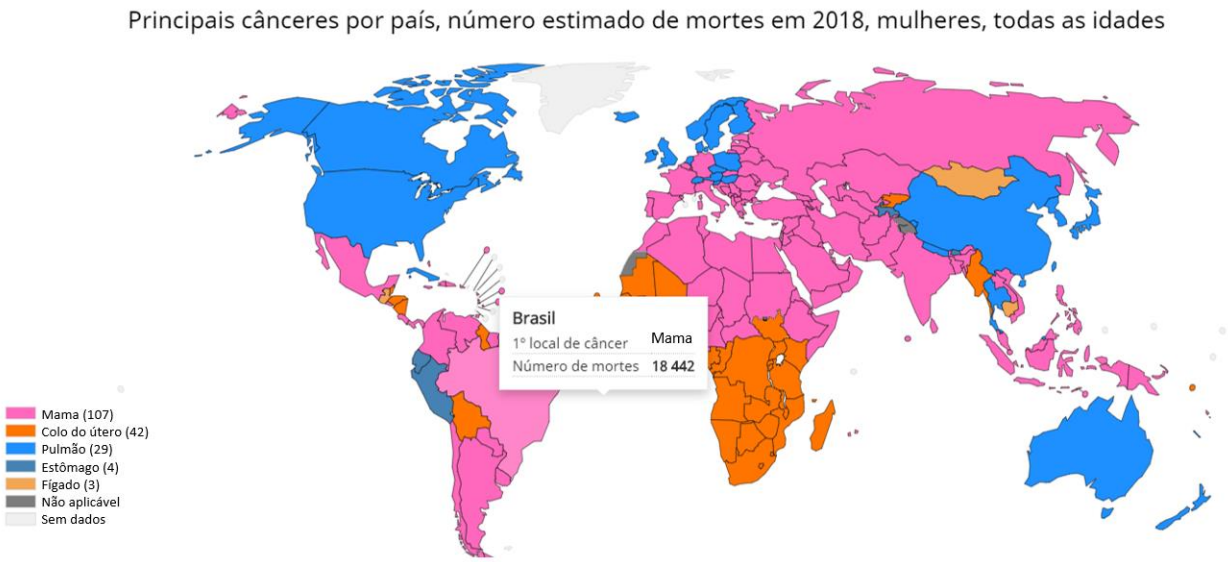

Figura 1. Número estimado de mortes, em 2018, em relação aos principais cânceres por país em mulheres de todas as idades. Imagem adaptada, retirada do observatório global do câncer realizado pela IARC, mostrando que no Brasil o câncer de mama é a principal causa de morte em mulheres com uma estimativa de 18.442 número de mortes. Produção gráfica: IARC (http://gco.iarc.fr/today). Organização Mundial da Saúde. Data de acesso: 28/12/2019.

2.2. Modelos experimentais *in vivo* para estudo do câncer de mama

Devido à alta incidência do câncer de mama no mundo, estudos científicos utilizam modelos in vivo para identificar, classificar e caracterizar tumores, a fim de se conseguir melhor tratamento e prevenção contra essa doença. Os modelos in vivo podem ser aplicados em diversas espécies animais, mas os camundongos são os mais utilizados por serem de fácil manuseio, ocupar pouco espaço, ter um custo relativamente baixo e possuir uma fisiopatologia bem descrita e semelhante a dos humanos (CEKANOVA & RATHORE, 2014). Em geral, os modelos mais comuns são os de enxerto - derivados de linhagens celulares de camundongo ou de humano - e os modelos geneticamente modificados (SAKAMOTO *et al.*, 2015).

Nos modelos de enxerto derivados de linhagens celulares há uma injeção de células tumorais imortalizadas no animal. Por causa da alta agressividade dessas células, esse modelo tem o desenvolvimento tumoral rápido, e deste modo gera uma dificuldade de se estudar a evolução da doença desde o seu início. Ainda, grande parte dos estudos injetam as células tumorais imortalizadas no flanco ou em outra região que não é o ambiente nativo da célula, o que pode influenciar no desenvolvimento e resposta tumoral (HOLEN

et al., 2017). Por sua vez, os modelos que utilizam células humanas ou fragmentos de tumores humanos, denominados de xenoenxertos, necessitam realizar essa técnica em camundongos imunodeprimidos, o que pode alterar a fisiopatologia da resposta tumoral e análises de imunoterapias (SAKAMOTO *et al.*, 2015)

Os modelos de camundongo geneticamente modificados (GEMMs) são desenvolvidos no intuito de avaliar toda a evolução do tumor desde os eventos iniciais até os finais, devido a sua progressão mais lenta. O início espontâneo do tumor ocorre dentro do microambiente próprio, a partir de uma célula mamária normal, que possui uma alteração no oncogene ou gene supressor de tumor (HOLEN *et al.*, 2017). Neste modelo é possível estudar alterações genéticas análogas àquelas que ocorrem em humanos (SWIATNICKI & ANDRECHEK, 2019).

Existem mais de 30 tipos de GEMMs para o estudo do câncer de mama, sendo os principais promotores utilizados para o desenvolvimento desses modelos o promotor da proteína ácida do soro de leite (WAP), a repetição terminal longa do vírus do tumor mamário de camundongo (MMTV-LTR) e a proteína de ligação a esteroides da próstata de rato [C3(1)]. Esses promotores são comumente usados para orientar a expressão de oncogenes, como *neu/ErbB2*, *ciclina D1*, *Ras*, *Myc e Wnt1*, ou inibir genes supressores de tumor como *p53 e pRb* (CARDIFF *et al.*, 2000; PARK *et al.*, 2018).

2.3. Parênquima Mamário de Camundongos

O camundongo possui cinco pares de mamas localizadas na camada adiposa subcutânea, onde três pares estão situados na região superior e dois pares na região inferior (RILLEMA, 1994; RICHERT *et al.*, 2000). Anatomicamente as glândulas mamárias podem ser identificadas pela numeração de 1 a 5 ou por região (Fig. 2) (PLANTE *et al.*, 2011; HONVO-HOUETO & TRUCHET, 2015). Normalmente as glândulas mamárias abdominais (número 4) são melhores para análises, porém as glândulas torácicas (números 2 e 3) também podem ser utilizadas mesmo apresentando alguns músculos interdigitados. As glândulas mamárias cervicais (número 1) são menos utilizadas devido à dificuldade de retirá-las, pela forte associação com os músculos (PLANTE *et al.*, 2011).

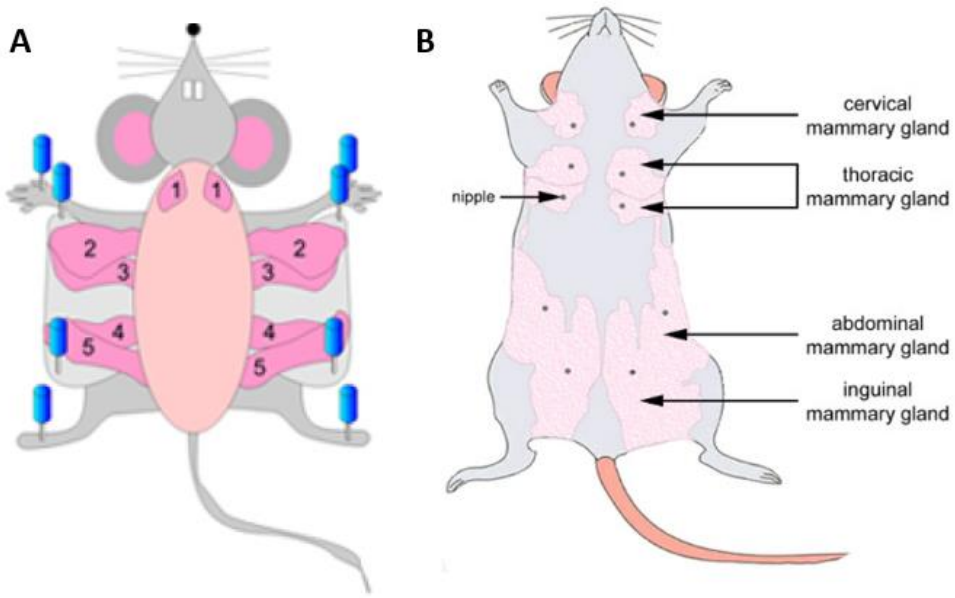

Figura 2. Identificação das glândulas mamárias de camundongos (A) por números (imagem retirada de *Plante, I., et al. (2011))* e (B) por região (imagem retirada de *Honvo-Houeto, E. and S. Truchet (2015)).*

Ao nascimento, as mamas possui uma estrutura ductal rudimentar composta por brotos terminais (TEB) (RICHERT *et al.*, 2000). Os TEBs são estruturas bulbosas com alta taxa de proliferação celular localizados nos ductos mamários em crescimento (RUSSO & RUSSO, 1996). Eles são formados por várias camadas de células epiteliais envoltas por uma camada de células-tronco pluripotentes denominadas de "*cap cells*" que futuramente podem se diferenciar em células epiteliais ou mioepiteliais (RUSSO & RUSSO, 1996; RICHERT *et al.*, 2000). Nessa fase, há uma ausência de tecido conjuntivo

no estroma permitindo uma maior expansão desses ductos na bolsa gordurosa (em inglês, denominado de *fat pad*) (RICHERT *et al.*, 2000).

O desenvolvimento das glândulas mamárias inicia-se a partir de 3 semanas de vida devido à secreção de hormônios sexuais (RICHERT *et al.*, 2000). Esse desenvolvimento é caracterizado por um processo em que o epitélio invade o estroma circundante, composto principalmente por adipócitos (HONVO-HOUETO & TRUCHET, 2015). Com 12 semanas o camundongo tem uma extensão dos ductos mamários em toda a bolsa gordurosa formando estruturas denominadas de lóbulos, identificados como primário, secundário e terciário (RICHERT *et al.*, 2000). O lóbulo primário possui poucos ductos com uma única camada de célula epitelial e um estroma esparso com tecido conjuntivo mais frouxo. Já os lóbulos secundários e terciários possuem um maior número de ductos que são mais arredondados, possuem uma camada epitelial cuboide, seguida de uma mioepitelial, e possui um estroma mais denso, rico em fibroblastos (RICHERT *et al.*, 2000). As principais diferenças entre o desenvolvimento da mama murina e da humana é pelo fato que a mama de camundongos possui pouco tecido conjuntivo ao redor dos ductos mamários, enquanto que o estroma mamário humano é rico em tecido conjuntivo. Além do fato de que camundongos fêmeas somente apresentam lóbulos terciários em fase de gestação, lactação ou sob estimulação hormonal, enquanto que em mulheres esses lóbulos estão presentes independentemente de gestação ou lactação (CARDIFF *et al.*, 2000).

As células epiteliais das glândulas mamárias são capazes de sintetizar e secretar produtos lácteos. Elas formam estruturas com lúmens e são envoltas externamente por uma camada basal de elementos contráteis, que são as células mioepiteliais (RICHERT *et al.*, 2000; HONVO-HOUETO & TRUCHET, 2015). O desenvolvimento do câncer de mama pode ocorrer devido a mutações nas células epiteliais mamárias ou nas células-tronco mamárias (BOMBONATI & SGROI, 2011). Apesar da classificação do câncer de mama ser distinta entre ductal e lobular, grande parte da comunidade científica concorda que esses dois tipos de tumores surgem na Unidade Lobular Ductal Terminal (TDLU). Porém, progressão neoplásica em camundongos ainda não está bem elucidada, já que são poucos os estudos que fornecem análises detalhadas da histopatologia evolutiva da doença (CARDIFF *et al.*, 2000). Geralmente, em camundongos, o estágio de DCIS é denominado neoplasia intra-epitelial mamária (MIN) (CARDIFF *et al.*, 2000)

2.4. Animal C3(1)-TAg

Em 1999, o Instituto Nacional de Saúde dos Estados Unidos (NIH) convocou uma reunião de médicos patologistas e veterinários para avaliar a evolução de modelos de câncer de mama em camundongos. Em poucos modelos foi encontrada similaridade com a evolução da doença em humanos. Neste consenso, destacou-se a semelhança dos tumores do animal C3(1)-TAg com os tumores humanos, inclusive produzindo lesões do tipo DCIS e com estroma esclerosante (CARDIFF *et al.*, 2000).

Maroulakou e colaboradores (1994) foram pioneiros na descrição do animal C3(1)-TAg. Em 1994, os pesquisadores descrevem por meio de análises histológicas e histoquímicas a progressão do tumor mamário no camundongo fêmea da linhagem FVB (MAROULAKOU *et al.*, 1994). Os autores identificam que os animais desenvolvem hiperplasia mamária aos 3 meses de idade (12 semanas) com subsequente desenvolvimento de adenocarcinoma mamário aos 6 meses de idade (24 semanas) em 100% dos animais fêmeas (MAROULAKOU *et al.*, 1994). Metástases pulmonares são raramente vistas, assim como lesões proliferativas da tireoide, glândulas salivares e epitélio nasal, além do desenvolvimento de osteossarcoma e condrodisplasia que também são pouco encontradas (MAROULAKOU *et al.*, 1994). Duas décadas após este estudo, Fan e colaboradores (2014) analisaram os animais C3(1)-TAg fêmeas, na linhagem FVN/NJ, por ressonância magnética. Com a técnica mais apurada, os pesquisadores identificaram hiperplasia ductal atípica com 8 semanas, neoplasia intraepitelial mamária com 13 semanas e carcinoma ductal invasivo com 18 semanas (FAN *et al.*, 2014).

Os camundongos transgênicos C3(1)-TAg são assim denominados por possuírem uma superexpressão do antígeno T (TAg) sob o controle regulador da proteína de ligação de esteroide prostático de rato C3(1) 5' (LIU *et al.*, 1998). Essa superexpressão de TAg gera uma inibição dos genes supressores de tumor p53 e pRB, especificamente no parênquima mamário, alterando o ciclo celular e favorecendo a proliferação celular (HERSCHKOWITZ *et al.*, 2007). A deficiência desses genes supressores de tumor é característica dos principais tumores mamários encontrados em mulheres e alguns estudos já demonstraram que pacientes humanos com pior prognóstico de câncer de mama possuem maior expressão do antígeno T (PHILLIPS *et al.*, 1999; CHANG *et al.*, 2005; DEEB *et al.*, 2007; HERSCHKOWITZ *et al.*, 2007). Ainda, o antígeno T desregula a família da proteína fosfatase 2A das fosfatases serina/treonina que está relacionada com a transformação celular e tumorigênese, juntamente com a *hTERT* e o *H-ras* oncogênico

(HAHN *et al.*, 2002; ARROYO & HAHN, 2005). A expressão tecidual do TAg é detectável por análise imuno-histoquímica a partir de 3 semanas de idade (YOSHIDOME *et al.*, 2000).

