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DISSERTAÇÃO DE MESTRADO

ANÁLISE DA EXPRESSÃO GÊNICA EM SUÍNOS NORMAIS E AFETADOS COM HÉRNIA ESCROTAL

WILLIAM RAPHAEL LORENZETTI

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Zootecnia, Área de Concentração Ciência e Produção Animal, da Universidade do Estado de Santa Catarina (UDESC), como requisito parcial para obtenção de grau de **Mestre em Zootecnia**

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ANÁLISE DA EXPRESSÃO GÊNICA EM SUÍNOS NORMAIS E AFETADOS COM HÉRNIA ESCROTAL

Elaborada por
WILLIAM RAPHAEL LORENZETTI

como requisito parcial para obtenção do grau de
Mestre em Zootecnia

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RESUMO
Dissertação de Mestrado
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

ANÁLISE DA EXPRESSÃO GÊNICA EM SUÍNOS NORMAIS E AFETADOS COM HÉRNIA ESCROTAL

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Chapecó, 28 de Fevereiro de 2018

A hérnia escrotal é uma malformação congênita frequente na produção de suínos, caracterizada pela abertura anormal do anel inguinal e que permite a passagem de alças intestinais ao saco escrotal. Essa condição inicia-se no período fetal e envolve modificações anatomofisiológicas da descida testicular. A hérnia causa dor, desconforto e reduz o desempenho do animal afetado, acarretando grandes perdas econômicas na suinocultura. Buscou-se, portanto, identificar genes relacionados com a ocorrência desta patologia por meio de estudos de expressão gênica quantitativa (qPCR) no tecido do anel inguinal de suínos normais e afetados com hérnia escrotal. Primeiramente, a estabilidade de expressão de 10 genes normalmente utilizados como referência foi analisada em diferentes condições experimentais: 1) leitões com 30 dias de idade da linhagem MS115 e 2) suínos de 60 dias de idade da raça Landrace. Amostras teciduais do anel inguinal foram colhidas após eutanásia dos leitões, congeladas em nitrogênio líquido e submetidas à extração do RNA total e síntese de cDNA. Os resultados de expressão gênica dos grupos amostrais foram analisados por meio das ferramentas geNorm (SLqPCR), NormFinder, BestKeeper e método ΔCt e uma listagem geral foi estabelecida com o uso da ferramenta BruteAggreg. Os genes *RPL19, RPL32* e *H3F3A* demonstraram as melhores estabilidades de expressão aos 30 dias e o *PPIA* e *RPL19* aos 60 dias de idade e foram considerados adequados genes de referência para as condições estudadas. Posteriormente, foram avaliados 17 genes candidatos para a ocorrência da hérnia escrotal, a partir de amostras teciduais de 18 suínos machos inteiros da raça MS115 com 30 dias de idade, agrupados em normais (n=9) e afetados (n=9) com hérnia escrotal. Os genes escolhidos para a análise de expressão foram identificados em estudo prévio do transcriptoma do anel inguinal de suínos com 60 dias de idade, normais e afetados com hérnia escrotal. Considerando o nível de expressão no transcriptoma e algumas funções biológicas, 17 genes alvos foram avaliados: *MYH1, DES, TNNI1, ACAN, ACTG2, ACTA1, FGF1, MMP1, FMOD, FBN2, ADCY5, MYBPC1, MAP1LC3C, GUSB, MYH7, COL23A1* e *CNN1*. Os *primers* para cada gene foram desenhados a partir da sequência do genoma suíno (*Sus scrofa*) depositada no GenBank. A quantificação relativa dos genes alvos foi realizada por qPCR e os valores do ciclo de amplificação (Cts) dos genes foram obtidos. O programa REST® (Relative Expression Software Tool) foi utilizado para comparar a expressão gênica entre os grupos experimentais por meio de uma análise não paramétrica e os genes *RPL19, RPL32* e *H3F3A* foram utilizados como constitutivos. Os genes *MYH1, DES* e *TNNI1* ($p \leq 0,05$) e *ACTA1* e *MYH7* ($p \leq 0,08$) apresentaram menores níveis de expressão no grupo afetado comparado ao grupo controle. A menor expressão destes genes nos animais afetados pode estar relacionada ao aparecimento da hérnia escrotal em suínos pela reduzida capacidade de sustentação na região inguinal. Esses resultados contribuem para o melhor entendimento do mecanismo genético envolvido no aparecimento da hérnia escrotal.

Palavras-chave: Genes, Canal inguinal, qPCR, Descida testicular.

ABSTRACT
Master's Dissertation
Programa de Pós-Graduação em Zootecnia
Universidade do Estado de Santa Catarina

GENE EXPRESSION ANALYSIS OF NORMAL AND SCROTAL HERNIA-AFFECTED PIGS

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Chapecó, 28 de Fevereiro de 2018

The scrotal hernia is a common congenital malformation in pig production, characterized by the abnormal opening of the inguinal ring, which allows the passage of intestinal loops to the scrotal sac. This condition begins in the fetal period and involves anatomopathological changes of the testicular descent. Hernia causes pain, discomfort and reduces the performance of the affected animal, causing great economic losses in pig production. Therefore, we aimed to identify genes related to the occurrence of this pathology through quantitative gene expression (qPCR) in the inguinal ring/canal tissue of normal and scrotal hernia-affected pigs. Firstly, the expression stability of 10 genes normally used as reference was analyzed under different experimental conditions: 1) MS115 30 days-old piglets and 2) Landrace 60 days-old pigs. After the piglets euthanasia, the inguinal tissue samples were collected, frozen in liquid nitrogen and subjected to total RNA extraction and cDNA synthesis. The gene expression results were analyzed using the geNorm (SLqPCR), NormFinder, BestKeeper and the ΔCt method tools, and a general rank was established using the BruteAggreg tool. The *RPL19, RPL32* and *H3F3A* genes demonstrated the best expression stability at 30 days and the *PPIA* and *RPL19* at 60 days of age, being considered suitable reference genes for the conditions studied. Moreover, 17 candidates genes for the occurrence of scrotal hernia were evaluated from tissue samples of 18 30-days-old MS115 entire male piglets, grouped in normal (n=9) and affected (n=9) with scrotal hernia. The genes chosen for the expression analysis were identified in a previous study of the inguinal ring transcriptome of 60 days-old pigs, normal and affected with scrotal hernia. Considering the level of expression in the transcriptome and biological functions, 17 genes were evaluated: *MYH1, DES, TNNI1, ACAN, ACTG2, ACTA1, FGF1, MMP1, FMOD, FBN2, ADCY5, MYBPC1, MAP1LC3C, GUSB, MYH7, COL23A1* end *CNN1*. The primers for each gene were drawn from the swine genome sequence (*Sus scrofa*) deposited in the Ensembl. The relative quantification of the target genes was performed by qPCR and the genes amplification cycle (Cts) values were obtained. The REST® program (Relative Expression Software Tool) was used to compare the experimental groups with a non-parametric analysis and the genes *RPL19, RPL32* and *H3F3A* were used as housekeeping genes. The *MYH1, DES* and *TNNI1* ($p \leq 0.05$) and *ACTA1* and *MYH7* ($p \leq 0.08$) genes were downregulated in the affected compared to the control group. The lower expression of these genes in the affected animals can be related to the appearance of scrotal hernia in pigs by the reduced support in the inguinal region. These results contribute to a better understanding of the genetic mechanism involved in the development of scrotal hernias.

Key words: Genes, Inguinal canal, qPCR, Testicular Descent.

SUMÁRIO

1. CAPÍTULO I

REVISÃO DE LITERATURA

1.1 SUINOCULTURA

O suíno doméstico, uma das mais importantes espécies de produção, após o sequenciamento do seu genoma, vem sendo amplamente utilizado como modelo biológico para compreensão de doenças na área humana (GROENEN et al., 2012). A domesticação do suíno, iniciada gradativamente a cerca de 10 mil anos em regiões da Europa e China, gerou uma gama de genótipos e, consequentemente, de fenótipos, observados nos suínos modernos que são essencialmente diferentes dos animais selvagens, mas que visava a seleção de genes que afetam, por exemplo, imunidade, docilidade, comportamento e crescimento (GROENEN, 2016). As preferências humanas, por meio de seleção artificial, alteraram significativamente a frequência de determinados alelos e atributos de interesse em populações domesticadas. Atualmente, a exploração de características fenotípicas através da identificação de genes ou QTL (*quantitative trait loci*) por meio de tecnologias genômicas possibilitam rápidas aplicações na produção animal como forma de facilitar a melhoria genética (ERNST; STEIBEL, 2013).

Quanto ao mercado brasileiro de produção suinícola, no último relatório anual da ABPA (2017) consta que o ano de 2016 encerrou com a produção de carne suína em cerca de 3.731 milhões de toneladas. No entanto, mesmo com aumento nas exportações, observou-se quedas no consumo per capita interno, atualmente estimado em 14,4 Kg/hab. Por outro lado, o USDA (2017) apontou uma expansão para o ano de 2018 de 2,5 %, ou seja, a produção de carne suína brasileira pode ultrapassar 3.9 milhões de toneladas. Contudo, foi observada uma redução geral de exportações da carne suína brasileira, especialmente para a Rússia, mas houve melhora na remuneração em R$/Kg vendido (CEPEA/ESALQ, 2017). Existe, portanto, a necessidade de maiores esforços para estancar perdas produtivas rotineiras.

A combinação da genética molecular com o melhoramento genético de suínos ampliou consideravelmente a compreensão dos mecanismos envolvidos em características de interesse, como por exemplo, a qualidade de carne (DAVOLI; BRAGLIA, 2007). Nessa concepção, com o aperfeiçoamento das tecnologias de seleção busca-se constante

avanço em índices produtivos e reprodutivos, bem como, solucionar problemas anatômicos para atender exigências de mercado (ABCS, 2017). No entanto, vários problemas ainda afetam a produção de suínos, entre eles o criptorquidismo e hérnias escrotais e umbilicais, considerados alguns dos defeitos congênitos mais frequentes nas criações comerciais (MATTSSON, 2011) e causam dor, desconforto, estresse e comprometem o desempenho zootécnico dos animais acometidos (GRINDFLECK et al., 2006) que se manifesta, geralmente, durante a idade de desmame (MANALAYSAY et al., 2016). Assim, compreender os mecanismos embriológicos envolvidos na formação, descida e posicionamento final dos testículos no interior do escroto podem contribuir de maneira significativa para o esclarecimento das possíveis alterações genéticas que levam ao aparecimento da hérnia escrotal em suínos.

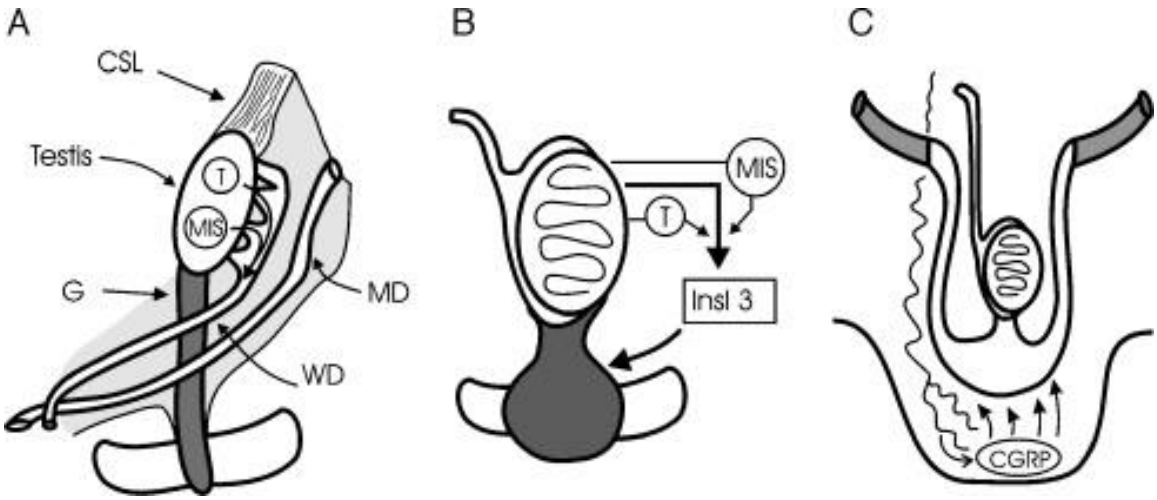
1.2 HÉRNIAS E DESCIDA TESTICULAR: EMBRIOLOGIA

De maneira geral, a formação das gônadas é inicialmente semelhante para machos e fêmeas logo nas primeiras semanas de gestação. As estruturas reprodutivas surgem na parede corporal dorsal do concepto (PATTEN, 1948), junto aos órgãos excretores do mesoderma intermediário (folheto embrionário) durante o estádio indiferenciado da organogênese (NODEN; LAHUNTA, 1990). Em seguida, um grupo de células invade a crista gonadal dos mesonefros, uma estrutura que origina os testículos (HUTSON et al., 2015). As gônadas aparecem como sulcos germinativos adjacentes aos mesonefros e a diferenciação celular proporciona o estabelecimento dos aparelhos urinário e genital e demais estruturas reprodutivas (NODEN; LAHUNTA, 1990). A partir deste estágio, os eventos relacionados à descida testicular são formados por fases anatômicas e hormonais conhecidas como transabdominal e inguinoescrotal (HUGHES; ACERINI, 2008; HUTSON et al., 2015; HUTSON; HASTHORPE, 2005a; MAMOULAKIS et al., 2015), atuantes de forma independente para cada gônada (HUTSON; HASTHORPE, 2005b). Estas etapas são melhor estudadas em humanos e ratos (Figura 1A), principais modelos utilizados para o entendimento do processo na espécie suína.

A etapa embriológica anterior ao movimento dos testículos até sua posição no saco escrotal consiste da diferenciação sexual. A produção dos hormônios INLS3 (*insulin-like 3*) pelas células de Leydig (AMANN; VEERAMACHANENI, 2007; MAMOULAKIS et al., 2015) e AMH (anti-Mulleriano) e testosterona pelas células de Sertoli do testículo em formação, provocam o desenvolvimento dos ductos de Wolff

(EMMEN et al., 2000; HUGHES; ACERINI, 2008), que se conectam às gônadas e originam o epidídimo. Essas estruturas são ancoradas ao diafragma do embrião pelo ligamento crânio-suspensório e, a nível caudal, pelo gubernáculo (AMANN; VEERAMACHANENI, 2007). O primeiro ligamento regride, enquanto o segundo se expande e invade a musculatura inguinal através de um "inchaço" (AMANN; VEERAMACHANENI, 2007; HUTSON et al., 2015) regulado por *INSL3* (EMMEN et al., 2000), o qual também medeia a descida testicular transabdominal (BAY; ANDERSSON, 2011; HUTSON; HASTHORPE, 2005b).

Figura 1: Etapas embriológicas dos eventos relacionados à descida testicular e passagem dos testículos através do canal inguinal.



Legenda: (A) Fase de surgimento das gônadas e diferenciação sexual; CSL: ligamento suspensório craniano; T: testosterona; G: gubernáculo; MD: ducto Mulleriano; WD: ducto de wolff; MIS: hormônio anti-mulleriano; (B) Fase transabdominal; INSL3: hormônio insulin-like 3; (C) Fase inguinoescrotal; CGRP: neurotransmissor calcitonin gene-related peptide.

Fonte: HUTSON, J. M.; HASTHORPE, S. Testicular descent and cryptorchidism: The state of the art in 2004. **Journal of Pediatric Surgery**, v. 40, n. 2, p. 297–302, 2005.

1.2.1 Fase intra-abdominal ou transabdominal

Preso à parede abdominal inguinal, a região distal gubernacular, conhecida como bulbo gubernacular engrossa com a rápida multiplicação das células mesenquimais e acúmulo de moléculas hidrofílicas de glicosaminoglicanos (MAMOULAKIS et al., 2015)

e ácido hialurônico (BECK et al., 2006) em seu interior. O gubernáculo apresenta um conteúdo mesenquimatoso ao centro, sendo recoberto por uma camada de mioblastos (EMMEN et al., 2000). Nesse sentido, os músculos que rodeiam o bulbo se modificam para formar o canal inguinal (HUTSON et al., 2015). Juntamente ao processo de expansão do bulbo gubernacular, ocorre uma herniação do peritônio, ou seja, a formação do *processus vaginalis* (HUTSON et al., 2015; MAMOULAKIS et al., 2015) que irá conceber as gônadas (Figura 1B). As modificações estruturais atravessadas pelo gubernáculo durante a migração testicular direcionam à redução de seu tamanho e composição tecidual e o tornam menos rígido (COSTA et al., 2002), controladas por hormônios produzidos pelo próprio testículo em desenvolvimento (BAY et al., 2011).

A presença de andrógenos pode modificar a composição do gubernáculo, bem como influenciar a liberação de metaloproteases pelos fibroblastos gubernaculares que atuam na degradação da matriz extracelular do gubernáculo durante a descida testicular (VIGUERAS et al., 2004). Essas mudanças são fortemente relacionadas à presença do hormônio anti – Mulleriano (KISTAMAS et al., 2013) e produção de testosterona pela gônada em desenvolvimento (AMANN; VEERAMACHANENI, 2007; CLARNETTE; HUTSON, 1996). É também postulado que a descida testicular envolve a pressão abdominal criada e exercida pela expansão de órgãos em conjunto com a atividade e mudanças do gubernáculo (FIEGEL et al., 2011; HUGHES; ACERINI, 2008; HUSMANN; LEVY, 1995; MAMOULAKIS et al., 2015).

