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"MODELO ANIMAL GENETICAMENTE MODIFICADO DE CARCINOMA DE CÉLULAS ESCAMOSAS DE VULVA COM DELEÇÃO DO GENE ARID1A E ATIVAÇÃO DO ONCOGENE KRAS"

Programa de Pós-graduação da Saúde da Mulher

Área de concentração: Patologia Ginecológica e Reprodução

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"Optimism is essential to achievement and

it is also the foundation of courage and true progress."

Nicholas M. Butler

Dedicatória

À minha família querida, vocês são os meus "sunshines"! É por vocês que tudo vale a pena!

Ao meu Praça boy, Thomás, e à minha boneca sapeca, Maria Fernanda, meus maiores aprendizados, meus verdadeiros desafios e minhas diárias lições de amor.

Ao meu amado companheiro, Edu, por quem "eu largo tudo: dinheiro, carreira e canudo". Você é brilhante e nós somos para sempre! Muito obrigada por nossa família.

Ao meu amado irmão Lucas, sem você minha vida não seria completa. Obrigada por ser tão "team leader" e por nunca ter saído do meu lado.

À minha mãe querida, por ser quem ela é, pela sua energia e por sempre me mostrar o lado positivo da vida.

Ao meu pai, por me brindar com sua religiosidade e carinho.

Aos meus avós, meus grandes exemplos.

Agradecimentos

Agradeço aos meus queridos orientadores, Prof. Agnaldo Lopes e Profª Shannon Hawkins, pela oportunidade, pelos ensinamentos, pela inestimável confiança e, sobretudo, pelo exemplo de profissionais, seja na assistência, na docência ou na pesquisa. Vocês são incríveis. Foi um privilégio ter vocês como orientadores. Muito obrigada!

Obrigada aos meus queridos colegas de laboratório, Xiyin Wang e Jillian Hufgard. Foi essencial e fundamental poder contar com vocês. Obrigada pela paciência chinesa, pela retidão americana e por toda a "ajuda internacional". Somente foi possível porque vocês me acolheram na bancada e me permitiram dividi-la com vocês. Sorte a minha por vocês estarem sempre lá quando eu precisei;

Obrigada a Indiana University por me receber tão bem e se tornar um capítulo inesquecível e tão importante na minha vida pessoal e acadêmica.

Obrigada à UFMG, minha casa.

Obrigada a todos meus queridos amigos, por estarem sempre presentes, de longe, de perto, e para sempre. Em especial àqueles que torcem por mim, desmedidamente, compartilham de todas as alegrias, me ajudam nas eventuais dificuldades e me encorajam no meu caminho: Schi e Henderson, minhas também referências profissionais, Loyolinas, Mafê, Juju, Cris, Nikoletinha, Lu Loures, ter vocês em minha vida é impagável;

Obrigada Lê Trivellato, minha querida amiga de anos, pelo generoso auxílio anátomo-patológico no desafio diagnóstico dos tumores;

Obrigada ao Hospital Mater Dei, tão bem representado nas pessoas do Carlos Henrique Mascarenhas e Cláudia Laranjeiras, pela compreensão da minha ausência e pela oportunidade de seguir como membro da equipe de Ginecologia e Obstetrícia desse grande hospital;

Obrigada a Pós – graduação da Saúde da Mulher, Prof. Selmo Geber, Prof. Fernando Reis e secretária Caroline Alves, pela assistência primordial;

Muito obrigada a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior **(**CAPES), que me permitiu, através do Programa de Doutorado Sanduíche no Exterior, a viabilização de um sonho;

Meu muito obrigada aos mestres do DGO/UFMG e da Pós – graduação da saúde da mulher, no maior âmbito da palavra obrigada.

Meus agradecimentos aos membros da banca da qualificação e aos membros da banca examinadora da defesa. Vocês têm minha admiração e todo o meu respeito. Muito obrigada pela disponibilidade e pelas valiosas contribuições.

A todos que, de alguma forma, me ajudaram a realizar e a concretizar esse trabalho. Esse é o resultado do auxílio de todos vocês. Nomear é limitante e não faz jus a todos inestimáveis apoios e queridos afetos que tenho em minha vida. Muito obrigada!

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LISTA DE SIGLAS

ACCO: Adenocarcinoma de Células Claras do Ovário

ACOG: The American College of Obstetricians and Gynecologists

AEO: Adenocarcinoma Endometrióide do Ovário

AEU: Adenocarcinoma Endometrióide Uterino

AKT: Proteína Quinase B

Amhr2: Receptor do Hormônio Anti-mulleriano tipo 2

Arid1a ou ARID1A : AT-Rich Interactive Domain-Containing Protein 1A

CCE: Carcinoma de Células Escamosas

FIGO: Federação Internacional de Ginecologia e Obstetrícia

HPV: Papilomavírus Humano

INCA: Instituto Nacional do Câncer

Kras ou KRAS: Kirsten Rat Sarcoma

LS: Líquen Escleroso

MAGM: Modelo Animal Geneticamente Modificado

MAPK: Mitogen-Activated Protein Kinase

NIC: Neoplasia intraepitelial cervical

dNIV: Neoplasia Intraepitelial Vulvar diferenciada

uNIV: Neoplasia Intraepitelial Vulvar usual

Pgr: Receptor de Progesterona

PI3: Fosfoinositida 3-Quinase

SWI/SNF: SWItch/Sucrose Non-Fermentable

VSCC: Vulvar squamous cell carcinoma

WHO: World Health Organization

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Resumo

O câncer ginecológico é um permanente problema de saúde pública. Em 2017, mais de 100,000 mulheres serão diagnosticadas com câncer ginecológico, e mais de 31,000 morrerão nos Estados Unidos. Dentre os tumores ginecológicos, o câncer de vulva representa cerca de 5% do total dos tumores diagnosticados. O tratamento cirúrgico curativo padrão para tumores invasivos localmente avançados tem morbidade considerável. Por ser um tumor infrequente, estudos randomizados de abordagens terapêuticas para os tumores de vulva são incomuns. Assim, modelos animais são ferramentas necessárias e interessantes para melhor compreender a fisiopatologia da doença e desenvolver melhores opções terapêuticas. Para estudar os cânceres do trato reprodutivo feminino, em particular o câncer endometrial, nós criamos um modelo de camundongo geneticamente modificado usando o receptor de progesterona como promotor (Pgr^{Cre}) da ação da Cre recombinase. Com a deleção do *Arid1a*, um gene supressor tumoral que é considerado uma mutação "driver" no câncer endometrial, os camundongos fêmeas se tornaram inférteis, mas não desenvolveram câncer endometrial. Foi adicionada a mutação do oncogene gene *Kras* ($Kras^{G12D}$). Surpreendentemente, os camundongos fêmeas ($Pgr^{Cre}Arid1a^{f/f}Kras^{LSL\text{-}G12D}$) desenvolveram grandes tumores de vulva, com penetrância de 100% na 8ª semana de vida. Foram realizados estudos histológicos e imunohostoquímicos de fragmentos da vulva, vagina, colo uterino, útero e ovários dos camundongos, com o objetivo de caracterizar as vias de carcinogênese do carcinoma de células escamosas da vulva. Exame histológico uterino e ovariano revelou estruturas normais, sem malignidade ou tumores benignos. Esse modelo de camundongo, geneticamente modificado, recapitula os tumores vulvares humanos e pode promover um melhor entendimento dos mecanismos moleculares envolvidos na transformação genética do câncer de vulva e pode ser usado para desenvolver novos alvos – terapêuticos personalizados.

Abstract

Gynecological cancer remains a significant public health issue. In 2017, over 100,000 women will be diagnosed with a gynecologic cancer and more than 31,000 will die in the United States. Primary vulvar cancer accounts for less than 5% of gynecological cancers diagnosed, but the curative surgical treatment for deeply invasive tumors has considerable morbidity. Since vulvar cancer is an infrequent tumor, randomized trials of therapeutic approaches in women are uncommon. Thus, model systems are needed to understand the pathophysiology of the disease to develop improved therapies. To study female reproductive tract cancers, in particular endometrial cancer, we created a genetically engineered mouse model using the progesterone receptor-Cre (Pgr^{Cre}), which conditionally deletes in the uterus. When we deleted *Arid1a*, the proposed tumor suppressor that is thought to be a driver mutation in endometrial cancer, the female mice were infertile but did not develop endometrial cancer. Thus, we added oncogenic *Kras* ($Kras^{G12D}$). Surprisingly, these female mice ($Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$) developed large vulvar tumors, with 100% penetrance by 8 weeks of life. Sections of fixed tissue from vulva, vagina, cervix, uterus and ovaries of the mice were submitted to histological and immunohistochemistry studies to evaluate the $Kras^{G12D}$ mutation, *Arid1a* deletion and other biomarkers to characterize the pathways of oncogenesis in vulvar squamous cell carcinoma. Histological examination of uterine and ovarian tissues revealed normal structures without malignancy or benign tumors. This mouse model may allow a better understanding of the molecular mechanisms underlying the genetic transformation of vulvar cancer and can be used to develop targets for therapy.

INTRODUÇÃO

1. Introdução

1.1 Câncer de Vulva

1.1.1 Epidemiologia

O Câncer ginecológico configura uma importante questão pública de saúde. Nos Estados Unidos (EUA), estima – se que, em 2017, mais de 100.000 mulheres serão diagnosticadas com tumores do trato reprodutivo feminino e 31.000 irão a óbito devido a essas neoplasias[1]. Dentre os tumores ginecológicos, o câncer de vulva representa cerca de 5% do total dos tumores diagnosticados[2] e tem o carcinoma de células escamosas (CCE) como o tipo histológico mais comum, representando mais de 80% dos casos[3]. Para 2017, são esperados 6.020 novos casos de tumores de vulva nos EUA[1,4]. Apesar de ser considerado um tumor de mulheres idosas, com idade média de incidência na sétima para oitava década de vida[5], nas duas últimas décadas, têm – se observado um aumento da incidência das lesões precursoras dos tumores vulvares em pacientes mais jovens. O aumento da incidência dessas lesões pode ser um dado real ou apenas refletir uma melhora no reconhecimento e diagnóstico das mesmas[6,7]. No Brasil, não existem dados sobre a incidência do câncer de vulva pelo Instituto Nacional do Câncer (INCA)[8], mas parece existir uma tendência ao aumento da taxa de mortalidade por esses tumores no país (Gráfico 1).

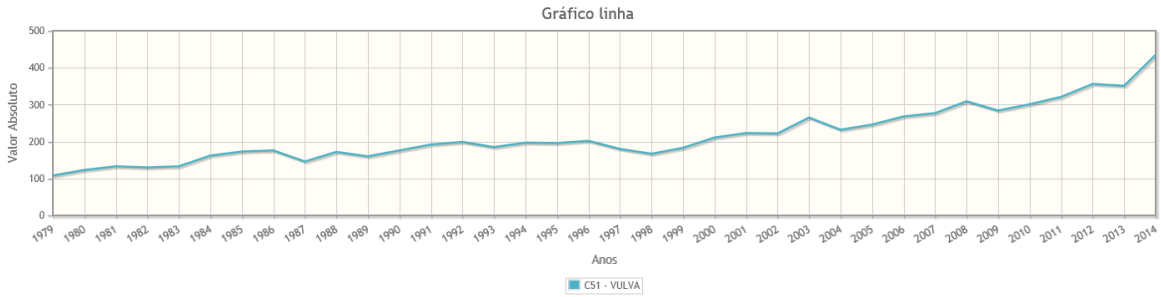

Gráfico 1: Taxa de mortalidade por câncer de vulva no Brasil.

Fontes: MS/SVS/DASIS/CGIAE/Sistema de Informação sobre Mortalidade – SIM MP/Fundação Instituto Brasileiro de Geografia e Estatística – IBGE MS/INCA/Conprev/Divisão de Vigilância

1.1.2 Etiopatogênese do câncer de vulva

O câncer de vulva pode ser dividido em 2 tipos, com etiopatogênese distinta. O primeiro tipo acontece associado à infecção pelo Papilomavírus Humano que leva a neoplasia intraepitelial vulvar usual (uNIV) e representa 95% dos casos dos CCE de vulva. O segundo tipo, HPV – independente, representa 5% dos tumores e leva a neoplasia intraepitelial vulvar diferenciada (dNIV)[9,10]. Esses dois tipos de tumores possuem epidemiologia diferente, características histológicas e moleculares distintas e também diferentes prognósticos[2,10].