Um estudo utilizando hibridização genômica comparativa (CGH) verificou um aumento do número de cópias cromossômicas, nos tumores dos animais C3(1)-TAg, nos cromossomos 1, 6, 11 e X (LIU et al., 1998). Essa amplificação foi mais intensa no cromossomo 6 que contém o proto-oncogene *K-ras*, também muito encontrado em tumores humanos, sendo este detectado com alta expressão nos tumores do modelo C3(1)-TAg, especialmente os mais tardios (entre 5 a 6 meses). Outros genes, como o *c-myc* e o *ErbB2/neu/HER2* também foram analisados e não houve amplificação deles (LIU et al., 1998; HERSCHKOWITZ et al., 2007).

Metadados de análises de expressões gênicas demonstram que os modelos que utilizam o TAg são compostos aproximadamente de 150 genes relacionados a *p53, pRb, E2F e myc* que estão associados ao dano de DNA e vias de apoptose (SHIBATA *et al.*, 1996; DEEB *et al.*, 2007). Outros, como o fator de crescimento tumoral-α (TGF-α) e o receptor de fator de crescimento epidérmico (EGFR) também são expressos em tumores mamários dos animais transgênicos C3(1)-TAg, sendo esses mais expressos em lesões iniciais (YOSHIDOME *et al.*, 2000). Além de tais expressões, há uma alta correlação positiva para a imunomarcação de Ki67, marcador de proliferação celular, em tumores mamários dos animais C3(1)-TAg (DEEB *et al.*, 2007). Esse camundongo possui, ainda, uma expressão gênica semelhante aos animais *knockout* de *p53* e *BRCA1* (DEEB *et al.*, 2007).

Outra vantagem dos animais C3(1)-TAg é não ter o sistema imune comprometido como os animais nudes. Estes apresentam imunodeficiência de células T para aceitar enxertos de vários tipos celulares tumorais, inclusive de humanos. Porém devido a essa imunodeficiência, estes animais apresentam uma resposta imune alterada, dificultando a comparação dos resultados obtidos com dados em pacientes humanos (APRELIKOVA et al., 2016). Por isso e por todas as características citadas anteriormente, o desenvolvimento do animal C3(1)-TAg gerou grande impacto nos estudos do desenvolvimento do câncer de mama.

Todavia, linhagens diferentes podem apresentar variações na resposta imune e perfil comportamental (CRAWLEY *et al.*, 1997; SELLERS *et al.*, 2012). Isso faz com

que diferentes linhagens de camundongos possuam diferentes respostas a diversas doenças, incluindo o câncer (NISHINA *et al.*, 1993; ROSSMEISL *et al.*, 2003; WOODWORTH *et al.*, 2004). Desse modo, é interessante pesquisar qual linhagem possui o melhor fenótipo para estudar determinada doença humana. Por exemplo, Davie e colaboradores (2007) mostraram que a análise da evolução tumoral da mama é melhor realizada na linhagem C57BL/6 do que na linhagem FVB. Isso porque animais da linhagem C57BL/6 possuem uma resposta pró-inflamatória mais intensa que animais FVB favorecendo um desenvolvimento mais lento do tumor e permitindo uma melhor análise dos estágios do desenvolvimento do câncer (DAVIE *et al.*, 2007; SZADE *et al.*, 2015; SONG & HWANG, 2017).

2.5. Inervação Sensorial e Câncer

O sistema nervoso periférico é composto por nervos periféricos, gânglios nervosos e órgãos terminais. Esse sistema conecta-se ao sistema nervoso central por meio de inervações motoras e sensoriais (CARRIEL *et al.*, 2014). Nervos sensoriais são nervos aferentes, que transportam informações sensoriais dos tecidos periféricos para o sistema nervoso central. Esses neurônios são responsáveis principalmente pela sensação de dor, temperatura e pressão. Seu corpo celular está nos gânglios da raiz dorsal e os axônios têm suas extensões especialmente por toda a periferia do corpo (NASCIMENTO *et al.*, 2018). As fibras sensoriais possuem muitos canais iônicos que apresentam alterações em seu funcionamento durante processos como câncer, neuropatia, artrite e lesão tecidual (CAMERLINGO *et al.*, 1998; MUKOUYAMA *et al.*, 2002; PARADA *et al.*, 2003; SCHAIBLE, 2014; BOOTH *et al.*, 2015).

Em 1948, Denny Brown associou a neuropatia sensorial periférica com o aumento da incidência do câncer (DENNY-BROWN, 1948). Atualmente, alguns estudos indicam que quase 35% dos pacientes com neuropatia sensorial periférica desenvolvem câncer (CAMERLINGO *et al.*, 1998). Crescentes estudos vêm evidenciando, por análise celular, que o sistema nervoso está associado à progressão do câncer, como o de mama, próstata, pâncreas, colorretal e gástrico (AYALA *et al.*, 2008; ONDICOVA & MRAVEC, 2010; ALBO *et al.*, 2011; ZHAO, C. M. *et al.*, 2014). Inclusive, pesquisadores acreditam que anticorpos contra neuropeptídios ou células neurais podem ser um grande detector de mau

prognóstico da doença (GRABOWSKI et al., 2002; GRABOWSKI et al., 2005; ENTSCHLADEN et al., 2006).

Neste sentido, recentes pesquisas vêm destacando a atuação da inervação autônoma no microambiente tecidual (MAGNON *et al.*, 2013; MAGNON, 2015). Porém, o papel da inervação sensorial no tumor ainda precisa ser estabelecido. De fato os estudos mostram que as fibras nervosas sensoriais podem liberar muitos fatores reguladores que possuem papéis importantes na progressão do câncer (PUNDAVELA *et al.*, 2015). Essas fibras podem regular a função vascular através da substância P e do peptídeo relacionado ao gene da calcitonina (CGRP) (AUSTIN *et al.*, 2017). A substância P está presente nos nervos periféricos e está relacionada à migração de células de carcinoma de cólon, pulmão e mama, sendo útil para metástases (RUFF *et al.*, 1985; DRELL *et al.*, 2003; ENTSCHLADEN *et al.*, 2004). O CGRP tem uma função pró-inflamatória e ajuda na migração de células tumorais (FOSTER *et al.*, 1992; NAGAKAWA *et al.*, 2001). Além disso, os peptídeos opióides atuam nos receptores dos nervos sensoriais periféricos e desempenham papel de quimioatração das células do carcinoma pulmonar de pequenas células e auxiliam a migração das células tumorais (RUFF *et al.*, 1985; ENTSCHLADEN *et al.*, 2004; ENTSCHLADEN *et al.*, 2006).

É válido ressaltar a diferença entre a inervação tumoral acima discutida e o processo conhecido como invasão perineural, considerada como um indicador de mau prognóstico e metástase (HUANG *et al.*, 2014; ZHAO, Q. *et al.*, 2014). Este processo é caracterizado quando células tumorais migram ao longo das fibras nervosas para infiltrar no sistema nervoso central, fazendo com que os nervos se tornem um guia para as células tumorais fornecendo uma rota alternativa para a disseminação metastática (CEYHAN *et al.*, 2008; BAPAT *et al.*, 2011; MAGNON *et al.*, 2013).

2.6. Inervação Sensorial e Câncer de Mama

A relação entre o sistema nervoso e o câncer de mama também tem despertado a curiosidade de alguns pesquisadores, já que o órgão é bastante inervado por fibras sensoriais (mamilo e pele) e simpáticas (vaso sanguíneo e ductos) (SARHADI *et al.*, 1996; HUANG *et al.*, 2014; ZHAO, Q. *et al.*, 2014; PUNDAVELA *et al.*, 2015). Alguns trabalhos já demonstraram que a presença de fibras nervosas está relacionada a um

prognóstico ruim câncer de mama e ao aumento do potencial metastático, com maior taxa de invasão de linfonodos (PUNDAVELA *et al.*, 2015). De acordo com Pundavela et al. (2015) e Zhao et al. (2014), quanto maior a agressividade do tumor, maior o número de fibras nervosa, estando estas localizadas principalmente no estroma do tumor, perto de adipócitos e vasos sanguíneos (ZHAO, Q. *et al.*, 2014; PUNDAVELA *et al.*, 2015). Por outro lado, Erin *et al.* (2004 e 2008) demonstraram em seu trabalho que as fibras nervosas sensoriais sensíveis à capsaicina poderiam inibir metástases do câncer de mama (ERIN *et al.*, 2004; ERIN *et al.*, 2008)

Em um modelo de xenoenxerto e em cortes histológicos de tumores humanos foi demonstrada imunomarcação em tumores mamários para neurônios sensoriais por meio de produto proteico do gene 9.5 (PGP9.5), CGRP e neurofilamento 200 (NF200). Os trabalhos sugerem que o número de fibras nervosas pode estar relacionado com uma alta taxa de densidade de microvasos, assim como uma alta expressão do fator de crescimento endotelial vascular-A (VEGF-A) (ZHAO, C. M. *et al.*, 2014; AUSTIN *et al.*, 2017). Já estudos *in vitro* mostraram que as células tumorais mamárias aumentam a neuritogênese, o número de ramos colaterais dos neurônios sensoriais e as respostas dos canais iônicos nos neurônios sensoriais do gânglio da raiz dorsal (DRG) (AUSTIN *et al.*, 2017). Sendo que esse aumento da sensibilidade dos canais iônicos pode favorecer o aumento da dor relacionada ao câncer, assim como a alta expressão de neurotrofinas pode favorecer a angiogênese tumoral (TODA *et al.*, 2008; AUSTIN *et al.*, 2017).

Alguns mediadores, como o fator de crescimento nervoso (NGF) e o VEGF, podem ser liberados pelas células tumorais, que podem ajudar na invasão neural no tumor (ADRIAENSSENS *et al.*, 2008; PUNDAVELA *et al.*, 2015). Enquanto que a noradrenalina, fator neurotrófico derivado do cérebro (BDNF), dopamina e substância P secretado pelas fibras nervosas podem ter relação direta com a proliferação e o potencial migratório das células tumorais mamárias (ENTSCHLADEN *et al.*, 2004; ONDICOVA & MRAVEC, 2010; VANHECKE *et al.*, 2011). A discussão sobre a interação entre a inervação sensorial e o câncer de mama pode ficar mais interessante, haja vista que *in vitro,* foi demonstrado que diferentes linhagens de células de câncer de mama têm diferentes níveis de secreção de NGF e efeitos neurotróficos (PUNDAVELA *et al.*, 2015).

Entretanto, os estudos supracitados que discutem a relação entre fibras nervosas e câncer de mama foram realizados apenas em modelos *in vitro* ou *in vivo* com células tumorais injetadas (ZHAO, Q. *et al.*, 2014; PUNDAVELA *et al.*, 2015; AUSTIN *et al.*,

2017). Por isso, estudos em modelos geneticamente modificados podem auxiliar uma melhor compreensão sobre o papel da inervação sensorial no câncer de mama.

2.7. Modelo Nav1.8-Cre

Para estudar a importância de genes específicos, criaram-se modelos nocautes que são GEMMs com mutações silenciando um único gene ou uma combinação de vários genes (CAPECCHI, 2005). Todavia esse silenciamento normalmente é feito em células da linhagem germinativa o que pode gerar, dependendo do nocaute, uma alta letalidade embrionária ou pós-natal, além de diminuir a veracidade da análise do desenvolvimento de algumas doenças. Para contornar essas dificuldades foi criado um sistema de nocaute específico denominado como sistema Cre-loxP. que permite que camundongos que possuem a enzima Cre recombinase em tecidos específicos sejam cruzados com camundongos contendo genes floxeados (flanqueados por loxP) para produzir mutantes nulos específicos (DENG, 2014).

O animal Nav1.8-Cre foi criado para estudar um gene específico de nociceptores expresso apenas em um subconjunto de neurônios sensoriais (STIRLING *et al.*, 2005). Gautron (2011) cruzou o animal Nav1.8-Cre com outro camundongo geneticamente modificado, TdTomato, para identificar neurônios sensoriais em diversos órgãos. O camundongo TdTomato possui um cassete de Stop flanqueado por loxP o que impede a expressão da proteína de fluorescência TdTomato. Porém, neurônios que expressam a enzima Cre (associada aos canais Nav1.8) tem essa sequência retirada, permitindo a expressão da fluorescência. Portanto, utilizando microscópio de fluorescência é possível identificar somente as fibras nervosas sensoriais que estarão emitindo a fluorescência em vermelho (GAUTRON *et al.*, 2011).

Os canais de sódio regulados por voltagem estão associados a respostas térmicas, mecânicas e químicas (FELTS *et al.*, 1997; ISOM, 2001; DJOUHRI *et al.*, 2003; MIAO *et al.*, 2010). O Nav1.8 é uma isoforma da família Nav amplamente expressa no sistema nervoso periférico (ERICKSON *et al.*, 2018). Ele está associado a nervos periféricos que estão exclusivamente em neurônios sensoriais, mas não no sistema nervoso central

(AKOPIAN *et al.*, 1996; MIAO *et al.*, 2010). O Nav1.8 é principalmente expresso no DRG e está presente em cerca de dois terços dos corpos celulares aferentes sensoriais do gânglio nodoso (DJOUHRI *et al.*, 2003; STIRLING *et al.*, 2005; GAUTRON *et al.*, 2011). Além do gânglio, o Nav1.8 é encontrado em muitas terminações sensoriais em órgãos como língua, pulmões, canal alimentar, pâncreas, bexiga, coração, rins, supra-renais, pele, vesícula biliar, fígado, tecido adiposo branco e linfonodos (GAUTRON *et al.*, 2011). Todavia, ainda não se sabe se a glândula mamária também apresenta essas fibras nervosas sensoriais no modelo Nav1.8-Cre/TdTomato.

3. JUSTIFICATIVA

O câncer de mama, exceto pele, é o tipo com a maior taxa de incidência e mortalidade em mulheres em todo o mundo. Para tentar diminuir esses números é necessário estudar, caracterizar e classificar os tumores mamários, a fim de se conseguir melhores resultados na prevenção e tratamento desta doença. Neste intuito, a comunidade científica faz o uso de alguns modelos animais. Porém poucos são os estudos que fornecem análises detalhadas da histopatologia evolutiva da doença. Deste modo este projeto avalia a progressão tumoral mamária em um modelo de camundongo geneticamente modificado, assim como identifica a presença de inervações sensoriais no tumor de mama.