1.2.2 Fase extra-abdominal ou inguinoescrotal

No momento em que o testículo é posicionado em seu trajeto natural de descida, modificações estruturais ocorrem no espaço que a gônada percorre. Nessa fase que é dependente de andrógenos (HUGHES; ACERINI, 2008; HUTSON et al., 2015; MAMOULAKIS et al., 2015) e da atividade do nervo genitofemoral (LIE; HUTSON, 2011), células do mesênquima extra-abdominal do gubernáculo continuam a se proliferar em resposta ao efeito do neurotransmissor do nervo genitofemoral, o CGRP (Peptídeo relacionado com o gene da calcitonina) (NG et al., 2009; SHENKER et al., 2006) e direcionam testículo e epidídimo a deslizarem pelo trajeto inguinal (PATTEN, 1948), motivado pelo encurtamento do ligamento gubernacular e sob possível influência do hormônio anti-Mulleriano (HUTSON; LOPEZ-MARAMBIO, 2017). A consequência dessa multiplicação colapsa o *processus vaginalis* e, como resultado, tem-se um

engrossamento do saco escrotal e a formação das túnicas vaginais (membranas de revestimento) que recobrem internamente o saco escrotal (NODEN; LAHUNTA, 1990), formadas pelo tecido remanescente resultante da degradação parcial do gubernáculo na porção extra-abdominal (CHURCHILL et al., 2011). Nos suínos, a etapa inguinoescrotal de descida testicular ocorre por volta dos 100 – 110 dias de gestação. Assim, ao fim do processo, os testículos são dispostos em sua posição anatômica natural (Figura 1C). Importantes modificações são necessárias para que o saco escrotal receba os testículos em desenvolvimento. Contudo, juntamente ao processo fisiológico natural, existem outros fatores pouco conhecidos que podem desencadear a hérnia escrotal.

1.2.3 Fatores predisponentes à herniação

Segundo Brandt (2008), as hérnias inguinais são provocadas pela menor integridade estrutural da parede abdominal e fraqueza da região inguinal, atrelados à descida testicular e obliteração incompleta da invaginação no processo embriológico. Em humanos, o fechamento do anel inguinal esquerdo precede o direito e por vezes a doença da hérnia pode ser unilateral (BRANDT, 2008). Além disso, certos distúrbios ligados ao metabolismo e hidrólise de colágeno, estrutura de fibras musculares e demais componentes da matriz extracelular (BENDAVID, 2004) poderiam comprometer a reparação de tecido conectivo pelos fibroblastos (FRANZ, 2008) e essa pouca resistência na região inguinal evoluir para hérnia escrotal (BEUERMANN et al., 2009).

Entretanto, as informações anteriores são relacionadas à estrutura da parede abdominal que podem explicar, apenas em parte, a ocorrência da hérnia escrotal propriamente dita. Provavelmente, a manifestação da hérnia escrotal esteja fundamentada no *processus vaginalis*. É aceito que o fechamento do processo vaginal seja controlado pela atividade do nervo genitofemoral através de seu neurotransmissor CGRP (AMANN; VEERAMACHANENI, 2007; HUGHES; ACERINI, 2008; HUTSON et al., 2015; LIE; HUTSON, 2011). Em tecidos gubernaculares de ratos tratados com agentes bloqueadores da sua atividade, o neurotransmissor CGRP regulou a multiplicação celular do bulbo gubernacular (NG et al., 2005; SHENKER et al., 2006; YONG et al., 2008) e evitou a ocorrência da apoptose (CHAN et al., 2009). Além disso, é possível que a atrofia gerada possa comprometer as inervações e causar danos musculares progressivos na musculatura da região inguinal (AMATO et al., 2011; AMATO et al., 2012) e essa particularidade poderia influenciar a atividade das inervações que controlam o *processus vaginalis*.

Beuermann et al. (2009) demonstraram, a partir de tecidos urogenitais de leitões herniados ou criptorquidas que níveis reduzidos de cálcio poderiam interferir nos processos de sinalização celular, encerramento da invaginação e apoptose incompleta. A presença de células não completamente diferenciadas conhecidas como miofibroblastos na região inguinal também pode ser um indício da tentativa de apoptose da musculatura inguinal durante o período embriológico, que parece ser indispensável à oclusão do processo vaginal (TANYEL et al., 2001; MOURAVAS et al., 2010).

1.3 COMPONENTES GENÉTICOS ASSOCIADOS ÀS HÉRNIAS EM SUÍNOS

Os componentes genéticos envolvidos na manifestação da hérnia escrotal precisam ser melhor desvendados. Foram relatadas frequências de ocorrência para esta malformação de 0,6 a 1,5 % entre algumas raças puras, especialmente Landrace (MATTSSON, 2011; SEVILLANO et al., 2015; VOGT; ELLERSIECK, 1990) e embora a herdabilidade para a hérnia escrotal tenha sido estimada entre 0,15 a 0,86 (VOGT; ELLERSIECK, 1990; TAYLOR, 1995; MIKAMI; FREDEEN, 1979; MAGEE, 1951; SEVILLANO et al., 2015), dependendo do cruzamento, podem ocorrer oscilações entre as populações ao longo dos anos. Além disso, apesar da hérnia escrotal ocorrer somente em machos, os genes potencialmente relacionados a anomalia também são transmitidos através das fêmeas de forma hereditária, sendo necessário um processo de seleção contra essas matrizes (MAGEE, 1951; MANALAYSAY et al., 2016).

De maneira geral, é indicado como estratégia para redução da hérnia escrotal a castração cirúrgica ou mesmo a eliminação de reprodutores cujas progênies são altamente afetadas, desde que a incidência seja suficientemente elevada (TAYLOR, 1995). Anotações sobre leitegadas afetadas, acompanhamento nas fases subsequentes de criação e informações sobre reprodutores machos e fêmeas dentro da produção têm relevância direta para elaboração de estudos aplicados ao melhoramento genético e formas de redução do problema. No entanto, a manifestação clínica da doença é variável e a confirmação da anomalia logo nos primeiros dias de vida do suíno pode ser confusa.

Estudos de associação genômica ampla (GWAS) antes conduzidos averiguaram a ocorrência de defeitos congênitos que são de baixa incidência. Grindflek et al. (2006) descreveram que regiões de QTLs detectadas em 7 cromossomos (SSC1, 2, 5, 6, 15, 17 e X), bem como alguns genes (*INLS3, MIS* e *CGRP*), foram significativamente envolvidos com a ocorrência de hérnia escrotal, confirmados por meio de testes de desequilíbrio de

transmissão (TDT) e pares de meio-irmãos afetados (ASP). Já Sevillano et al. (2015), a partir da genotipagem em alta densidade de suínos Landrace e Large White e a fenotipagem de descendentes puros e mestiços, constataram que a ocorrência da hérnia escrotal/inguinal em Large White foi superior ao Landrace (0,42 % vs. 0,34 %), além de uma maior frequência em leitegadas de raça pura. No entanto, Du et al. (2009) apontaram que as taxas de hérnias foram menores nas linhas puras. Ainda, Sevillano et al. (2015) detectaram que 10 polimorfismos de base única (SNPs) em cromossomos da raça Large White (SSC 3, 5, 7, 8 e 13) e 22 SNPs em cromossomos da raça Landrace (SSC 1, 2, 4, 10 e 13), presentes em diferentes regiões de QTLs, podem influenciar a incidência de hérnia escrotal nas populações estudadas e, dessa forma, contribuir para seleção genética, a fim de se evitar tal segregação. Adicionalmente, regiões significativas nos cromossomos SSC 2, 4, 8 (lócus *SW 933*), 13 e 16 já foram associadas como suscetíveis à ocorrência de hérnia escrotal, mas interações entre genes, populações e ambiente podem prejudicar a detecção de QTLs (DING et al., 2009).

Além de regiões cromossômicas e QTLs relacionados à hérnia escrotal, alguns genes que poderiam estar envolvidos na manifestação da doença foram previamente investigados, incluindo *GUSB* (β-glucuronidase) (BECK et al., 2006), *HOXA10* (Homeobox A10), *ZFPM2* (Zinc finger protein multitype 2), *COL2α1* (Collagen type II α1) e *MMP2* (Matrix metallopeptidase 2) (ZHAO et al., 2009), *COL23A1* (Collagen type XXIII alpha 1 chain) e *ELF5* (E74 like ETS transcription factor 5) (DU et al., 2009) e *INSL3* (Leydig insulin-like hormone) (KNORR et al., 2004). Recentemente, identificou-se uma maior frequência da hérnia escrotal em reprodutores da raça Pietran, portadores de uma mutação no gene *BAX* (proteína X associada ao *BCL2*) envolvido em apoptose (MANALAYSAY et al., 2016). Por ser uma doença de caráter hereditário, a regulação e ocorrência pode ter origem poligênica (ELANSARY et al., 2015; VOGT; ELLERSIECK, 1990; TAYLOR, 1995; THALLER; DEMPFLE; HOESCHELE, 1996). Portanto, novas buscas por mais genes candidatos podem contribuir significativamente para revelar as associações genéticas que causam o fenótipo da hérnia escrotal.

1.4 GENES CANDIDATOS À HERNIA ESCROTAL

Os genes candidatos são conhecidos por estarem envolvidos no desenvolvimento e fisiologia de determinadas características. Ao serem identificados, é possível associar as funções desempenhadas às características de interesse, importantes para o melhoramento

genético. Em geral, caracteres quantitativos têm grande interesse econômico para indústria de suínos, regulados por complexos sistemas que podem ser influenciados por inúmeros genes ou QTLs (ERNST; STEIBEL, 2013). Porém, apenas poucos genes são conhecidos, bem como os efeitos gerais em meio às possíveis variantes (COUTINHO et al., 2010), o que motiva a busca por uma maior compreensão dos processos fisiológicos vinculados aos genes.

A partir de um estudo desenvolvido na Embrapa Suínos e Aves, onde foram gerados os transcriptomas de suínos normais e afetados com hérnia escrotal aos 60 dias de idade, foi identificado um conjunto de genes expressos na região inguinal. Com base nas diferenças de expressão gênica entre animais normais e afetados e seu envolvimento em funções biológicas, foram selecionados 17 genes para investigação do diferencial de expressão entre grupos de suínos normais e afetados aos 30 dias de idade, que possibilitem avançar no conhecimento dos mecanismos genéticos envolvidos na hérnia escrotal. A maioria dos genes selecionados e descritos a seguir ainda não foram relatados em estudos com suínos ou mesmo em casos de ocorrência da herniação.

1.4.1 Matriz metalopeptidase 1 *(MMP1*)

O gene da Metalopeptidase de matriz 1 *(MMP1)* está posicionado no cromossomo 9 de suínos (NCBI, 2018). O gene *MMP1* codifica um tipo de colagenase e é expresso em diversos tipos celulares como fibroblastos, osteoblastos e macrófagos de humanos e atua basicamente na clivagem de colágenos (ALA-AHO; KÄHÄRI, 2005; BENDAVID, 2004). As *MMPs* são proteases pertencentes a uma família de endopeptidases dependentes de zinco para sua atividade, que atuam no *turnover* e degradação proteica da matriz extracelular (MANNELLO; MEDDA, 2012; TOCCHI; PARKS, 2013). Estas proteases estão relacionadas a inúmeros processos fisiológicos de sinalização celular, reparação e remodelação de tecidos e exibem atividade catalítica de substratos específicos (RA; PARKS, 2007), onde a regulação e ativação pode estar atrelada aos glicosaminoglicanos (GAGs) (TOCCHI; PARKS, 2013), como o ácido hialurônico. Essas proteases degradam os colágenos, elastina, fibronectina e conteúdos de matriz extracelular (BENDAVID, 2004) durante a remodelação do gubernáculo e podem enfraquecer sua estrutura (CHURCHILL et al., 2011), que é semelhante a um esfíncter (AMATO et al., 2009). A expressão em tecidos normais é reduzida e pouco detectável, mas se torna aumentada em situações de cicatrização ou patológicas (PARDO; SELMAN,

2005). Um outro gene dessa mesma família, o *MMP2,* foi considerado potencialmente associado à ocorrência da hérnia escrotal em suínos (ZHAO et al., 2009).

1.4.2 Beta glucoronidase (*GUSB*)

O gene da protease que codifica a β-glucuronidase *(GUSB)* está localizado no cromossomo 3 do suíno (BECK et al., 2002; NCBI, 2018). A protease β-glucuronidase presente em lisossomas de muitos tecidos que atuam na hidrólise de glicosaminoglicanos (GAGs), tais como o ácido hialurônico (NAZ et al., 2013). A deficiência da hidrolase β-glucuronidase é associada ao acúmulo de GAGs e também à Síndrome de Sly - mucopolissacaridose VII, uma doença autossômica recessiva causada pelas várias possíveis mutações (TOMATSU et al., 2009) que desestabilizam a estrutura e reduzem a atividade da protease β-glucuronidase (KHAN et al., 2016) e inclui, entre outros sinais clínicos, o acúmulo de GAGs no líquido amniótico fetal durante a gestação em indivíduos humanos (KUBASKI et al., 2017). Segundo Beck et al. (2006), o gubernáculo infla com a deposição de ácido hialurônico (HA) em seu interior e, dessa forma, facilita a passagem do testículo por abrir o canal inguinal, ao passo que possíveis acúmulos ou falhas na degradação enzimática de HA pela β-glucuronidase aumentariam a abertura normal do canal inguinal, predispondo à hérnia inguinal/escrotal. Os mesmos autores confirmaram que o gene *GUSB,* anteriormente tratado como candidato hereditário posicional à malformação, pôde ser isentado como agente causal da doença de hérnia escrotal e ressaltam que a avaliação de doenças complexas a partir de um único gene pode reduzir a capacidade analítica.

1.4.3 Actina gama 2 do músculo liso entérico (*ACTG2*)

O gene Actina gama 2 (*ACTG2*) está envolvido na síntese de proteínas da classe das actinas, importantes para movimentação das células e manutenção do citoesqueleto (GENECARDS, 2017). Este gene é expresso em células musculares e não musculares (DOMINGUEZ; HOLMES, 2011) e está mapeado no cromossomo 3 de *Sus scrofa* (NCBI, 2018). O gene foi associado por Wangler et al. (2014) à doenças do músculo liso em humanos, por causarem desordens que afetam o intestino e bexiga. Alterações nas sequências de aminoácidos causam mutações no interior da proteína expressa pelo gene, dificultam a polimerização dos filamentos finos de actina e tornam a musculatura lisa

pouco contrátil na ocorrência de doenças viscerais, como a miopatia visceral familiar (LEHTONEN et al., 2012) e a síndrome de megabexiga microcolon e hipoperistaltismo intestinal em humanos (THORSON et al., 2014).

1.4.4 Miosina de cadeia pesada 1 (*MYH1*)

Identificado no cromossomo 12 de *Sus scrofa, o* gene *MYH1* codifica um tipo de cadeia pesada de miosina (NCBI, 2018). A miosina é uma proteína envolvida na contração muscular que apresenta isoformas nos variados tipos celulares (GENECARDS, 2017). O *MYH1* é também conhecido como *MyHC-2x* e demonstra características intermediárias entre fibras musculares rápidas tipos 2A e 2B (DENARDI et al., 1993) que exibem metabolismo glicolítico ou oxidativo. Além disso, a cadeia pesada de miosina interage com os filamentos finos de actina no movimento muscular (LIE; HUTSON, 2011), sendo a proteína de maior quantidade no músculo esquelético (LEFAUCHEUR, 2010). A expressão do gene *MYH1* foi maior na musculatura esquelética de leitões desmamados provenientes de uma linhagem suína Landrace que apresentava menor taxa de crescimento comparado a uma linhagem de elevada taxa crescimento da mesma raça (KOMATSU et al., 2016). A expressão desse gene está relacionada ao tipo de miofibra, que é mais oxidativa e com maior presença de MyHC-2x em raças de crescimento lento, como Meishan e Lantang, e glicolítica (maior incidência de fibras musculares MyHC-2b) em raças com maior taxa de crescimento, tal como Large White e Landrace (XU et al., 2009; LI et al., 2013), entre àqueles genótipos asiáticos ou ocidentais. Mudanças estruturais musculares são relacionadas aos padrões de expressão e síntese da miosina, os quais se alteram e adaptam-se ao tipo de desafio exposto na formação de tecidos especializados, de acordo com as funções e metabolismo muscular (EIZEMA et al., 2007), sendo a diversidade funcional das fibras musculares puras e híbridas afetadas por vários fatores, conexas às isoformas da cadeia pesada de miosina (PETTE; STARON, 2000; WEISS; LEINWAND, 1996).

1.4.5 Fator de crescimento de fibroblastos 1 (*FGF1*)

O gene *FGF1* está mapeado no cromossomo 2 do genoma suíno (NCBI, 2018) e que forma uma subfamília com *FGF2* (ORNITZ; ITOH, 2015). Ambos os genes pertencem a uma ampla família de fatores de crescimento de fibroblastos (*FGFs*) que

estão envolvidos em inúmeros processos biológicos como proliferação, crescimento, sobrevivência celular e morfogênese, induzidas através de ligações aos seus receptores de tirosina quinase (FGFRs 1-4) presentes na superfície celular (DOREY; AMAYA, 2010; ORNITZ; ITOH, 2015; THISSE; THISSE, 2005) e que podem ser regulados por micro RNAs (miRNA) que afetam a expressão de *FGFs* ou *FGFRs* durante a diferenciação celular (ORNITZ; ITOH, 2015). A sinalização celular defeituosa pode anular a diferenciação das células embrionárias e assim comprometer a correta montagem da matriz extracelular (LI et al., 2001). Além disso, por meio de suas proteínas, *FGF1/FGF2* integram um grupo de proteínas extracelulares que agem de forma parácrina e que são biologicamente ativas por meio dos receptores *FGFRs* (ITOH; ORNITZ, 2011). Para tal, a ativação dos *FGFRs* é dependente de heparina/sulfato de heparano como cofatores (ITOH; ORNITZ, 2011; ORNITZ; ITOH, 2015). Mutações envolvidas nesse grupo de genes (*FGFs*) também podem causar doenças ósseas (DU et al., 2012).

1.4.6 Desmina (*DES*)

O gene Desmina *(DES)* está presente no cromossomo 15 na espécie suína. (NCBI, 2018). A proteína desmina, codificada pelo gene *DES*, é encontrada principalmente nos discos Z da musculatura estriada, envolvida no arranjo e organização celular e transmissão da força contrátil, além de estar associada a doenças musculares congênitas quando ausente (PAULIN; LI, 2004), onde os tecidos musculares apresentam-se desalinhados. Por ser componente dos filamentos intermediários do citoesqueleto, sugere-se que a desmina, em suas interações, possa influenciar a sobrevivência celular, bem como as funções e distribuição de mitocôndrias (CAPETANAKI, 2002; PAULIN; LI, 2004). Mutações do gene *DES*, que estão vinculadas à natureza de herança e localização da molécula, reduzem a capacidade da desmina em interagir com diferentes estruturas celulares (VAN SPAENDONCK-ZWARTS et al., 2011) e estas mutações estão atreladas à fraqueza muscular progressiva (GOLDFARB et al., 2004). Segundo Capetanaki et al. (2015), a desregulação da proteína desmina pode causar diferentes miopatias e cardiomiopatias, devido aos muitos processos biológicos de integração e coordenação que desempenha no músculo estriado.