O uNIV e o CCE de vulva associado a infecção pelo HPV, possuem as mesmas características epidemiológicas que os demais tumores ginecológicos associados à infecção viral, como os CCE de colo uterino e as neoplasias intraepiteliais cervicais (NIC): possuem maior incidência em mulheres com média de idade mais jovem (3ª a 5ª década de vida) e têm o tabagismo, a presença de múltiplos parceiros sexuais e condições imunossupressoras como importantes fatores de risco[10]. O HPV 16 é o tipo mais comumente encontrado na uNIV e no CCE de vulva associado ao HPV, entretanto, outros tipos como o 18, 31, 33 e 45 também são observados em lesões vulvares pré-invasoras e invasoras[2]. Nas lesões dependentes da infecção pelo HPV, a patogênese envolve a ação das oncoproteínas E6 e E7 que causam a inativação de genes supressores tumorais. Enquanto a oncoproteína E6 leva a degradação da proteína codificada pelo gene p53 e estimula a atividade da telomerase, a oncoproteína E7 se liga a proteína codificada pelo gene do retinoblastoma (Rb) que leva à ativação de fatores de transcrição celular. Associado a outras alterações moleculares, com ativação de oncogenes e inativação de genes supressores tumorais, essas proteínas, promovem a perda do controle do ciclo celular e dão início à carcinogênese vulvar[11,12].

O dVIN e o CCE de vulva HPV independente relacionam – se a presença de doenças dermatológicas vulvares crônicas, como o líquen escleroso, em pacientes idosas (6ª a 8ª década de vida)[2,5]. Entretanto, os mecanismos envolvidos na progressão do líquen

escleroso (LS) e demais dermatoses inflamatórias para o CCE invasor não são claras e precisam serem melhor determinadas. Apesar da presença dessas lesões serem frequentemente encontradas em concomitância, em amostras de biópsias vulvares[2], e alguns estudos de coorte terem apontado a presença de líquen escleroso como um fator de alto risco para desenvolvimento de CCE de vulva, a maioria das mulheres com diagnóstico dessas lesões, não evoluem para a doença invasora[13,14]. Embora um estudo prospectivo anterior tenha estimado que o risco de uma paciente com LS desenvolver CCE de vulva era de 300 vezes maior que de pacientes que não apresentavam essas lesões[15], a estimativa do risco não foi baseada em avaliações comparativas entre lesões tratadas, não tratadas ou não diagnosticadas e nem baseadas em estimativas do risco considerando o tempo da presença das lesões[14].

1.1.3 Características histológicas das lesões vulvares

Por ser uma lesão associada com a infecção pela HPV, a uNIV possui características histológicas semelhantes as outras lesões secundárias a infecção viral e são mais facilmente identificadas[2]. Observa – se espessamento da camada epitelial, frequentemente associada a hiperceratose e/ou paraceratose. Ocorre a perda da maturação celular, aumento da relação núcleo – citoplasma. Há presença de hipercromasia e pleomorfismo celular e presença de figuras de mitoses em toda a extensão da camada epitelial. Células apoptóticas, com núcleos picnóticos e citoplasma eosinofílico também são achados comuns[16]. A uVIN foi subdivididaa em tipos condilomatosa e basaloide, embora muitos casos apresentem morfologias misturadas[17].

O diagnóstico histológico da dNIV e lesões invasoras que evoluem dela não é fácil devido às sutis alterações histológicas. Essa definição de lesão invasora pode ser um desafio e possui uma baixa reprodutibilidade entre os patologistas[18,19]. Por essa dificuldade e pelas frequentes incongruências encontradas no diagnóstico dessas

lesões vulvares[20], um estudo avaliou as características histológicas que seriam mais úteis na definição de malignidade. Foram definidos 5 melhores critérios histológicos diagnósticos: (1) presença de figuras de mitose atípica na camada basal; (2) atipia celular basal; (3) presença de disceratose; (4) presença de nucléolo proeminente e (5) espessamento epitelial e alongamento das cristas epiteliais descendentes. Essas cinco características mostraram aumentar a concordância entre a avaliação dos patologistas e auxiliar no diagnóstico dessas lesões vulvares[18,19].

1.1.4 Avaliação imunohistoquímica das lesões vulvares

Os estudos imunohistoquímicos ajudam na melhor caracterização tumoral e são ferramentas interessantes no estudo das vias envolvidas na carcinogênese[19].

1.1.4.1 Coloração imunohistoquímica para p16

A coloração imunohistoquímica positiva para a proteína p16 está associada a presença de infeccção pelo HPV em mais de 90% dos casos[21,22]. O padrão de coloração, quando presente, deve ser difuso, intenso e contínuo (podendo ser nuclear e citoplasmático) e deve envolver a camada basal e pelo menos um terço da espessura epitelial total[23]. Por esse motivo, observa – se um resultado positivo para imunohistoquímica das lesões vulvares do tipo usual (uVIN).

1.1.4.2 Coloração imunohistoquímica para p53

Apesar da coloração imunohistoquímica positiva para o p53 ter uma boa associação com os casos de dVIN, a imunomarcação para p53 possui limitações[3,24,25]. Existem dois padrões de resultados imunohistoquimicos que dependerão do tipo de mutação do p53. A imunomarcação aberrante com coloração intensa e difusa pode acontecer devido as mutações do tipo missense e a coloração completamente negativa (padrão nulo) pode acontecer secundária as mutações do tipo nonsense[26].

1.1.4.3 Coloração imunohistoquímica para o Ki-67

A imunomarcação para o Ki-67, um marcador de proliferação celular, pode ser um útil

para distinguir dVIN de uVIN. Enquanto e dVIN mostram coloração positiva para Ki-67 nas camadas basal e suprabasilar, a coloração positiva para Ki-67 nas uVIN pode ser observada em toda a espessura do epitélio vulvar[3].

1.1.5 Manejo terapêutico

Apesar de etiopatogênese e apresentações clínicas diferentes, os dois tipos de CCE de vulva não diferem quanto ao estadiamento tumoral (Anexo I) e, até o momento, ao tratamento[27]. Será o estadiamento do tumor que determinará as opções terapêuticas e definirá o prognóstico da doença[28-30]. Por esse motivo, é de extrema importância um correto reconhecimento da lesão para que o tratamento adequado seja realizado. O tratamento de escolha das lesões vulvares é a abordagem cirúrgica[31]. O procedimento cirúrgico curativo padrão, a vulvectomia, para tumores localmente agressivos, possui limitações devido à proximidade da vulva a órgãos e estruturas nobres[32,33]. Para as doenças vulvares recorrentes, especialmente as recorrências linfonodais, esse manejo é ainda mais difícil, as opções terapêuticas são mais restritas e o prognóstico é ainda pior[27,28,34].

1.2 Modelos animais geneticamente modificados

O desenvolvimento de modelos animais geneticamente modificados (MAGM), que reproduzem as alterações moleculares dos tumores humanos, permitiu grandes avanços nas pesquisas oncológicas, principalmente no que tange às opções de tratamento direcionadas à alvos terapêuticos tumorais[35,36]. Esses modelos pré-clínicos, revolucionaram a compreensão dos mecanismos genéticos envolvidos na carcinogênese. Os MAGM permitem a avaliação dos genes envolvidos no início e na

progressão dos tumores e, por conseguinte, a descoberta de potenciais biomarcadores tumorais, com valores diagnóstico e prognóstico[37].

Os modelos condicionais de camundongos transgênicos, que utilizam o sistema Cre – Lox para inserir oncogenes e inativar os genes supressores tumorais[38], fornecem interessantes modelos de tumores humanos e são importantes métodos para o estudo dessas doenças. O sistema Cre – Lox, com a utilização da técnica com a enzima Cre recombinase especificamente localizada[39,40], é particularmente interessante, uma vez que geram camundongos com mutações determinadas, em locais específicos, com expressão gênica temporalmente controlada[41]. Nessa tecnologia, a ativação da Cre recombinase é realizada por um promotor, como genes de receptores celular ou sistemas virais, como o adenovírus[42].

1.2.1 Geração de Camundongos condicionais, Knock-out e Knock-in

É possível a criação de camundongos condicionais knock-out e/ou knock-in utilizando o sistema Cre - Lox. Nos camundongos knock-out, a Cre recombinase requer um sítio curto (loxP com 34 pares de bases (pb)) para catalisar a recombinação. O alelo é alterado pela inserção de loxP em dois íntrons ou em extremidades opostas de um gene. O promotor ativa a ação da Cre recombinase, que catalisa a recombinação entre os loxP e inativa o gene[40] (Figura 1).

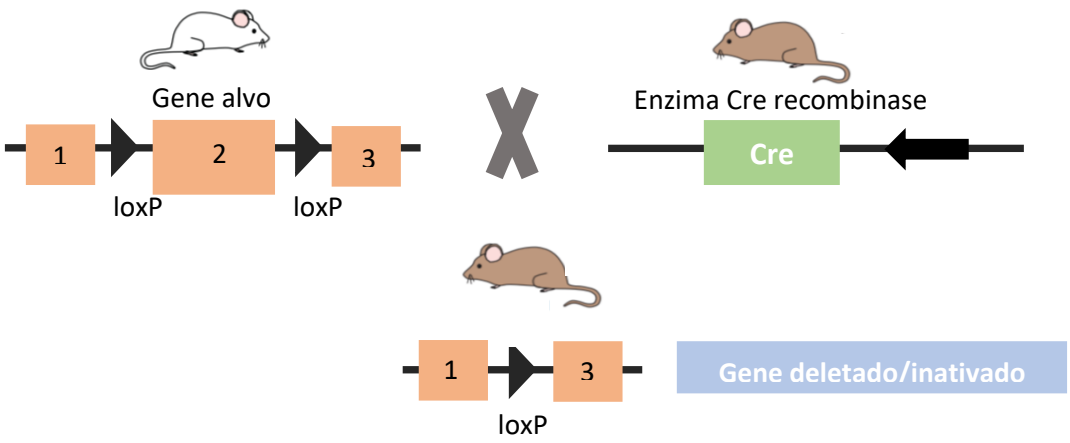

Figura 1: Esquema de camundongos geneticamente modificados, condicional knock-out. Observe que o gene de interesse está flanqueado com duas sequencias loxP, necessárias para a ação da enzima Cre recombinase. O gene de interesse (gene alvo) é inativado nas células somáticas que expressam a enzima através de um promotor específico.

Para a geração de camundongos knock-in, em que o objetivo é a ativação gênica, a ação da Cre recombinase constitui em remover um stop códon, LoxP-stop-LoxP (LSL), e permitir a expressão do gene determinado no tecido ou célula específicos[37] (Figura 2). A combinação dessas técnicas, com a criação de modelos ani

mais modificados condicionalmente, reproduz o processo de tumorigênese humana de maneira mais fidedigna e são úteis para estudar genes, que inseridos ou deletados de maneira convencional, levariam a letalidade embrionária.

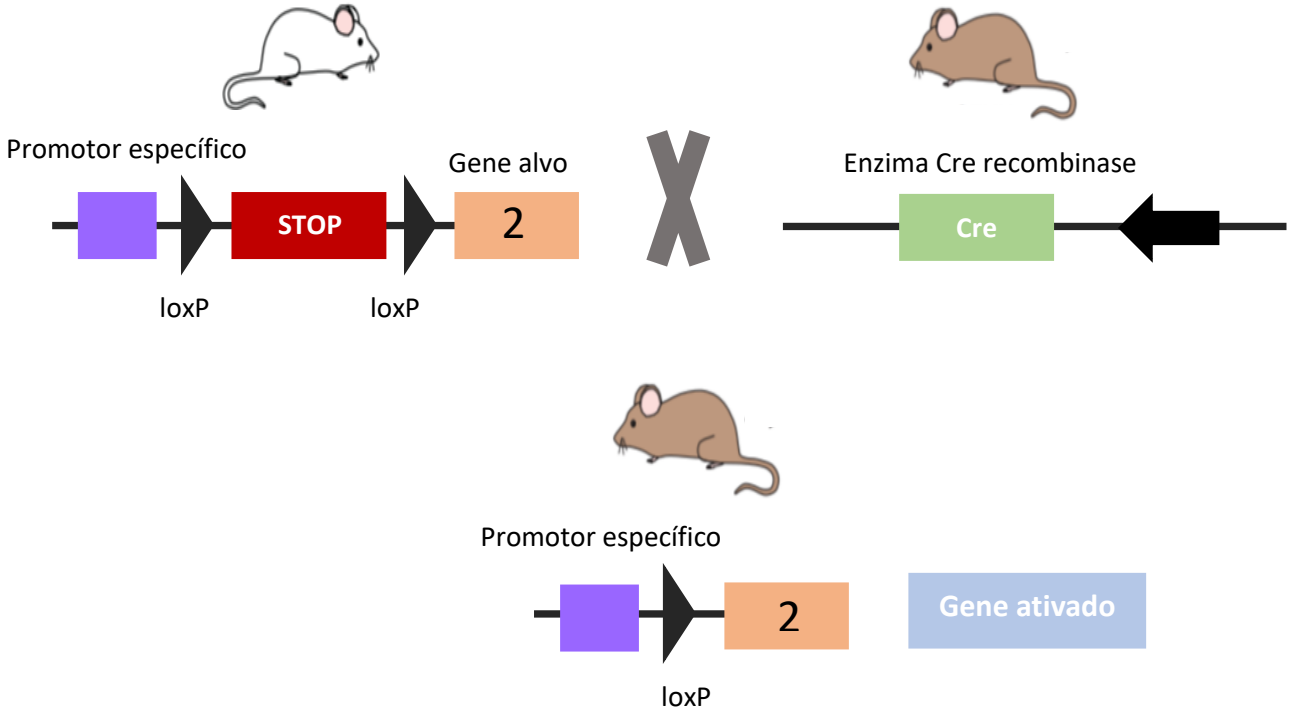

Figura 2: Esquema de camundongos geneticamente modificados, condicional knock-in. Observe a presença do códon STOP flanqueado com duas sequencias loxP, necessárias para a ação da enzima Cre recombinase. O gene de interesse (gene alvo) é ativado nas células somáticas que expressam a enzima através de um promotor específico.Essa técnica permite a expressão da versão selvagem do gene ou do gene mutado.