4. HIPÓTESE

O modelo geneticamente modificado C(3)1-TAg na linhagem C57BL/6 é adequado para se estudar o desenvolvimento do câncer de mama e no modelo Nav1.8-Cre/TdTomato/C(3)1-TAg é possível identificar e estudar a inervação sensorial nas mamas.

5. OBJETIVO

5.1. Objetivo Geral

Identificar o desenvolvimento do câncer de mama no camundongo C(3)1-TAg na linhagem C57BL/6 e identificar nervos sensoriais, por meio de da presença de canais Nav 1.8, no tumor mamário.

5.2 Objetivos Específicos

- Estabelecer a taxa de sobrevida dos animais C(3)1-TAg na linhagem C57BL/6.
- Descrever as características tumorais dos animais C(3)1-TAg na linhagem C57BL/6.
- Identificar o perfil de receptores hormonais e de HER-2 do tumor mamário do animal C(3)1-TAg na linhagem C57BL/6.
- Verificar a proliferação celular do tumor mamário do animal C(3)1-TAg na linhagem C57BL/6.
- Caracterizar a evolução fibrótica no tumor mamário do animal C(3)1-TAg na linhagem C57BL/6.
- Analisar a disposição dos adipócitos no tumor mamário do animal C(3)1-TAg na linhagem C57BL/6.
- Localizar os nervos sensoriais no tumor mamário do animal C(3)1-TAg na linhagem C57BL/6.

- Quantificar a área de inervação e identificar os aspectos dos nervos sensoriais nos animais controle em relação aos animais com câncer de mama.
- Correlacionar a presença de vasos sanguíneos com a inervação sensorial em animais controle e com câncer de mama.

6. MATERIAL E MÉTODOS, RESULTADOS E DISCUSSÃO

Estes tópicos serão apresentados na forma de 2 artigos científicos que se encontram nas normas dos respectivos periódicos a que serão submetidos.

Artigo 1: Mammary tumor staging in the genetically modified mouse model C(3)1-TAg in C57BL/6 strain

Este trabalho será submetido a revista *Journal of Mammary Gland Biology and Neoplasia* que possui fator de impacto de 2.758.

Artigo 2: Identification Of Sensory Nerves In The Mammary Tumor Using The Nav1.8-Cre/TdTomato/C(3)1-TAg model

Este trabalho será submetido a revista *Experimental and Molecular Pathology* que possui fator de impacto de 2.350.

Mammary tumor staging in the genetically modified mouse model C(3)1-TAg in C57BL/6 strain

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Abstract

Worldwide breast cancer is one of the most prevalent in women and the leading cause of death in this population. The diagnosis and prognosis of breast cancer is based on the staging of the disease that is identified through histopathological techniques and molecular biology. Several types of animal models are used to better understand the development of breast cancer. One of this models is the C(3)1-TAg that is a genetically modified animal that develops endogenous mammary gland cancer and is characterized in FVB. However, this strain has a difficulty in performing tumor response analysis due to faster cancer development. Therefore, we aimed to establish cellular alterations resulting from the C3(1)-TAg animal in C57BL/6 mouse strain that has a more pro-inflammatory pattern than FVB. Our results showed that C3(1)-TAg animal in C57BL/6 strain has a solid-basaloid adenoid cystic carcinoma characteristic, an increased fibrosis, decreased area of adipocytes and is ER and PR positive and HER2 negative. This make it an advantageous model to study the development of basaloid-type and B-luminal-type breast cancer, allowing perform analysis from the beginning of tumor development and in important stages of breast cancer.

Keywords: mammary tumor; genetically engineered mouse model; breast cancer; tumor development

Introduction

Breast cancer is the one that most affects women in the world and it is one of the most aggressive cancer for this population [1-3]. According to the International Cancer Research Agency, in 2018, the number of new cases of breast cancer exceeded two million and the number of deaths due this disease exceeded 600.000 cases worldwide. This makes breast cancer the second most common cancer in the world population [4].

The diagnosis and prognosis of breast cancer is based on the staging of the disease that is identified through histopathological techniques and molecular biology. Tumors can be classified according to the expression of hormone receptors, oncogenes, tumor suppressors and cellular alterations [5]. Due to this range of variables to identify and classify the tumor, and also to test the effectiveness of various therapies, several types of animal models are used to better understand the development of breast cancer. Commonly used, the genetically engineered model is designed to evaluate the entire evolution of the tumor allowing the analysis in the early stages of cancer. Also, it is a good model for the fact that it has the tumor development in the natural microenvironment, has an intact immune system and develops histopathological cellular alterations similar to those in humans [6, 7].

The C(3)1-TAg is a genetically modified animal model that develops endogenous mammary cancer. The model has an expression of T Antigen (TAg) under the regulatory control of the rat prostate prostate steroid binding protein C3(1)5' [8]. This TAg expression inhibits the expression of tumor suppressor proteins, such as p53 and pRB, specifically in breast tissue, altering the cell cycle and favoring cell proliferation [9]. The C3(1)-TAg is a model very used and characterized in FVB strain due its higher fertility [10-13]. However, some studies demonstrate greater difficulty in performing tumor response analysis in FVB strain due to faster cancer development. Since FVB strain mice present a less pro-inflammatory pattern than C57BL/6 mouse [14-16]. As it is known that different mouse strains have different responses to many pathologies, including cancer [17, 18], we aimed establishing cellular alterations resulting from the C3(1)-TAg in C57BL/6 mouse strain could also yield a good model for analyzing tumor development, especially between stages.

One commonly classification of breast cancer is as non-invasive or invasive and in stages between 0-IV [19]. The mainly non-invasive breast cancer is the ductal

carcinoma in situ (DCIS), which in mice this stage is named as mammary intraepithelial neoplasia (MIN), that have a classic characteristic of mammary glands with the basement membrane intact, preventing tumor cells from invading adjacent tissues [20]. This stage is critically important for establishing a therapeutic strategy for the disease not to advance to the invasive stage, which tumor cells invade in others tissues and the survival rate is lower [19, 21]. For these facts, the aim of this study is to analyze the development of mammary cancer in genetically modified C(3)1-TAg model, in the C57BL/6 strain, focusing on the analyzes of two major stages of the disease: DCIS/MIN and invasive carcinoma..

Materials and methods

Ethical aspects

All procedures were performed under the guidelines and with the approval of the Animal Use Ethics Commission of the Federal University of Minas Gerais (CEUA/UFMG) (Protocol 204/2017).

Animals

Wild type C57BL/6 mice were obtained from the Central Animal Housing of the Federal University of Minas Gerais and the C3(1)-TAg transgenic animals on the C57BL/6 strain were obtained from the Tissue Microenvironment Laboratory of the Federal University of Minas Gerais. The animals were kept under controlled temperature and light (12h/12h light/dark cycle). They fed on standard feed and water available *ad libitum*.

Genotyping of TAg

DNA extraction was performed using the phenol-chloroform method [22]. Then, a conventional polymerase chain reaction (PCR) was performed to amplify the gene of interest using the Applied Biosystems MiniAmp thermocycler (Fisher Scientific Term). The primers (Invitrogen) used were: Forward: 5 'CAGAGCAGAATTGTGGAGTGG-3' and Reverse: 5'-GGACAAACCACAACTAGAATGCAGTG -3'. The amplified product was 500bp and identified by 1% agarose gel electrophoresis.

Experimental Design

For staging analyzes, C(3)1-TAg males crossed with wild-type females and littermates were used in the experiments. The females positive for C(3)1-TAg were used as

experimental group and the negatives were used as control group. The animals were euthanized between 4-weeks and 36-weeks and the analysis performed with animals with 20-weeks, for presenting characteristics of neoplasia in situ, and 32-weeks for presenting characteristics of invasive carcinoma.

Tissue Processing

The animals were anesthetized intramuscularly with an injection of 10% ketamine (80mg/kg body weight) and 2.3% xylazine (12mg/kg body weight), euthanized by cervical dislocation and all mammary glands collected. The tissues were fixed in 10% (v/v) buffered formalin, embedded in paraffin blocks, sectioned into a 4μm thickness, placed onto glass slides, and stained with hematoxylin-eosin or Masson's trichrome.

Microscopic Analysis

The BX51 microscope (Olympus) and ImageJ software were used to quantify fibrosis area and area of adipocytes. The area of fibrosis was measured at 10x magnification in ten fields of mammary tissue of each animal. Ten adipocytes were measured at 20x magnification in ten fields, generating a total of 100 adipocytes analyzed, in mammary tissue of each animal.

Immunohistochemistry

Immunohistochemistry were performed as previously described on mammary glands tissue mounted on gelatinized slides [23, 24]. The antibodies used were CDC047 (47DC141, Neomarkers, 1/300), pan-cytokeratin (AE1/AE3, Dako, 1/100), cytokeratin 14 (LL002, Abcam, 1/100), p63 (4A4, Abcam, 1/100), HER2 (c-erB-2, Dako, 1/200), estrogen receptor (1D5, Dako, 1/50) and progesterone receptor (hRPa2, Neomarkers, 1/50). A polymer detection system was used for the identification of the secondary antibody (ADVANCE HRP-ready to use, DakoCytomation). Diaminobenzidine (DAB) was used as a chromogen and sections were counterstained with Mayer's hematoxylin. Negative controls were obtained by substitution of primary antibody by normal serum. Tissue obtained were used as positive controls. Adjacent normal mammary gland and skin were used as internal positive control. Pan-cytokeratin and cytokeratin 14 (CK14) was considered positive when cells revealed a dark-brown cytoplasmatic staining. CDC047, p63, estrogen receptor (ER) and progesterone receptor (PR) was considered positive when cells revealed a dark-brown nuclear staining. HER2 was considered

positive when cells revealed a dark-brown membrane staining. The percentage of positive cells immunostained for CDC047, RP, RE and HER2 was calculated in a total of 500 tumor cells [24, 25].

Statistical analysis

The results were analyzed by statistical tests that were applied over each case with the GraphPad Prism 7 software (Prism Software, Irvine, CA, USA). Data normality was accessed by the Shapiro-Wilk test and parametric data was performed using the T test and non-parametric data using the Mann Whitney test. The percentage survival was analyzed using a Kaplan-Meier survival analysis and Log-rank (Mantel-Cox) statistic was used for comparison of the curves between C3(1)-TAg and control animals. In all cases, we consider $p < 0.05$ as statistically significant and exhibit the mean ± standard error.

Results

Survival Curve

The survival curve of C3(1)-TAg animals showed an average survival of about 32 weeks. Between four-week and sixteen-week the survival rate was 100%. At twenty-week the survival rate was 95.00%; at twenty-four-week it was 86.06%; at twenty-eight-week it was 74.22%; at thirty-two-week it was 46.39%; and at thirty-six-week it was 23.20%. The Log-rank (Mantel-Cox) test showed that there was a significant difference between the survival curves between C3(1)-TAg and control animals ($p=0.0061$) (Fig. 1).

Staging of model C3(1)-TAg, C57BL/6 strain

First, we histologically analyzed the development of cancer in the C3(1)-TAg model C57BL/6 strain and identified that in four-week and eight-week age there were no cellular alterations indicative of tumoral lesions (Fig. 2A and 2B). In the twelve-week it was possible to identify hyperplasia in the mammary glands which presented some supernumerary projections of epithelial cells, which were morphologically similar to normal cells of the duct, with mild nuclear pleomorphism. Disorganized or irregular bridges formed by intraductal papillary projections were also observed. (Fig. 3A).

In the sixteen-week and twenty-week hyperplasia progressed to mammary intraepithelial neoplasia presenting filling mammary glands with epithelial cells, but without disruption of the basement membrane (Fig. 3B). From twenty-four-week there was an evolution to invasive tumor (Fig. 3C). Interestingly, we identified that tumors of animals aged over

24 weeks are histologically similar exhibiting a solid arrangement (Fig. 3D). The tumor cells have rounded nucleus and scant cytoplasm and hyperchromatic peripheral cells were arranged in a palisade pattern forming a basaloid carcinoma morphological profile.

To ratify the mammary epithelial profile of tumor, we performed an immunohistochemistry for pan-cytokeratin, an epithelial cell marker, and CK14, a basal cell marker, and we identified that there were an intense cytoplasmic labeling in the tumors of C3(1)-TAg, C57BL/6 strain (Fig. 4A and 4B). This confirms the presence of epithelial cells of basal origin. We also performed a p63 immunohistochemistry, a myoepithelial cell marker, and identified that there was no nuclear staining in the tumor areas (Fig. 4C). In addition, we analyzed the hormone profile of the tumor by immunohistochemistry for PR, ER and HER2. We verified that PR has an intense nuclear labeling in over 75% of cells in tumor, ER has a weakly nuclear labeling in over 75% of cells in tumor and HER2 has an incomplete membrane labeling with less than 10% positive cells (Fig. 5).

Cell Proliferation

Numerous mitosis figures were observed in the tumor of C3(1)-TAg animals. The C3(1)-TAg animals presenting mammary intraepithelial neoplasia had an average of 6.96 ± 3.207 number of mitoses per field, while the C3(1)-TAg animals presenting invasive carcinoma had an average of 18.06 ± 1.519 (Fig. 6A and 6B). This showed a significant increase in the number of mitoses between C3(1)-TAg and control animals and demonstrated a higher number of mitosis in animals with invasive carcinoma when compared to the animals with MIN. An immunohistochemistry of CDC47, which is a cell proliferation marker, was also performed and there was a significantly higher number of positive cells compared to negative cells in the tumor (Fig. 6C and 6D).

Fibrosis area

We performed a Masson trichrome stain to identify the area of fibrosis. The C3(1)-TAg animals presenting mammary intraepithelial neoplasia had a higher fibrosis area (3072.0 ± 769.8 μm^2) compared to control animals (927.9 ± 378.2 μm^2). The C3(1)-TAg animals presenting invasive carcinoma also had a higher fibrosis area (3738.0 ± 1038.0 μm^2) compared to control animals (981.2 ± 212.2 μm^2). We did not note a significantly difference in fibrosis area between animal presenting MIN and invasive carcinoma (Fig. 7).