1.4.7 Actina alfa 1 do músculo esquelético (*ACTA1*)

Anotado no cromossomo 14 (NCBI, 2018) da espécia suína, o gene da Actina alfa 1 do músculo esquelético (*ACTA1*) codifica uma isoforma de actina, a qual desempenha funções na motilidade, estrutura e integridade celular do músculo esquelético em associação à miosina (GENECARDS, 2017). Essa proteína é abundante no músculo *longissimus* de suínos da raça Meishan em razão da montagem e regulação da característica miofibrilar (XU et al., 2009). Este gene (*ACTA1*) foi indicado como potencial marcador molecular para programas de melhoramento de frangos de corte, visto que apresentou associação significativa com características de órgãos, carcaça e desempenho (VENTURINI et al., 2012). Por ser um gene com sequências altamente conservadas, quaisquer alterações nos aminoácidos no *ACTA1* podem resultar em inúmeras miopatias, motivadas pelas possíveis variantes do gene (LAING et al., 2009), bem como os fenótipos resultantes dessas desordens, que afetam todas as possíveis associações e interações funcionais intrínsecas da actina (FENG; MARSTON, 2009). A maioria das doenças associadas ao *ACTA1* são ligadas a mutações autossômicas dominantes com substituição de aminoácidos (*missense*), embora possam ocorrer variados tipos de mutações em menor intensidade, porém, em todos os casos a actina esquelética é ausente ou existe a incorporação de peptídeos defeituosos que comprometem as propriedades normais dos filamentos finos de actina, com forte propensão a ocorrência de doenças musculares (LAING et al., 2009; NOWAK et al., 2013).

1.4.8 Calponina 1 (*CNN1*)

O gene da Calponina 1 (*CNN1*) está localizado no cromossomo 2 (NCBI, 2018) e compõe uma família proteica de três isoformas, sendo a calponina 1 específica da musculatura lisa (LIU; JIN, 2016). A calponina apresenta capacidade de ligar-se à actina de várias formas em função de suas características moleculares (FERJANI et al., 2010), induz a polimerização e inibem a despolimerização de monômeros nos filamentos finos de actina de forma Ca^{+} dependente (EL-MEZGUELDI, 1996), modulam funções contráteis da musculatura lisa (JIN et al., 2003; TANG et al., 2006; WU; JIN, 2008) e podem desempenhar funções organizacionais do citoesqueleto na musculatura lisa e/ou células não musculares (WU; JIN, 2008; LIU; JIN, 2016). Além disso, a calponina pode se associar com várias outras proteínas do citoesqueleto, como exemplo a desmina, na

montagem dos filamentos intermediários (ROZENBLUM; GIMONA, 2008), os quais conferem resistência mecânica/estrutural às células e sua participação na fisiologia da contração muscular é relacionada ao estado de fosforilação. A interação da calponina com uma PKC (proteína quinase C) resulta em fosforilação, capaz de inibir o fator de supressão à miosina (SOMARA; BITAR, 2008), que facilita a contração do músculo (LIU; JIN, 2016).

1.4.9 Proteína C de ligação com miosina, tipo lento (*MYBPC1*)

O gene da Proteína C de ligação à miosina (*MYBPC1*), presente no cromossomo 5 de *Sus Scrofa* (NCBI, 2018), pertence a uma família de proteínas acessórias (proteína C) de ligação à miosina codificadas por diferentes genes (WEBER et al., 1993). O gene *MYBPC1* (ou sMyBP-C, tipo lento) é expresso tanto em músculos esqueléticos de contração lenta quanto rápida, onde compõem a montagem estrutural das bandas A e M sarcoméricas (ACKERMANN; KONTROGIANNI-KONSTANTOPOULOS, 2010), e realiza interações com os filamentos de actomiosiona (ACKERMANN; KONTROGIANNI-KONSTANTOPOULOS, 2011). Existem pelo menos 14 variantes em humanos (ACKERMANN et al., 2013) e é possível que as variações proteicas dessa família encontradas na musculatura estejam relacionadas com as isoformas da cadeia pesada de miosina (ACKERMANN; KONTROGIANNI-KONSTANTOPOULOS, 2013). A ocorrência de *splicing* causa alterações nas porções terminais (NH_2 e COOH) e internas da molécula e afeta negativamente as interações com os complexos de actomiosina (ACKERMANN et al., 2013).

O MYBP-C tipo lento (*MYBPC1*) é o único gene da família proteica *mybpc* expresso no músculo esquelético antes do nascimento (GAUTEL et al., 1998). Juntamente à falta de movimento articular durante o desenvolvimento esquelético embriológico, o gene pode ser um fator determinante na ocorrência de contraturas congênitas (GURNETT et al., 2010), tal como a miopatia da artrogripose distal 1 (AD-1) (GEIST; KONTROGIANNI-KONSTANTOPOULOS, 2016), uma doença que causa malformações musculares e articulares. A não expressão do gene *MYBPC1* tem como resultado tecidos estruturalmente desorganizados e anomalias graves, como evidenciado por Ha et al. (2013), que através de ensaios com peixe-zebra (*Danio rerio*) demonstrou que a supressão do gene (*knockdown*) durante a embriologia causa anomalias na formação dos tecidos musculares, resposta a estímulos retardada e curvaturas corporais. Em

bovinos jovens com diferentes taxas de crescimento, Tong et al. (2015) discutem que a expressão do gene pode acelerar o crescimento muscular que é induzido pela proliferação de células satélites.

1.4.10 Proteína de ligação aos microtúbulos 1, cadeia leve 3 gama (*MAP1LC3C*)

Posicionado no cromossomo 10 do genoma suíno (NCBI, 2018), o gene *MAP1LC3C* compõe uma variante de proteínas homólogas a Atg8 (proteínas relacionadas à autofagia) da levedura, mas que em mamíferos é subdividida em subfamílias de proteínas LC3 e GABARAP, envolvidas em processos autofagossomais (LEE; LEE, 2016; SHPILKA et al., 2011) ou não-autofagossomais (HUANG; LIU, 2015), que ocorrem de acordo com as interações de um complexo regulatório nucleocitoplasmático, mediadas por condições celulares de estresse nutricional ou excesso de proteínas dobradas, onde o sistema LC3/Atg8 atua como fator crítico na indução de autofagia (HUANG et al., 2015; LEE; LEE, 2016). A autofagia contribui para homeostase metabólica, porém, alterações em moléculas de sinalização poderiam prejudicar tal homeostase, pelo qual algumas patologias podem estar relacionadas a esses processos (SCHNEIDER; CUERVO, 2014). O *knockout* de genes relacionados a autofagia em células provenientes de humanos do tipo HeLa, demonstrou que os autofagossomos se formam em taxas mais lentas na ausência de LC3/GABARAP (PADMAN et al., 2017).

Em comparação com as demais isoformas (*MAP1LC3 A,B*), o *MAP1LC3C* é em geral a forma menos expressa no organismo, porém, os maiores níveis observados por He et al. (2003) encontram-se na placenta, pulmão e ovário de tecidos humanos. Segundo os mesmos autores, a diferença presente entre locais de maior expressão, juntamente à modificações pós-traducionais entre todas as isoformas, pode ser acompanhada de funções diferenciadas em células ou tecidos. Em estudo com diferentes células tumorais, Koukourakis et al. (2015) demonstraram que as variantes LC3A e C estão presentes em grande quantidade no núcleo celular, embora suas funções neste compartimento permaneçam indefinidas. As atividades desempenhadas de algumas variantes dependem das modificações pós traducionais, e a estabilidade dos microtúbulos celulares é especialmente ligada a ação prejudicial da colchicina (NOIGES et al., 2002), um agente antimitótico.

1.4.11 Miosina de cadeia pesada 7 (*MYH7*)

O gene da cadeia pesada da miosina 7 (*MYH7*), mapeado no cromossomo 7 dos suínos, é expresso em fibras musculares lentas esqueléticas e cardíacas de tipo I/β (NCBI, 2018). Uma doença que acomete suínos conhecida como 'síndrome de Campus' (CPS) é relacionada a *MYH7* e foi inicialmente reconhecida em proles de um reprodutor suíno de raça Pietran que demonstravam tremores musculares progressivos e suscetibilidade ao estresse. Tammen et al. (1999) buscaram identificar esses efeitos e demonstraram que a taxa de segregação dessa doença aos descendentes de um reprodutor Pietran com CPS foi de 8,7 %, ou seja, 26 leitões dos 300 desmamados a partir do acasalamento daquele reprodutor com matrizes saudáveis apresentavam a doença. Murgiano et al. (2012) confirmaram em seus testes com suínos o modo de herança como autossomal dominante, onde a inserção dos aminoácidos prolina e alanina no éxon 30 da proteína de miosina prejudica as interações motoras envolvidas. Em geral, os fenótipos associados às mutações dos genes de miosina, como o *MYH7* e descritos para humanos são variados (OLDFORS, 2007), entre os quais estão a cardiomiopatia hipertrófica (MARON et al., 2012; VAN DER LINDE et al., 2017) e a miopatia distal de Laing (DUBOURG et al., 2011; MEREDITH et al., 2004). Além disso, a expressão de *MYH7* foi reduzida no músculo *longissimus dorsi* de leitões da raça Landrace que apresentam menor taxa de crescimento em comparação a um grupo de elevada taxa de crescimento (KOMATSU et al., 2016).

1.4.12 Troponina I tipo 1 (*TNNI1*)

O gene *TNNI1* está posicionado no cromossomo 10 do genoma suíno (NCBI, 2018) e codifica a troponina I (TnI), uma proteína do complexo de troponinas da musculatura esquelética lenta, inibidora da ATPase de actomiosiona que é mediada por cálcio durante a excitação, contração e acoplamento (GENECARDS, 2017). Apesar de ser predominantemente expresso em fibras musculares de tipo lento, foi observada uma elevada expressão de *TNNI1* em músculos *longissimus dorsi* (fibras de contração rápida) e semitendinoso (contração lenta) entre 35 e 60 dias pós-natal para suínos da raça Yorkshire, e aos 120 e 180 dias pós-natal em suínos Meishan, além da grande participação de *TNNI1* no desenvolvimento do coração, co-expresso ao gene *TNNI3* (YANG et al., 2011). Já, no músculo *bíceps femoralis* de suínos Meishan, foi observada

alta expressão de *TNNI1* no período pré-natal, com subsequente queda no período pós-natal, inversamente ao ocorrido em suínos da raça Yorkshire (XU et al., 2010), enquanto que em ovelhas, foi observada uma maior expressão na musculatura intercostal (SUN et al., 2016). Essas diferenças de expressão podem, em parte, ser alguns dos fatores que determinam as características fenotípicas do crescimento dessas espécies.

É bem possível que as relações entre todas as isoformas de troponina tenham um princípio funcional conservado durante o processo evolutivo, uma vez que variações nas porções NH2 e C-terminal da cadeia peptídica, *splicing* alternativo, modificações pós-traducionais (fosforilações, acetilglucosaminações, glutationilações) e demais mutações podem afetar as interações com outras proteínas, especialmente a tropomiosina (SHENG; JIN, 2016; WEI; JIN, 2016). Além disso, o *TNNI1* demonstrou estar envolvido com a ocorrência de determinados tipos de cânceres e é geralmente super expresso por desempenhar funções regulatórias em processos celulares junto a actina (CASAS-TINTÓ et al., 2016). Constatou-se também, por meio de análises *in silico*, variadas mutações nos éxons que codificam a proteína de troponina I (prolina82serina, arginina98glicina, arginina141glicina e arginina162glicina), que podem afetar as associações com outras proteínas e prejudicar a estabilidade de complexos sarcoméricos, importantes para o correto funcionamento do músculo cardíaco, observados em doenças como a cardiomiopatia hipertrófica (RAMACHANDRAN et al., 2013).

1.4.13 Fibrilina 2 (*FBN2*)

O gene da Fibrilina 2 (*FBN2*) está presente no cromossomo 2 dos suínos (NCBI, 2018). As fibrilinas são glicoproteínas colágeno-resistentes componentes das microfibrilas existentes dentro da matriz extracelular e que mantém a elasticidade daquela estrutura, além de contribuírem para o sequestro do fator de transformação do crescimento (TGFβ), formando um complexo com proteínas de ligação ao TGFβ que controlam proliferação e diferenciação celular, especialmente em tecidos de origem mesenquimatosa (DAVIS; SUMMERS, 2012). You et al. (2017), Guo et al. (2016) e Deng et al. (2016) relataram em seus trabalhos mutações nesse gene do tipo *missense* nas posições p.G1145D, p.C1077G e p.C1257R, respectivamente, como causadoras da doença da aracnodactilia contratual congênita (CCA) em famílias chinesas com longo histórico. Essa doença é um distúrbio autossômico dominante do tecido conjuntivo que causa deformidades corporais variadas, como contraturas nas articulações e escoliose

(TUNÇBILEK; ALANAY, 2006). Adicionalmente, as fibrilinas se organizam em feixes e formam microfibrilas em associação a outras proteínas e glicoproteínas. Portanto, modificações nesse conjunto podem prejudicar as estruturas fundamentais da matriz extracelular e os tecidos ancorados por essa estrutura.

1.4.14 Fibromodulina (*FMOD*)

O gene da fibromodulina (*FMOD*) está mapeado no cromossomo 9 do suíno (NCBI, 2018). Esse gene codifica uma proteína citosólica rica em leucina que regula a montagem e composição da matriz extracelular e está envolvida na formação das fibras colágenas, desenvolvimento muscular, reprogramação celular e angiogênese (JAN et al., 2016), presente em vários tecidos conectivos e componente de um grupo de proteoglicanos funcionalmente relacionados (OLDBERG et al., 1989). No relato de Mormone et al. (2012), foram analisados os processos regulatórios de expressão de fibromodulina através de lesões (colestases) induzidas no fígado de ratos normais pela estrangulação do ducto biliar comum, administração de tiocetamida (TTA) ou tetracloreto de carbono (CCl_4) e em fígados humanos lesionados por cirrose associada à hepatite C. Neste caso, o *FMOD* foi superexpresso e ativou a deposição de colágeno tipo I ao exibir efeitos sobre o comportamento profibrinogênico de células estelares hepáticas (HSCs), um dos tipos celulares presentes no fígado. Essas células podem perder suas funções normais no processo de fibrose hepática e serem afetadas pela elevada liberação de radicais livres gerados pela inflamação.

Lee et al. (2016) investigaram a participação de *FMOD* no processo de formação muscular (miogênese) em células cultivadas. Os autores descrevem que esse gene é um modulador essencial da proliferação e diferenciação celular de mioblastos durante a miogênese, especialmente nas primeiras semanas após o nascimento, etapa em que é altamente expresso. Proteoglicano da matriz extracelular, fornece o suporte mecânico aos tecidos em desenvolvimento. Em células satélites musculares *FMOD-knockdown,* a baixa expressão de *FMOD* limitou a formação de miotubos e a fusão destes e suprimiu a expressão de genes regulatórios miogênicos *MYOD* (*myogenic differentiation*), *MYL2* (*myosin light chain 2*) e *MYOG* (*myogenin*), bem como *COL1α1*, um dos muitos colágenos que compõem a matriz extracelular e que proporcionam integridade estrutural aos tecidos. Concomitante, houve elevada expressão de miostatina *(MSTN*). A *FMOD* se liga à miostatina e reduz a afinidade dessa ao seu receptor activina tipo IIB (ACVRIIB),

um complexo regulatório que inibe fatores de transcrição e pode alterar as cascatas de sinalização do crescimento muscular em células-alvo.

1.4.15 Adenilato ciclase 5 (*ADCY5*)

O gene da adenilato ciclase 5 (*ADCY5*), presente no cromossomo 13 do suíno (NCBI, 2018), codifica uma proteína enzimática de membrana que converte ATP em cAMP, um segundo mensageiro, após a estimulação por proteína G. Os tipos de adenilato ciclases (AC) são variados e sua distribuição no organismo, bem como as funções que desempenham, possibilitam que determinados grupos celulares respondam de maneira distinta à estímulos bioquímicos similares, sendo a isoforma AC5 encontrada principalmente no coração (VATNER et al., 2013). A menor expressão de AC5 em ratos *ADCY5-knockout* reduziu a produção de cAMP e aumentou a resistência do organismo ao estresse oxidativo, através da expressão mais elevada de enzimas que combatem radicais livres, os quais tem efeitos sobre a expressão gênica, apoptose e ciclo celular (VATNER et al., 2015). Concentrações de cálcio/calmodulina, fosforilação por proteínas quinases, óxido nítrico ou mesmo algumas subunidades das próprias proteínas G podem alterar e regular a atividade das AC (SUNAHARA, 2002), embora existam sinalizações e sistemas regulatórios específicos de cada isoforma (OSTROM et al., 2012). Ademais, é aceito que o Ca^{+}, de acordo com sua concentração intracelular, funciona como inibidor, enquanto a proteína quinase C (PKC), por exemplo, funciona como ativador de AC5 (HALLS; COOPER, 2011; MOU et al., 2009).

1.4.16 Agrecano (*ACAN*)

O gene *ACAN* está presente no cromossomo 7 da espécie suína (NCBI, 2018) que codifica a proteína agrecana. Esta proteína é um proteoglicano de sulfato de condroitina e queratano que forma grandes agregados com glicosaminoglicanos (GAGs), presentes em elevadas concentrações nos discos vertebrais e cartilagens articulares, onde criam grande pressão osmótica capaz de adsorver e armazenar água no interior da matriz extracelular que compõem as cartilagens e proporciona alta viscosidade e resistência à compressão, mas que podem ser degradadas por metaloproteases entre domínios interglobulares da molécula (KIANI et al., 2002; SIVAN et al., 2014). Doenças ligadas ao gene *ACAN*, agrecanopatias, ainda são pouco conhecidas, mas em geral se manifestam com

anormalidades esqueléticas (GIBSON; BRIGGS, 2016). Mutações sem sentido (*missense* e *nonsense*) que ocorreram no gene *ACAN* foram associadas à baixa estatura hidiopática em indivíduos humanos, no qual as variantes impedem a correta interação entre a proteína agrecana e o hialuronano (HAUER et al., 2017), criticamente importante ao desenvolvimento esquelético no processo de ossificação (ASPBERG, 2012), expresso em vários tecidos, mas componente principal da cartilagem (GIBSON; BRIGGS, 2016). A alta concentração de agrecanos exibe uma característica aneural e avascular no interior do disco intervertebral, normal nas regiões periféricas ao anel de colágeno circundante, mas que pode permitir o crescimento daquelas estruturas (vasculares e neurais) conjuntamente à calcificação em caso de degeneração do disco (SIVAN et al., 2014).