1.2.2 Modelo de camundongo usando o receptor de progesterona como promotor específico

O modelo de camundongo usando o receptor de progesterone como promotor da ação da enzima Cre recombinase (*Pgr*^{Cre}) foi desenhado para elucidar questões envolvendo a biologia mamária e aspectos reprodutivos. Também é uma via extremamente útil para

avaliação de potenciais genes envolvidos na carcinogênese dos tumores do trato reprodutivo feminino e das mamas[43].

Para criação desse modelo, a Cre recombinase foi inserida no exon 1 do gene do receptor de progesterona de células tronco – embrionárias. A ativação da enzima ocorrerá apenas em células de órgãos específicos que expressam os receptores de progesterona, tais como as células de todas as camadas uterinas, mamas, células granulosas do ovário logo após a ovulação e hipófise anterior.

1.3 Os genes envolvidos na geração do modelo condicional de camundongos

O processo de carcinogênese é complexo e envolve alterações genéticas sequenciais e somatórias, incluindo ativação de oncogenes e inativação de genes supressores tumorais. Essas alterações são essenciais tanto nos estágios iniciais da neoplasia como na progressão tumoral[44,45]. Os genes supressores tumorais regulam a divisão, a proliferação e a apoptose celular e, desse modo, impedem o desenvolvimento de tumores[46]. O envolvimento específico de determinados genes supressores tumorais e oncogenes durante o processo do desenvolvimento e a progressão neoplásica, podem originar tumores de diferentes comportamentos clínicos, respostas terapêuticas heterogêneas, e, portanto, de prognósticos variáveis[47-49].

Para a geração do modelo de camundongo condicional do estudo em questão, foram escolhidos dois genes comumente envolvidos em tumores ginecológicos. Um gene com potencial ação supressora tumoral, o *Arid1a* (AT-rich interactive domain-containing protein 1A)[50], e um oncogene mutado, o oncogene *Kras* com uma mutação pontual no códon 12 (G12D)[51].

1.3.1 O gene *ARID1A*

O gene ARID1A está localizado no cromossomo 1p36.11 e codifica o complexo remodelador da cromatina SWItch/sucrose non-fermentable (SWI/SNF)[52], com

características que sugerem que esse gene seja um gene suppressor tumoral na espécie humana. Diversos estudos já mostraram que ele está envolvido tanto no processo inicial quando na progressão de inúmeros tumores[53,54].

Mutações do *ARID1A*, levam ao silenciamento desse gene e a perda da expressão da proteína codificada por ele[55]. Essas alterações são encontradas em diversos tumores, especialmente em tumores ginecológicos, como os tumores endometriais e ovarianos[55-60]. A mutação do *ARID1A* pode ser observado em 46 a 57% dos Adenocarcinomas de Células Claras do Ovário (ACCO)[55,59], em aproximadamente 30% dos Adenocarcinomas Endometrioides do Ovário (AEO)[57] e em cerca de 40% nos Adenocarcinomas Endometrioides Uterinos (AEU)[60]. Esses tumores estão associados a endometriose e compartilham os mesmos mecanismos envolvido com a mutação do *ARID1A*. Dentre eles, está presente a alteração na via PI3/AKT. Inúmeros outros genes supressores tumorais alteram essa via, contudo, o mecanismo detalhado ainda não foi totalmente elucidado[56,61]. Inúmeros estudos sugerem que a mutação do gene *ARID1A* está envolvida tanto no surgimento quanto na progressão do câncer. Foi observado que a ativação do tipo selvagem do *ARID1A* em células de câncer de ovário foi suficiente para inibir a proliferação celular e o crescimento desses tumores em camundongos, enquanto que o silenciamento de *ARID1A* em células epiteliais normais promoveu a proliferação celular e a tumorigenicidade em camundongos[62].

O gene *Arid1a* também possui importante função na reprodução. A deleção condicional do *Arid1a* no trato reprodutivo feminino em modelos de camundongos usando o receptor do hormônio anti – Mülleriano do tipo II (*Amhr2*) como promotor leva a subfertilidade nos camundongos fêmeas devido a defeitos na implantação embrionária e a disfunções placentárias[63]. Também já foi mostrado, em um outro modelo usando o Pgr^{Cre} com promotor das expressões gênicas, que os camundongos fêmeas knock – out condicional para o *Arid1a* eram completamente inférteis devido a disfunções endometriais[50].

Apesar das evidências do papel do *ARID1A* como gene supressor tumoral na espécie humana[56], a inativação isolada do gene *Arid1a* nos modelos de camundongos não é

suficiente para iniciar o processo de carcinogênese. Estudos prévios sugerem que outras mutações gênicas são necessárias para o início do desenvolvimento e progressão tumorais [50,62].

1.3.2 O oncogene *KRAS*

O oncogene *KRAS* (Kirsten Rat , identificado em 1982[64], *KRAS* é uma guanina nucleotídeo transferase, que permite a ligação de receptores de superfície celular com vias de sinalização intracelulares, incluindo cascatas de cinase, tais como a via MAPK, cinases lipídicas, tais como fosfoinositida 3-quinase (PI3K), e outras pequenas GTPases. Através desses efetores, *KRAS* regula numerosos processos, incluindo a proliferação, sobrevivência e diferenciação celulares, migração e comunicação extracelular[65]. Esse oncogene é frequentemente encontrado mutado nas neoplasias. Nos adenocarcinomas pancreáticos, essa frequência chega a 95%, sendo também encontrado em 50% das neoplasias de cólon e em 30% nos adenocarcinomas de pequenas células do pulmão[66]. As mutações que levam a ativação do oncogene *KRAS* geralmente estão associadas a tumores clinicamente agressivos, a uma pior resposta terapêutica e logo a um pior prognóstico tumoral.

Os modelos animais que possuem o oncogene *KRAS* ativado possuem grande importância nas pesquisas do câncer e na compreensão da biologia tumoral. Elucidar as neoplasias que possuem mutações com ativação do oncogene *KRAS* e compreender as demais interações gênicas envolvidas podem trazer grandes avanços no tratamento desses tumores. Entender qual a vulnerabilidade tumoral permite a obtenção de uma droga mais específica e possibilita melhores resultados terapêuticos[65].

Assim, a melhor compreensão dos mecanismos moleculares envolvendo a deleção do gene *ARID1A* e ativação do oncogene *KRAS* pode ser a chave que permitirá personalizar o tratamento do câncer cervical e que ajudará na melhora do prognóstico dessa neoplasia, principalmente em estádios avançados [54].

OBJETIVOS

2. Objetivos do estudo

1. Caracterizar histologicamente e molecularmente os tumores vulvares que se desenvolveram no modelo de camundongos geneticamente modificado.

2. Validar esse modelo de carcinoma de células escamosas de vulva comparando - o com as características histológicas e moleculares encontradas nos tumores de vulva da espécie humana.

ARTIGO

3. Artigo

3.1 Title: Vulvar squamous cell carcinoma developing in a mouse model with of *Arid1a* and gain of oncogenic *Kras*

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3.2 Abstract

Gynecological cancer remains a significant public health issue. In 2017, over 100,000 women will be diagnosed with a gynecologic cancer and more than 31,000 will die in the United States. Primary vulvar cancer accounts for less than 5% of gynecological cancers diagnosed, but the curative surgical treatment for deeply invasive tumors has considerable morbidity. Since vulvar cancer is an infrequent tumor, randomized trials of therapeutic approaches in women are uncommon. Thus, model systems are needed to understand the pathophysiology of the disease to develop improved therapies. To study female reproductive tract cancers, in particular endometrial cancer, we created a genetically engineered mouse model using the progesterone receptor-Cre (Pgr^{Cre}), which conditionally deletes in the uterus. When we deleted *Arid1a*, the proposed tumor suppressor that is thought to be a driver mutation in endometrial cancer, the female mice were infertile but did not develop endometrial cancer. Thus, we added oncogenic *Kras* ($Kras^{G12D}$). Surprisingly, these female mice ($Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$) developed large vulvar tumors, with 100% penetrance by 8 weeks of life. Sections of fixed tissue from vulva, vagina, cervix, uterus and ovaries of the mice were submitted to histological and immunohistochemistry studies to evaluate the $Kras^{G12D}$ mutation, *Arid1a* deletion and other biomarkers to characterize the pathways of oncogenesis in vulvar squamous cell carcinoma. Histological examination of uterine and ovarian tissues revealed normal structures without malignancy or benign tumors. This mouse model may allow a better understanding of the molecular mechanisms underlying the genetic transformation of vulvar cancer and can be used to develop targets for therapy.

3.3 Introduction

Gynecological cancer is still a public health issue. It is estimated that in 2017 over a hundred thousand women will be diagnosed with reproductive tract cancer and more than 31000 will die in the United States[1,2]. Genetically engineered mice model (GEMM) is a very interesting tool to better understand the molecular mechanisms underlying the genetic transformation of cancer[3,4], particularly when the Site-Specific Recombinases (SSR) technique is employed[5,6]. To study female reproductive tract cancers, in particular endometrial cancer, we created a GEMM using the progesterone receptor-Cre (*Pgr*^{Cre}). The *Pgr*^{Cre} mouse model was previously described and was developed to be a powerful approach to evaluate the potential oncogenic pathways in tissues that specifically express progesterone receptor (*Pgr*)[7]. The *loxP-Cre* system allows for the creation of conditional mice with tissue and time specific expression (insertion or deletion) of oncogenes or tumor suppressor genes. This method permits a better control over frequency, timing and spatial distribution of the gene expression and provides uniqueness to the mouse model[8,9].

In order to generate this conditional mouse model, we first deleted AT-rich interactive domain 1A (SWI-like) gene (*Arid1a)*[10]. ARID1A is a gene that encodes a protein which is one of the subunits in the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex[2]. This gene has been studied and seems to be involved in the onset and progression of several human cancers[11,12]. The loss of expression of the protein encoded by ARID1A is frequently found in gynecologic cancer, and the ARID1A mutations are present especially in ovarian and endometrial tumors[13]. In ovarian cancer, the mutation is present in 46-57% of cases of Ovarian Clear Cell Adenocarcinoma (OCCC)[14,15], and in approximately 30% of cases of Ovarian Endometrioid Adenocarcinoma (OEA)[16]. ARID1A mutation also occurs in about 40% of cases of Uterine Endometrioid Adenocarcinoma (UEA)[17]. These tumors are endometriosis-associated carcinomas and share the common mechanism of ARID1A mutation with alteration of the PI3K/AKT pathways. A variety of other tumor suppressor genes have

been found to also alter these pathways, though the detailed mechanisms remain unclear[13,18]. *Arid1a* is also very important for female reproduction. The conditional deletion of *Arid1a* in the female reproductive tract using the anti-Müllerian hormone receptor type 2 (*Amhr2)*-Driven Cre mouse model leads to subfertility due to defects in embryo implantation and placental dysfunction[19]. As well, we previously showed that the conditional *Arid1a* knock-out mice developed using the Pgr^{Cre} mouse model had infertility due to endometrial dysfunctions[10].

Despite the evidences that ARID1A has a role as a tumor suppressor gene in human cancer[13], inactivation of A*rid1a* alone is not sufficient for tumor initiation in the female reproductive tract of mice. $Arid1a^{d/d}$ mice showed aberrant active epithelial proliferation, but developed neither endometrial hyperplasia nor endometrial or ovary cancer. These studies suggested that other gene mutations are necessary for tumorigenesis and tumor development[10,20].

