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Regulação da divergência folicular: Sinalização Intracelular e Hormônio Anti-mülleriano

TESE DE DOUTORADO

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Regulação da divergência folicular: Sinalização Intracelular e Hormônio Anti-mülleriano

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
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REGULAÇÃO DA DIVERGÊNCIA FOLICULAR: SINALIZAÇÃO INTRACELULAR E HORMÔNIO ANTI-MÜLLERIANO

Elaborada por
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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
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REGULAÇÃO DA DIVERGÊNCIA FOLICULAR: SINALIZAÇÃO INTRACELULAR E HORMÔNIO ANTI-MÜLLERIANO
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O desenvolvimento folicular inicial em espécies monovulatórias ocorre no padrão de ondas foliculares e é regulado por uma complexa interação entre gonadotrofinas e moléculas regulatórias intrafoliculares. No primeiro estudo, a indução de folículos codominantes *in vivo* através do tratamento com FSH foi utilizada para o estudo dos mecanismos de seleção do folículo dominante em bovinos. Foi determinado o status funcional dos padrões de sinalização STAT3, AKT e MAPK nas células da granulosa de folículos dominantes (DF), subordinados (SF) e codominantes (co-DF). Os resultados deste estudo demonstraram que comparados com células da granulosa de DFs, a expressão relativa de RNAm para *MAPK1/3* e *AKT1/2/3* foram significativamente maiores em células da granulosa de SFs. Contudo, houve uma tendência para menor abundância de proteína fosforilada de MAPK3/1 e AKT em células da granulosa de SFs. A abundância relativa de RNAm e a proteína fosforilada de STAT3 foram significativamente maiores em células da granulosa de SFs em comparação com DFs e co-DFs. Em linha com esses resultados, células da granulosa de SFs tiveram maiores níveis de RNAm para *LIFR* e *IL6ST*, dois receptores envolvidos na ativação do STAT3. Neste primeiro estudo concluímos que a atresia de SFs está associada ao aumento das expressões de *LIFR* e *IL6ST* e consequente ativação da STAT3 nas células da granulosa. Essas características moleculares estavam ausentes nos co-DF2s sugerindo que o FSH resgata co-DF2s através da ativação do MAPK e AKT, e inibição do padrão STAT3. No segundo estudo, investigamos em dois modelos bovinos *in vivo* a regulação do AMH e seu receptor (AMHR2) durante a divergência folicular e o efeito da indução de folículos codominantes com FSH na produção de AMH. Este estudo demonstrou que as expressões de RNAm para AMH foram similares em F1s e F2s antes da divergência folicular (Dia 2). Por outro lado, foram maiores em células da granulosa de DFs em relação a SFs no momento esperado (Dia 3) e após (Dia 4) a divergência folicular. Não houve diferença nos níveis de RNAm para *AMHR2* durante o processo de divergência folicular. Contudo, após a divergência (Dia 4) houve uma tendência de aumento nos níveis de RNAm para *AMHR2* em DFs em relação a SFs. No modelo de codominância, as expressões de RNAm para AMH foram similares entre os folículos dentro dos grupos. No entanto, folículos suplementados por FSH apresentaram maiores níveis de RNAm para AMH. Esses dados foram complementados pela abundância da proteína AMH nas células da granulosa, a qual foi maior em co-DFs e DFs em comparação a SFs. Por outro lado, os níveis de RNAm para AMHR2 foram maiores em DFs em relação a SFs e similares entre co-DFs. Neste segundo estudo, os resultados sugerem que o AMH é regulado na divergência folicular, sendo estimulado por FSH, enquanto que o AMHR2 é regulado na atresia folicular.
Palavras chave: ovário, granulosa, padrões de sinalização, AMH, FSH, folículo codominante

ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

REGULATION OF FOLLICULAR DEVIATION: INTRACELULLAR SIGNALING AND ANTI-MULLERIAN HORMONE

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Santa Maria, December 09th, 2014.