1.4.17 Colágeno tipo XXIII cadeia alfa 1 (*COL23A1*)

Mapeado no cromossomo 2 do suíno (NCBI, 2018), o gene *COL23A1* foi associado, juntamente a outros genes em uma região significativa de QTL no SSC2, ao desenvolvimento da hérnia escrotal (DU et al., 2009). Entre os muitos tipos de colágenos conhecidos, o colágeno XXIII está incluído em um grupo de proteínas transmembranares que pode estar envolvido na adesão celular (FRANZKE et al., 2005) e é expresso, em grande parte, na epiderme para formar ou manter o contato entre células (KOCH et al., 2006). Na doença de cardiomiopatia dilatada (DCM), vários genes de colágeno apresentaram maiores níveis de expressão no coração de indivíduos com tal anomalia, onde a maior deposição de colágeno aumenta a rigidez do miocárdio como forma de remodelação do tecido em condições patológicas, no qual o colágeno XXIIIα1 pode estimular a migração de células cardíacas e, dessa forma, aumentar a expressão de metaloproteinases (*MMPs*) que reduzem a rigidez e alteram a estrutura ventricular (GIL-CAYUELA et al., 2016). Além disso, o *COL23A1* pode servir como um mediador principal da adesão e potencial metastático de células tumorais pulmonares (SPIVEY et al., 2012). Recentemente, foi demonstrado que a elevada expressão de *COL23A1* em carcinoma de células renais (ccRCC) torna esse tipo de câncer mais agressivo, mas o *knockdown* do gene em linhas celulares atenuou a capacidade de adesão e migração e demonstrou efeito bloqueador à progressão do ciclo celular ainda na fase G1 (XU et al., 2017).

1.5 GENES REFERÊNCIA PARA QUANTIFICAÇÃO RELATIVA

Após a identificação do perfil de transcritos com tecnologias de transcriptoma como a utilização do RNA-Seq (WANG et al., 2009), a análise de expressão gênica necessita de uma etapa para validação dos resultados e, nesse caso, pode-se utilizar a técnica de reação em cadeia da polimerase em tempo real (qPCR). Essa prática permite uma rápida e confiável obtenção de dados a partir da amplificação do DNA/cDNA de interesse. Entre os métodos de quantificação da expressão gênica, a quantificação relativa é comumente utilizada para mensurar diferenças de níveis de expressão de mRNA em experimentos que investigam mudanças fisiológicas no organismo. Neste método, a curva de calibração não é necessária, pois são utilizados genes controles, constitutivamente expressos no organismo, de forma que os genes de interesse são comparados com os genes de referência através de um método comparativo, referido como $2^{-\Delta\Delta Ct}$ (SCHMITTGEN; LIVAK, 2008). Geralmente o nível de expressão é determinado pelas diferenças entre os ciclos de amplificação (*cycle thresholds, Cts*) mensurados entre os genes constitutivos e os genes alvo (PFAFFL et al., 2002).

Genes referência/constitutivos, mais conhecidos como *housekeeping*, são utilizados como normalizadores das reações para quantificação relativa e, de maneira geral, não podem apresentar variações significativas (KOZERA; RAPACZ, 2013). Contudo, a escolha desses genes deve ser determinada nas condições experimentais específicas de cada ensaio (WANG et al., 2012), como tipo de tecido, estágio de desenvolvimento ou doença (KOZERA; RAPACZ, 2013). Portanto, é importante realizar testes prévios que conduzam à correta seleção, a fim de minimizar a possibilidade de erro experimental. Diversos genes constitutivos já foram avaliados e são utilizados como normalizadores de expressão relativa em inúmeras espécies, porém, para suínos, os estudos conduzidos envolveram diferentes tecidos musculares (FENG et al., 2010; NIU et al., 2016; NYGARD el al., 2007; ZHANG et al., 2012) ou estágios de desenvolvimento (NIU et al., 2016), não sendo encontrados genes próprios para o tecido do anel inguinal na ocorrência da hérnia escrotal.

1.6 OBJETIVOS

1.6.1 Objetivo geral

Analisar o perfil de expressão de genes candidatos à ocorrência da hérnia escrotal, identificados no tecido do anel inguinal de suínos normais e afetados.

1.6.2 Objetivos específicos

- Averiguar a estabilidade de expressão de genes referência para servirem como normalizadores de ensaios de expressão relativa em qPCR para estudos com suínos de diferentes raças (Landrade e MS115) e idades (30 e 60 dias), normais e afetados com hérnia escrotal.
- Quantificar o nível de expressão relativa de 17 genes candidatos à ocorrência da hérnia escrotal a partir de amostras de tecido do anel inguinal de suínos normais e afetados aos 30 dias de idade.
- Examinar se esses genes são diferencialmente expressos no tecido do anel inguinal de suínos normais e afetados com hérnia escrotal aos 30 dias de idade.

2. CAPÍTULO II

MANUSCRITOS

Os resultados desta dissertação são apresentados na forma de dois manuscritos, com sua formatação de acordo com as orientações da revista PLoS ONE.

2.1 MANUSCRITO I

Identification of reliable endogenous genes in the inguinal ring tissue for scrotal hernia expression studies in pigs

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Identification of reliable endogenous genes in the inguinal ring tissue for scrotal hernia expression studies in pigs

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Abstract

The use of reference genes is required for relative quantification in gene expression analysis and since the stability of these genes could be variable depending on the experimental design, it has become indispensable to test the reliability of endogenous genes. Therefore, this study evaluated 10 reference candidate genes in two different experimental conditions in order to obtain stable genes to be used as reference in expression studies related to scrotal hernias in pigs. Two independent experiments were performed: one with 30 days-old MS115 pigs and the other with 60 days-old Landrace pigs. The inguinal ring/canal was collected, frozen and further submitted to real-time PCR analysis (qPCR). For the reference genes stability evaluation, four tools were used: GeNorm in the SLqPCR, BestKeeper, NormFinder and Comparative CT. A general ranking was generated using the BruteAggreg function of R environment. In this study, the *RPL19* was one of the most reliable endogenous genes for both experiments. The breed/age effects influenced the expression stability of candidate reference genes evaluated in the inguinal ring of pigs. A consensual set of reference genes was not obtained for the two experimental conditions, evidencing the importance of evaluating the stability of several endogenous genes previous their use. Therefore, two set of genes are recommended: *RPL19*, *RPL32* and *H3F3A* for 30-days MS115 and *PPIA* and *RPL19* for the 60 days-old Landrace pigs.

Keywords: reference genes, housekeeping genes, inguinal canal, qPCR, swine

Introduction

The real time PCR (qPCR) is one of the main approaches used for gene expression studies, being highly sensitive [1]. However, many factors related to this technique, since the quality of biological material up to the laboratorial procedures, might compromise the reliability of the qPCR results [2]. Furthermore, qPCR is a powerful technique to validate differentially expressed genes from global expression approaches, such as microarrays and, more recently, RNA-Seq [3]. Therefore, it is essential to standardize the methodologies to be used, and specifically considering gene expression studies using qPCR, the correct choice and use of reference genes, also known as housekeeping genes, avoid mistaken results. Thus, knowing the behavior of these genes in each experimental design is crucial to obtain reliable results [1,4]

A gene can be considered as reference or endogenous when its expression is not variable in different experimental conditions, tissues or physiological state of the tissue or organism [2]. In relative gene expression analyses, the use of reference genes is required to normalize and obtain the fold-change, through mathematical algorithms, such as those described previously by Pfaffl (2001), Livak & Schmittgen (2001) and Schmittgen & Livak (2008) [5–7]. Some of the most well-known housekeeping genes are *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase), *PGK* (Phosphoglycerate kinase), *UBQ (*ubiquitin), *RPL19* (RPL19 ribosomal protein L19), *18S rRNA* (ribosomal RNA 18S), *β-actin* and *β-tubulin* [1] and they have been used in several studies, in many species, including pigs. However, the stability of the reference genes can be altered depending on the tissue, age, treatment and other conditions, which makes indispensable to test the stability of several genes before using those as reference [8–10]. Several studies searching for reliable housekeeping genes in pigs have been reported [11–14] with different breeds, tissues and conditions. However, studies aiming to verify stable reference genes in the inguinal ring for scrotal hernia studies have not been reported to date.

The scrotal hernia is a malformation whereby intestinal loops traverse the abnormally open inguinal ring [15]. Although there are indications about the involvement of genetic components in the occurrence of this anomaly in humans [16,17] and other species [16], including the pig [18,19], the genes affecting this condition remain unknown. Therefore, expression studies are required to clarify the genetic mechanism involved in this malformation. Most of the expression studies searching for reference genes are based on muscular tissues with better characterized anatomy, such as *longissimus dorsi* [10,20–23] or even with a broader set of tissues [9,12,24]. No gene

expression study is available in livestock using inguinal ring tissue, which is composed by connective and muscular tissues, and it is the site of occurrence of the scrotal hernia. Thus, knowing reliable reference genes for the inguinal canal is essential to obtain accurate gene expression assays with this tissue. Therefore, to obtain stable genes to be used as reference in expression studies related to scrotal hernias in pigs, 10 endogenous candidate genes were evaluated in the present study in two different experimental conditions.

Materials and Methods

Animals and sample collection

This study was performed with the approval of the Embrapa Swine and Poultry Ethical Committee of Animal Use (CEUA) under the protocol number 011/2014. Two experiments were carried out to detect the best housekeeping genes in two different ages: at 30 and 60 days of age. The details of each experiment are presented below:

Experiment 1 (E1): Animals were raised at the Embrapa Swine and Poultry National Research Center farm until 30 days of age. A total of 18 entire male pigs of the MS115 synthetic line were used. The animals were grouped in normal (n= 9, absent from malformations and coming from litters with no history of hernias) and affected (n= 9, from litters with the presence of more than one animal with scrotal hernia).

Experiment 2 (E2): eight Landrace pigs with 60 days of age from the same nucleus farm, located in Santa Catarina State, Brazil, were used in this study. These animals were transported from the farm to the necropsy room at the Embrapa Swine and Poultry. As in the experiment 1, the animals were grouped in normal (n= 4) and affected (n= 4) with scrotal hernia**.**

For both experiments, the euthanasia was performed by electrocution for 10 seconds, followed by immediate exsanguination, according to the practices recommended by the Ethics Committee. The necropsy was performed for the evaluation of possible problems and additional characteristics that could interfere in the accuracy of the data, as well as for the correct characterization of the hernia phenotype. Tissue samples from the inguinal ring/canal of normal and scrotal hernia-affected groups were collected and immediately frozen in liquid nitrogen and then stored at -80° C for subsequent RNA extraction. After necropsy and tissue collection, the piglets' carcasses were destined for composting.

RNA extraction

Tissue RNA extraction was performed according to the Trizol Reagent® (Invitrogen, Carlsbad, CA) protocol. Samples containing about 100 mg of tissue were initially macerated in liquid nitrogen with mortar and pistil, properly treated for this procedure. After maceration, the generated contents were placed into 1.5 mL micro tube containing 1 mL of the Trizol® reagent, vortexed and then incubated for 5 minutes at room temperature (25 °C). Next, 200 µl of chloroform was added to the tube, shacked vigorously with the hands for 15 seconds, and finally incubated at room temperature for 5 minutes. After incubation, centrifugation was performed at 11,000 rpm (rotations per min) at 4 °C for 15 minutes. Thereafter, the aqueous phase was removed into a clean polypropylene tube and 500 µl of isopropanol was added. The tube was stirred and subsequently incubated for 10 minutes at room temperature. After 10 minutes, the tubes containing the sample were centrifuged for 10 minutes at 10,000 rpm at 4 ° C. The supernatant was discarded and the pellet washed with 1 mL of 75% ethanol and homogenized in vortex. This was centrifuged at 9,000 rpm for 5 minutes at 4 °C. The supernatant was discarded and the pellet dried for 15 minutes at room temperature, resuspended in DEPC water and heated at 55 ° C for 10 minutes. The quality and quantity of the total RNA were evaluated by spectrophotometer (Biodrop, UK) and also in 1% agarose gel. Finally, the total RNA extracted was conserved in ultrafreezer - 80 ºC.

Complementary DNA (cDNA) synthesis

For the synthesis of complementary DNA (cDNA), the SuperScript III ™ First-Strand Synthesis Supermix Kit (Invitrogen, USA) was used. For each 3µg of total RNA, 1µL of Annealing buffer, 1µL of oligo dT 0.5µg / µL and water until the volume was completed in 10µL were added, incubated at 65ºC for 5 minutes and then cooled in ice for 1 minute. Then, 10 µL of 2X First-Strand reaction mix and 2 µL of SuperscriptIII/RNAseOUT enzyme mix (Invitrogen, USA) were added to the mixture, being incubated for 50 minutes at 50 °C and subsequently inactivated for 5 minutes at 85 °C, and then stored at -20 ºC.

Relative quantification using qPCR

The relative quantification of each putative reference gene was performed by qPCR. The expression pattern of the following genes was evaluated: Hydroxymethylbilane synthase (*HMBS*), Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein

zeta (*YWHAZ*), Succinate dehydrogenase complex flavoprotein subunit A (*SDHA*), Topoisomerase (*DNA*) II beta (*TOP2B*), Ribosomal protein 13A (*RPL13A*), H3 histone, family 3A (*H3F3A*), Eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*), Ribosomal protein 32 (*RPL32*), Ribosomal protein L19 (*RPL19*) and Peptidyl prolyl cis-trans isomerase A (*PPIA*). The sequences and annotations for these 10 genes were obtained in the swine genome (*Sus scrofa*, v. 10.2) available in GeneBank (https://www.ncbi.nlm.nih.gov/genbank/) and Ensembl 86 (http://www.ensembl.org/index.html). Primers were designed in exon-exon junction regions, in order to avoid the genomic DNA amplification, using the Primer-Blast program [25] and are shown in Table 1. The qPCR reactions were carried out in duplicate in 15 µL final volume containing 1X of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, USA), 0.05 to 0.13 µM of each primer and ~20 ng of cDNA. Reactions were performed in the Quantstudio 6 equipment (Thermo Fisher Scientific, USA) using SYBR Green as fluorescence dye with the following cycling condition: 95º for 10 min, 40 cycles of 15 seconds at 95ºC and 30 seconds 60ºC. In addition, the melting curve stage of 70°C to 95°C at 0.1°C/s for all genes studied were included to verify the primers specificity.

Reference gene stability evaluation

A total of four algorithms widely used to identify the most stable expressed genes: the geNorm [26], NormFinder [27], BestKeeper [28] and Comparative ΔCt [29] were used to evaluate the housekeeping candidate genes in the present study. The geNorm is a robust software that calculates an internal control gene-stability measurement (*M*) for each combination of two control genes tested, obtaining a transformed expression ratio and then, calculates a standard deviation of these pairwise gene combinations. The two most stable genes are determined based on the lowest *M* value [26]. The NormFinder is a visual basic application for Microsoft Excel that also calculates a stability value based on intragroup and intergroup variation of genes tested, ranking the genes according to their expression stability and similarity [27]. In the NormFinder, the data used was transformed in log2, as suggested by the developer [27]. The BestKeeper is also an Excel-based tool for scoring the best genes using an index (power of the gene) composed by the lowest values of ΔCt, standard deviation and coefficient of variation [28]. The Comparative ΔCt [29] uses a basic ΔCt approach to compare the relative expression of pairs of genes, creating a stability rank based on the ΔCt and standard deviations means.

Table 1: Primers for the 10 reference candidate genes for the qPCR analysis in the inguinal ring of pigs.

Gene	Function	Primer Sequences (5' – 3')	Ensembl ID
HMBS Hydroxymethylbilane synthase	Third enzyme of the biosynthetic pathway of the Heme group	F: AGGATGGGCAACTCTACCTGA R: ATGGATGGTGGCCTGCATAG	ENSSSCG00000015108
RPL19 Ribosomal protein L19	Ribosomal protein 60S subunit component, L19E family	F: ACCGCCACATGTATCACAGTC R: TGTGCTCCATGAGAATCCGC	ENSSSCG00000017509
RPL32 Ribosomal protein 32	Ribosomal protein 60S subunit component, L32E family	F: CAAAATTAAGCGGAACTGGCGG R: GCACATTAGCAGCACTTCAAGC	ENSSSCG00000027637
EEF1A1 Eukaryotic translation elongation factor 1 alpha 1	Enzymatic delivery of aminoacyl tRNAs to the ribosome.	F: CCGCCAGGACACAGGT R: TTCCCATCTCCGCAGCCT	ENSSSCG00000004489
H3F3A H3 histone, family 3A	3rd component of nuclear histones	F: CTTTGCAGGAGGCAAGTGAG R: TGGCATGGATAGCACACAGG	ENSSSCG00000023971
RPL13A Ribosomal protein 13A	Ribosomal protein 60S subunit component, L13A family	F: CCAAGCAGGTACTTCTGGGC R: GGCAGCATGCCTCGCA	ENSSSCG00000003166 ENSSSCG00000003167
TOP2B Topoisomerase (DNA) II beta	DNA transcription and replication	F: AGAAGAGCTGCTGCTGAAAGG R: TCCCCGTCATTTGTCACAGG	ENSSSCG00000011213
SDHA Succinate dehydrogenase complex flavoprotein subunit A	Encodes a major catalytic subunit of succinate-ubiquinone oxidoreductase, in the mitochondrial respiratory chain	F: TTGTACGGAAGGTCTCTGCG R: GATGACTCCACGACACTCCC	ENSSSCG00000020686
YWHAZ Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	Regulation of signal transduction pathways through binding phosphoserine proteins	F: ATCAGATTGGGTCTGGCCCT R: GGTATCCGATGTCCACAATGTC	ENSSSCG00000006062
PPIA Peptidyl-prolyl cis-trans isomerase A	Accelerate the folding of proteins	F: GCGTCTCCTTCGAGCTGTTT R: ACTTGCCACCAGTGCCATTA	ENSSSCG00000016737

F: forward; R: reverse.