Area of Adipocytes

We analyzed the area of adipocytes present in the mammary tissue and identified that animals presenting mammary intraepithelial neoplasia had a smaller area of adipocytes (166.6 ± 20.15 μm^2) compared to control animals (341.6 ± 51.59 μm^2). The C3(1)-TAg animals presenting invasive carcinoma also had a smaller area of adipocytes (123.5 ± 28.44 μm^2) when compared with the control animals (280.6 ± 35.05 μm^2). We did not note a significantly difference in the area of adipocytes animal presenting MIN and invasive carcinoma (Fig. 8).

Discussion

Maroulakou *et al.* was pioneer on the description of the C3(1)-TAg animal in the FVB strain. In 1994, the researcher describes through histological and histochemical analyzes the progression of the mammary tumor in the female mouse. The author identified that the animals develop mammary hyperplasia at twelve weeks of age with subsequent development of mammary adenocarcinoma at twenty-four-weeks [10]. Interestingly, our data corroborate with this study demonstrating histologically that development of mammary cancer in the C3(1)-TAg model in the C57BL/6 strain also promotes hyperplasia of the mammary glands at twelve-weeks, progressing to mammary intraepithelial neoplasia at sixteen-weeks and the development of adenocarcinoma starting at twenty-four-weeks.

However, we identified that C3(1)-TAg animals on C57BL/6 strain has a longer percent of survival than the animals on FVB strain. The research of Maroulakou *et al.* shows that the survival percentage of female C3(1)-TAg animals of the FVB strain is up to twenty-four-weeks, while our study shows that C3(1)-TAg on C57BL/6 have an average survival of thirty-two-weeks [10]. Interestingly comparative analyzes of another transgenic breast cancer model, PyMT and MMTV-neu, also show a tumor development delay of approximately six weeks in the C57BL/6 strain compared to the FVB strain [14, 26]. Likewise, Yoshidome *et al.* using C3(1)/TAg-ER$\alpha^{+/+}$ females in the FVB-C57BL/6 mixed background suggested that this strain lived significantly longer than C3(1)/TAg-ER$\alpha^{+/+}$ females in the FVB background [13]. This may correspond to the fact that C57BL/6 mouse strain has a more pro-inflammatory pattern than FVB which favor a slower mammary tumor development compared to FVB strain [14-16].

We demonstrated that the tumor of C3(1)-TAg animals in C57BL/6 has a solid arrangement with cells organized in a palisade pattern, a high index of proliferating cells, an expression of pan-cytokeratin and CK14 and a lack of expression of p63. Some pathologists classify these morphological characteristics as classic of the basaloid tumor [27-29]. On the other hand, by analyzing the ER, PR and HER2 expression, C3(1)-TAg animals in C57BL/6 strain can be classify as luminal-B-HER2-negative, a very prevalent subtype in many countries Some studies demonstrated that tumor development of animal C(3)1-TAg is hormone-independent and loses ER expression during tumor development [30, 31], others demonstrate that ER can be detected weakly in basal cells in mammary ducts and in invasive carcinomas [13]. Our result corroborates with these data, since we identified that C3(1)-TAg animals in C57BL/6 strain have ER labeling in over 75% of cells. Also, we identified an incomplete membrane labeling with less than 10% HER2-positive cells, but a high nuclear labeling of PR.

For the fact that tumor microenvironment is extremely rich, being composed of tumor cells, fibroblasts, blood vessels, nerves, inflammatory cells, adipocytes, extracellular matrix and several other important components for tumor growth, we decided to analyze two important cell components in this microenvironment [32]. Fibroblasts, for example, are abundant cells in the tumor microenvironment and can synthesize collagen fibers and secrete various growth factors favoring the proliferation of tumor cells [33, 34]. It is known that increased fibrosis is usually correlated with a poor cancer prognosis and that around 80% of fibroblast present in breast tumor promote tumor growth [32, 33, 35]. This is consistent with the results of our study that demonstrated that mammary tissue of C3(1)-TAg in the C57BL/6 strain have a significantly greater fibrosis area than the control animals.

Another interesting cell to study in the tumor microenvironment is the adipocyte. Adipocytes can be classified into white, beige or brown adipocytes. White adipocytes are those uniloculars responsible for storing energy, while brown adipocytes are those multiloculars responsible for energy expenditure [36]. In cancer, tumor cells spend a lot of energy to proliferate and consequently use some energy sources such as adipocytes [37]. Because of this, a decrease in lipid droplet size in the presence of tumor cells has already been demonstrated *in vitro* and *in vivo* [38, 39]. Petruzzelli and colleagues have shown that animals with skin, liver, pancreatic, and lung cancer have regions composed of small adipocytes with larger nuclei and multilocular cytoplasm [39]. These findings

are very similar to our breast cancer analyzes, which we identified a significant reduction of the area of adipocyte in C3(1)-TAg animals. However, to confirm the classification of adipocytes as brown, immunohistochemical analyzes for UCP1 need to be performed [40, 41].

In conclusion, our study showed that the genetically modified model C3(1)-TAg in the C57BL/6 strain is a good model to study the development of a solid-basaloid adenoid cystic carcinoma type and luminal B mammary cancer, allowing perform analysis from the beginning of tumor development and in important stages of progression tumor of breast cancer (Fig 9). The model has classic features of a breast tumor, such as cellular alterations, increased fibrosis, decreased adipocytes, as occurs in several types of cancer.

Conflict of interest

The authors declare no conflict of interest.

Fundings

CAPES, Instituto Serrapilheira (Serra-1708-15285) and CNPq (CNPq-28/2018)

Figures

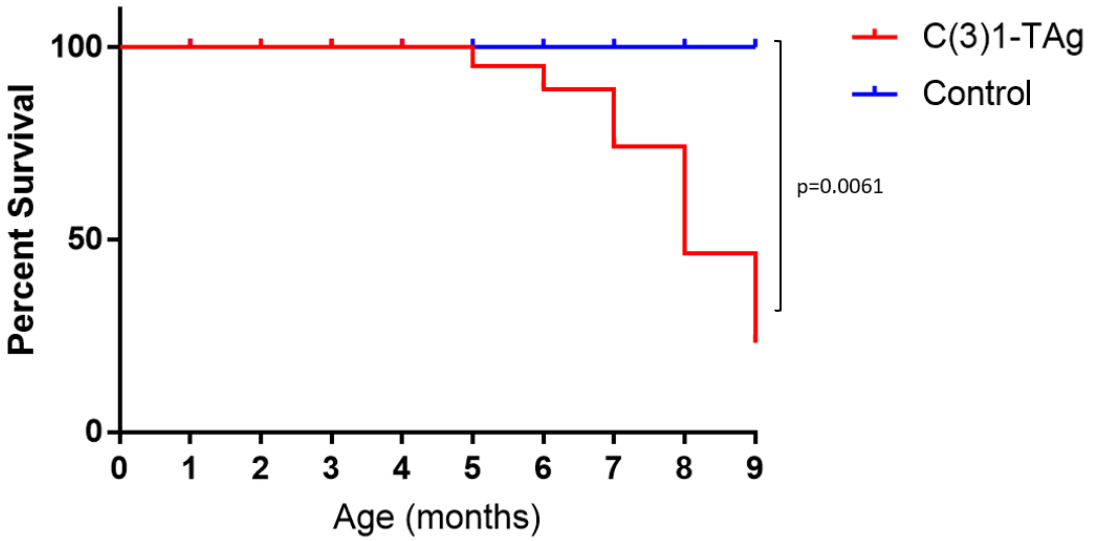

Figure 1. Survival curve of C3(1)–TAg, C57BL/6 strain, and the control group. The C3(1)-Tag has an 8-month (thirty-two-weeks) survival media. Log-rank (Mantel-Cox) analysis showed a significant difference between the C3(1)-TAg (n=16) and control curves (n=16).

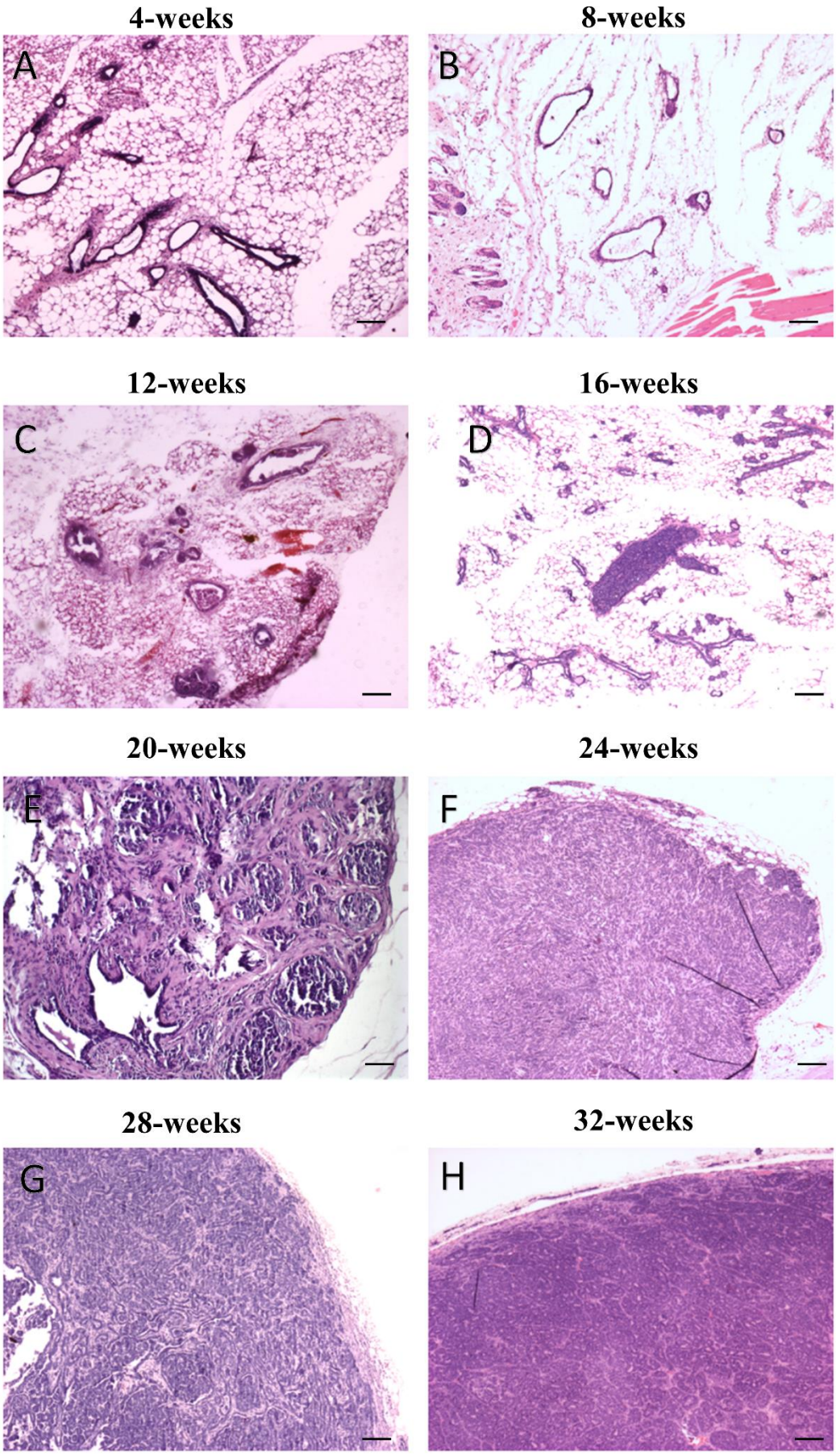
4-weeks
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Figure 2. Descriptive image of tumor development of C3(1)-TAg mouse, C57BL/6 strain. Photomicrograph of HE stained slides of mammary tissue of the C3(1) –TAg at (A) 4-week; (B) 8-weeks; (C) 12-weeks; (D) 16-weeks; (E) 20-weeks; (F) 24-weeks; (G) 28-weeks; (H) 32-weeks. Scale bar: 50μm.

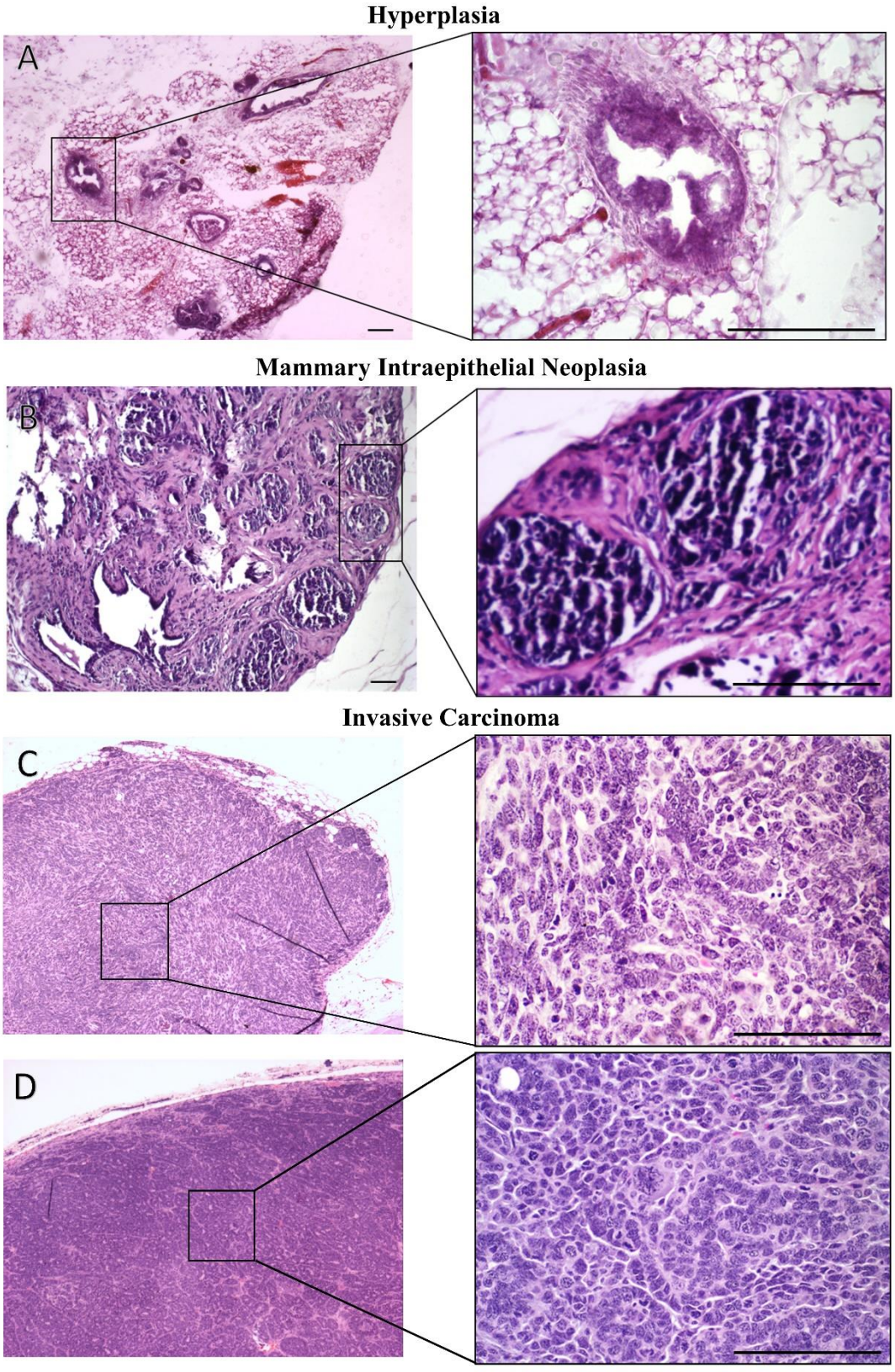

Figure 3. Main stages of breast cancer development in C3(1)-TAg mouse, C57BL/6 strain. Photomicrograph of HE-stained slides of mammary tissue showing (A) hyperplasia in mammary gland presenting some layers of disorganized and atypical epithelial cells; (B) mammary intraepithelial neoplasia with tumor cells filling mammary

gland, but with the basement membrane intact; (C) invasive carcinoma in which tumor cells had already taken all the surrounding tissue. (D) Interestingly breast tumors of animals aged over 24 weeks with invasive carcinoma stage show a similar pattern with cells with rounded nucleus, moderate cytoplasm and arranged in a palisade pattern. Scale bar: 50μm.