Therefore, we added the Kirsten rat sarcoma viral oncogene homolog (*Kras*) G12D mutation to our mice model, using the Cre/Lox system, with the activation of this oncogene only in the cells where Cre is active (Figure 1). $Kras^{G12D}$ was conditionally expressed in *Pgr* containing tissues, such as the uterus, the ovarian granulosa cells just before ovulation and the anterior pituitary and mammary glands[7]. The RAS family of oncogenes are frequently mutated in human cancer and play a critical role in the regulation of cellular proliferation, differentiation and survival[21]. The KRAS oncogene mutation is associated with the development of numerous endometrial malignancies, with an average incidence varying between 10-30%[22,23]. The Cancer Genome Atlas (TCGA) Research Network is the most comprehensive genomic analysis of this heterogenic group of tumors. This classification divided the endometrial cancer in four distinct molecular subtypes and described the KRAS mutations as one of the five main molecular alterations in type I endometrioid carcinomas. TCGA data also showed nearly 40% ARID1A mutation rate in endometrial cancers, out of which 22% also contained mutations in KRAS[23].

Because these two molecular alterations (deletion of *Arid1a* with oncogenic *Kras* insertion) are considered driver mutations in human endometrial cancer, we reproduced them in our conditional knock-in - knock-out mouse model and expected to induce endometrial carcinoma in the $Pgr^{Cre/+}$ $Arid1a^{f/f}$ $Kras^{LSL\text{-}G12D/+}$ female mice. However, surprisingly, these mice developed large vulvar tumors with 100% penetrance by 8 weeks of life and did not develop endometrial cancer.

Primary vulvar cancer accounts for less than 5% of gynecological cancers diagnosed[24], and vulvar squamous cell carcinoma (VSCC) is the predominant type, representing over 80% of cases[25]. Two distinct etiological pathways can lead to VSCC: (1) Human Papillomavirus (HPV) dependent, which leads to the usual type of Vulvar Intraepitelial Neoplasia (uVIN) and account for 95% of the cases of VSCC; (2) HPV independent, which leads to the differentiated type of VIN (dVIN) and accounts for 5% of VSCC cases[26,27]. These types have different epidemiology, histology, molecular features and prognosis[24,27]. In the past two decades, the incidence of VIN is rising and the mean age of disease onset is becoming earlier[28,29]. Thus, it is important to correctly recognize and promptly treat these tumors, since the delay in the diagnosis leads to disease progression, and the tumor stage defines the treatment options and prognosis[30,31]. The most common treatment is surgery, but the curative surgical standard for deeply invasive tumors has considerable morbidity and is limited by proximity of the vulva to vital organs and tissues[31,32]. For the recurred vulvar disease, especially inguinal lymph node recurrence, the treatment options are even more restricted and the prognosis is poorer[33-35]. Because vulvar cancer is an infrequent tumor, randomized trials of therapeutic approaches are uncommon and small[36,37], and most studies are based on retrospective reviews[38-41].

So, the main objective of this study was to characterize this novel mouse model of vulvar squamous cell carcinoma. Aside from basic tumor characterization, we attempted to validate this mouse model by comparing it with the histologic and genetic features of human vulvar cancer types. We expect to use this mouse model to better understand

the pathophysiology of this disease and to develop improved target therapies for the vulvar carcinoma.

3.4 Materials and Methods

3.4.1 Creation of *Pgr^{Cre};Arid1a $^{f/f}$;Kras $^{G12D-LSL}$* mouse model

Arid1a conditional allele (*Arid1a $^{f/f}$*)[42], oncogenic *Kras* conditional allele (*Kras $^{G12D-LSL}$*)[43], and *Pgr^{Cre}* mice[7] were mated to obtain *Pgr^{Cre};Arid1a $^{f/f}$;Kras $^{G12D-LSL}$* female mice (designated as *Arid1a-Kras* cKO throughout). Mice were genotyped from tail biopsies by PCR analyses as described. All mice were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals under an approved protocol. Laboratory Animal Resource Center, Indiana University - School of Medicine (LARC, IU - SM) performed rederivation and initial breeding.

Pgr $^{cre/+}$ mice were bred to *Arid1a$^{f/f}$* mice (*f*=floxed allele) to generate *Pgr $^{cre/+}$ Arid1a$^{f/f}$* male mice. We used the Cre-Lox system of the Progesterone receptor (Pgr) – driven Cre Knockin mouse to construction of a conditional expressing *Kras* mouse*. Pgr^{Cre}* is used to conditionally delete the floxed alleles in tissues expressing the Progesterone receptor. *Kras$^{SLS-G12D}$* contains *loxP* sites around a stop codon in front of the oncogenic *Kras^{G12D}* allele. In the presence of the Cre recombinase, in the tissues that have the expression of *Pgr gene*, we removed the stop codon and lead to the expression of the oncogene *Kras* in these tissues. The male mice *Pgr $^{cre/+}$ Arid1a$^{f/f}$ Kras$^{LSL-G12D/+}$* or *Pgr $^{cre/+}$ Arid1a$^{f/f}$ Kras$^{+/+}$* were bred to female mice *Arid1a$^{f/f}$ Kras$^{+/+}$ or Arid1a$^{f/f}$ Kras$^{LSL-G12D/+}$* respectively, to generate f*emale mice Pgr$^{Cre/+}$ Arid1a$^{f/f}$ Kras$^{LSL-G12D/+}$*. These breeders were used as experimental mice (termed *Arid1a* conditional knockout, Kras knockin female mice, Arid1a Kras cKO) and *Arid1a$^{f/f}$* mice (termed control mice). Our studies with *Pgr^{Cre}Arid1a$^{f/f}$* female mice showed a complete infertility phenotype, so the *Cre* allele was maintained on the male for breeding purposes.

Mice were genotyped at 12–14 days of postnatal life from tail biopsies by PCR analyses using specific primers. For the *Arid1a floxed allele*, the following conditions were used with *Arid1a* forward and reverse primers: 2 min at 50ºC, 10 min at 95ºC, followed by 40 cycles of 15 sec at 95ºC (denaturation), 45 sec at 59ºC (annealing), and 45 sec at 72ºC (extension). For the Cre allele, the following conditions were used with Pr-Cre forward and reverse primers: 2 min at 50ºC, 10 min at 95ºC, followed by 40 cycles of 15 sec at 95ºC (denaturation), 45 sec at 60ºC (annealing), and 45 sec at 72ºC (extension). For the genotyping of the oncogenic Kras ^{G12D} mutation, total RNA was isolated from uterus according to, Qiagen minieasy kit protocol. cDNA was generated from RNA samples by reverse transcription, followed by PCR amplification using primers *Kras*1S, 5_-GCCATTTCGGACCCGGAGCGA and *Kras*1A, 5_-CCTACCAGGACCATAGGCACATC. RNA expression of wild-type *Kras* and mutant Kras ^{G12D} was determined by digestion of 5 μL of the reverse transcription-PCR products with HindIII for 1 hour at 37◦C. The restriction products were resolved in a 2% agarose gel. The mutant Kras ^{G12D} allele contains a HindIII restriction site engineered in exon 1, which is absent in the wild-type allele. Therefore, digestion of the 488-bp products generates 300-bp and 148-bp restriction fragments in the mutant but not in the wild-type PCR product.

3.4.2 Tissue collection and histological analysis

At times listed in experimental design, mice were euthanized, and reproductive organs were dissected and fixed for histology or snap frozen for RNA isolation. Uteri, vaginal/vulvar tissue, cervix, and tumors were fixed in 4% paraformaldehyde (Sigma), and ovaries were fixed in 10% neutral buffered formalin (EMD Millipore). The Histology Core of the Department of Anatomy & Cell Biology of Indiana University, School of Medicine performed tissue processing and paraffin embedding. Sections were cut at 5 µm and stained with hematoxylin (VWR) and eosin (VWR) or periodic acid-Schiff (Sigma) using standard techniques.

3.4.3 Immunohistochemical staining

Four sections from each of three independent control and *Arid1a-Kras* cKO tissues samples were analyzed in parallel. Paraffin-embedded tissues were cut at 5 μm, mounted on silane-coated slides, deparaffinized, rehydrated in graded alcohol series, and boiled in citrate buffer, pH 6.0 (Abcam) prior to blocking with 10% normal goat serum and incubating in primary antibody diluted in 10% normal goat serum (Vector Laboratories) in phosphate-buffered saline (PBS), pH 7.5 (Life Technologies, Inc.) overnight at 4ºC. Primary antibodies and conditions are listed Table 1.

Sections were washed with PBS and incubated with the appropriate species-specific horseradish peroxidase-conjugated secondary antibody (2 lg/ml; Vector Laboratories) for 1 h at room temperature. Immunoreactivity was detected using the Vectastain Elite DAB kit (Vector Laboratories) and the development of the colour reaction was monitored. Sections were then briefly counterstained with hematoxylin, dehydrated, and mounted. Slides were analyzed by light microscopy.

3.4.4 Serum analysis

Adult control and *Arid1a - Kras* cKO female mice were anesthetized by Tribromoethanol [Avertin®], 125-250mg/kg intraperitoneal or isoflurane inhalation, and blood was collected in microtainer tubes (Becton Dickinson) by closed cardiac puncture. Serum was separated by centrifugation and stored at 20ºC until use. The University of Virginia Ligand Assay and Analysis Core performed measurements of follicle stimulating hormone (FSH) and luteinizing hormone (LH).

3.5 Results

We evaluated experimental and control mice at 3, 4, 8 and 12 weeks of age. Long-term survival studies were set up using *Arid1a – Kras* cKO ($Pgr^{Cre}Arid1a^{f/f}Kras^{LSL\text{-}G12D}$) and control mice ($Pgr^{+/+}$). At least, 6 female mice were used in each group by genotype and age. Defined endpoints for humane euthanasia were followed including palpable or

visible mass of >1.5 cm. To confirm tissue-specific recombination, ablation of *Arid1a* and insertion of *Kras* mutation, we performed qPCR and immunohistochemical staining on tissues from female mice of all ages mentioned above.

3.5.1 *Arid1a - Kras* cKO Mice did not develop endometrial cancer

Since the loss-of-function mutations in ARID1A and KRAS oncogene are frequently mutated in endometrial cancers in women, we were expected to see the development of endometrial cancer in these mice. However, the histology studies of the uterus in this mouse model did not show any signal of endometrial neoplasia neither hyperplasia nor dysplasia (Figure 2). Instead, the sections showed an aspect of atrophic uterus, with a thin uterine endometrium and lower number of glands when compared with uterus of the controls mice. This was a consistent pattern regardless of ages of the mice.

3.5.2 Arid1a - Kras cKO Mice developed large vaginal - vulvar tumors

These experimental female mice ($Pgr^{Cre}Arid1a^{f/f}Kras^{LSL\text{-}G12D}$) developed large vulvar tumors with 100% penetrance by 8 weeks of age (Figure 4, C - F). Macroscopically, the ovaries and uterine cervix were normal, which has been confirmed by histological examination (Figure 3, B, D). In the Control mice, the external genitalia and also the female reproductive tract were normal, gross and histologically without malignancy or benign tumors (Figures 4, A, B) (Figure 3, A, C and E).

In the histological studies, we observed a progression of the tumors with important morphological changes by aging of the mice. The arrangement of the epithelium lesion become abnormal and it was possible to see features of the loss of cell maturation and cellular atipia.

The tumor size was also correlated with age of the mice (Figure 4). In the survival study, all of experimental female mice reached the endpoint for euthanasia (Figure 7). The median survival *Arid1a-Kras* cKO mice was 103 days of life. Beside the large vaginal – vulvar tumors, none of these mice exhibited moribund behavior.

The female reproductive tract weight was measured in the groups. We observed differences between *Arid1a-Kras* cKO and control female reproductive tracts at 8 weeks (*p=0.018*). We had *p=0.055* and *p=0.971* for 3 and 12 weeks, respectively. (Figure 6).

3.5.3 The immunohistochemical staining showed the pathways of oncogenesis in vulvar squamous cell carcinoma

The immunohistochemical of the tumor tissues samples showed negative staining to *Arid1a* and positive staining to *Kras* in all of tested samples.

The immunostaining to p16 and p53 found in vulvar tumors revealed results typically presents in the dVIN. The expression of p53 was positive in cells extending above the basal layer. In contrast, p16 was not expressed in tumor samples.

The *Pgr* staining showed a diffuse expression of progesterone receptor in tumor samples and vulvar control mice samples, with no differences in the intensity of the expression in these tissues.

The p63 expression appeared strongly positive in all extension of the tumor and proliferated epithelium. As expected, in vulvar samples of control mice, the staining can be observed only in basal epithelial cells.