In addition, once all of the stability values for all tools were obtained, the BruteAggreg function, a weighted rank aggregation tool from the RankAggreg package [30] of R environment [31], was used to determine a general ranking of the most stable genes for each experiment. The BruteAggreg function was used twice for each experiment. This had to be done because the geNorm software ranks the two best genes at the same time. Then, these genes were both put in the 1st and 2nd positions for each experiment in BruteAggreg to improve the prediction of the best endogenous control gene.

Results

The total RNA average concentration was 1,033.19 ng/µL for the normal and 1,052.66 ng/µL for the affected group in the Experiment 1, and 918.55 ng/µL for the normal and 995.03 ng/µL for the affected group, in the Experiment 2. Regarding the RNA quality, the average A260/280 ratio was 1.90 ± 0.04 and 2.06 ± 0.01 for the unaffected pig samples and 1.92 ± 0.05 and 2.07 ± 0.02 for herniated pig samples in the E1 and E2, respectively, evidencing a good quality of the RNA samples to be used in the further analysis.

The mean Ct values (± SD) of the reference candidate genes were 21.73 (± 0.66) and 21.95 (± 0.41) for *HMBS*, 15.14 (± 0.65) and 13.63 (± 0.41) for *RPL19*, 13.00 (± 1.57) and 14.05 (± 0.71) for *RPL13A*, 18.13 (±1.45) and 18.89 (± 0.45) for *TOP2B*, 14.09 (± 0.96) and 13.39 (± 0.65) for *RPL32*, 20.37 (± 1.45) and 20.84 (± 1.24) for *SDHA*, 10.88 (± 1.89) and 12.62 (± 1.08) for *EEF1A1*, 16.12 (± 0.76) and 18.75 (± 1.34) for *H3F3A*, and 19.91 (± 2.32) and 15.58 (± 0.47) for *YWHAZ* (Fig 1, S1 Table) for E1 and E2, respectively. The *PPIA* gene was removed from the Experiment 1 analysis since there was no amplification for some of the samples, differing from the Experiment 2, where all samples amplified for this gene, with Ct mean of 15.98 ± 0.34. Most of the genes started the amplification between cycles 10 to 20 cycles (Fig 1), indicating high levels of expression. Also, it was possible to identify a higher dispersion of the Cts for the *YWHAZ* gene in E1 compared to E2. According to the melting curve analysis, all genes presented a specific amplification (Fig 2).

Fig 1. Cycle threshold (Ct) variation in normal and hernia-affected pigs in the two experiments. CG: control group; AG: affected group; 1 – experiment 1 and 2 – experiment 2. *PPIA: just the information about the experiment 2 was plotted, since there was no amplification for some samples in the experiment 1.

Fig 2. Melting curve analyzes of the 10 reference candidate genes evaluated in this study.

Regarding the experiment 1, it was possible to observe a similar expression profile among *RPL19, RPL32, H3F3A* and *HMBS* genes obtained with the several evaluated tools (Table 2). These were the first four genes ranked with the BestKeeper (S2 Table) and with the geNorm (Fig 3A) tools and also presented the smallest dispersion of Ct values (Fig 1). The geNorm classified the *RPL19 / H3F3A* and *HMBS* genes with the

lowest M values: M = 0.620 and M = 0.659, respectively (Fig 3A). The NormFinder program included the *TOP2B* among the most stable genes, while BestKeeper, geNorm and DeltaCt ranked those genes in the last five positions, showing a reduced stability (Table 2). Another important observation was that the *RPL13A, SDHA, EEF1A1* and *YWHAZ* genes demonstrated the lowest stability values according to the BestKeeper, geNorm and NormFinder tools (Table 2). However, when the ΔCt comparative approached was evaluated, the *YWHAZ* gene was scored as the most stable, differing from the other three tools previously mentioned (Table 2).

Table 2: Gene classification values and ranking (in parenthesis) according to the four algorithms analyzed and the general score generated by the BruteAggreg for experiments 1 (E1) and 2 (E2). 1st and 2nd are the rank after running the BruteAggreg twice.

	BestKeeper (Power of the gene)		**DeltaCt**		**NormFinder (S)**		**geNorm (M)**		**BruteAggreg**			
									E1		E2	
Gene	E1	E2	E1	E2	E1	E2	E1	E2	1st	2nd	1st	2nd
HMBS	1.334 (1)	0.000 (1)	1.202 (4)	0.811 (5)	0.542 (4)	0.205 (4)	0.659 (3)	0.478 (2)	3	3	3	4
RPL19	1.386 (2)	1.242 (4)	1.145 (2)	0.796 (4)	0.477 (2)	0.220 (6)	0.620 (2)	0.478 (1)	**1**	**1**	**2**	**2**
H3F3A	1.393 (3)	5.416 (10)	1.214 (5)	1.081 (10)	0.551 (5)	0.270 (9)	0.620 (1)	1.097 (10)	4	**2**	10	10
RPL32	1.632 (4)	1.549 (6)	1.157 (3)	0.871 (6)	0.434 (1)	0.192 (3)	0.736 (4)	0.646 (6)	**2**	4	6	6
TOP2B	2.244 (5)	0.963 (2)	1.259 (6)	0.909 (8)	0.498 (3)	0.321(10)	0.884 (5)	0.599 (5)	5	5	7	7
RPL13A	2.325 (6)	2.485 (7)	1.338 (7)	0.724 (3)	0.633 (6)	0.118 (1)	0.995 (6)	0.761 (7)	6	6	5	5
SDHA	2.351 (7)	4.652 (9)	1.376 (8)	1.023 (9)	0.660 (7)	0.264 (8)	1.089 (7)	1.081 (9)	7	7	9	9
EEF1A1	2.742 (8)	4.002 (8)	1.584 (9)	0.897 (7)	0.900 (8)	0.217 (5)	1.192 (8)	1.001 (8)	9	9	8	8
YWHAZ	3.204 (9)	1.225 (3)	1.141 (1)	0.715 (2)	1.175 (9)	0.238 (7)	1.348 (9)	0.566 (4)	8	8	4	3
PPIA	-	1.422 (5)	-	0.701 (1)	-	0.218 (2)	-	0.492 (3)	-	-	**1**	**1**

Fig 3: Ranking of reference candidate genes based on the average expression stability using the geNorm software. A: results obtained in the Experiment 1. B: results obtained in the Experiment 2.

When the analysis was performed in the experiment 2, several differences among the most suitable genes were found in comparison to experiment 1. Also, it is interesting to note that each algorithm/tool indicated one different gene as most stable (Table 2). Using geNorm, the *RPL19/HMBS* and *PPIA* genes presented the lowest M value, of 0.478

and 0.492, respectively, which suggest that those genes should be used as housekeeping in E2 (Table 2, Fig 3B). The best genes according to BestKeeper were *HMBS*, *TOP2B* and *YHWAZ* (Table 2), respectively, while the *RPL13A*, *RPL32* and *PPIA* genes were listed by NormFinder. Furthermore, for the ΔCt method, the *PPIA*, *YHWAZ* and *RPL13A* were the top three reliable genes (Table 2).

A great variation on the rank of the best housekeeping genes were observed, depending on the evaluated tool. Therefore, a general rank considering those four tools was performed using the BruteAggreg function. For the experiment 1, *RPL19* and *RPL32*, and *RPL19* and *H3F3A* were pointed out as the first and second most stable genes, followed by *HMBS*, after performing the BruteAggreg function twice (Fig 4A and 4B, respectively). The results from the BruteAggreg function are similar to those obtained with the geNorm evaluation, including for the genes *EEF1A1* and *YWHAZ*, which were the worst genes evaluated (Fig 4). For the experiment 2, the *PPIA* and *RPL19* were scored as the best genes in both BruteAggreg analyses, while the *H3F3A* and *SDHA* were the most variable genes (Fig 5A and 5B, respectively).

Fig 4. Suitable genes ranked by the BruteAggreg tool in the two simulations for Experiment 1. A: simulation 1, genes *RPL19, RPL32* and *HMBS*; B: simulation 2, genes *RPL19, H3F3A* and *HMBS* (Table 2).

Fig 5. Suitable genes ranked by the BruteAggreg tool in the two simulations for Experiment 2. A: Simulation 1, genes *PPIA*, *RPL19* and *HMBS*; B) Simulation 2, genes *PPIA, RPL19* and *YWHAZ* (Table 2).

The *RPL19* was ranked as one of the less variable genes, showing a similar classification (Table 2) for both experiments, which were run independently. Also, the *HMBS* gene was classified as the 3rd most stable in the general rank for E1 and the 3rd and 4th for E2 (Figs 4 and 5). However, despite of these similarities, there were two important differences in the general score: one related to the *H3F3A* gene, that was the 2nd/4th most stable gene in experiment 1, but was the worst gene evaluated in the experiment 2, and the *PPIA* gene, which was the best gene to be considered as reference in the experiment 2, while several samples did not amplify in the experiment 1.

Discussion

The studies using gene expression methodologies have been increasing and the use of qPCR for mRNA quantification might be highlighted [32]. Although the qPCR analysis is widely disseminated, some concerns are always important to improve the quality of the laboratory analyses. One of them is related to the RNA amount and integrity, which helps in achieving high accuracy, sensitivity and reproducibility of the further analysis. In this study, the total RNA with good quality according to the usually recommended [32,33] and an amount necessary for all the expression analysis in just one batch was obtained.

The evaluation of a large set of reference candidate genes is essential to obtain reliable data in qPCR studies [28,34]. For this purpose, 10 putative reference genes were evaluated according to their expression stability and consistency with four different specific tools: geNorm, NormFinder, BestKeeper and ΔCt method (Table 2), which are widely used in similar studies. In the last years, several studies have been published discovering many candidate genes that might be used as internal control [28,34]. However, the search for the best reference gene is not trivial, since there are many approaches available and no standard methodology is established. Moreover, each experiment/condition requires a specific search for genes with non-variable expression patterns to be used as control [34].

In this study, two independent experiments were carried out in pigs from two different lines and ages. In general, it was possible to observe a discordance of the best normalizer genes chosen among the four methodologies in both experiments for the inguinal ring tissue (Table 2). These results reinforce the need for checking a certain number of reference candidate genes before initiating a gene expression analysis, in order to have an appropriate normalization of the transcript level [35].

In pigs, there are some studies validating internal control genes in multiple tissues, such as backfat, muscle, heart, adipose, skin, liver, pancreas, lung, within others [10,20,24] and also, in various developmental stages [22]. Although several tissues have already been evaluated, no information about expression profile of the inguinal ring has been reported, especially considering the presence or absence of scrotal hernia phenotype. In our study, 10 reference candidate genes were tested. However, some issues to select the best genes were encountered, since the most stable reference genes varied when each tool was evaluated separately. For example, while the *HMBS* was the best ranked in the BestKeeper in both experiments, it was the 2nd and 3rd in the geNorm (Fig 3), the 4th and 5th for Delta Ct, and 4th in the NormFinder, for E1 and E2, respectively. Other studies,

such as the one reported by Perez, Tupac-Yupanqui & Dunner (2008) [36], also found a divergent pattern among the tools evaluated for internal control genes in bovine muscle tissues. Obviously, since the algorithms and data transformation of those tools are different, it is possible that this could happen. However, when such a great variation is observed on the genes scored by different tools, no recommendation of the best method to select the genes exists. Mosley et al. (2017) [37], after analyzing 5 tools (BestKeeper, geNorm, NormFinder, DeltaCt and RefFinder), concluded that the geNorm seems to be the best tool for choosing the most reliable genes. Some studies have generated a rank when several approaches are used as an alternative to choose the best normalizer genes [38–40]. Therefore, the validation with another tool is essential to improve the quality of the genes to be chosen [41].

Therefore, a general ranking obtained with the BruteAggreg function pointed out that for the E1, *RPL19, H3F3A/RPL32* and *HMBS* (Fig 4) were the most stable genes, while for the E2 the most stable genes were *PPIA, RPL19* and *HMBS/YWHAZ* (Fig 5, Table 2). The *H3F3A* and *HMBS* have been previously described as reference genes in swine tissues, where the *H3F3A* was the most stable and the *HMBS* was regulated in some of the evaluated tissues [23,24]. Few studies have been performed using the *RPL19* as reference gene in pigs [42], but it has been considered as a good internal reference gene in other livestock species [43–46]

Regarding the best endogenous genes for the inguinal ring tissue, the *RPL19* showed the highest uniformity in its expression within the tools and experiments (Table 2, S1 Table). Ribosomal proteins have been suggested as good reference genes in many studies [47], because of their function on ribosome production [48]. Schulze et al. (2017) [45] and Lenart, Kogut & Salinska (2017) [46] also found stable expression of this gene on sheep bone cells and in chick brain, respectively. In pigs, *RPL19* was recommended as endogenous gene in studies using peripheral blood mononuclear and dendritic cells [42]. The *RPL19* amplified in early Cts (before 20) and had small coefficient of variation in each experiment (Fig 1, S1 Table), which can indicate that this gene would be a good housekeeping. The *RPL32, H3F3A* and *RPS18* (ribosomal protein 18S), involved in the development of cellular machinery, have also been chosen as endogenous gene for multiple tissues and swine breeds [14,23]. In addition, Zhang et al. (2012) [23], testing six endogenous genes in the *longissimus dorsi* of pigs, found differences on the best genes according to the breeds studied, where *RPL32* / *RPS18* were the most stable in the Landrace and *H3F3A* / *RPS18* in the Toncheng breed. In our study, both *RPL32* and

H3F3A genes were the 2nd most stable genes in the E1 (Table 2, Fig 4), endorsing the results obtained by Zhang et al. (2012) [23]. On the other hand, for the E2, the *RPL32* and *H3F3A* were not considered as stable genes.

The *PPIA* gene, that is involved in protein folding [49], has also been recommended for being used as endogenous control in several tissues, species and ages [9,14,21]. In our study, the *PPIA* was ranked as the most invariable gene in the E2, where Landrace pigs with 60 days of age were evaluated, being one of the most indicated as endogenous gene from the geNorm, NormFinder and Comparative Ct tools (Table 2, Fig 3). A similar pattern was observed when several tissues of Berskshire, Duroc, Landrace and Yorkshire pigs were evaluated [14], suggesting that *PPIA* is a reliable gene for expression studies in adult pigs. However, in our study, a variation in the *PPIA* expression between the two experiments was observed (Table 2). Although the *PPIA* was the best normalizer gene in the E2, for E1, in which the samples were obtained from 30 days-old MS115 pigs, the expression of this gene was impossible to be analyzed, since many samples did not amplify. This might be due to the different ages and breeds used in each experiment. Uddin et al. (2011) [9] observed that distinct genes should be used as reference gene depending on the pig's age. In addition, stability differences can also occur among the tissues analyzed [12]. Here, samples of the inguinal ring were collected and, albeit a unique technician had collected all samples in a specific anatomical region, it could be possible that the tissues were slightly unequable among samples and experiments, since this tissue is highly complex to collect. Therefore, studies evaluating the distinction among breeds, phenotypes and age of the animals are essential [9].

The *HMBS* gene had a good score in the general rank and in most of the other tools for both experiments (Table 2). This gene has been used as housekeeping in many species, pig lines, tissues and ages [24,38,50,51]. However, the regulation of this gene depends on the muscle tissue, sex, age and experimental conditions [38,50].

The *H3F3A* and *YWHAZ* were the most variable genes between both experiments. For instance, *H3F3A* was considered reliable in the experiment 1, with 30 days-old MS115, while it was the least reliable in the experiment 2, with 60 days-old Landrace pigs. The same pattern was observed with the *YWHAZ*, which in this case was stable with 60 days-old Landrace samples and variable with the 30 days-old MS115, reinforcing the statement that there are no general reference genes that might be used in all situations. The *SDHA*, *TOP2B*, *EEF1A1* and *YWHAZ* genes were highly variable regarding the general score in both experiments, possibly because of the late Ct and its variation

between and within groups. Furthermore, the variability presented by these genes could be possibly due to the non-homogeneity of the tissue used in this study.

Although more than two genes should be used as housekeeping in gene expression studies [33], the average number of genes used is only 1.2, which means, below the recommendation [33,34]. Moreover, it is usual studies with relatively common genes such as *GAPDH, β-actin* and *18S RNA*, without testing for stability. Given the complexity of the experimental designs and tissues to be evaluated, a broad panel of genes and tools should be used to search for the best reference genes [34]. The use of more than three genes is indicated to reduce the selection of false endogenous genes that may impact on the reliability of the results [52]. One example could be observed in our study, where the same tissue was collected from animals of two different lines and ages and, despite of being from the same species, two sets of genes should be used as reference: the *RPL19, RPL32 and H3F3A* for 30-days MS115 (E1) and *PPIA* and *RPL19* for the 60 days-old Landrace pigs (E2).

In this study, even though there was a confounding between age and breed effects, the experimental conditions influenced the stability of the evaluated genes. Therefore, further studies are recommended to clarify the isolated contribution of age and breed to variations on the genes' expression profile in the inguinal ring tissue of pigs. The effect of breed is expected to influence scrotal hernia congenital anomaly. Vogt & Ellersieck (1990) [18] found significant differences in frequency of this defect among Duroc, Landrace and Yorkshire male lines. Sevillano et al. (2015) [19] observed a slightly higher incidence of scrotal hernia in Large White (0.42%) compared to Landrace breeds (0.34%). In addition, these authors mapped distinct regions associated to scrotal hernia between Landrace e Large White pigs. Probably, intrinsic conformation and anatomical differences of each breed could affect the inguinal ring tissue composition causing variation in the expression profile of the endogenous candidate genes. Regarding the age effect, since hernias are related to development, usually resulting from failed obliteration of the *processus vaginalis* after descent of the testis, it is expected that the age would be important to this malformation. As evidence, most scrotal hernias are diagnosed at the time of castration, an early phase in the pig's life [53]. Therefore, the age effect should be evaluated independently in different ages, especially in early stages of life.