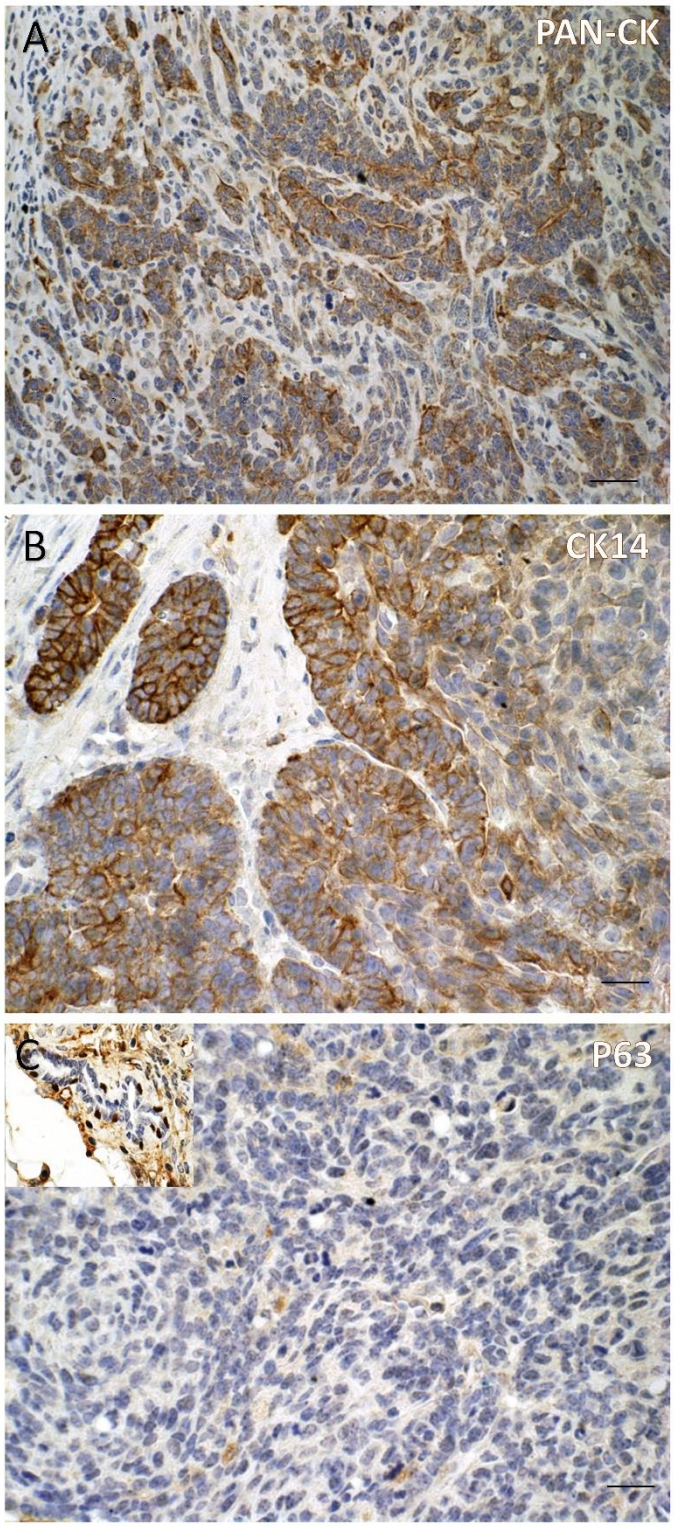

Figure 4. Tumors of C3(1)-Tag, C57BL/6 strain, have a solid-basaloid adenoid cystic carcinoma type phenotype. Representative images of breast tumor sections stained with (A) pan-cytokeratin, an epithelial cell marker, and with (B) CK14, a basal cell marker, with intense cytoplasmic marking arranged in a palisade pattern. (C) Representative

image of breast tumor sections stained with p63, showing central region of tumor with no positive nuclear marking for p63. (*Inset*, positive control). Scale bar: 50μm.

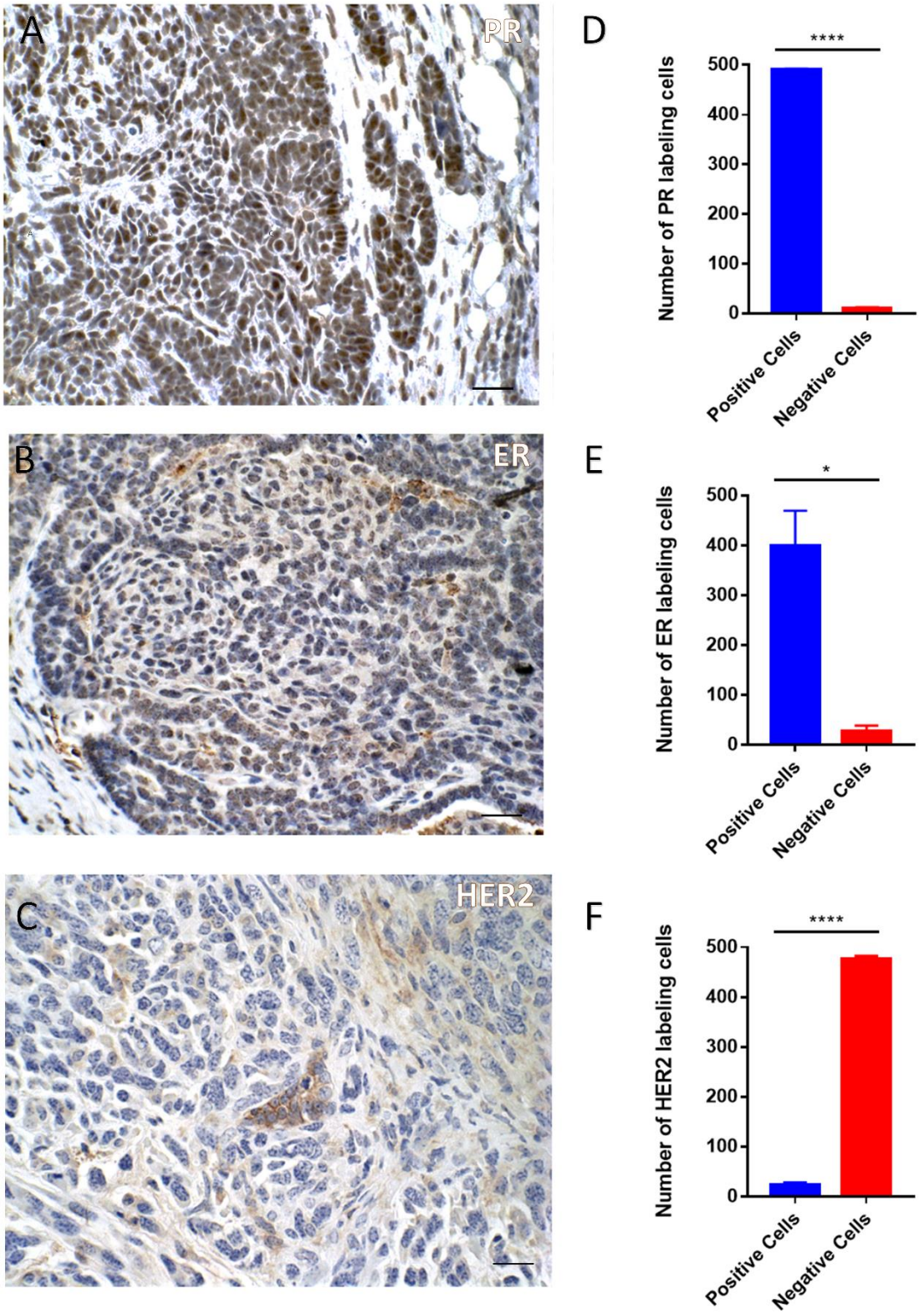

Figure 5. Hormone profile of tumors of C3(1)-TAg animals, C57BL/6 strain. Representative image of breast tumor sections stained with (A) PR, with intense nuclear labeling; (B) ER, with considerable nuclear labeling; and (C) HER2, with sparse

membrane labeling in tumor. Quantification of positive cells number for (D) PR labeling, (E) ER labeling, and (F) HER2 labeling, showing significant differences in number of negatively-labeled cells compared to positively-labeled cells (n=3 for each staining). T-test, ****$p < 0.0001$ and *$p < 0.05$. Scale bar: 50μm.

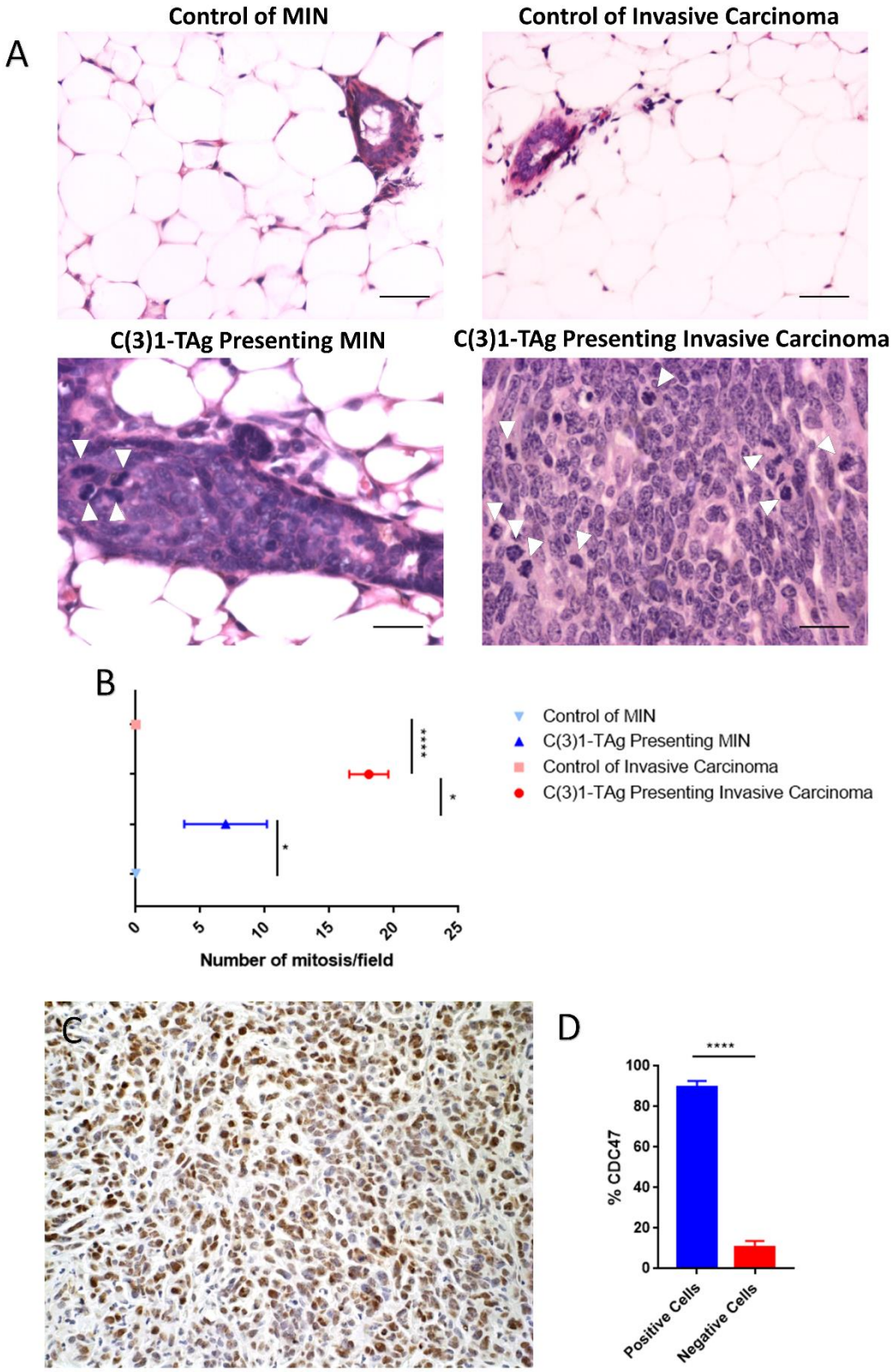

Figure 6. Cell proliferation in the tumors of C3(1)-TAg animals, C57BL/6 strain. (A) Representative photographs of HE stained slides of mammary tissue sections from control and C3(1)-TAg animals. White arrow heads indicate mitotic cells. (B) Quantification of the number of mitotic cells, showing a significantly greater number of mitosis per field

in the mammary tissue of C3(1)-TAg animals compared to respective control groups with MIN (n=3) and invasive carcinoma (n=4). Wilcoxon Test, ****p<0.0001 and *p <0.05. (C) Representative image of mammary tumor stained with the proliferation marker CDC47 (brown) and nuclei (hematoxylin, blue). (D) Quantification of the number of CDC47 labeling cells, showing a significantly greater number of positively-labeled cells compared to negatively-labeled cells (n=3). T-test, ****p <0.0001. Scale bar: 50μm.