3.6 Discussion

This study presented a mouse model of vulvar squamous cell carcinoma and documented all different stages of the progression from vulvar intraepithelial neoplasia (VIN) to invasive cancer. The $Pgr^{Cre/+}$ $Arid1a^{f/f}$ $Kras^{LSL\text{-}G12D/+}$ female mice developed these vulvar tumors with 100% penetrance, after sexual maturity, but at an early age (8 weeks). Our results strongly suggested that mutation in the Kras oncogene was a driver mutation in this mouse model, since in our previous studies, with the conditional *Arid1a* knock-out mice using the Pgr^{Cre} mouse model[10] or using the $Amhr2^{cre}$ mouse model[19], we did not see these vulvar squamous tumors. Vulvar tumors are rare in mice, and the creation of animal models to study vulvar lesions is not common. This mouse model is unique,

reproduced the histology and molecular features of the human vulvar tumors and could be a valuable tool for the study of these cancers.

Two other studies that used mice models with the *Kras*^{G12D} oncogene mutation observed the development of vulvar tumors. One publication used the pancreatic and duodenal homeobox 1 (Pdx1) promoter-driven transgene, and the other used the *Pgr*^{Cre} mouse model with phosphatase and tensin homologue (PTEN) ablation. Neither of them had the vulvar tumor penetrance of our study, described a correlation of the tumor size and abnormal histologic architecture with aging of the mice, or observed malignant tumors in the vulvar lesions of their mice.[44,45]. However, the number of mice affected in these studies was small and maybe not representative enough to define tumor invasion. In the first study, the proposed explanation for the development of spontaneous vulvar papilloma was the strong activation of the Pdx1 promoter in vulvar cells, given that Pdx1 is a developmental transcription factor. Therefore, the transcription of the *Kras*^{G12D} oncogene in these cells could have resulted in proliferation and benign papilloma formation[44]. The second study did not explain the pathway for development of the vulvar lesion[45].

The most accepted theory about the origin of the mouse vaginal and vulvar epithelium is the female reproductive tract of mice is derived from Müllerian ducts (MDs)[46]. This theory justifies the expression of the *Pgr* gene in the lowest third of the vaginal and vulvar tissues and consequently the alteration of the expression of *Arid1a* gene and *Kras* oncogene in these mice tissues. In mouse cervical, vaginal and vulvar epithelial cells, Pgr is upregulated by estrogen through the estrogen receptor α (ERα) as in most progesterone target tissues[47]. After mice puberty, the estrogen stimulated the epithelium differentiation and the cells started to express the *Pgr* and the Conditional harbored mice developed vulvar tumors.

The biggest challenge of our vulvar cancer mouse model was to verify if the vulvar squamous cell tumor was malignant. For this end, we tried to delineate the natural history of this tumor, which like many others, begins with epithelium proliferation, hyperplasia,

then dysplasia and finally progression to an invasive tumor. The definition of malignance in vulvar human tumors is not easy, and the diagnosis reproducibility among pathologists of high - grade vulvar lesions is poor[48,49]. This definition is particularly challenging in the dVIN, which has very subtle histopathological changes. The difficulty in the recognition of these lesions leads to them being often misdiagnosed as benign tumors[48,50]. Previous publications defined the five most useful histological features for diagnosis of dVIN: 1) atypical mitosis in the basal layer; 2) basal cellular atypia; 3) dyskeratosis; 4) prominent nucleoli; and 5) elongation and anastomosis of rete ridges. These histological characteristics proved to increase the agreement between pathologists' judgment[48,49]. To help us elucidate the diagnosis of vulvar lesions, we applied these criteria in the tumor histological studies with good reproducibility. To our knowledge, the genetically engineered mouse model of VSCC described in our study is the one that best reproduced the features of vulvar cancer (Figure 5).

In addition, we attempted to characterize the pathways of oncogenesis in vulvar squamous cell carcinoma through immunohistochemistry staining. For usual vulvar intraepithelial neoplasia, HPV infection guided the molecular mechanisms responsible for the induction of precancerous vulvar lesions and the evolution to VSCC[51]. The carcinogenesis is linked to the E6 and E7 oncoproteins that inactivate the p53 and retinoblastoma protein (pRb) tumor suppressors following infection and viral integration[52]. Immunostaining for p16 detects a protein overexpressed in host cells infected by HPV, so high levels of expression of this protein have a strong association with HPV status and could be an accurate marker to use in the classification of the two histologic subtypes of VSCC[53,54]. All of our tumor samples were negative for p16 immunostaining. This result strengthens the likelihood of the subtype not associated with HPV infection. For differentiated vulvar intraepithelial neoplasia, the HPV – independent lesion, the pathways are distinct and remain incompletely characterized[55]. However, a proportion of cases harbor p53 mutations as a potential-driving event. This missense mutated and nonfunctional p53 protein often accumulates in the cell and can be detected

by immunohistochemistry[56,57]. Some of our tumor samples were positive to p53 staining, but it was not a consistent find. In our mouse model, invasive tumor was observed in some mice, but the majority had lesions characterized as high grade dysplasia, which may explain the p53 staining results. The positivity of p53 staining is associated with an aggressive tumor, with high rates of recurrence[58], which suggests that the majority of VSCC in our mice were tumors with a better prognosis. Apart from big vulvar tumors and unequivocal evidence of tumor invasion, these mice did not have cachexia signs, which support this. The second common pattern in HPV-independent VSCC is p53-null staining, which is characteristic of nonsense mutations and leads to an undetectable p53 protein in the tumor cells. Previous studies of p53 mutation in HPV-independent vulvar cancer did not carefully separate tumors on the basis of HPV status or use methods to identify missense or nonsense mutations in this gene, making them difficult to interpret[59]. Our results for Ki-67 staining are consistent with the current literature and can help distinguish dVIN and uVIN from normal epithelium and squamous cell hyperplasia. Both subtypes show positive staining for Ki-67, but the major difference is that in uVIN, the full thickness of the epithelium is usually stained, whereas in dVIN, Ki-67 staining is usually positive only in the basal layer and in a thin parabasal layer[25]. The p63 expression appears to be essential for differentiation of all squamous epithelial tissues in female reproductive tract and the may be useful to evaluate the differences between preinvasive and invasive lesions[60]. In cervical intraepithelial neoplasia (CIN) I, the p63 staining is typically localized to the basal and parabasal cells whereas in CIN II and CIN III, p63-positive nuclei extended into the middle and upper layers[61]. In CIN III, p63 expression can be uniformly observed in all dysplastic cells[62]. It was exactly the pattern of expression that we observed in the preinvasive and invasive lesion of this VSCC. These results helped to define the grade of invasion in the mice tumors an confirm the squamous differentiation of the lesions.

In spite of the role of the aforementioned driver mutations in human endometrial cancer, the possible reason our mice did not develop the endometrial tumor was the premature emergence of the vulvar lesions. The mice were euthanized when they reached the endpoint of the study. If we could have waited and observed these mice for longer, they may have developed the endometrial tumor.

This study has one limitation. Despite the great valuable of this mouse model of VSCC to mimic the human vulvar tumor, we detected considerable heterogeneity in the tumor phenotype. Maybe, with more tumor samples, these histological differences could be clarified.

Although the external location of the vulva supports an early identification, vulvar cancers are often advanced at the time of diagnosis and have high rates of recurrence[37,39]. The lack of preclinical models that accurately reflect this tumor is a barrier to the development of new approaches for personalized treatment[38,39,55]. Therefore, moving forward, this mouse model may allow for better understanding of the molecular mechanisms underlying the genetic transformation of vulvar cancer and can be used to test and develop target treatments for VIN and invasive tumors of vulva.

The authors have nothing to disclose. This work was supported by 1R03 CA19127 (to SMH) and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Capes (to MSLP).

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CONCLUSÕES

4. Conclusões

Os tumores vulvares desenvolvidos com o modelo de camundongos utilizado no estudo foram histologicamente e molecularmente caracterizados. As características histológicas e moleculares observadas, recriaram, com grande similaridade, o carcinoma de células escamosas diferenciado de vulva (CCE) da espécie humana, em todos os seus estádios. Por esse motivo, esse modelo possui grande potencial para ser usado como modelo pré-clínico para elucidar questões relacionadas ao câncer vulvar.

A originalidade desse modelo de CCE de vulva é o aspecto mais forte do estudo. Não há na literatura, outro modelo geneticamente modificado, que recapitule, de maneira tão fiel, esses tumores, em especial as lesões do tipo diferenciado, cuja etiopatogênese é independente da infecção pelo HPV.

Esse modelo de camundongo poderá ser útil na elucidação dos eventos moleculares envolvidos na progressão dos tumores vulvares e melhor caracterizar geneticamente essas lesões. Por se tratar de um câncer relativamente raro, a existência de um modelo que reflita, molecularmente esse tumor, poderá permitir o desenvolvimento de opções terapêuticas personalizadas, baseadas na assinatura genômica tumoral, para um tumor cujo tratamento para a doença avançada e/ou recorrente é muito limitado.

CONSIDERAÇÕES FINAIS

5. Considerações finais

Com a ampliação da cobertura da vacinação para o HPV e a queda da incidência das lesões genitais associadas à infecção viral, o foco das pesquisas para esclarecer os mecanismos, genéticos e epigenéticos, envolvidos na carcinogênese dos tumores de vulva deve ser a doença HPV independente. Esses tumores, de diagnósticos clínico e histológico desafiadores e de alta taxa de progressão para doença invasora, precisam de marcadores diagnósticos e prognósticos acurados. Para isso, os genes específicos envolvidos na carcinogênese desses tumores vulvares deverão ser avaliados e definidos.

Com o estabelecimento de subtipos tumorais mais precisos, será possível o desenvolvimento de tratamentos personalizados, e talvez a redução da morbidade associada ao tratamento cirúrgico padrão desses tumores.

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ANEXOS

7. Anexos

Anexo I: ESTADIAMENTO DO CARCINOMA DA VULVA (FIGO -2009)

Estádio I	Tumor confinado à vulva e/ou períneo, linfonodos negativos	**Ia:** Lesões com 2 cm ou menos confinados a vulva ou períneo com invasão de estroma de 1 mm ou menos. (Carcinoma epidermoide superficialmente invasivo/ microinvasivo); **Ib:** Lesões maiores que 2 cm ou com invasão do estroma maior que 1 mm, confinadas à vulva ou ao períneo.
Estádio II	Tumor com extensão para estruturas perineais (1/3 inferior uretra, 1/3 inferior vagina, ânus) com qualquer tamanho e linfonodos negativos.	
Estádio III	Tumor de qualquer tamanho com ou sem extensão para estruturas perineais (1/3 inferior uretra, 1/3 inferior vagina, ânus) e linfonodos inguinofemorais positivos	**IIIa:** (i) 1 metástase linfonodal (≥ 5mm), ou (ii) 1-2 metástases linfonodais (<5mm); **IIIb:** (i) 2 ou mais metástases linfonodais (≥ 5mm), ou (ii)3 ou mais metástases linfonodais (<5mm); **IIIc:** Presença de extravasamento capsular.
Estádio IV	**IV a**: Tumor invadindo qualquer estrutura de que se segue: (i) 2/3 superior de uretra, 2/3 superior de vagina, mucosa vesical, mucosa retal, fixo ao osso pélvico (ii) linfonodos inguinofemorais fixos ou ulcerados **IV b**: Qualquer metástase à distância, incluindo linfonodos pélvicos.	

Anexo II: PROTOCOLO DO ESTUDO APROVADO PELA INDIANA UNIVERSITY

Indiana University School of Medicine
Institutional Animal Care and Use Committee (IACUC) Animal Protocol Form

For IACUC Office Use Only			
Protocol Number:	11016 MD/R /MSS	Old Protocol Number:	
Approval Date:	10/21/2015	3-Year Expiration Date:	10/21/2018
Amendment #:	1	Amendment Approval Date:	2/1/2016

Section A
General Information

THIS FORM MUST BE SUBMITTED AS A WORD FILE VIA EMAIL TO: somiacuc@iupui.edu

Approval is renewable annually for up to an additional two years.

Continuation of the approved animal usage beyond three years requires completion

of a new application form and a complete IACUC review.

Principal Investigator and General Information

Title of Project:	Deletion of Arid1a and Kras as a model of endometriosis-associated ovarian cancer

Principal Investigator:	Shannon M Hawkins	Degree(s):	MD, PhD
Campus Address:	R3 C221	Department	Ob/Gyn
Campus Phone:	317-274-8225	IU e-mail address:	shhawkin@iu.edu

Protocol Type - Check all that apply				
X	**Research Protocol**		**Teaching Protocol**	
	Pilot Protocol		**Protocol Other (Explain)**	
	Replacement Protocol: Please provide the expiring protocol number this will replace			
X	**Amendment**: Proceed to the "**Amendment Summary of Changes**" section below			

Current Funding

This section is required because the Institution/IACUC must implement a process for ensuring that sponsored program support is consistent with the approved activities involving animals.