Follicular development occurs in wave like patterns in monovulatory species and is regulated by a complex interaction of gonadotropins with local intrafollicular regulatory molecules. In the first study, induction of co-dominant follicles *in vivo* with FSH treatment was used to study morphological mechanisms of selection of the dominant follicle in cattle. We determined the functional status of the STAT3, AKT and MAPK signaling pathways in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles. We observed that the relative mRNA abundance of *MAPK1/3* and *AKT1/2/3* was significantly higher in granulosa cells of SFs than in DFs. However, there was a tendency for lower abundance of phosphorylated *MAPK3/1* and AKT proteins in SFs granulosa cells. Relative abundance of mRNA and phosphorylated isoform of STAT3 was higher in granulosa cells of SFs than DFs and co-DFs. In line with this, SF granulosa cells had higher mRNA levels of *LIFR* and *IL6ST*, the two receptors involved in STAT3 activation. In summary, in the first study, we showed that atresia of SFs is associated with increased expression of *LIFR* and *IL6ST*, and activation of STAT3 in granulosa cells. These molecular features were absent in co-DF2, suggesting that FSH rescues co-DF2s through activation of MAPK and AKT, and inhibition of STAT3 pathways. In the second study, we investigated the regulation of AMH and its receptor (AMHR2) during follicular deviation and the effect of FSH-induced codominant follicles on AMH production in two *in vivo* models. In this study, we observed that *AMH* mRNA expression was similar in F1 and F2 before deviation (Day 2). On the other hand, the *AMH* mRNA levels were higher in DFs than SFs at the expected time (Day 3) and after (Day 4) deviation. There was no statistical difference at *AMHR2* mRNA expression during the deviation process. However, *AMHR2* tended to be more expressed in DFs than SFs after deviation (day 4). In the co-dominant model, *AMH* mRNA levels in granulosa cells were similar among the follicles within the groups. However, FSH supplemented follicles had more *AMH* abundance than control follicles. These data were complemented by AMH protein which was higher in FSH-supplemented follicles (co-DFs) and DFs than SFs. On the other hand, *AMHR2* mRNA was higher in DFs than in SFs and similar between co-DFs. Our results from the second study suggest that AMH expression is regulated during follicular deviation, being stimulated by FSH, whereas *AMHR2* is downregulated during advanced atresia.

Keywords: ovary, granulosa, signaling pathways, AMH, FSH, co-dominant follicle

LISTA DE FIGURAS

ARTIGO 1
Figure 1 39
Figure 2 40
Figure 3 41
Figure 4 42
Figure 5 43
Figure 6 44
ARTIGO 2
Figure 1 62
Figure 2 63
Figure 3 64

LISTA DE TABELAS

ARTIGO 1
Table 1 36
Table 2 37

SUMÁRIO

1. INTRODUÇÃO **11**
2. REVISÃO BIBLIOGRÁFICA **14**
2.1. Desenvolvimento folicular antral em bovinos **14**
2.2. Rotas de sinalização durante a divergência folicular **15**
2.3. Hormônio Anti-mülleriano **18**
3.ARTIGO 1 .... **22**
3.1. Introduction **25**
3.2. Materials and methods **26**
3.3. Results **28**
3.4. Discussion **30**
3.5. Acknowledgements **32**
3.6. References **33**
4.ARTIGO 2 .... **45**
4.1. Introduction **48**
4.2. Materials and methods **49**
4.3. Results **52**
4.4. Discussion **54**
4.5. Acknowledgements **57**
4.6. References **57**
5. DISCUSSÃO **65**
CONCLUSÃO **70**
REFERÊNCIAS **71**

1. INTRODUÇÃO

O ciclo estral em mamíferos é caracterizado por ondas de crescimento folicular e culmina com a dominância de um folículo e a ovulação de um oócito. O controle endócrino desse processo é conhecido e determinado pelos hormônios hipofisários folículo estimulante (FSH) e hormônio luteinizante (LH) juntamente com esteroides ovarianos (BEG et al., 2002). Porém, a complexa dinâmica parácrina e autócrina que envolve o crescimento, desenvolvimento e maturação do folículo ovariano em espécies monovulares ainda não está totalmente esclarecida.

As gonadotrofinas, LH e FSH, exercem seus efeitos nas células foliculares através dos seus receptores ligados à proteína G, os quais aumentam a produção de AMPc e ativam a *protein kinase* A (PKA) (SEGER et al., 2001). Esses hormônios também podem ativar outros padrões de sinalização que incluem AKT (*protein kinase B*) e MAPK (*Mitogen-activated protein kinase*) (BABU et al., 2000; ZELEZNIK et al., 2003). Muitos estudos demonstraram as funções da sinalização via AKT e MAPK nas células da granulosa, principalmente em modelos *in vitro* e parecem ser importantes para a dominância folicular (EVANS; MARTIN, 2000).

Muitos fatores de sinalização atuam nas células da teca, da granulosa e no oócito contribuindo para a proliferação e diferenciação folicular (GLISTER et al., 2010). Atualmente, o foco da pesquisa nessa área é entender os estágios iniciais do desenvolvimento folicular, com a finalidade de buscar quais os fatores responsáveis pelo recrutamento e dominância folicular (FORTUNE et al., 2011). As pesquisas sobre esse processo, também chamado de divergência folicular, onde um folículo segue em crescimento e o restante se torna atrésico, podem contribuir para o aprimoramento de biotécnicas da reprodução, contracepção e controle de patologias ovarianas.