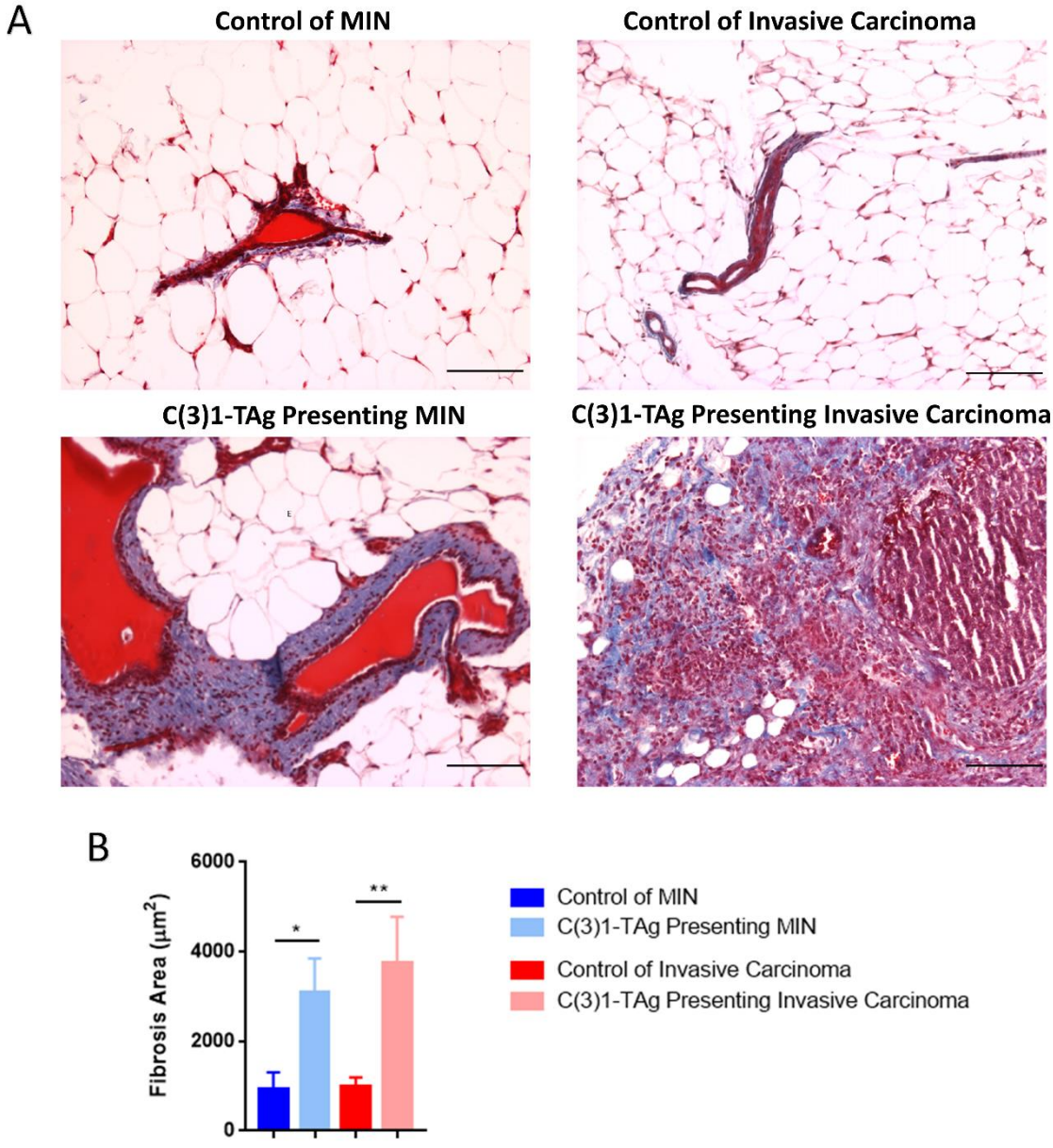

Figure 7. Evolution of fibrosis in breast tumor in model C(3)1-TAg, C57BL/6 strain. (A) Representative photographs in Masson's trichrome stained slides of mammary tissue sections from control and C3(1)-TAg animals. Area of collagen is in blue. (B) Quantification of the fibrosis area in mammary tissue of control group and C3(1)-TAg

group, presenting MIN (n=3) and invasive carcinoma (n=4), showing a significant greater fibrosis area when compared with the control group. T-Test, ** p <0.01 and *p <0.05. Scale bar: 50µm.

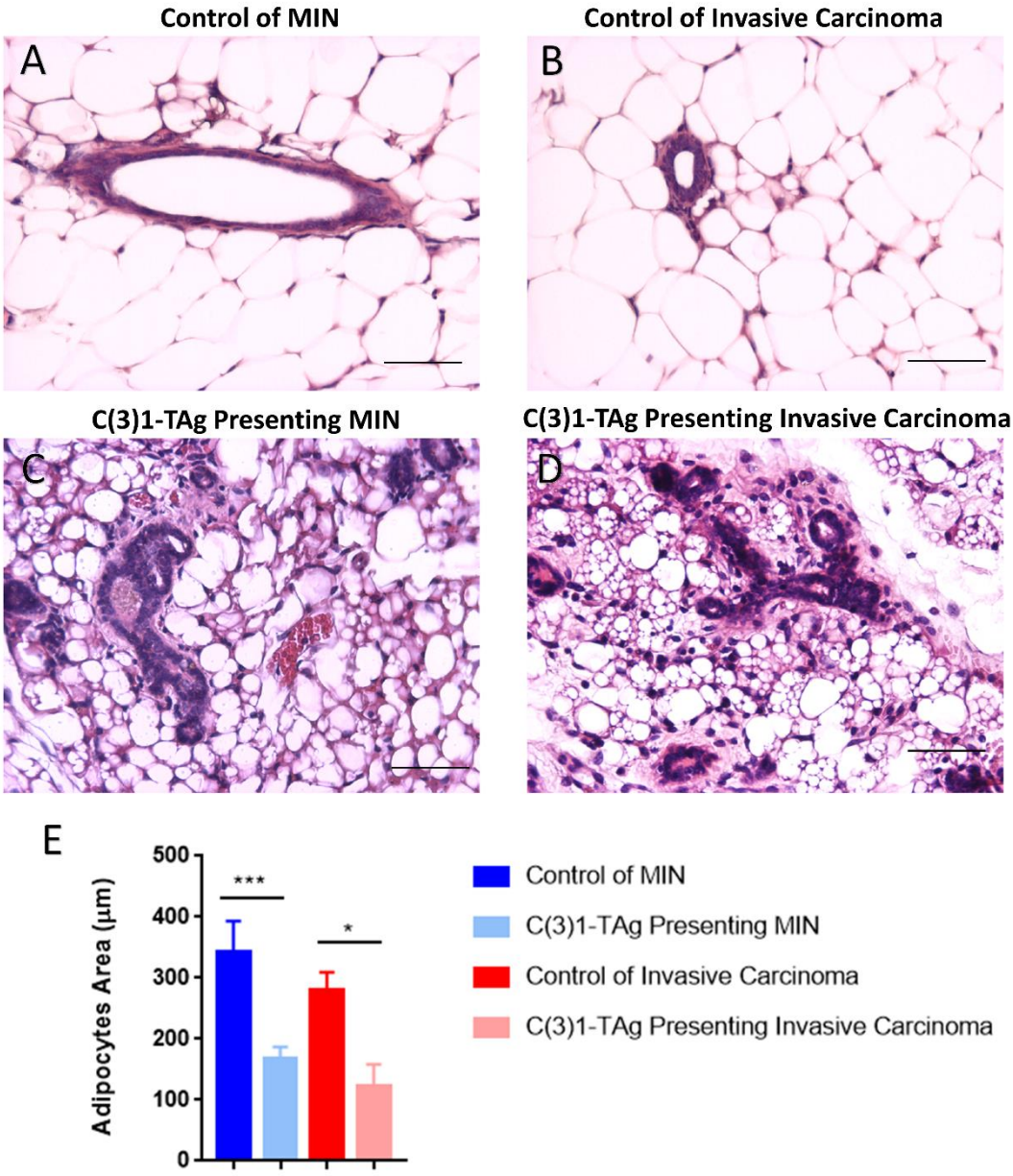

Figure 8. Monitoring of adipocytes area in model C(3)1-TAg, C57BL/6 strain. Representative photographs in HE stained slides of mammary tissue from (A) control animal of MIN (B) control animal of invasive carcinoma, both showing a mammary fat pad with wide adipocytes. In (C) note mammary tissue of C3(1)-TAg animal presenting MIN and (D) presenting invasive carcinoma, both showing a mammary fat pad with small adipocytes. (E) Quantification of the adipocytes area in mammary tissue of control group and C3(1)-TAg group with MIN (n=3) and invasive carcinoma (n=4), showing a

significant decrease in adipocyte area in relation to the control group. T-Test, *** p <0.001 and *p <0.05. Scale bar: 50µm.

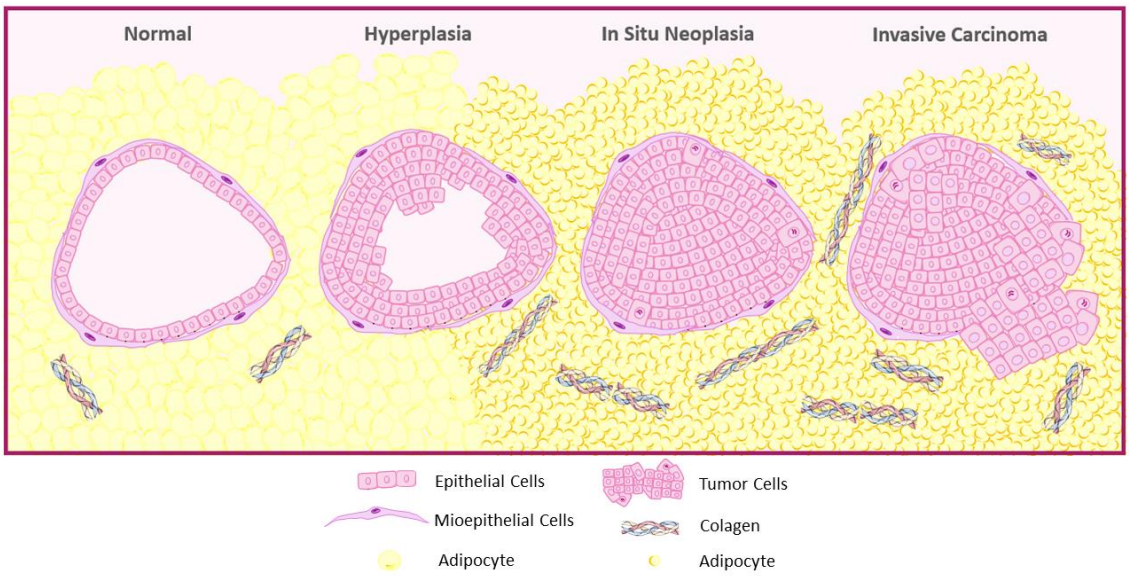

Figure 9. Graphical Abstract showing the development of the main stages present in the animal C(3)1-TAg, C57BL/6 strain. Normal mammary gland evolves to hyperplasia, follow by in situ neoplasia and invasive carcinoma. The model has classic characteristics of a mammary tumor, such as cellular changes, increased fibrosis and decreased adipocyte area.

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Identification of sensory nerves in the mammary tumor using the Nav1.8-Cre/TdTomato and C(3)1-TAg model

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Abstract

Breast cancer is the one that most affects women worldwide and is the leading cause of death in this population in several countries. Because of this, several studies on the tumor microenvironment are carried out to identify a possible therapeutic target. Research on cells such as fibroblasts, blood vessels and immune cells are already well discussed. However, the study on sensory innervation and breast cancer is still scarce. It is known that on the one hand sensory nerves can release important factors for cancer progression and on the other tumor cells can induced sensory neuronal growth. However, these studies on sensory innervation and breast cancer have only been carried out in *in vitro* models or *in vivo* xenograft models. For these facts, this study analyzed the sensory nerves in mammary tumor through a genetically engineered mouse model Nav1.8-Cre/TdTomato/C(3)1-TAg that develop endogenously mammary tumor and expresses a fluorescent endogenously only on sensory nerves. Interestingly, we identified that animals with mammary cancer have a larger area of sensory innervation and, in turn, these nerves are closer to blood vessels.

Keywords: Mammary tumor, sensory innervation, genetically engineered mouse model, breast cancer

Introduction

Each year there are about one million new cases of breast cancer in the world [1]. Due to genetic, hormonal, environmental and behavioral factors and due to the increase in studies on the disease, data on the incidence of breast cancer is increasing every day [2]. For example, in the past, it was believed that the tumor was a homogeneous mass composed of tumor cells, today it is known that the tumor has a complexity as high as other organs of the body [3]. Cancer has interactions between malignant and non-malignant cells, forming the tumor microenvironment (TME) [4]. Because of this communication, many studies show the importance of several cell types in the development of cancer [4, 5].

Several researches try to understand the tumoral microenvironment of the breast in order to identify new therapeutic targets [4, 6]. Analysis on the presence and influence of fibroblasts, adipocytes, endothelial cells and immune cells are well discussed in the scientific community [7-9]. However, knowledge of the role of the nervous system, especially of the sensory nerves, is still scarce.

It is known that sensory innervation influences the pathophysiology of processes such as neuropathy, arthritis and tissue damage [10-13]. Currently, some research shows that the nervous system is associated with the progression of cancer, such as breast, prostate, pancreas, colorectal and gastric [14-18]. Specially, some studies show that sensory nerves can release important factors for cancer progression, such as the release of substance P and the calcitonin gene-related peptide (CGRP). Both help in the migration of tumor cells and are important for vascular function [19-21]. Others have shown that breast tumor cells can induced sensory neuronal growth [22-24]. However, these studies on sensory innervation and breast cancer have only been carried out in *in vitro* models or *in vivo* xenograft models [19, 20, 25].