Conclusions

The breed/age effects influenced the expression stability of candidate reference genes evaluated in the inguinal ring of pigs. A consensual set of reference genes was not obtained for the two experimental conditions, evidencing the importance of evaluating the stability of several endogenous genes previous their use. In this study, only the *RPL19* was one of the most reliable endogenous genes for the two experiments. Therefore, two set of genes are recommended: *RPL19*, *RPL32* and *H3F3A* for 30-days MS115 and *PPIA* and *RPL19* for the 60 days-old Landrace pigs.

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Competing Interests

The authors declare that they have no competing interests.

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Supporting information

S1 Table. Ct means for the 10 reference genes by group and experiment.

S2 Table. Results from the Bestkeeper tool.

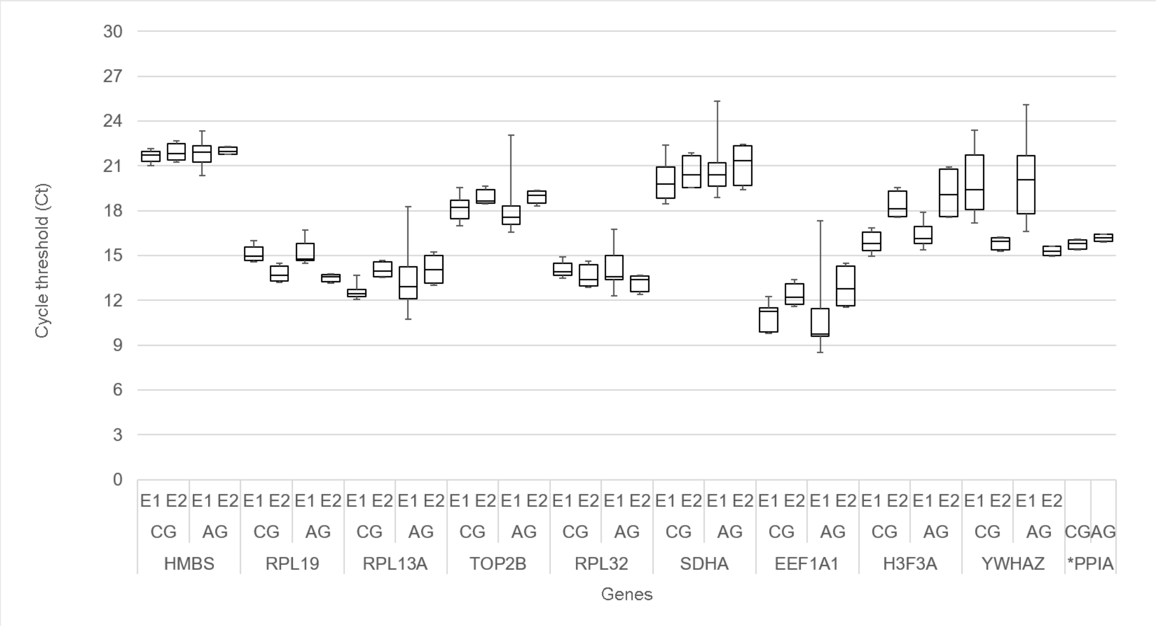

Fig 1. Cycle threshold (Ct) variation in normal and hernia-affected pigs in the two experiments. CG: control group; AG: affected group; 1 – experiment 1 and 2 – experiment 2. *PPIA: just the information about the experiment 2 was plotted, since there was no amplification for some samples in the experiment 1.

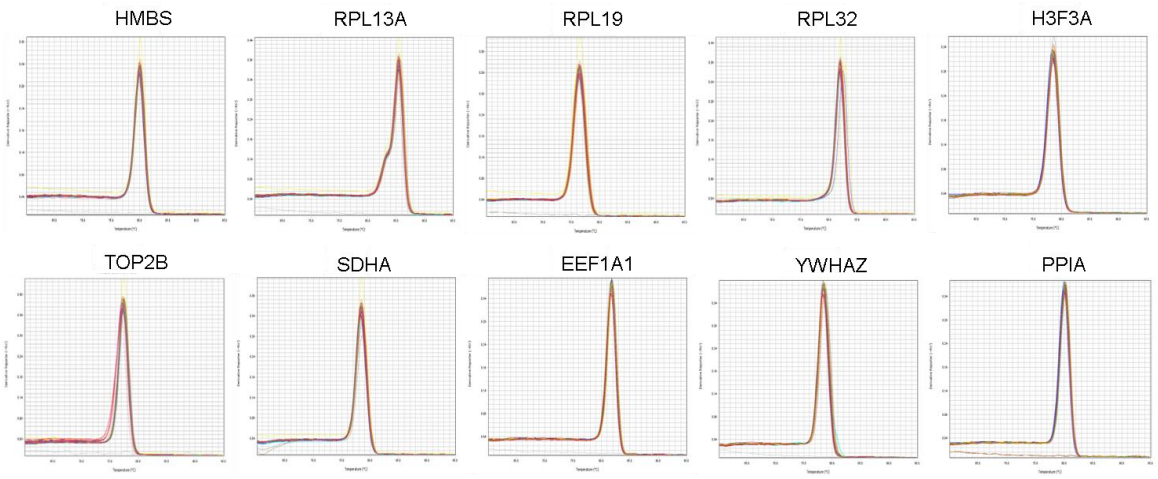

Fig 2. Melting curve analyzes of the 10 reference candidate genes evaluated in this study.

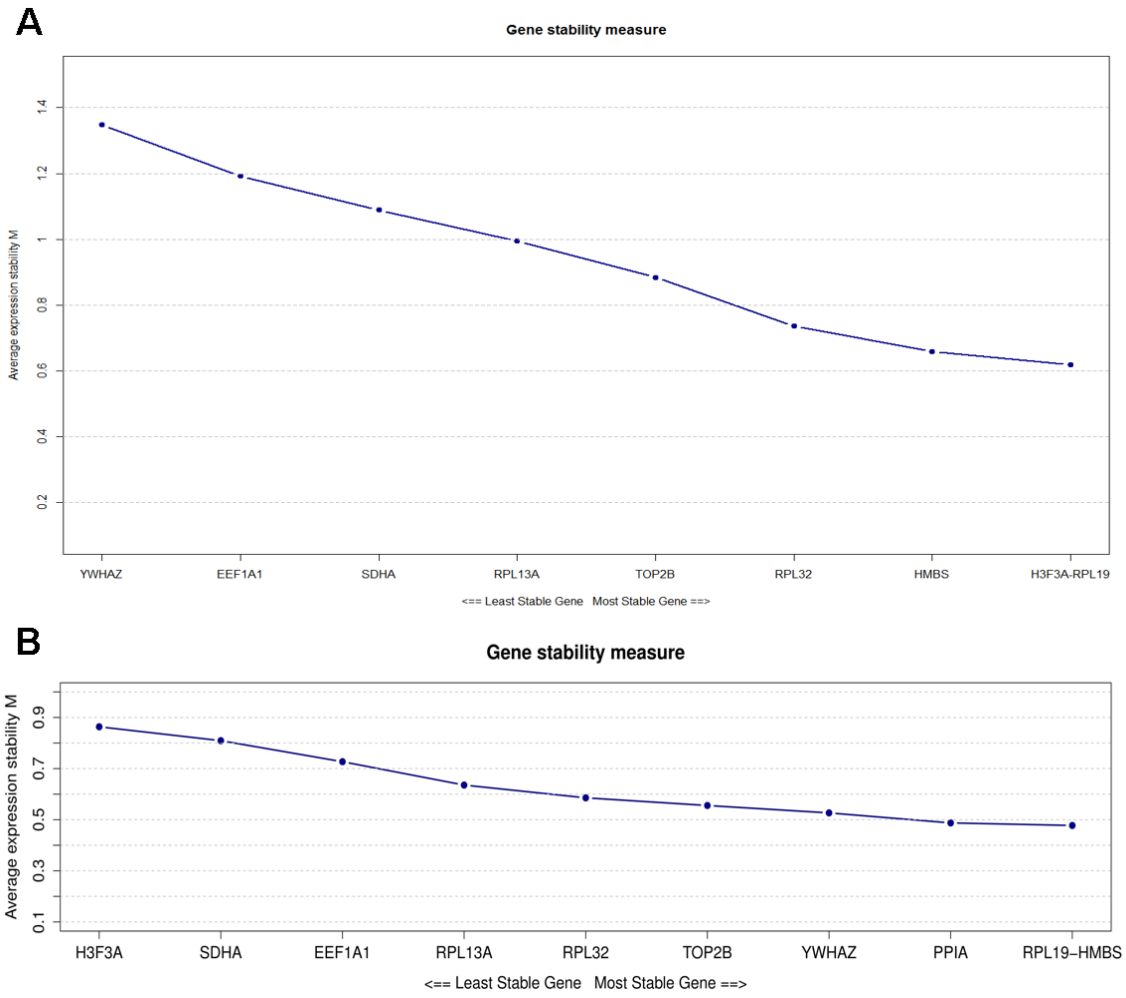

Fig 3: Ranking of reference candidate genes based on the average expression stability using the geNorm software. A: results obtained in the Experiment 1. B: results obtained in the Experiment 2.

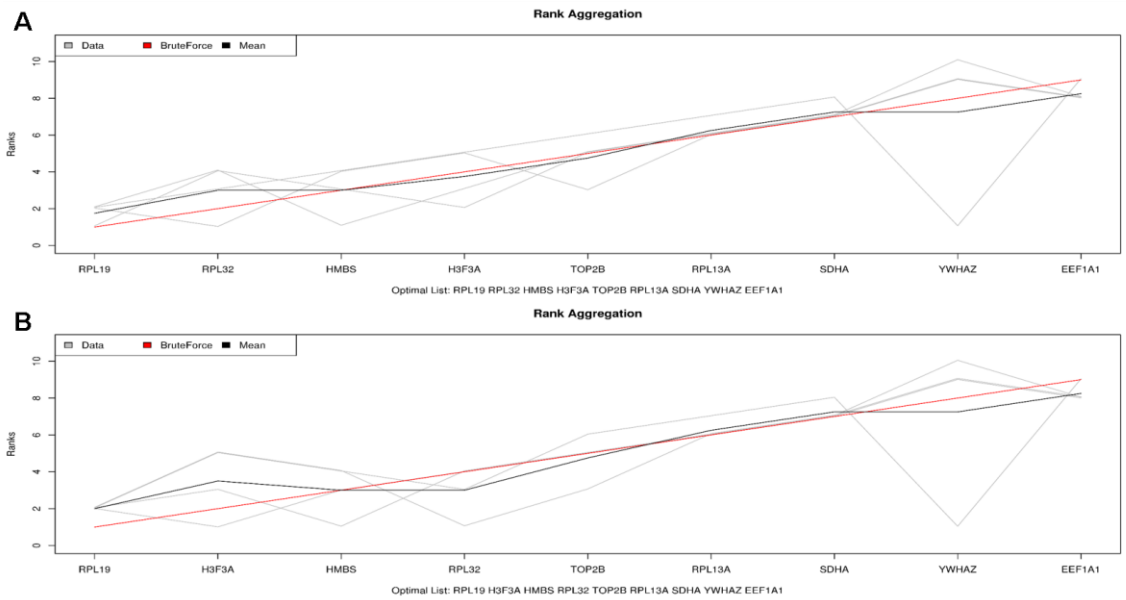

Fig 4. Suitable genes ranked by the BruteAggreg tool in the two simulations for Experiment 1. A: simulation 1, genes *RPL19*, *RPL32* and *HMBS*; B: simulation 2, genes *RPL19*, *H3F3A* and *HMBS* (Table 2).

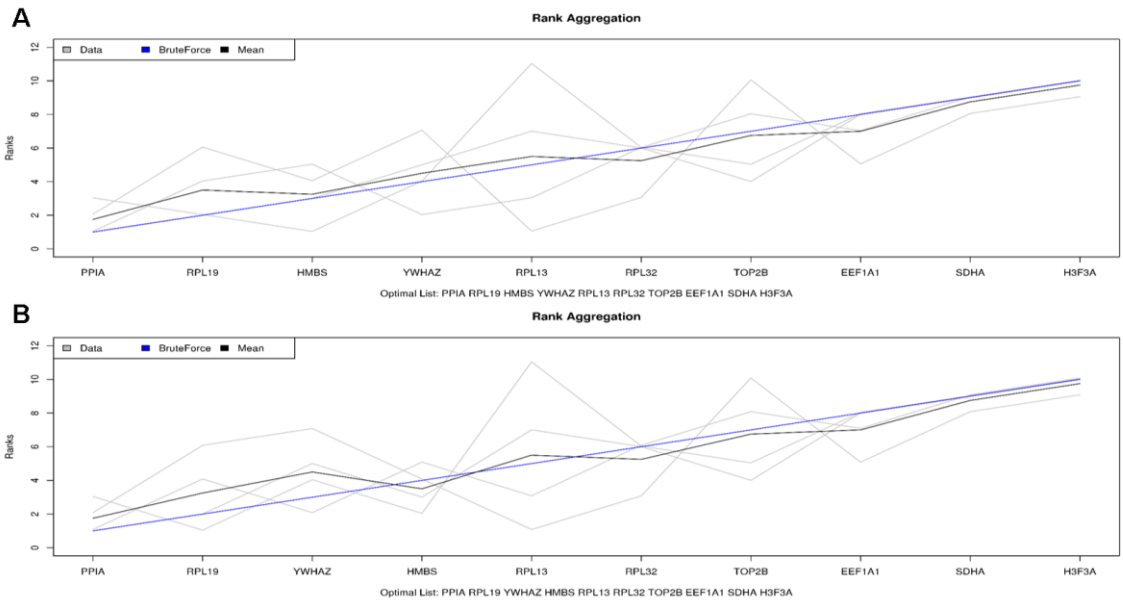

Fig 5. Suitable genes ranked by the BruteAggreg tool in the two simulations for Experiment 2. A: Simulation 1, genes *PPIA*, *RPL19* and *HMBS*; B) Simulation 2, genes *PPIA*, *RPL19* and *YWHAZ* (Table 2).

2.2 MANUSCRITO II

The downregulation of genes encoding muscle proteins have a potential role in the development of scrotal hernia in pigs

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The downregulation of genes encoding muscle proteins have a potential role in the development of scrotal hernia in pigs

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Abstract

Testicular descent is a physiological process regulated by many factors. Eventually, disturbances in this embryological/fetal development path may facilitate the occurrence of scrotal hernia, a malformation characterized by the presence of intestinal portions within the scrotal sac caused by the abnormal expansion of the inguinal ring. In pigs, some genes have been related to this anomaly, but the genetic mechanisms involved remain unclear. The aim of this study was to investigate the expression profile of a set of genes in the inguinal ring tissue that may be involved in the manifestation of scrotal hernia. Thus, tissue samples from the inguinal ring/canal of male pigs with 30 days of age, normal and affected with scrotal hernia were used. Relative expression analysis was performed using qPCR to confirm the expression profile of 17 candidate genes previously identified in a RNA-Seq study. Among them, the Myosin heavy chain 1 (*MYH1*), Desmin (*DES*), Troponin 1 (*TNNI1*), Actin alpha 1 skeletal muscle (*ACTA1)* and Myosin heavy chain 7 (*MYH7*) genes were differentially expressed between groups and had reduced levels of expression in the affected animals. These genes encode proteins involved in the formation of muscle tissue, important for increasing the resistance of the inguinal ring to the abdominal pressure, which is essential to avoid the occurrence of scrotal hernia.

Keywords: gubernaculum, inguinal ring, qPCR, testicular descent, swine.

Introduction

Domestic pigs are one of the most important livestock species, also considered a good biological model for the understanding of human diseases [1]. The rapid technological advance, for which genetics have contributed significantly, allowed the exploration of important attributes in pork production, such as meat quality [2], behavior and feed efficiency [3]. However, the genetic gain has been focused on productive characteristics of great economic interest, while the selection for animals more resistant to diseases was not prioritized [4]. As consequence, physiologic and metabolic disturbances have increased in pigs, which limit the industry's progress [5]. Among these production problems, scrotal hernia disease can be highlighted as one of the problems affecting the pig development and welfare [6,7]. The hernia-affected pigs suffer from pain, discomfort, reduced welfare and, consequently, have a drop in their performance [7,8].

The scrotal hernia is a congenital malformation, characterized by the presence of intestinal loops inside the scrotum, resulting from the abnormal opening of the inguinal ring after the testicular descent [7]. This pathology may occur due to failures during the inguinoscrotal stage of the testicular descent. During this phase, the gubernaculum expands and invades the inguinal musculature through a swelling caused by glycosaminoglycans (GAGs) accumulation and proliferation of mesenchymal cells in the gubernaculum distal region, known as gubernacular bulb [9–13]. As a result of the bulb expansion, a herniation of the parietal peritoneum forms the *processus vaginalis* to receive the gonads [9]. Normally, the vaginal process is closed after testicular migration to separate the vaginal tunic and the abdominal cavity, preventing the entrance of the intestinal loops into the scrotum. However, in scrotal hernia-affect animals, the intestinal loops traverse the abnormally open inguinal ring [7]. Moreover, stimuli for vaginal process closure are controlled by the genitofemoral nerve (GFN) and its neurotransmitter calcitonin gene-related peptide (CGRP) [9–11]. Thus, dysfunctions in this mechanism may be associated to failure during the testicular descent, possibly, being a of the causes of hernias.

In pigs, heritability estimates for scrotal hernia disease range from 0.15 to 0.86 [6,15–18], indicating the influence of a genetic component in the occurrence of this malformation. Furthermore, several QTLs (quantitative trait loci) located in different chromosomes have been associated to scrotal/inguinal hernia [7,17,19–21].

Although, there are evidences of the involvement of genetic components in the occurrence of this anomaly in the pigs, the genes affecting this condition remain

unknown. Some genes previously investigated that possibly have influence on the manifestation of scrotal hernia are: *BAX* (BCL-2 associated X protein) [22], *GUSB* (β-glucuronidase) [23], *HOXA10* (Homeobox A10), *ZFPM2* (Zinc finger protein multitype 2), *COL2α1* (Collagen type II α1) and *MMP2* (Matrix metallopeptidase 2) [20], *INSL3* (Insulin-like receptor 3), *MIS* (Mullerian inhibiting substance) and *CGRP* (calcitonin gene- related peptide) [7], *COL23A1* (Collagen type XXIII alpha 1 chain) and *ELF5* (E74 like ETS transcription factor 5) [21]. These evidences suggest that the regulation and occurrence of scrotal hernia is of a polygenic nature [24].