Funding Sources	Grant Title(s)	Name of PI(s) on Grant

NCI **1 R03 CA191527-01A1**	Deletion of Arid1a and Kras as a model of endometriosis-associated ovarian cancer	Shannon Hawkins
Ob/Gyn	Departmental Startup Funds	Shannon Hawkins

Note: If funded by VA, you must complete the Animal Component of the Research Protocol Form instead of this form.

Amendment Summary of Changes

In the text box below; please summarize all proposed changes to the protocol and follow these guidelines:

1. Use track changes to modify the document.
2. **Do not delete prior text** from this amendment summary. Place new text for the amendment summary above any previous amendment text.
3. Include the amendment number (i.e. A01, A02) and the date to distinguish from previous approved amendments.
4. Modify the appropriate protocol sections with the information relevant to the changes requested in the amendment.
5. Update the Procedures Check List as needed and complete corresponding subsection of the protocol.
6. The Purpose, Goals, and Hypothesis section should be updated with the added amendment changes.
7. If a new procedure is added which may cause pain or distress, a new literature search for the consideration of alternatives is required. .
8. If you are requesting additional animals, ensure that you have justified the additional number of required animals.

A1- APPROVED 2/1/2016

1) Addition of re-genotyping (if initial genotyping fails) after age 21 days with use of analgesia (carprofen) and anesthesia (Isoflurane) and Quik-Stop for any bleeding; Sections modified = D01, D02, D03. The PA has also been modified to include re-genotyping as a procedure. No additional animals are needed for this.
2) Addition of additional strain of mice. The current protocol is for using Amhr2-Cre, to study endometriosis and ovarian cancer. The cell of origin of endometriosis and/or ovarian cancer is unknown but could be endometrial in origin. Thus, we will perform similar studies using and progesterone receptor Cre to delete in the uterus. Sections modified = .B, D01, D02. This will essentially double the number of mice required for these studies. This has been indicated under each section with the totals updated.
3)

Administrative Update 2/10/2016

Addition of Zhaomin Li to protocol. The PA form has been modified.

Administrative Update 2/27/2017

Addition of Mariana Seabra Leite Praça to protocol. The PA form has been modified.

Procedures Check List

This Checklist is part of your application.

For New Protocols:

The "Core Sections" and Protocol Associates Supplement are required.

For Section D, "Procedures", place an X next to each procedure to be performed in the protocol and

complete the corresponding supplemental sections to be included in this application.

For Amendments: List which of the following sections is being modified in the "Amendment Summary" section, above. If you are adding a procedure, make sure to complete the supplemental section(s) and submit with the amendment. The IACUC Staff will insert the additional sections into the amendment document at the time of submission. As the PI, you do not need to combine the documents			
B: Summary, Experimental Design, Rational, and Animal Numbers			
C: Research Sites			
D: Procedures (Check all that apply)			
	X	D 01: Breeding, Weaning, and Genotyping	
	X	D 02: Anesthesia, Sedation, Analgesia	
		D 03: Surgical Categories	
		X	Non-survival Surgery
		X	Single Survival Surgery
		X	Multiple Survival Surgery
	X	D 04: Agent Administration	
		D 05: Irradiation, Imaging with Ionizing Radiation, and Other Radioisotope Administration	
	X	D 06: Blood Sampling	
		D 07: Behavioral Testing	
		D 08: Special Caging, Husbandry, Food/Water Deprivation/Restriction	
		D 09: Immunization	
		D 10: Hybridoma	
		D 11: Restraint	
		D 12: Laser Use	
E: Potential Experimental Complications and Emergency Management Plan			
F: Euthanasia and Disposition			
G: Justification for the Use of Animals, Unnecessary Duplication & the Three R's			
H: PI Assurance			
Protocol Associates Supplement			

Species and Number of Animals

Provide the information requested in the table below.

- **Category B:** Animals that will be bred or purchased for breeding, but not used for experiments. This includes breeders, offspring that cannot be used because of improper genotype or gender and any other animals that will not participate in the research studies.
- **Category C:** Animals used in research, experiments, or tests which involve no pain or distress or only momentary or slight pain or distress that WOULD NOT REQUIRE anesthetic, analgesic or tranquilizing agents (examples: s.c., i.m., i.p. or percutaneous i.v. injection, a brief period of restraint, tissue harvesting after euthanasia has been performed).
- **Category D:** Animals used in research, experiments, or tests where appropriate anesthetic, analgesic, or tranquilizing agents are used to avoid pain or distress (examples: major and minor surgery, tissue or organ collection prior to euthanasia, retro-orbital blood collection, prolonged restraint accompanied by tranquilizers or sedatives). Animals used in research, experiments, or tests which, if they experience pain or distress cannot be treated with an anesthetic, analgesic or tranquilizer, but the agent or procedure producing the pain/distress is immediately discontinued or the animal is euthanized to prevent pain and/or suffering.
- **Category E:** Animals used in research, experiments, or tests involving pain or distress in which the use of appropriate anesthetic, analgesic or tranquilizing agents would have adversely affected the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests (examples: studies which allow endpoints that are painful or stressful, addictive drug withdrawals without treatment, pain research, noxious stimulation). ***IF YOU LIST ANIMALS IN THIS CATEGORY YOU MUST PROVIDE A DETAILED JUSTIFICATION***

Note for Breeding Protocols:

The experimental timeline (above) should include all animals involved in experimental procedures. Animals that are generated and genotyped and found to fit the needs of the experiment should be included in the experimental timeline (column B from breeding section-D 01). Animals that are generated and genotyped, but found not to fit the needs of the experiment, do not need to be in the experimental timeline (column C from the breeding section – D 01). Breeders needed to generate pups for experimentation do not need to be in the experimental timeline, but need to be accounted for in the below table. The number of breeders needed comes from the breeding section (D 01) table (# males and # female). **The breeding schemes are to be explained in Section D 01, not the experimental timeline above.**

Species	Strain/ Nomenclature/ Genotype	*tg/KO/KI (check all that apply)			Weight or Age	Source ("LARC Vendor" is acceptable)	Total Number of Animals per Category			
		tg	KO	KI			B	C	D	E*
Mouse	$Amhr2^{Cre/+}Arid1a^{f/f}Kras^{SLS\text{-}G12D/+}$		X X	X	adult	LARC Vendor or Import from Texas through quarantine		3		
Mouse	$Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$		XX	X	Adult	LARC Vendor or Import from Texas through quarantine		3		
Mouse	$Amhr2^{Cre/+}Arid1a^{f/f}Kras^{SLS\text{-}G12D/+}$		X X	X	Weanlings (3 weeks)	In house breeding		130		

Mouse	$Amhr2^{Cre/+}Arid1a^{f/f}Kras^{SLS\text{-}G12D/+}$		X X	X	adult	In house breeding		30		
Mouse	$Amhr2^{Cre/+}Arid1a^{f/f}Kras^{SLS\text{-}G12D/+}$		X X	X	Weanlings (3 weeks)	In house breeding		588		
Mouse	$Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$		X X	X	Weanlings (3 weeks)	In house breeding		130		
Mouse	$Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$		X X	X	adult	In house breeding		30		
Mouse	$Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$		X X	X	Weanlings (3 weeks)	In house breeding		742		
Mouse	$Amhr2^{Cre/+}Arid1a^{f/f}Kras^{SLS\text{-}G12D/+}$		X X	X	adult	In house breeding		84	108	
Mouse	$Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$		XX	X	Adult	In house breeding		42	54	
Mouse	$Amhr2^{Cre/+}Arid1a^{f/f}Kras^{SLS\text{-}G12D/+}$		X X	X	Weanlings (3 weeks)	In house breeding		20		
Mouse	$Pgr^{Cre}Arid1a^{f/f}Kras^{SLS\text{-}G12D}$		XX	X	Weanlings (3 weeks)	In house breeding		10		
Mouse	+/+ males C57BL/6J;129S5/Brd mixed hybrid background (fertility studies)				adult	LARC Vendor or Import from Texas through quarantine	30			
Mouse	$Amhr2^{+/+}Arid1a^{f/f}Kras^{SLS\text{-}G12D/+}$ (fertility studies)		X X	X	Weanlings (3 weeks)	In house breeding	580			
						Total Numbers of Animals	610	1812	162	
NOTE: These totals should match the number of animals needed for experiments and those generated from breeding (used and not used from the breeding table in the breeding section)										
* tg = transgenic animal; KO = Knockout animal; KI = Knockin animal										

Do you have an IBC approved protocol for tg/KO/KI animals listed above?		
X	Yes. Please provide the IBC protocol #	1826
	No. Please contact the IBC http://researchadmin.iu.edu/Biosafety/IUPUI/bio_contacts.html	

Number Justification **(address each species individually by copying/pasting this table)** **Note: statistical estimates are expected when possible**	
Species:	Mouse
The number of animals requested for this protocol is based on the following (select all that apply):	
X	A statistical estimate (power analysis) is used to estimate the number of animals and experimental groups. Please provide the justification, calculations, and details below.
All animal experiments will be performed with 6 mice per genotype, treatment, and time point except where noted. For the proposed studies of 6 samples in each group, if we anticipate a standard deviation of 0.25, the power should be 0.95 at 0.05 significance. For fertility and other studies with 10 samples in each group, using data from our *Amhr2^{Cre}Arid1a$^{f/f}$* studies, if we anticipate a standard deviation of 5, the power should 0.94 at 0.05 significance. For survival studies, 20 mice in each arm will give us a 90% probability that the study will detect a difference at a two-side 5.0 percent significance level, with median survival assumed to be 6 months.	
	The estimated minimum number necessary to achieve the goals of the research in the absence of a statistical estimate. Explain:
	The number necessary to obtain sufficient tissue or other material for testing or analysis, i.e. collection of cells for in vitro experiments. Explain:
	The number required to provide sufficient technical training or practice for the number of trainees expected. Explain:
	Other. Explain:

Anexo III – Table 1 - Primary antibodies

Name	*Company*	*Category*	*Isotype*	
Ariad1a	Santa Cruz	SC-98441	rabbit polyclonal lgG	1:100
p53(FL-393)	Santa Cruz	SC-6243	rabbit polyclonal lgG	1:50
Anti - Ras (G12D Mutant Specific)	Abcam	ab221163	rabbit monoclonal lgG	1:100
ERa(HC-20)	Santa Cruz	SC-543	rabbit polyclonal lgG	1:100
Pgr	Santa Cruz	SC-7280	rabbit polyclonal lgG	1:100
P63	Genetex	GTX102425	rabbit polyclonal lgG	1:100
Ki-67	Millipore	AB9260	rabbit polyclonal lgG	1:300
P16	Abcam	Ab108349	rabbit polyclonal lgG	1:100

FIGURAS

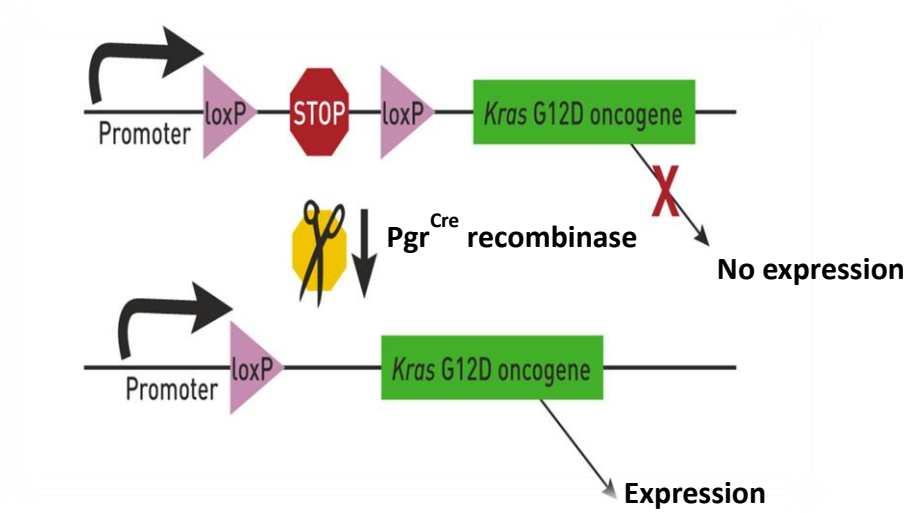

Figure 1: Conditional *Kras* knock-in mouse model: The stop sequence is flanked by *loxP* sites

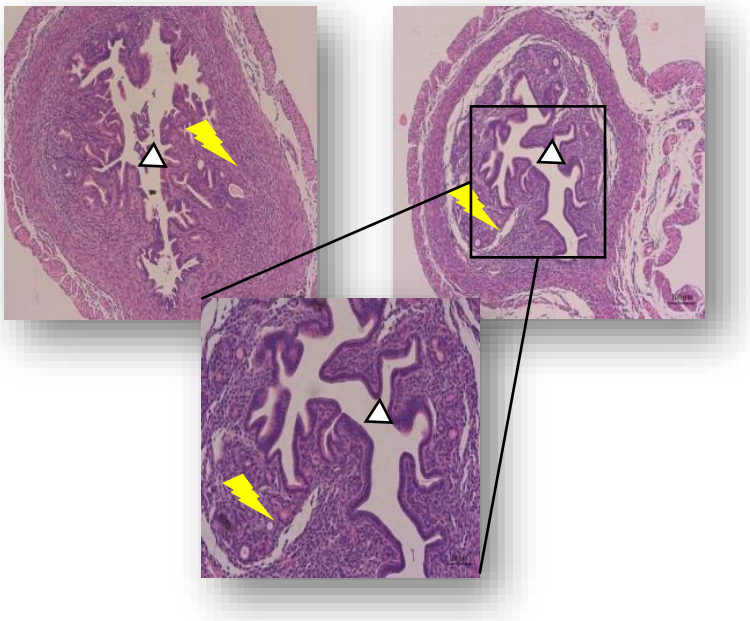

Figure 2: Atrophic uterus signs. Observe the thin endometrium (white narrow) and the lower number of glands (yellow lightning)..