The emergence of genetically engineered mouse models (GEMMs) has modified cancer studies, since these models have numerous benefits for studying the disease. The GEMMs are developed in order to assess the whole evolution of the tumor from the initial to the final events, due to its slow tumor progression. Spontaneous tumor onset occurs within the correct microenvironment from a normal mammary cell that has a change in oncogene or tumor suppressor gene [26]. Therefore, it is necessary to carry out a study on sensory nerves in mammary cancer in GEMMs that allow an analysis of the most

reliable to those that occur in humans [27]. For these facts, the aim of this study is to analyze the sensory nerves in mammary tumor through a GEMM Nav1.8-Cre/TdTomato/C(3)1-TAg that develop endogenously mammary tumor and expresses a fluorescent endogenously only on sensory nerves.

Materials and methods

Ethical aspects

All procedures were performed under the guidelines and with the approval of the Animal Use Ethics Commission of the Federal University of Minas Gerais (CEUA/UFMG) (Protocol 172/2018).

Specimens

Nav1.8-Cre/TdTomato and C3(1)-TAg transgenic animals on the C57BL/6 strain were obtained from the Tissue Microenvironment Laboratory of the Federal University of Minas Gerais. The animals were kept under controlled temperature and light (12h/12h light/dark cycle). They fed on standard feed and water available *ad libitum*.

Genotyping for C3(1)-TAg

DNA extraction was performed using the phenol-chloroform method [21]. Then, a conventional polymerase chain reaction (PCR) was performed to amplify the gene of interest using the Applied Biosystems MiniAmp thermocycler (Fisher Scientific Term). The primers (Invitrogen) used were: Forward: 5 'CAGAGCAGAATTGTGGAGTGG-3' and Reverse: 5'-GGACAAACCACAACTAGAATGCAGTG -3'. The amplified product was 500bp and identified by 1% agarose gel electrophoresis.

Phenotyping for Nav1.8Cre/TdTomato

Phenotyping was performed by analyzing a small animal tissue fragment using a fluorescence microscope (EVOS FL Cell Im). Animals were considered positive for the Nav1.8-Cre/TdTomato when it had a phenotype represented by figure 1B.

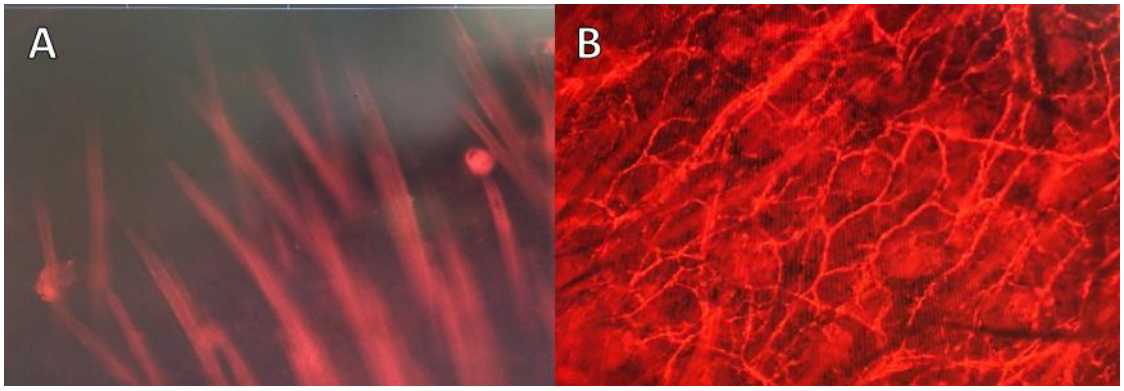

Figure 1. Representative image of the phenotyping of Nav1.8Cre/TdTomato animals. (A) Nav1.8Cre/TdTomato negative animal showing only autoflowering hair. (B) Nav1.8Cre/TdTomato positive animal showing the presence of endogenously labeled sensory fibers.

Experimental Design

Nav1.8-Cre/TdTomato males crossed with C(3)1-TAg females and littermates were used in the experiments. The females positive for C(3)1-TAg and Nav1.8-Cre/TdTomato were used as experimental group and the females Nav1.8-Cre/TdTomato positive and C(3)1-TAg negative were used as control group. The animals were euthanized with 32-weeks for presenting characteristics of invasive carcinoma.

Tissue Processing

The animals were anesthetized intramuscularly with an injection of 10% ketamine (80mg/kg body weight) and 2.3% xylazine (12mg/kg body weight), euthanized by cervical dislocation and all mammary glands collected as described [22]. The tissues were fixed in 4% paraformaldehyde (Sigma-Aldrich), pH 7.4, for 24 hours. Tissues were then transferred to 30% sucrose solution (Sigma-Aldrich) for 48 hours and included in OCT (Tissue-Tek® O.C.T. Compound, Sakura® Finetek) and frozen in -80°C. Histological sections were performed on cryostat (Leica CM1850) with a thickness of 12 µm.

Macroscopic Analysis

Before the mammary glands were fixed, the tissue was analyzed at the stereoscopic microscope SMZ745T (Nikon) to capture fluorescence images. All the mammary glands (cranial, thoracic and inguinal) on the right and left side were analyzed. ImageJ software was used to quantify the innervation parameters.

Microscopic Analysis

The Apotome.2 microscope (ZEISS) was used to capture fluorescence images and ImageJ software was used to quantify the innervation and blood vessel parameters. These parameters were measured at 20x magnification in ten fields of cranial, thoracic and inguinal mammary tissue of each animal as previously described [23, 24]. The microscopic data shown in this work was obtained using the microscopes and equipment in the Centro de Aquisição e Processamento de Imagens (CAPI- ICB/UFMG).

Immunofluorescence

The tissue sections were washed with phosphate buffered saline (PBS) then permeabilized with 0.5% Tritox x-100 for 5 minutes and blocked with 1% BSA for 45 minutes. Tissue sections were incubated overnight at 4°C with primary antibody against CD31 (Ab28364, Abcam, 1:50) and secondary antibody Alexa fluor 488 (Ab150077, Abcam, 1:100) was incubated for sixty minutes. The nuclei were stained with DAPI (ThermoFisher).

Statistical analysis

The results were analyzed by statistical tests that were applied over each case with the GraphPad Prism 7 software (Prism Software, Irvine, CA, USA). Data normality was accessed by the Shapiro-Wilk test and parametric data was performed using the T test and non-parametric data the Mann Whitney test. In all cases, we consider $p < 0.05$ as statistically significant and exhibit the mean ± standard error.

Results

Identification of Sensory Innervation in Macroscopic Analysis

Analyzing the mammary tissues in the stereoscopic microscope, we identified that the proportion of innervation area of mammary tissue is significantly higher in C(3)1-TAg animals compared to control animals (Fig. 2A-C). Interestingly, neither C(3)1-TAg nor control animals demonstrated a significant difference between left and right mammary fat pad, as well as inguinal, thoracic and cranial regions (Fig. 2D). Analyzing the tumors, we identified that sensory innervations are located predominantly on the periphery of the tumor (Fig. 3).

Identification of Sensory Innervation in Microscopic Analysis

Analyzing the mammary tissue microscopically, we identified that the Nav1.8-Cre/TdTomato/C3(1)-TAg have a higher area of innervation compared to control animals (Fig. 4A-C). However, there is no difference in thickness of the nerve neither the distance between innervation and mammary gland on Nav1.8-Cre/TdTomato and Nav1.8-Cre/TdTomato/C3(1)-TAg (Fig 4D and 4E).

Correlation Between Blood Vessel And Sensory Innervation

We performed an immunofluorescence for CD31 and identified that there is no difference in microvessel density (MVD) between Nav1.8-Cre/TdTomato and Nav1.8-Cre/TdTomato/C3(1)-TAg groups (Fig. 5A-C). However, we identified that blood vessel diameter in Nav1.8-Cre/TdTomato/C3(1)-TAg animals is significantly smaller and closer to innervation than Nav1.8-Cre/TdTomato animals (Fig. 5D and 5E).

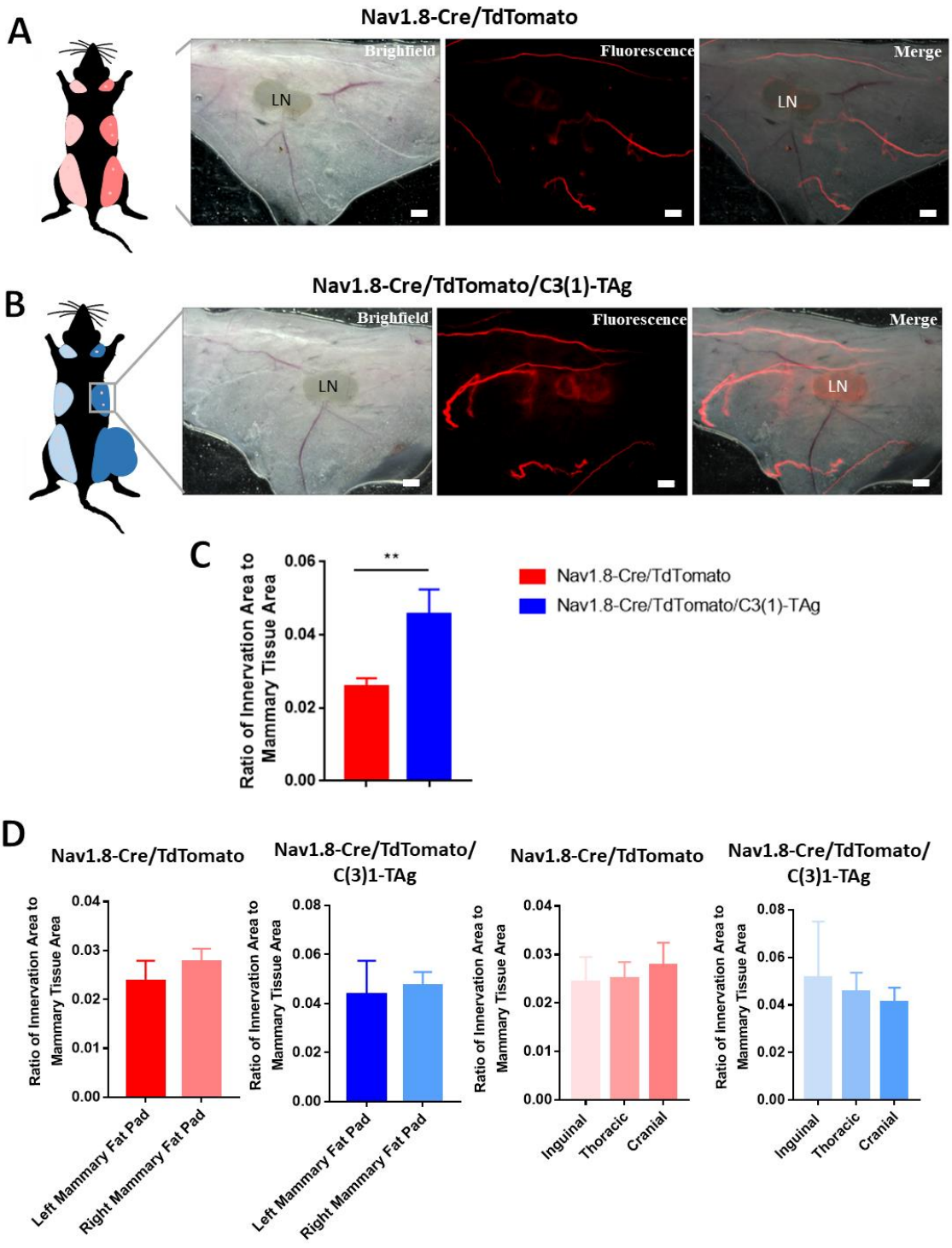

Figure 2. Sensory innervation in mammary tissue in macroscopy analyses. Macroscopic image of mammary gland of the animal Nav1.8-Cre/TdTomato showing sensory innervation in red (A). Macroscopic image of mammary gland of the animal Nav1.8-Cre/TdTomato/C3(1)-TAg showing sensory innervation in red (B). The ratio of innervation area to mammary tissue area in macroscopy analyses show higher sensory nerves density on Nav1.8-Cre/TdTomato/C3(1)-TAg mammary fat pad (C). Macroscopic

analysis of innervation of right and left mammary fat pad and inguinal, thoracic and cranial regions showing that there is no significant difference in these regions in both Nav1.8-Cre/TdTomato group and on Nav1.8-Cre/TdTomato/C3(1)-TAg (D). T test and One-way ANOVA. ** p< 0.01 (n=3). Scale bar: 1000µm. LN: Lymph Node.

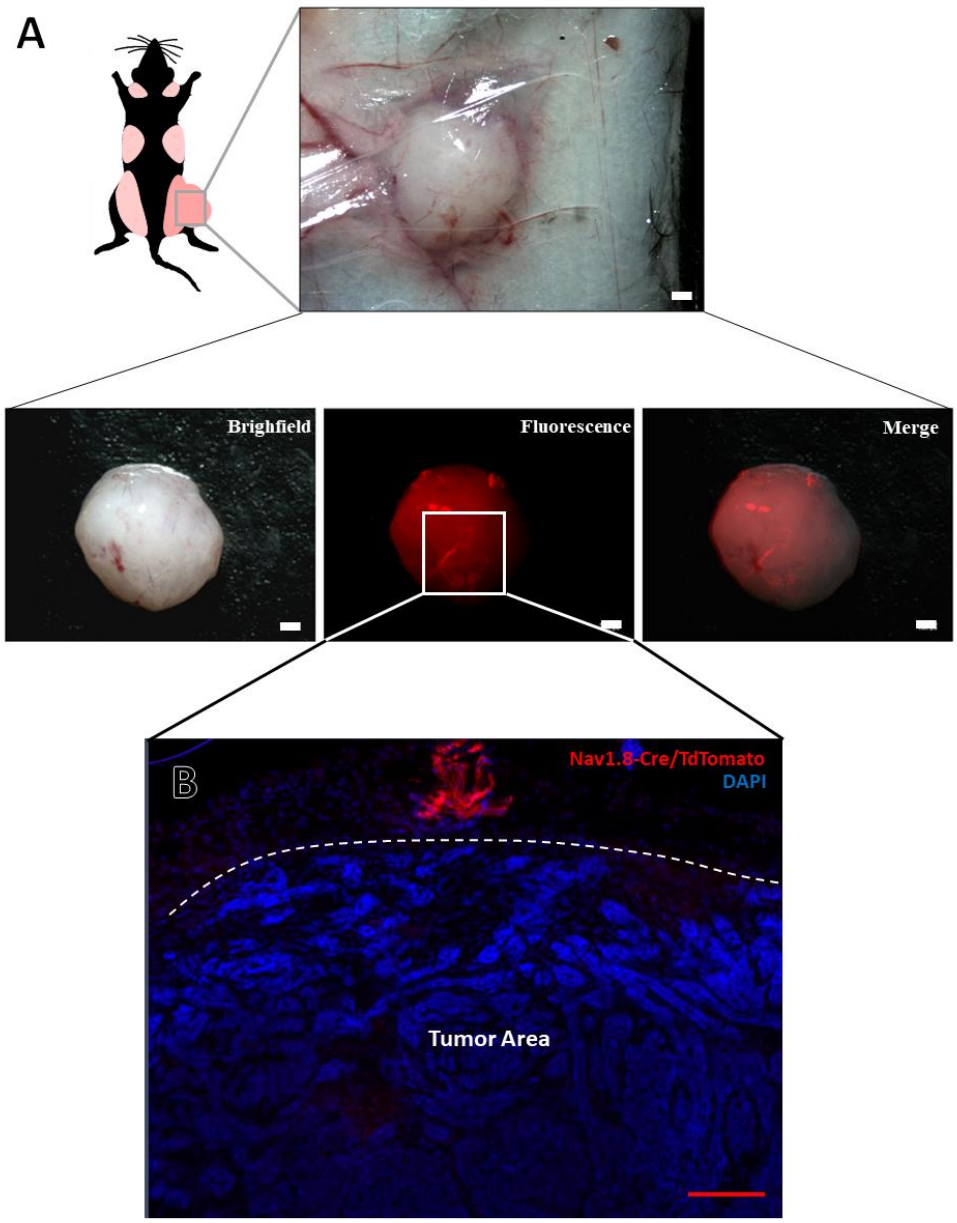

Figure 3. Identification of sensory fibers in mammary tumors. Macroscopic images of the mammary tumor of Nav1.8-Cre/TdTomato/C3(1)-TAg (A). Microscopic image of the same mammary tumor of Nav1.8-Cre/TdTomato/C3(1)-TAg (B). Note that sensory nerves in mammary tumors are located predominantly on the periphery of the tumor. White Scale Bar: 1000µm. Red Scale Bar: 100µm