Durante a divergência folicular, a apoptose é um processo fisiológico que está envolvido na mudança que ocorre no folículo para cessar seu crescimento e entrar em atresia. Esse processo possui um importante papel no desenvolvimento celular a partir de respostas intracelulares a sinais extracelulares. O fator de sinalização *Signal transducer and activator of transcription* (STAT) media a sinalização de citocinas, fatores de crescimento e pode ter relação com os padrões de sinalização AKT e MAPK (RAWLINGS et al., 2004; ABELL; WATSON, 2005). De fato, o efeito do padrão pró-apoptótico pelo qual STAT3 media uma mudança na sinalização da rota AKT já foi proposto (ABELL et al., 2005). Em um modelo de divergência folicular, nosso grupo de pesquisa demonstrou em bovinos um aumento agudo na proteína

STAT3 fosforilada em células da granulosa de folículos coletados no dia 4 da onda folicular e isso sugere que esse padrão possa estar envolvido na morte celular e consequente atresia do folículo subordinado (GASPERIN et al., 2014b). Desta forma, esclarecer a real participação do STAT3 no processo apoptótico em células foliculares, bem como o efeito do FSH nas principais vias de sinalização celular no folículo; STAT3, AKT e MAPK, torna-se iminente.

Os membros da superfamília do fator de crescimento e diferenciação β (TGFβ), incluindo inibinas, ativinas, proteínas morfogenéticas ósseas (BMPs) e, mais recentemente, o hormônio anti-mülleriano (AMH) vem sendo estudados por atuarem na proliferação e diferenciação folicular em várias espécies (KNIGHT; GLISTER, 2006). Estudos demonstram que ligantes associados à superfamília TGFβ são expressos no ovário, o que dá suporte a um possível papel em múltiplos aspectos do desenvolvimento folicular, recrutamento, proliferação celular/atresia, esteroidogênese, maturação do oócito, ovulação e formação do corpo lúteo (KNIGHT; GLISTER, 2006; CANTY-LAIRD et al., 2010; GLISTER et al., 2010; RICO et al., 2011).

O Hormônio anti-mülleriano (AMH) é uma glicoproteína, também pertencente à superfamília TGFβ (CATE et al., 1986), com conhecida importância no desenvolvimento gonadal, onde é responsável pela regressão dos ductos de müller em fetos masculinos. Recentemente, tem sido estudada a sua relação com o desenvolvimento e função reprodutiva em ambos os sexos. O AMH é descrito como potencial candidato intra-ovariano com função de inibidor da ativação folicular primordial (DURLINGER et al., 1999), sendo este o foco de muitos estudos que estão sendo desenvolvidos nessa área (MONNIAUX et al., 2012). Porém, uma possível participação do AMH na regulação do desenvolvimento de folículos antrais e na seleção do folículo dominante ainda não foi estudada.

Uma vez que todos os folículos estão sob o mesmo ambiente endócrino, faz-se necessário entender a participação de fatores locais no processo de diferenciação ou atresia folicular. O modelo bovino *in vivo* vem sendo utilizado por nosso grupo de pesquisa para a identificação de fatores envolvidos na seleção folicular, maturação oocitária, ovulação e luteólise sob o mesmo ambiente endócrino e preservando a interação entre as células ovarianas (FERREIRA et al., 2007; BARRETA et al., 2008; FERREIRA et al., 2011; GASPERIN et al., 2012; BARRETA et al., 2013; GASPERIN et al., 2014a). Por outro lado, desenvolver outro modelo *in vivo* a partir da suplementação de FSH possibilitará coletar folículos em um mesmo ambiente endógeno dessa gonadotrofina para o estudo dos eventos que ocorrem durante a divergência folicular.

Neste contexto, o primeiro estudo foi realizado a partir de um modelo de codominância folicular em bovinos *in vivo,* no qual objetivou avaliar os padrões de sinalização STAT3, AKT and MAPK em folículos codominantes (co-DFs) em contraste com folículos dominantes (DF) e subordinados (SF). Em um segundo estudo, utilizamos dois modelos *in vivo* para avaliar a participação do AMH e seu receptor (AMHR2) em células da granulosa durante a divergência folicular e a influência do FSH no perfil do AMH em células da granulosa de folículos codominantes.

2. REVISÃO BIBLIOGRÁFICA

2.1. Desenvolvimento folicular antral em bovinos

O controle endócrino do processo reprodutivo dos mamíferos é determinado pelos hormônios hipofisários folículo estimulante (FSH) e hormônio luteinizante (LH) juntamente com esteroides ovarianos (BEG et al., 2002). O crescimento, desenvolvimento e maturação do folículo ovariano são fundamentais neste processo.