Thus, to overcome the lack of information and improve the understanding about this disease in pigs, expression levels of a set of 17 genes previously identified in a RNA-Seq study of the inguinal region (results not shown) were analyzed by qPCR, in order to verify the possible involvement of these genes in the manifestation of scrotal hernia in 30-day-old pigs.

Material and methods

This study was performed with the approval of the Embrapa Swine and Poultry Ethical Committee of Animal Use (CEUA) under the protocol number 011/2014.

Experimental animals and sample collection

A total of 18 entire male non castrated pigs of the MS115 synthetic line with 30 days of age were used in this case-control study. Animals were raised at the Embrapa Swine and Poultry National Research Center Farm until 30 days of age, receiving feed and water *ad libitum*. The animals were grouped in control (n= 9, normal pigs with absence of malformations and coming from litters with no history of hernias) and case (n= 9, affected pigs from litters with the presence of more than one animal with scrotal hernia).

The piglets' euthanasia was performed by electrocution for 10 seconds, followed by immediate exsanguination, according to the practices recommended by the Ethics Committee. The necropsy of the animals was performed for the general evaluation of possible problems and additional characteristics for the correct characterization of the phenotype. Samples from the inguinal ring tissue of normal and hernia-affected pigs were collected for both RNA and histopathological analyses. For the RNA analysis, samples were immediately maintained in liquid nitrogen and then stored at -80° C for subsequent RNA extraction. For the histopathological analyses, samples were stored and fixed by

immersion in 4% paraformaldehyde. After necropsy, the piglet's carcasses were destined for composting.

Histopathological analysis

The histopathological analysis was performed to identify tissue alterations between case and control animals. For this, inguinal ring tissue was fixed in 4% buffered paraformaldehyde and routinely processed for histopathology. Tissue sections were obtained in an automatic microtome with 7 to 10 μm of thickness stained with hematoxylin and eosin methods and visualized in light microscopy.

RNA extraction and cDNA synthesis

Total RNA extraction from the 18 inguinal ring/canal tissue samples was performed using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Samples containing about 100 mg of tissue were initially macerated in liquid nitrogen with the aid of mortar and pistil, properly treated for use with RNA. After maceration, the generated contents were transferred to a 1.5 mL eppendorf containing 1 mL of TRIzol® reagent, vortexed and then incubated for 5 minutes at room temperature (RT, 25 ° C). After, 200 μL of chloroform was added, the tube was manually shaken vigorously for 15 seconds, and incubated at RT for another 5 minutes. After incubation, centrifugation was performed at 11,000 rpm (rotations per min) at 4 ° C for 15 minutes. Then, the aqueous phase was removed into a clean tube and 500 μl of isopropanol was added. The tube was stirred and subsequently incubated for 10 minutes at RT. After 10 minutes, the sample tubes were centrifuged for 10 minutes at 10,000 rpm at 4 ° C. The supernatant was discarded and the pellet washed with 1 mL of 75% ethanol and homogenized in vortex. This was centrifuged at 9,000 rpm for 5 minutes at 4 ° C. The supernatant was discarded and the pellet dried for 15 minutes at RT, resuspended in DEPC water and heated at 55 ° C for 10 minutes. The quality and quantity of total RNA were evaluated by quantification in Biodrop spectrophotometer and also in 1% agarose gel stained with ethidium bromide. Samples of RNA with 260/280 nm ratio greater than 1.8 were considered intact.

The cDNA (complementary DNA) synthesis was performed using the SuperScript III™ First-Strand Synthesis Supermix Kit (Invitrogen, EUA). For each 3μg of total RNA, 1μL of Annealing buffer, 1μL of oligo dT 0.5μg / μL and water until the volume of 10μL were added, incubated at 65ºC for 5 minutes and then cooled in ice for 1 minute. Then, 10

169 µL of 2X First-Strand reaction mix and and 2 µL of SuperscriptIII/RNAseOUT enzyme
170 mix (Invitrogen, USA) were added to the mixture, with subsequent incubation for 50
171 minutes at 50 °C and inactivation for 5 minutes at 85 °C, and then the cDNA was stored
172 at -20 ºC.
173
174 **Expression analysis by quantitative PCR (qPCR)**
175 The genes selected for the qPCR analysis were previously identified in a
176 transcriptome study of the inguinal ring of 60-day-old pigs (data not shown). The qPCR
177 analysis was used to evaluate the expression pattern of 17 candidate genes: Myosin
178 binding protein C slow type (*MYBPC1*), Myosin heavy chain 1 (*MYH1*), Desmin (*DES*),
179 Actin alpha 1 skeletal muscle (*ACTA1*), Actin gamma 2 (*ACTG2*), Matrix
180 metallopeptidase 1 (*MMP1*), Microtubule associated protein 1 light chain 3 gamma
181 (*MAP1LC3C*), Glucoronidase beta (*GUSB*), Calponin 1 (*CNN1*), Fibroblast growth factor
182 1 (*FGF1*), Troponin 1 (*TNNI1*), Collagen type XXIII alpha 1 chain (*COL23A1*),
183 Fibromodulin (*FMOD*), Fibrilin 2 (*FBN2*), Myosin heavy chain 7 (*MYH7*), Aggrecan
184 (*ACAN*) and Adenylate cyclase 5 (*ADCY5*). Primers for each gene (Table 1) were
185 designed in exon-exon junctions with the Primer-Blast online tool
186 (http://www.ncbi.nlm.nih.gov/tools/primer-blast, YE et al., 2012 [25]) using sequences
187 from the swine genome (Sus scrofa v10.2) on Genebank
188 (http://www.ncbi.nlm.nih.gov/gene/) and annotations from ENSEMBL 86. The NetPrimer
189 online software (http://www.premierbiosoft.com/netprimer/) was used to evaluate the
190 primers' quality.
191 The qPCR reactions were carried out in the QuantStudio 6 (Applied Biosystems,
192 USA) equipment, in a final volume of 15 µL containing 1X Maxima Mastermix SYBR
193 Green /ROX qPCR Master Mix (2X) (Thermo Fisher Scientific), 0.05 to 0.13 µM of each
194 primer and ~20 ng of cDNA. Reactions were performed following cycling condition: 95º
195 for 10 min, 40 cycles of 15 seconds at 95ºC and 30 seconds 60ºC. In addition, the melting
196 curve stage of 70°C to 95°C at 0.1°C/s for all genes studied were included to verify the
197 primers specificity. Samples were analyzed in duplicate and the cycle threshold (Ct) mean
198 for each sample was obtained and normalized for the reference genes. For normalization
199 of the qPCR analysis, the *RPL19* (Ribosomal protein L19), *H3F3A* (H3 histone, family
200 3A) and *RPL32* (Ribosomal protein L32) reference genes were used previously tested and
201 have demonstrated better stability in this experimental condition, as described by
202 Lorenzetti et al. (2018; submitted - chapter 2.1 of this thesis).

Table 1: Primers for the candidate genes used in qPCR analysis of the inguinal ring in pigs.

Gene	Chr.	Primer sequence	Nº bases	Final conc. (µM)	Ensembl ID
MYBPC1	5	F: CAAAAGGGGAGGCTGGAACT	20	0.13	ENSSSCG00000000866
		R: GCCCGACTACTCAAACCTGG	20		
MYH1	12	F: TACCAAACTGAGGAAGACCGC	21	0.05	ENSSSCG00000018005
		R: TTGGATTGTTCCTCCGCTTCC	21		
DES	15	F: ACTTCCGAGAAACAAGCCCT	20	0.05	ENSSSCG00000020785
		R: TGGCTTTAGAGCACCTCGTG	20		
ACTA1	14	F: TGAAGATCAAGATCATCGCCCC	22	0.13	ENSSSCG00000010190
		R: CAGCTGTTGGAATGGGGTTTAG	22		
ACTG2	3	F: CCTTCATCGGCATGGAGTCAG	21	0.13	ENSSSCG00000008294
		R: CAGCTGTTGGAATGGGGTTTAG	21		
MMP1	9	F: TCTATGGACCTTCCGAAAACCC	22	0.13	ENSSSCG00000014985
		R: GCTCCACTTCAGGGTAGAAGG	21		
MAP1LC3C	10	F: TGGAAACAGCTGGAGGAATGAG	23	0.13	ENSSSCG00000010870
		R: CCTCTCTTCTGGTTGCTAAGCTC	22		
GUSB	3	F: GACGGACACCTCCAAGTACC	20	0.13	ENSSSCG00000007739
		R: CAGTCCCGCGTAGTTGAAGAA	21		
CNN1	2	F: TGAGGTCAAGAACAAGCTGGC	21	0.13	ENSSSCG00000013614
		R: GGGTGGACTCATTGACCTTCTTC	23		
FGF1	2	F: CAGTGACAGCACAGAGCAGA	20	0.13	ENSSSCG00000024954
		R: GGTGCTTTCGAGGCTGAAGA	20		
TNNI1	10	F: CAGACCCGAGGCCTGTC	17	0.13	ENSSSCG00000024061
		R: GGTCCTTGATCTCCCTGGTG	20		
COL23A1	2	F: GCAATCAGGACGAGATGGCT	20	0.13	ENSSSCG00000014029
		R: TCTCCTGGTGCACCCTTTTC	20		
FMOD	9	F: CCCGCACACTCTCAGTAGAC	20	0.13	ENSSSCG00000015270
		R: CCACTGCATCTTGTATGTCTCG	22		
FBN2	2	F: AACAGTCCTGGGAGTTACCG	20	0.13	ENSSSCG00000014256
		R: ATTGCGATCCACACAGGCTC	20		
MYH7	7	F: AGGAGGCGGAGGAACAGG	18	0.13	ENSSSCG00000002029
		R: GGCAGATCAAGATGTGGCAA	20		
ACAN	7	F: CAGGAGGGGTTGTGTTCCATTA	22	0.13	ENSSSCG00000001832
		R: CCTCCTCGAAAGTCAGTGAGTAG	23		
ADCY5	13	F: CCTGCATGAAGCTTTTCCCG	20	0.13	ENSSSCG00000027952
		R: CACCAGGGTGCTGTTCATCT	20		

Chr: chromosome; F: forward; R: reverse.

Statistical analysis

The relative quantification analysis to verify the differences in gene expression level between case and control groups was performed using the Relative Expression Software Tool (REST©) [26]. The ratio of the relative expression is based on the amplification efficiency and the Ct variation of each treatment given by equation $R = (Etarget)^{\Delta CT\ target\ (control-sample)} / (E_{ref})^{\Delta CT\ ref\ (control-\ sample)}$ [27]. Significant expression differences were obtained after the application of the Pair Wise Fixed Reallocation Randomization Test©, a nonparametric statistical analysis [26]. The expression values for the Ct variation were transformed into $\log^2$ fold change.

Results

Phenotypic evaluation and histopathological analysis

The tissue sampled from the inguinal ring of the scrotal hernia-affected and control animals (Fig 1) were composed of irregular dense connective tissue (Fig 2). In the affected animal samples, the amount of fibers was slightly larger and the tissue was slightly thicker than the normal animal samples. In addition, peculiar characteristics were visually observed in the inguinal tissue according to the sample group. The tissue from affected animals presented a composition of muscular tissue with a great amount of collagen, while in the animals from control group, the tissue was more homogeneous and with a low collagenous fraction.

Fig 1. Animals affected and tissue collected. A) Scrotal hernia affected pigs. B) Intestinal loop traversing the inguinal ring. C) Inguinal ring tissue sampled.

Fig 2. Histological lamina of swine inguinal ring tissue stained with HE. A) Control sample; B) sample from a scrotal hernia affected animal.

Gene expression analysis

The inguinal ring RNA extraction presented good efficiency despite its tenacious structure. The mean RNA concentration was 1,033.19 and 1,052.66 ng/μL and the average A260/280 ratio was 1.90 ± 0.04 and 1.92 ± 0.05 nm for the normal and affected groups, respectively. All 17 genes were expressed in the evaluated experimental condition, where *ACTA1* (13.47 ± 6.43) and *FMOD* (20.05 ± 2.92) genes obtained the

lowest Ct mean in each group (CG and AG, respectively), while *ACAN* gene had the latest Ct means for both groups (Table 2).

Table 2: Mean Ct (± SD) of each gene for the normal (CG) and affected (AG) groups.

	Ct mean (± SD)	
Gene	**CG**	**AG**
MYBPC1	19.19 (5.84)	23.23 (6.79)
MYH1	17.07 (5.68)	23.63 (6.57)
DES	17.45 (3.35)	20.97 (3.98)
ACTA1	13.47 (6.43)	20.15 (7.93)
ACTG2	24.44 (2.90)	24.68 (2.08)
MMP1	27.88 (2.55)	27.55 (3.14)
MAP1LC3C	29.38 (2.71)	27.88 (3.22)
GUSB	20.82 (0.55)	20.76 (2.37)
CNN1	20.78 (2.12)	22.30 (2.36)
FGF1	28.04 (1.99)	28.14 (2.15)
TNNI1	18.60 (4.73)	25.12 (6.64)
COL23A1	28.75 (2.09)	27.65 (2.62)
FMOD	20.10 (1.65)	20.05 (2.92)
FBN2	20.79 (1.91)	21.74 (3.05)
MYH7	17.12 (5.35)	23.27 (6.56)
ACAN	30.18 (1.74)	28.61 (2.87)
ADCY5	25.83 (1.60)	25.78 (1.33)

Based on the relative expression levels of the 17 evaluated genes (Fig 3, Table 3), six of them were differentially expressed (DE) between groups. The *MYH1* ($p = 0.04$), *TNNI1* ($p = 0.05$), *DES* ($p = 0.05$), *MYH7* ($p = 0.07$) and *ACTA1* ($p = 0.08$) were downregulated in the affected compared to the control group, being, respectively, 90.91, 71.43, 11.11, 58.52 and 100.00 less expressed in the scrotal hernia affected group. On the other hand, the *ACAN* ($p = 0.07$) gene was upregulated (4.37 times) in the affected compared to the control group. In addition, there was no significant differences in the expression of the *MYBPC1* (-17.24, $p = 0.24$), *ACTG2* (-1.13, $p = 0.87$), *FBN2* (-1.96, $p = 0.39$) and *CNN1* (-2.76, $p = 0.13$) genes between normal and affected animals in our study. Nevertheless, these genes have also shown low levels of expression in the affected animals, following the same expression pattern obtained in the transcriptome of 60-days-old pigs (Table 3).

Table 3: Relative gene expression of 17 candidate genes between normal and scrotal hernia affected pigs obtained in a previous RNA-Seq study and in our qPCR analysis.

Gene	RNA-Seq		qPCR			
	logFC[1]	FDR[2]	Relative expression logFC	Standard error mín.	Standard error máx.	P-value
MAP1LC3C	7.00	$6.10E^{-35}$	2.92	0.25	39.10	0.26
ACAN	2.78	$3.90E^{-04}$	4.37	0.50	38.4	0.07
FGF1	1.88	$4.10E^{-03}$	-1.04	0.16	8.96	0.95
FMOD	1.33	$3.60E^{-02}$	1.19	0.20	5.59	0.80
GUSB	0.96	$3.60E^{-02}$	1.07	0.55	2.89	0.90
ADCY5	-1.13	$4.80E^{-02}$	1.05	0.61	2.44	0.83
FBN2	-1.88	$2.40E^{-02}$	-1.96	0.18	1.97	0.39
COL23A1	-2.99	$6.00E^{-03}$	2.33	0.25	20.00	0.25
CNN1	-3.75	$2.10E^{-08}$	-2.76	0.07	2.04	0.13
MMP1	-5.28	$1.60E^{-02}$	1.20	0.07	13.20	0.85
ACTG2	-5.96	$7.50E^{-08}$	-1.13	0.12	7.11	0.87
DES	-7.57	$6.60E^{-14}$	-11.11	0.00	2.20	0.05
TNNI1	-9.74	$2.20E^{-08}$	-71.43	0.00	2.75	0.05
MYH7	-10.27	$4.60E^{-06}$	-58.82	0.00	2.61	0.07
ACTA1	-11.83	$1.20E^{-10}$	-100.00	0.00	2.66	0.08
MYH1	-11.95	$1.40E^{-06}$	-90.91	0.00	2.05	0.04
MYBPC1	-14.59	$2.10E^{-10}$	-17.24	0.00	9.65	0.24

[1]logFC - fold change; [2]FDR - false discovery rate.

Fig 3: Relative expression levels of 17 hernia candidate genes in the inguinal ring of normal and scrotal hernia-affected piglets. *Significant at $p \leq 0.05$; †Significant at $p \leq 0.08$.

Discussion

Understanding the global physiological processes and protein interactions that coordinate testicular descent is necessary to clarify the factors triggering herniation. However, there are striking differences among mammals regarding testicular descent [9] and potential failures that can occur during phases related to scrotal hernia in pigs, which are under the control of several genes. Therefore, expression studies applied to the inguinal tissue, the primary site of the hernia malformation, are required to clarify the genetic mechanisms involved in this anomaly. Here, the expression profile of a set of genes previously identified in the inguinal ring tissue was evaluated. However, an obstacle for hernia expression studies is inherent to the composition of the herniated

tissue. According to Morsczeck et al. (2008) [28], no reliable method has been described for a quantitative gene expression analysis of hernia tissues. In rats, the RNA isolation from those tissues is difficult because of their tenacious structure and high amount of insoluble collagen [29]. Therefore, histological characterization of the inguinal ring tissue was performed to identify tissue alterations possibly related to the observed gene expression profile.

In the histological analysis, the inguinal tissue from the affected animals revealed the presence of a slightly larger amount of collagen fibers and denser connective tissue than the normal piglets (Fig 2). In human fetuses, this type of structure was reported to be the tissue resulting from extensive gubernacular remodeling after testicular migration [14]. During the progress of the gestational period, the concentrations of collagen within the gubernaculum increase, forming a dense fibrous tissue [29]. In addition, during the piglets necropsy and sample collection of the inguinal ring tissue, it was observed that all affected animals (n= 9) had unilateral occurrence of the hernia, specifically on their left side. Despite scrotal hernia can be both unilateral or bilateral, unilateral malformation has been reported on the left side as more frequent than on the right side [15]. The physiological causes of this preferential unilateralism are unknown to date. In humans, the closure of the left inguinal ring precedes the right [30]. Therefore, it is possible to assume that the testicular descent demonstrates an independent regulation for each gonad [12].