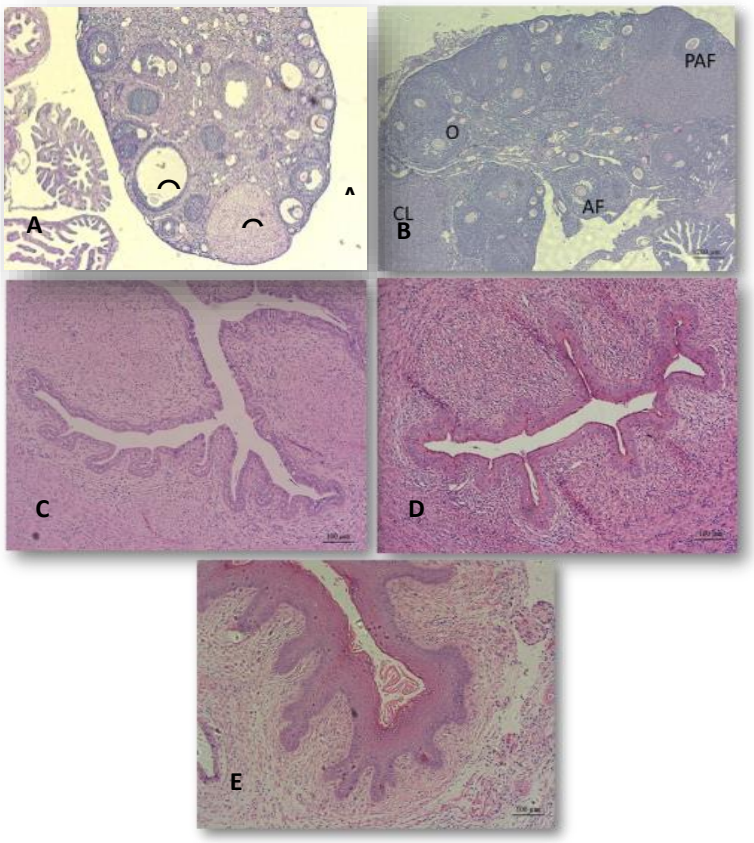

Figure 3: Normal aspects of the ovary (PAS staining) in histological studies of control mice (A) and *Arid1a-Kras* cKO mouse. This pictures also show the normal histology of cervix (H&E) of the control (C) and *Arid1a-Kras* cKO mouse (D). In (E), normal mice vulvar tissue.

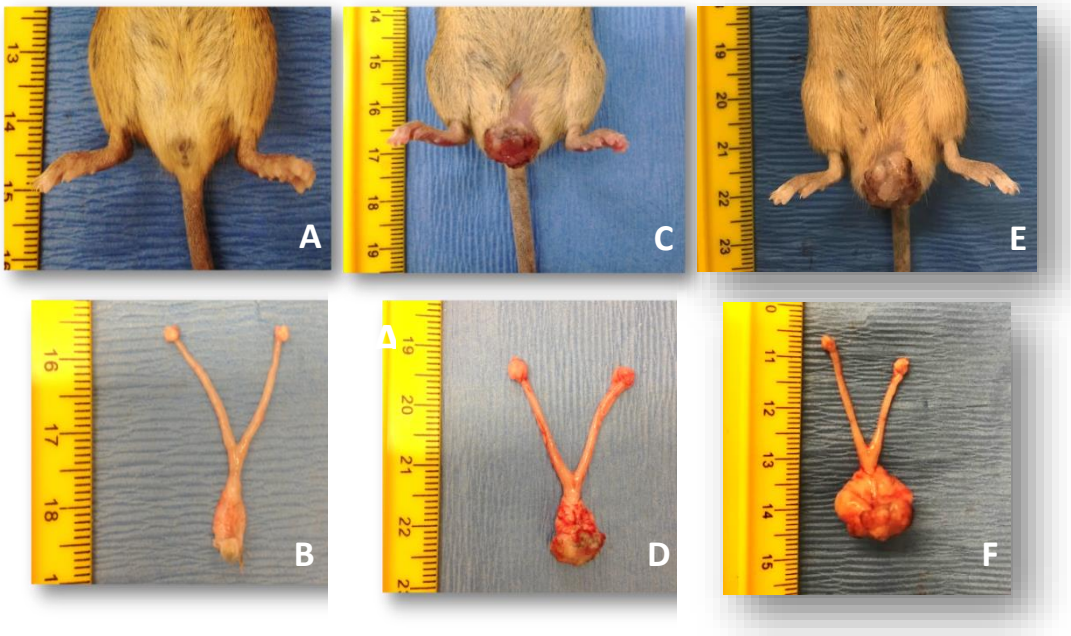

Figure 4: Large tumors in mice at 12 weeks of age and survival mice. Normal external genitalia and female reproductive tract is observed in control mice (**A, B**). Large tumors visible on gross inspection in Arid1a-Kras cKO female mice (C, E) with 100% penetrance by 8 weeks. Close examination of reproductive tract revealed tumors arising from either distal vagina or vulva area (**D, F**).

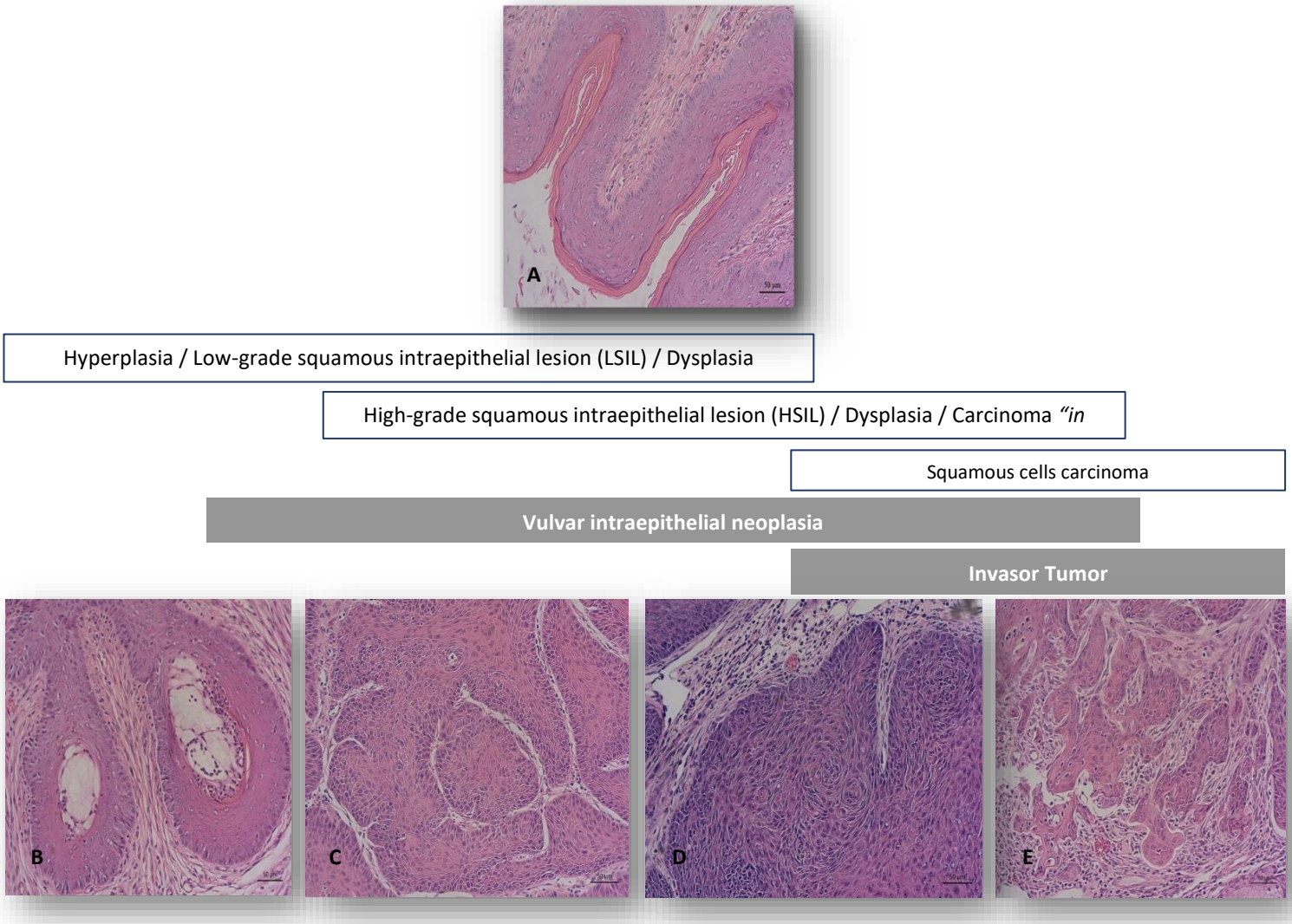

Figure 5: Observed the evolution of tumor, to preinvasive lesion (VIN), to vulvar squamous cell carcinoma. (A) Normal vulvar mice epithelium (Control mice). (B - E) Tumor samples. 4 weeks (B), 8 weeks (C), 12 weeks (D) and survival mice (E).

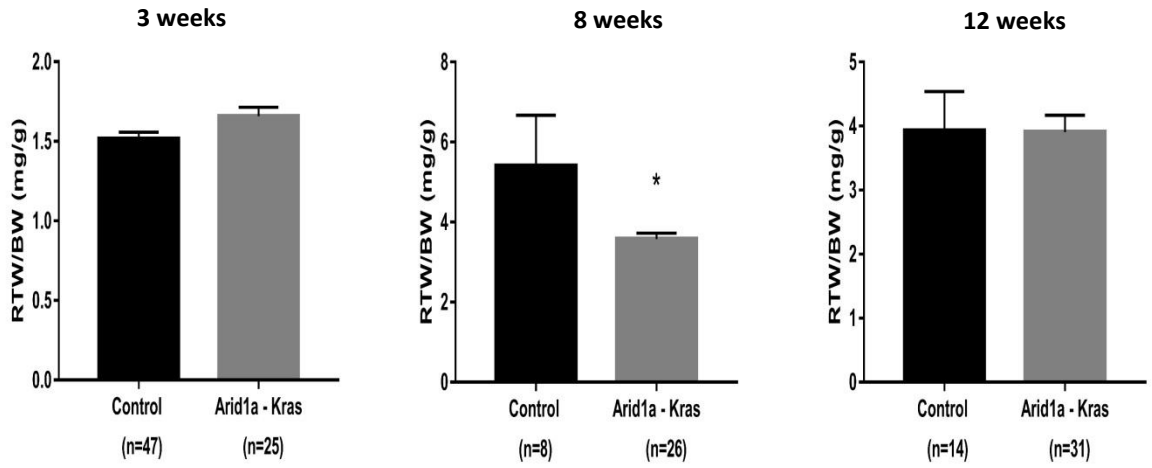

Figure 6: Female reproductive tract weight was measured at 3, 8, and 12 weeks. We observed differences between *Arid1a-Kras* cKO and control female reproductive tracts at 8 weeks (*p=0.018*). We had *p=0.055* and *p=0.971* for 3 and 12 weeks, respectively. No differences were observed in body weight (data not shown). The statistical significance (**P* < 0.05) was calculated by Student's *t* test.