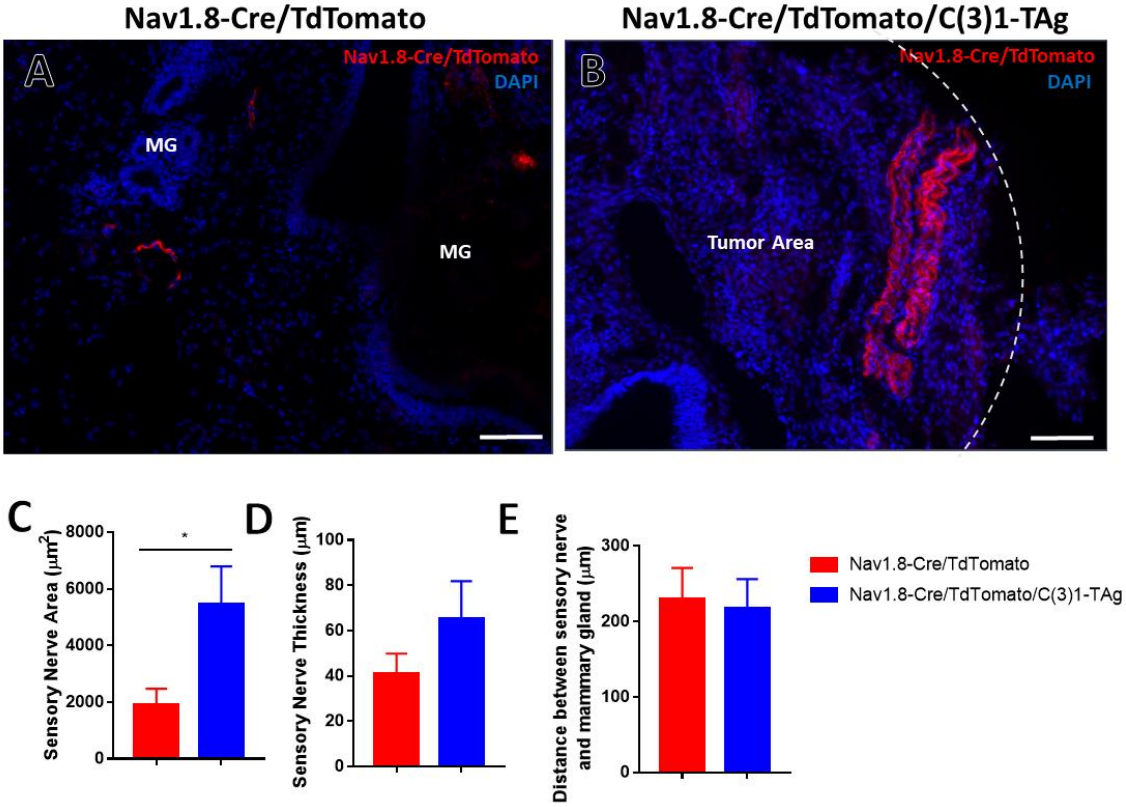

Figure 4. Sensory innervation in mammary tissue in microscopy analyses. Innervation on Nav1.8-Cre/TdTomato mammary tissue (A) and on Nav1.8-Cre/TdTomato/C3(1)-TAg mammary tissue (B). The innervation area is significant higher on Nav1.8-Cre/TdTomato/C3(1)-TAg mammary gland compared to the Nav1.8-Cre/TdTomato mammary gland (C). However, there is no significant difference in thickness of the sensory nerve and distance between the sensory nerve and mammary gland on Nav1.8-Cre/TdTomato and Nav1.8-Cre/TdTomato/C3(1)-Tag (D and E). T test. * $p < 0.05$ (n=3). Scale bar: 100μm. MG: Mammary Gland.

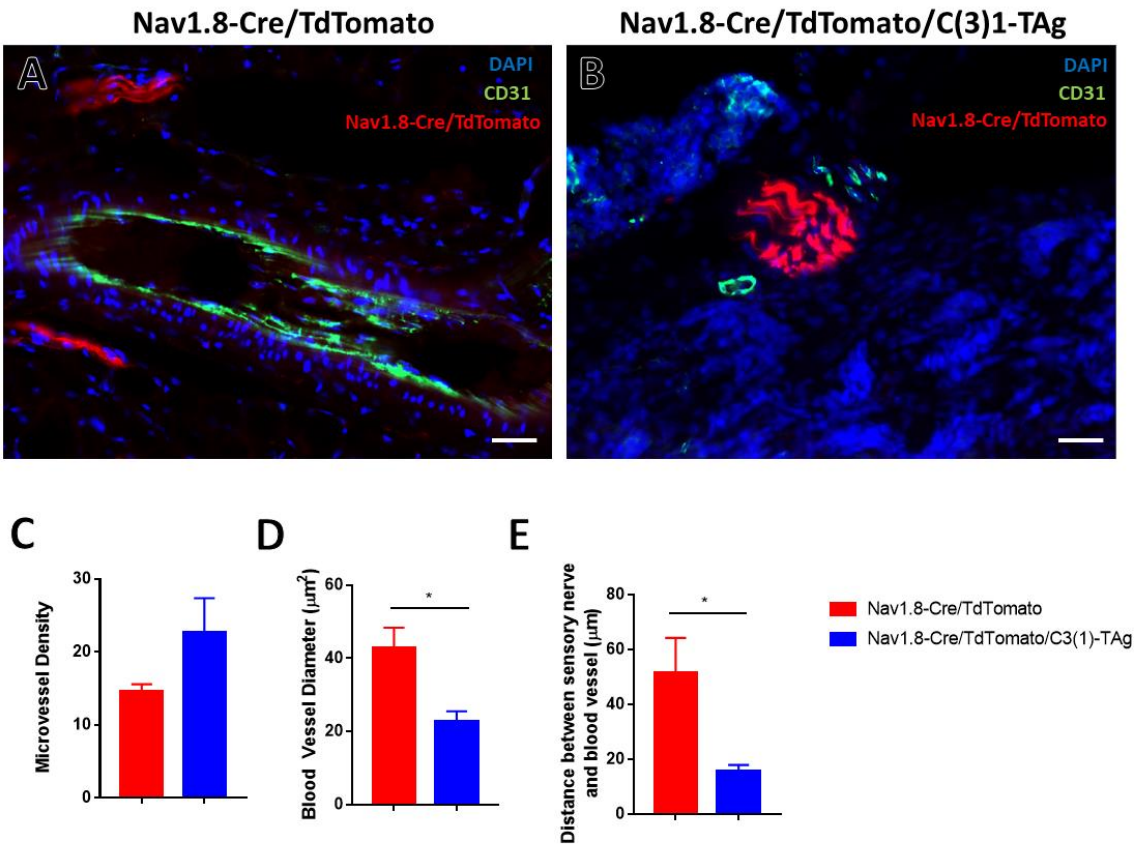

Figure 5. Blood vessel and sensory nerves. Blood vessel (green) and innervation (red) on Nav1.8-Cre/TdTomato mammary tissue (A) and on Nav1.8-Cre/TdTomato/C3(1)-TAg mammary tissue (B). There was no significant difference in MVD between the two groups (C). However, the blood vessel diameter is significant smaller and closer to sensory nerve on Nav1.8-Cre/TdTomato/C3(1)-Tag group compared to the Nav1.8-Cre/TdTomato group (D and E).

Discussion

Several researches have been analyzing the association between nervous system and cancer progression, especially about autonomic nerves [13-16, 26]. However, the role of sensory innervation in the tumor still needs to be clarified. Some studies, using *in vitro* methodologies and animal models with injected tumor cells, analyzed the presence of sensory innervations in mammary cancer [18, 19, 23]. Yet, our work is the first to use elegant techniques to prove these results. Using a transgenic model of the Cre-LoxP system, to identify sensory innervation, and the C3(1)-TAg model, to analyze breast cancer, we identified that animals with mammary cancer have a significantly larger area of innervation than animals without cancer. This identification of a greater number of sensory nerves in the tumor is the first step to discuss whether it is the tumor cells that recruits the sensory nerves in the tumor microenvironment or if it is the nerves that influence the outcome of breast cancer.

Some works showed that mammary tumor cells stimulates sensory neuron growth. Pundavela *et al.* demonstrated that breast tumor cells secrete nerve growth factor (NGF) favoring the infiltration of nerves in the tumor [18]. While Austin *et al.* discusses the increase in vascular endothelial growth factor-A (VEGF-A) in the breast tumor that may be related to the growth of sensory innervation [19]. Others showed that sensory nerves can release several substances that influence the progression of cancer. For example, Drell and colleagues showed that substance P has a pro-migratory effect on tumor cells [27]. While Nagakawa *et al.* demonstrated that CGRP can increase the invasive and migratory tumor cells [20].

Our study demonstrated that sensory nerves are present in the most peritumoral region and closest to the blood vessels in animals with mammary cancer. This consist with other studies that also state that sensory nerves are located mainly in the tumor stroma, close to adipocytes and blood vessels [18, 23]. Interestingly, Mukouyama *et al.* showed, in embryonic mouse limb skin, that arteries are correlated with sensory nerves. Including that the absence of sensory nerves generated an altered development of vasculature, suggesting that the innervation can determine the pattern of blood vessel branching [28].

Furthermore, our study also identified the presence of small diameter vessels in tumor. It is known that the development of blood vessels in the tumor occurs from established vessels located close to the tumor region that are infiltrating into the MTE [29]. This

development generates vessels of smaller diameter, tortuous and immature. This, in turn, favors the formation of a hypoxic environment that helps the proliferation of malignant cells [30, 31]. Studies with melanoma and multiple myeloma also identify the prevalence of smaller vessels in tumor tissue [30, 32].

In conclusion, these results demonstrate that sensory innervation may play an important role in the development of the breast tumor and that the animal Nav1.8-Cre/TdTomato/C(3)1-TAg can be a good model for these studies. We suggest that future research be carried out in the other stages of mammary cancer and that investigations about the mechanistic process between sensory innervation and the tumor be carried out using this model.

Conflict of interest

The authors declare no conflict of interest.

Fundings

CAPES, Serrapilheira (Serra-1708-15285) and CNPq (CNPq-28/2018).

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7. CONSIDERAÇÕES FINAIS

A partir dos resultados obtidos nesse estudo, podemos concluir que:

- O modelo C3(1)-TAg na linhagem C57BL/6 apresenta uma hiperplasia das glândulas mamárias com doze semanas, progredindo para neoplasia intraepitelial mamária com dezesseis semanas e o desenvolvimento de adenocarcinoma a partir de vinte e quatro semanas.
- O modelo C3(1)-TAg na linhagem C57BL/6 apresenta um tumor com característica sólido basalóide do carcinoma adenóide cístico e do tipo B-luminal.
- O tumor do modelo C3(1)-TAg na linhagem C57BL/6 apresenta um aumento da área de fibrose e uma redução na área de adipócitos.
- É possível realizar análises sobre inervação sensorial e câncer de mama no animal Nav1.8-Cre/TdTomato/C3(1)-TAg.
- Animais com câncer de mama têm maior densidade de inervação sensorial nas mamas do que os animais sem câncer. Sendo que esses nervos estão localizados mais na região periférica do tumor e próximos de vasos sanguíneos.

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9. ANEXOS

UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Prezado(a):

Esta é uma mensagem automática do sistema Solicite CEUA que indica mudança na situação de uma solicitação.

Protocolo CEUA: 204/2017
Título do projeto: Estagiamento de tumores de camundongos transgênicos cTag
Finalidade: Pesquisa
Pesquisador responsável: Alexander Birbrair
Unidade: Instituto de Ciencias Biologicas
Departamento: Departamento de Patologia

Situação atual: Decisão Final - Aprovado

Aprovado na reunião do dia 18/09/2017. Validade: 18/09/2017 à 17/09/2022
Belo Horizonte, 18/09/2017.

Atenciosamente,

Sistema Solicite CEUA UFMG
https://aplicativos.ufmg.br/solicite_ceua/

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UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Prezado(a):

Esta é uma mensagem automática do sistema Solicite CEUA que indica mudança na situação de uma solicitação.

Protocolo CEUA: 172/2018
Título do projeto: Identificação dos nervos sensoriais no microambiente tumoral da mama
Finalidade: Pesquisa
Pesquisador responsável: Alexander Birbrair
Unidade: Instituto de Ciencias Biologicas
Departamento: Departamento de Patologia

Situação atual: Decisão Final - Aprovado

Aprovado na reunião do dia 18/06/2018. Validade: 18/06/2018 à 17/06/2023

Belo Horizonte, 18/06/2018.

Atenciosamente,

Sistema Solicite CEUA UFMG
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