The inguinal ring is similar to a sphincter [31], being a muscular structure with the ability to sustain and contract. If there is a need for force generation to occlude the vaginal process (the inguinal ring must compress and 'close'), in this case, low concentrations of muscle proteins could be related to the weakness of the inguinal region. In addition, the passage of the testes from the intra-abdominal region to the scrotum appears to be a combined effect of changes in the gubernacular tissue and the expansion of the organs, which would increase abdominal pressure [9,13]. Consequently, a great pressure on the inguinal region could hinder the natural regression and closure of the inguinal ring, probably due to the reduced capacity of contraction, especially if the inguinal ring is flaccid. This could be related to the function of the desmin protein, coded by *DES*, which, in this study, was DE ($p \leq 0.05$) between groups, being downregulated in piglets affected with scrotal hernia. The desmin is involved in the arrangement, organization and cell survival, and in the transmission of the contractile force that focuses on the Z discs of the musculature [32]. However, it is necessary to consider the desmin

interactions with other proteins, such as myosin, actin, troponin and myosin binding protein.

In this study, with 30 days-old MS115 piglets, the *MYH1, TNNI1, DES, MYH7, ACAN* and *ACTA1* were DE in the qPCR analysis, being downregulated in affected piglets, except for the *ACAN*, which was upregulated in piglets with scrotal hernia (Fig 3, Table 3). The same expression pattern of these genes was observed in the RNA-Seq study with 60 days-old Landrace pigs (Table 1, unpublished data). Most of the evaluated genes in this study encode proteins that constitute the skeletal muscle or have functions in this tissue, being the reduced expression of the *MYH1, DES, TNNI1, MYH7* and *ACTA1* genes a possible implication for the appearance of the scrotal hernia. The observed DE genes evidence the importance of muscle strengthening in the inguinal region to prevent the manifestation of this disease.

In striated muscle, sarcomeric myosins consist of heavy, light and regulatory chains that are encoded by different genes [33]. All striated muscles express myosin heavy chain and troponin genes. Here, both *MYH1* and *TNNI1* were DE between groups (Fig 3, Table 3). Myosin Heavy Chain 1 (*MYH1*), also described as MyHC-2x, is an intermediate protein between muscle fibers of types 2A and 2B [34] and previous studies have described this type of myofibril as more frequent in swine breeds or lines with a slower growth rate [35–37]. Otherwise, the troponin protein is composed by three subunits that perform specific functions and maintains the troponin subunits linked to actin thought calcium-regulated interactions [38]. The Troponin I, encoded by the *TNNI1* gene, acts as an inhibitor of the ATPase of actomyosin [39]. Expressed in the skeletal muscle, this gene acts during myogenesis and skeletal muscle development [40]. High levels of *TNNI1* expression were reported in different pig muscle tissues, breeds and ages in association with growth rate and myofibril type [39,41]. Together with other muscle proteins, these set of proteins (myosins, actin and troponins) controls muscle contraction, in a process between thick filaments of myosin and fine actin filaments, which slide between them, activated by actomyosin and regulated by ions calcium [38]. The Ca, besides participating in regulatory systems for muscle contraction, may also be involved in the occurrence of hernias. Low levels of Ca in the urogenital tissue of piglets have been previously investigated and appear to influence the manifestation of scrotal hernia, by the participation of calcium in pathways related to cellular apoptosis and closure of the vaginal process [42]. However, in this study, levels of calcium in the inguinal tissue were

not measured in the moment of sample collection for RNA extraction to confirm this hypothesis.

An interesting gene involved in apoptosis is the *MAP1LC3C* gene. The *MAP1LC3C* gene is involved in autophagic processes [43,44], a cellular event capable of preventing apoptosis, which is regulated by several signaling pathways, such as calcium, although both autophagy and apoptosis can be regulated by the same proteins [45]. In addition, for obliteration of the vaginal process, smooth muscle apoptosis is required [46]. It is tempting to suggest that the upregulation of *MAP1LC3C* is a determining factor to prevent apoptosis, which would increase the possibility of herniation. On the other hand, the defect observed in the closure of the inguinal ring or of the vaginal process may be potentially due to the presence of non-differentiated cells, known as myofibroblasts [47]. According to Tanyel et al. (2001) [48], the existence of this cell type reflects an attempt of the smooth muscle apoptosis, which seems to be indispensable for occlusion of the vaginal process. Pietrain pigs carrying a mutation in the *BAX* gene (protein X associated with *BCL2*), that is involved in apoptosis, demonstrated a higher incidence of scrotal hernia [22]. This reinforces the concept about programmed cell death, which is ultimately a remodeling of the gubernacular connective tissue, in order to reduce its size after the testicular descent [14], a necessary process for the inguinal ring closure, thus preventing the intestinal loops from reaching the scrotum. Although the *MAP1LC3C* gene was upregulated in affected animals from the RNA-Seq study with 60-days-old pigs, no significant difference was observed between normal and affected piglets in our study, with 30 days of age (Table 3). The upregulation of this gene in the affected animals at 60 days of age may be due to a reflection of the maintenance of the apoptotic pathway. In other words, it is possible that with the advancement of age this expression intensifies to induce the establishment of muscle cells in the region of the inguinal ring.

The expression of the *ACAN* gene was upregulated in affected piglets ($p = 0.07$), presenting the same expression pattern as the one observed in the RNA-Seq study, with older pigs (Fig 3, Table 3). This might suggest that there is a large accumulation of proteoglycan and glycosaminoglycan molecules (GAGs) in the tissue that originate the inguinal ring. The Agrecan protein, coded by *ACAN*, promotes the aggregation of other proteins in its structure, mainly chondroitin and keratan sulfates (GAGs), being one of the types of extracellular proteoglycans (hialectanos) with high affinity to hyaluronic acid and together, they form complexes of high viscosity [49,50]. Chondroitin sulfate appears to be necessary for myogenesis initiation [51], but the amount of this GAG becomes

reduced with the advancement of the gestational period [29]. Furthermore, the hyaluronic acid (HA) accumulate in the gubernacular bulb to promote dilatation of the inguinal ring during the vaginal process and then, facilitate the passage of the testes [9,13]. Afterwards, HA is degraded by hyaluronidases, β-hexosaminidases and β-glucuronidases (*GUSB*) during the second phase of testicular descent [23]. However, it is possible that the swelling step during the vaginal process make the gubernaculum too dilated and flaccid. Abnormal opening of the inguinal ring, together with poor muscle protein performance and increased abdominal pressure, could be some of the primary factors of herniation. It is also possible that the reduced contents of chondroitin sulfate to initiate myogenesis could compromise the expression of muscle genes. The upregulation of *ACAN* in scrotal hernia-affected pigs at both ages, 30 and 60 days (Table 2), is possibly a way of the inguinal tissue cells in aggregating large amount of chondroitin sulfate to initiate the myogenesis, as a consequence of the failure of the apoptotic pathway.

All 17 evaluated genes were functional candidates to be associated to hernias since they were DE in a scrotal hernia case and control transcriptome study. Furthermore, those genes were also biological candidate due to their functions in the regulatory mechanisms potentially related to the development of hernias. However, most of the evaluated genes were not DE ($p>0.10$) in the qPCR analyses with 30 days-old piglets (*MAP1LC3C, FGF1, FMOD, GUSB, ADCY5, FBN2*, *COL23A1, CNN1, MMP1, ACTG2* and *MYBPC1*). This difference in results could be due to the distinct experimental conditions, which can influenced the expression variability of the genes. Scrotal hernia is a congenital disease related to malformations in animal development, so breed and age effects, which varied between the two studies (RNA-Seq and qPCR) are possibly important for variation on gene expression in the evaluated tissue. Some studies found significant differences in the frequency of this defect among breeds or genetic lines [6,17,21]. Besides the breed effect, age can have an important effect on conformation, tissue composition and anatomic differences in the inguinal ring, causing variation in the genes expression profile in the evaluated region. In humans, aging significantly alters the expression profile of genes and the occurrence of certain diseases are strongly linked to such changes [52]. Therefore, further studies are recommended to clarify the isolated contribution of age and breed to variations on the genes' expression profile of this tissue in pigs.

In this study, *MYH1, DES, TNNI1, ACTA1* and *MYH7* genes that encode muscle proteins were downregulated in scrotal hernia-affected pigs under the experimental conditions. These evidences point to the lower resistance of the inguinal tissue to support

the abdominal pressure generated during the period of testicular descent in the animals affected with scrotal hernia. The histological analysis demonstrated the presence of a collagen structure that may not provide the necessary support, but may also indicate disordered and incomplete events of anatomical and physiological changes required in the gubernaculum, involving complex regulatory cascades of apoptosis, cellular morphogenesis and other interactions for completing the occlusion process and the development of this type of hernia. This is in agreement with the hypothesis that the hernia development is a multifaceted process [24].

Conclusions

The downregulation of *MYH1, DES*, *TNNI1*, *ACTA1* and *MYH7* have a potential role in the anatomical constitution of the inguinal ring tissue being possibly involved with the development of scrotal hernia in pigs.

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Author Contributions

Conceived and designed the experiment: JOP MCL AMGI.

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Competing Interests

The authors declare that they have no competing interests.

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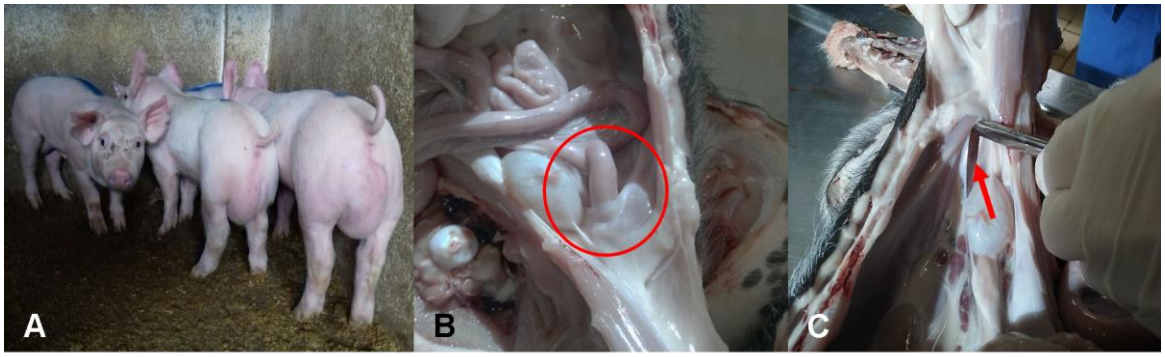


Fig 1. Animals affected and tissue collected. A) Scrotal hernia affected pigs. B) Intestinal loop traversing the inguinal ring. C) Inguinal ring tissue sampled.

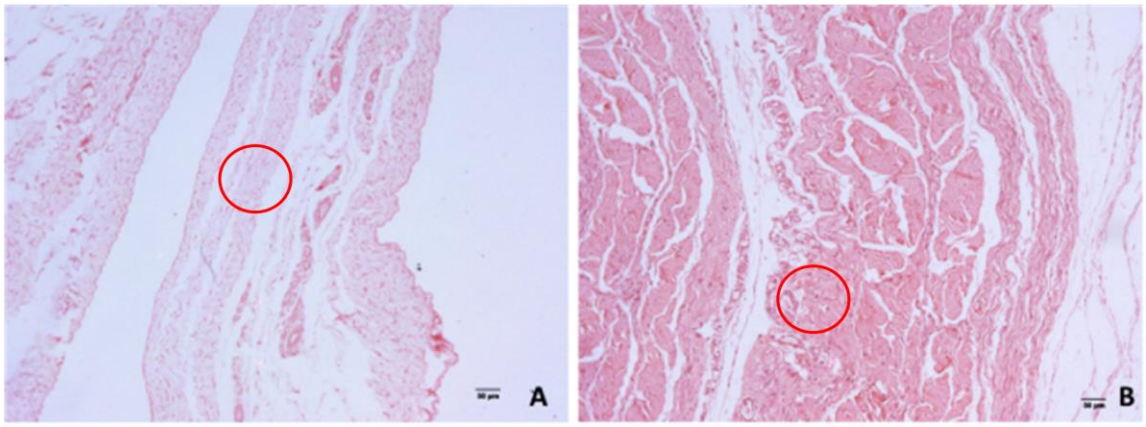
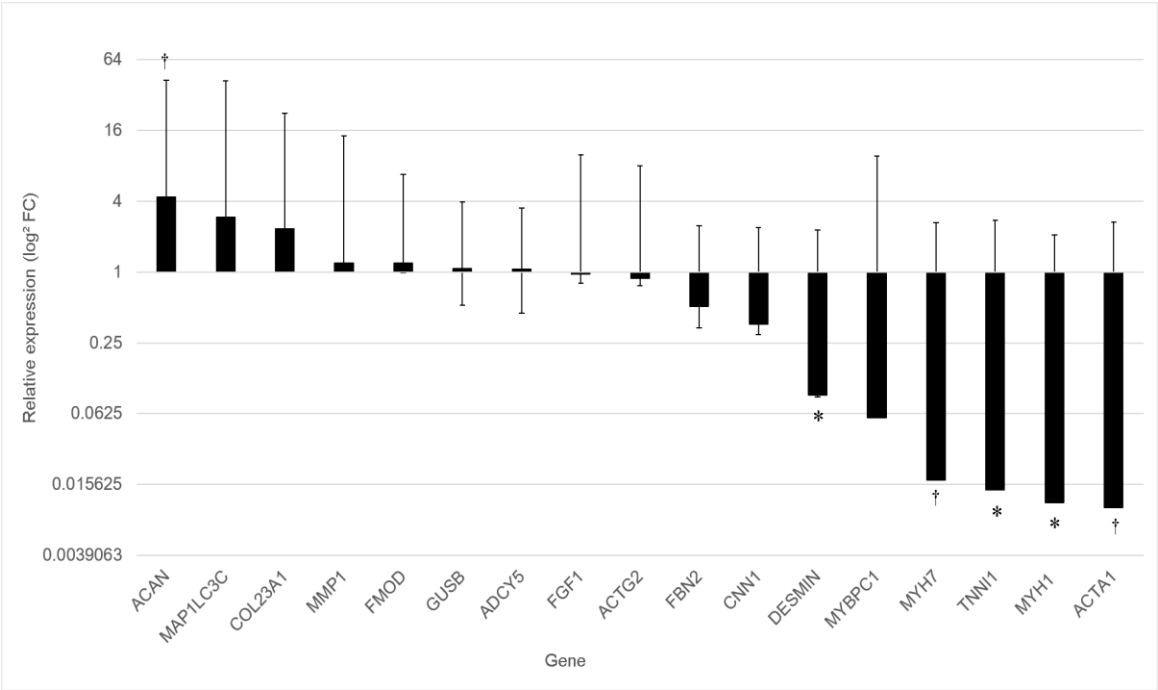


Fig 2. Histological lamina of swine inguinal ring tissue stained with HE. A) Control sample; B) sample from a scrotal hernia affected animal.



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676 **Fig 3. Relative expression levels of 17 hernia candidate genes in the inguinal ring of**
677 **normal and scrotal hernia-affected piglets.** *Significant at $p \leq 0.05$; †Significant at
678 $p \leq 0.08$.

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3. CONSIDERAÇÕES FINAIS

Neste estudo, foram conduzidos experimentos que averiguaram os níveis de expressão de genes no tecido do anel inguinal, onde foram realizadas comparações entre grupos de animais normais e afetados com hérnia escrotal.

Primeiramente, foram avaliados os padrões de expressão de 10 genes geralmente utilizados como normalizadores em estudos de expressão gênica. Foram observadas variações marcantes e fortemente afetadas por diferenças intrínsecas dos tecidos inguinais entre as idades (30 e 60 dias) e raças suínas utilizadas (MS115 e Landrace), bem como as ferramentas (BestKeeper, geNorm, NormFinder e método DeltaCt) empregadas para distinguir quais são os genes que demonstram elevada consistência de expressão. Para cada condição experimental foram recomendados diferentes conjuntos de genes para avaliação da expressão no tecido inguinal.

Posteriormente, os níveis de expressão relativa de 17 genes candidatos à ocorrência da hérnia escrotal foram avaliados e 6 destes foram diferencialmente expressos. Os genes *MYH1, DES, TNNI1, ACTA1* e *MYH7* foram menos expressos, enquanto o gene *ACAN* foi mais expresso no tecido inguinal de suínos afetados com hérnia escrotal aos 30 dias de idade quando comparado aos animais normais. Esses resultados indicam a possível atuação destes genes no desenvolvimento da patologia e contribuem de forma considerável para o avanço no conhecimento sobre o controle genético envolvido na ocorrência da hérnia escrotal.

Em suma, os efeitos de raças e idades dos suínos interferiram no perfil de expressão gênica, em razão às características teciduais do anel inguinal. Para tanto, com o avançar da idade, são esperadas alterações no padrão de expressão e propriedades funcionais de proteínas no tecido inguinal, o que torna difícil prever a origem da hérnia escrotal. Assim, futuras investigações durante o período gestacional ou em menores idades pós-natal podem facilitar a compreensão sobre a ocorrência da malformação.

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ANEXOS

Embrapa	Certificado de Conduta Ética	ETICA 1/1

CERTIFICADO

Certificamos que o Protocolo nº**(000/AAAA): 011/2014**, sob título **"Identificação de genes e polimorfismos associados à formação de hérnias em suínos pela combinação do sequenciamento exômico total e do RNA",** sob responsabilidade de **Mônica Ledur** está de acordo com os Princípios Éticos na Experimentação Animal, adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), **TENDO SIDO CONSIDERADO APROVADO PELA** Comissão de Ética no Uso de Animais (CEUA/CNPSA) em reunião realizada em **07/ 11/ 2014**.

CERTIFICATE

We certify that the Protocol nº **(000/YYYY): 011/2014**, under the following title **"Identification of genes and polymorphisms associated with formation of hernias in swines combining RNA and whole exons sequencing."** is in agreement with the Ethical Principles in Animal Research adopted by Brazilian College of Animal Experimentation (COBEA) and was approved by the Embrapa Swines and Poultry Ethical Committee for Animals utilization in experimentation (CEUA/CNPSA) in **11/07/2014**.

Concórdia, **07/11/2014**.

Presidente CEUA/CNPSA

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