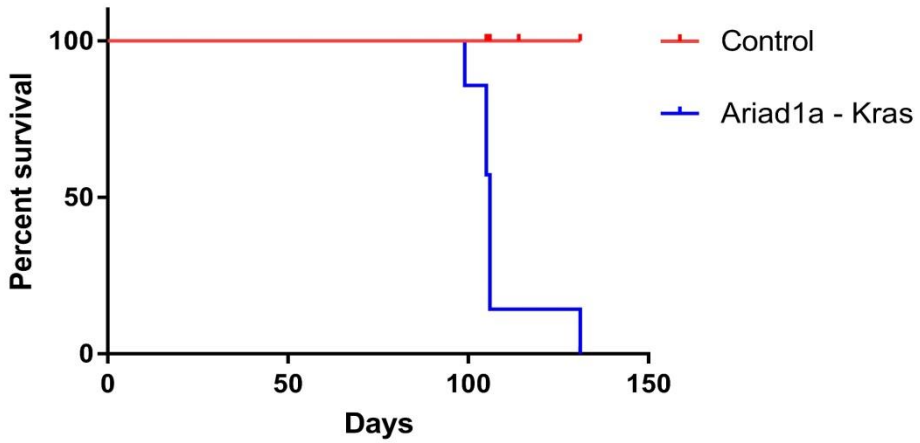

Figure 7: Kaplan-Meier survival curves. Female mice were euthanized due to disease (large vulvar tumors). Tumors were not observed in control mice. Median survival for Ariad1a – Kras was 103 days. (n = 10 each group) (*p = 0.0016*)

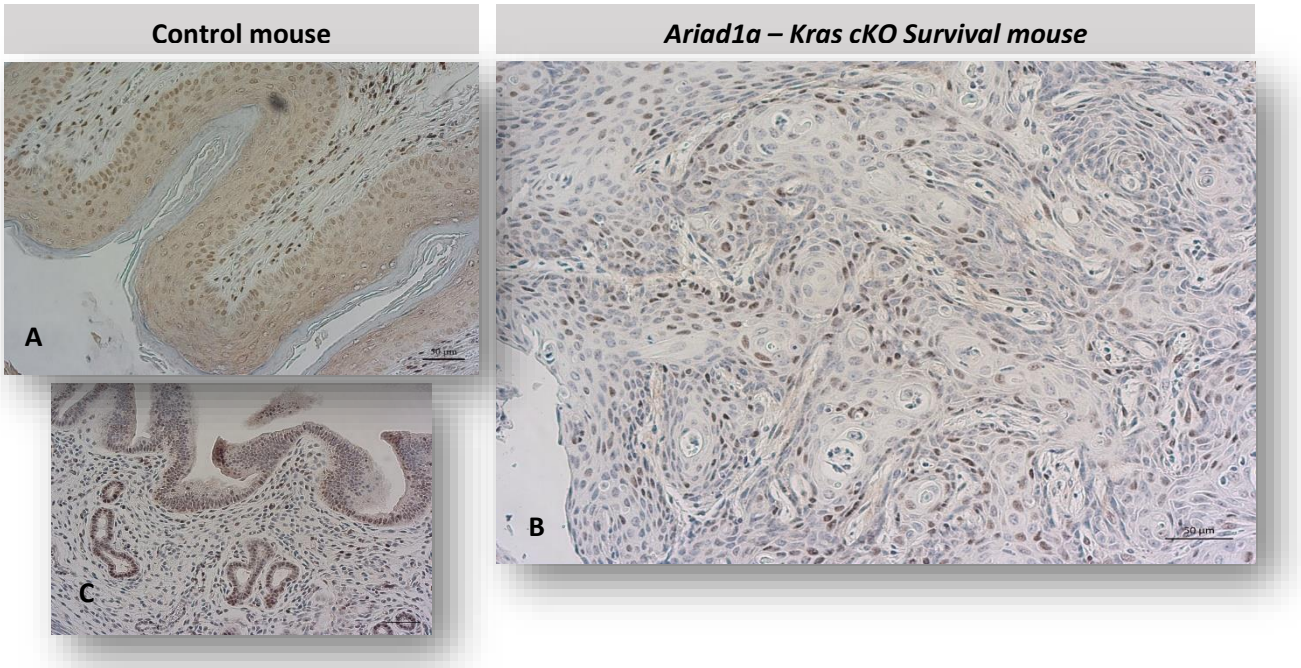

Figure 8: Immunostaining for progesterone receptor in vulvar and tumor samples. (A) Control mouse. (B) *Ariad1a – Kras cKO, Survival mouse* (C) mouse uterus as positive control for Pgr

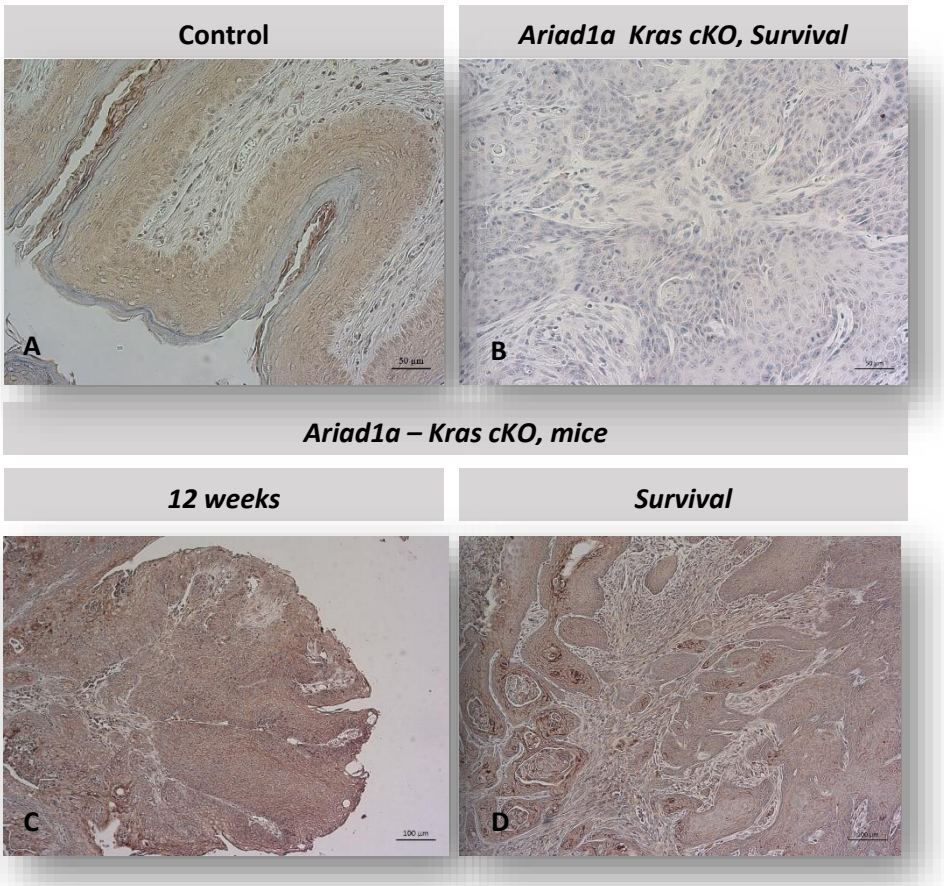

Figure 9: Immunostaining for *Arid1a and Kras^{G12D} oncogene* in vulvar and tumor samples. (A) Control mouse. (B) Negative staining for *Ariad1a Kras cKO, Survival mouse.* (C and D) Positive Imunostaining for *Kras^{G12D}* oncogene mouse uterus as

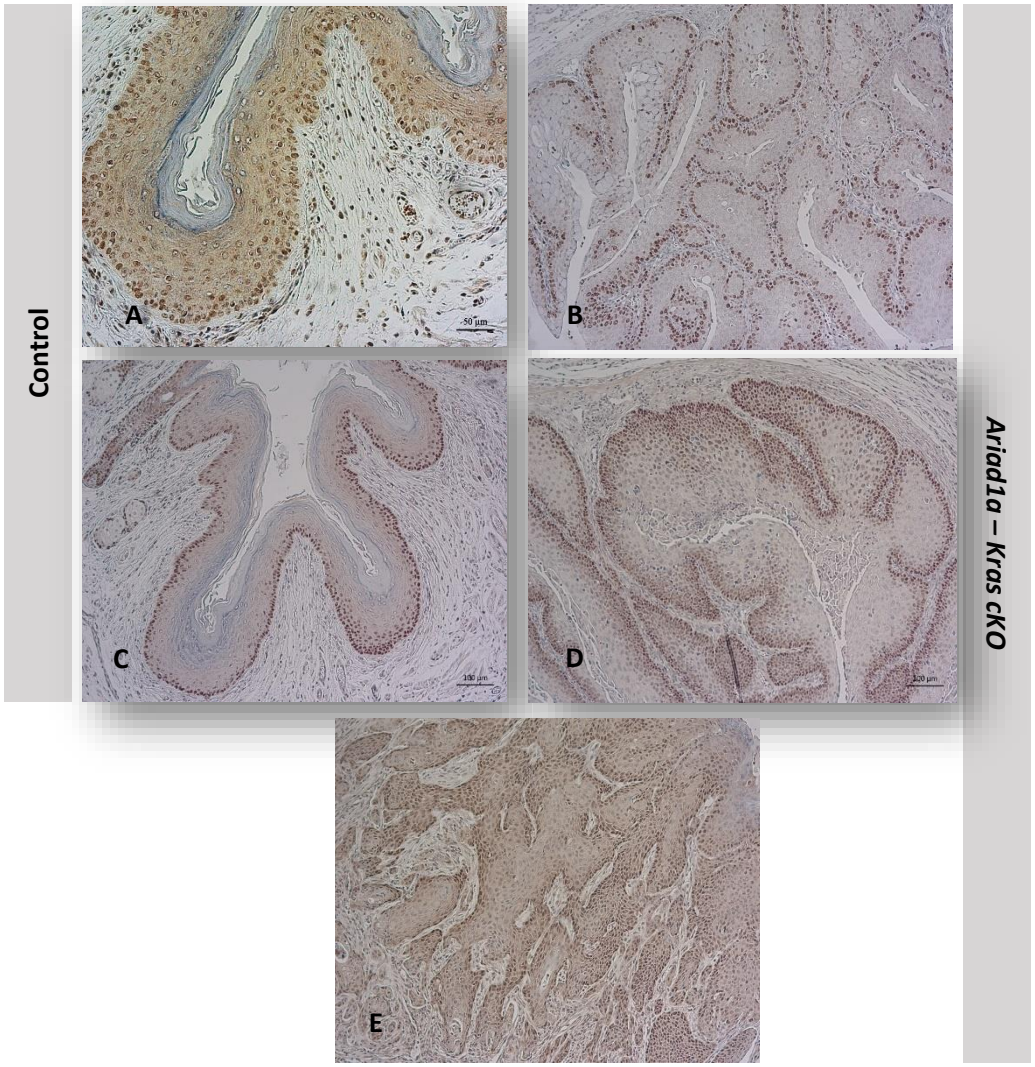

Figure :10 Vulvar and tumor samples Immunostaining for Ki-67, (A), control mice; (B), 8 weeks *Arid1a-Kras cKO mouse. (C – E),* observe positive immunostaining for p63 (C and D), (C) Control mouse, (D - E), 12weeks and survival mice, respectively.

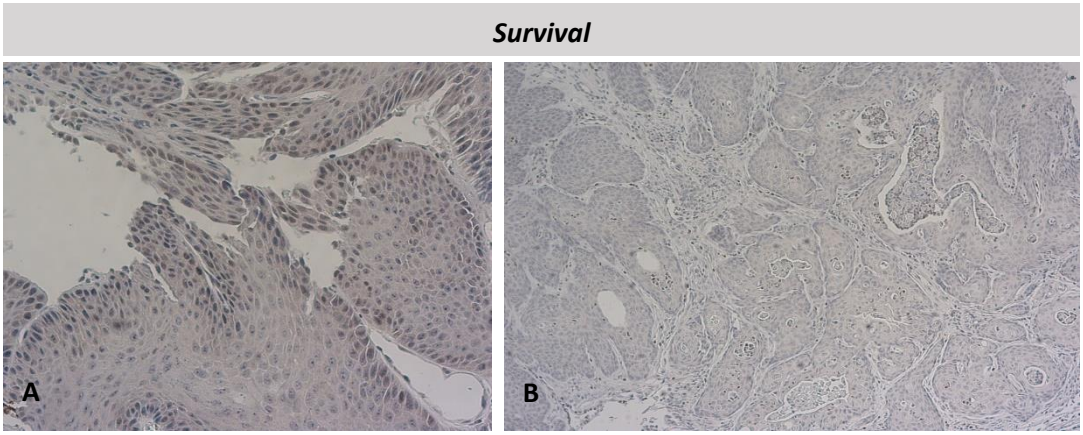

Figure 11: Tumor samples Immunostaining for p53, (A), positive; (B) negative. Both survival *Arid1a-Kras cKO mice.*