O ciclo estral é caracterizado por ondas de crescimento folicular e a emergência de uma onda culmina com a dominância de um folículo (GINTHER et al., 2001). O FSH atua nessas ondas foliculares, o que é demonstrado pelo aumento da concentração circulante desse hormônio no início de uma onda, e os seus níveis começam a diminuir nos dias 2 e 3 após a emergência. A partir desse evento, somente o folículo em dominância segue o seu crescimento, enquanto que os restantes entram em atresia via apoptose (BEG et al., 2002).

A mudança na qual somente um folículo segue em crescimento e o restante torna-se atrésico é denominada divergência folicular e esse mecanismo ainda precisa ser melhor elucidado. Raças bovinas taurinas e zebuínas possuem peculiaridades nesse processo onde a divergência ocorre quando o maior folículo se encontra com um diâmetro em torno de 8,5 e 6,0mm, respectivamente (BEG; GINTHER, 2006).

A habilidade com que o folículo dominante responde ao ambiente endócrino com padrões individuais de crescimento e desenvolvimento pode ser devido à aquisição precoce de receptores para LH nas células da granulosa, maior fluxo sanguíneo no folículo e/ou a maior disponibilidade de fatores produzidos localmente (EVANS; FORTUNE, 1997b). Além disso, folículos dominantes têm níveis mais elevados de RNAm para enzimas envolvidas na síntese de esteroides em comparação aos subordinados (FORTUNE et al., 2001).

Estradiol, fatores de crescimento semelhantes à insulina (IGFs), inibina e ativina, e angiotensina II são fatores candidatos a participar do desenvolvimento de folículos dominantes (GINTHER et al., 2001; FERREIRA et al., 2011). O FSH, mesmo em baixos níveis, parece ser importante na fase de dominância uma vez que a redução experimental de seus níveis está associada com uma parada no crescimento e, em alguns animais, com a perda da dominância (FORTUNE, 1994). O estradiol, nas células da granulosa, aumenta a atividade da aromatase (enzima conversora de andrógenos em estrógeno), promove expressão de receptores de LH, aumenta a sensibilidade ao FSH e LH, e aumenta a síntese de IGF-1 (BEG; GINTHER, 2006).

Após o estabelecimento da dominância, o folículo selecionado cresce até atingir diâmetro pré-ovulatório e é responsável pela alta produção de estradiol e inibina-A, que resultam em diminuição das concentrações de FSH, impedindo o crescimento de uma nova onda. Durante esse crescimento final, ocorrem as maiores taxas de atresia (FORTUNE, 1994; HUNTER et al., 2004), uma vez que com baixos níveis séricos de FSH, aqueles folículos que não expressam receptores LH em suas células da granulosa são incapazes de expressar a enzima aromatase, o que ocasiona uma queda dos níveis séricos de estradiol. A pulsatilidade aumentada do LH, induzida pelo estradiol, é necessária para a diferenciação final do folículo dominante, indução do pico de LH, ovulação e luteinização (FORTUNE, 1994; FORTUNE et al., 2001; MIHM et al., 2002).

Atualmente, o foco da pesquisa nessa área é entender os estágios iniciais do desenvolvimento folicular, com a finalidade de buscar quais os fatores responsáveis pelo recrutamento e dominância folicular (FORTUNE et al., 2011). A complexa dinâmica da onda folicular em espécies monovulares, no qual somente um folículo segue em crescimento enquanto os outros regridem, ainda não está totalmente esclarecida, principalmente em relação aos fatores locais que atuam no ovário.

2.2. Rotas de sinalização durante a divergência folicular

A família das *Signal transducer and activator of transcription* (STATs) formam um conjunto de sete proteínas com transcrição latente que mediam a sinalização de citocinas e fatores de crescimento que estimulam proliferação celular, diferenciação, migração e apoptose (LEVY; DARNELL, 2002; RAWLINGS et al., 2004). Essas proteínas dimerizam e acumulam no núcleo da célula onde ocupam lugares específicos do DNA estimulando o aumento da transcrição (BROMBERG; DARNELL, 2000). Após a ativação do receptor, as STATs tornam-se ativadas pela fosforilação da tirosina, a qual tipicamente ocorre através da associação das janus quinases (JAKs) (BROMBERG; DARNELL, 2000). Com isso, as proteínas STAT dimerizam e acumulam no núcleo onde se ligam em sequências específicas de DNA para alterar a transcrição (BROMBERG; DARNELL, 2000). A ativação dessa rota de sinalização na foliculogênese ainda não está claramente elucidada e pode ser um fator chave no controle da ativação e desenvolvimento folicular (SOBINOFF et al., 2013).

A mudança que ocorre no folículo para sessar seu crescimento e entrar em atresia envolve um processo fisiológico chamado de apoptose. O processo de apoptose possui um

importante papel no desenvolvimento e na regulação do processo celular a partir de respostas intracelulares a sinais extracelulares. Consequentemente, muitos desses sinais ativam fatores de transcrição específicos que modelam genes da maquinaria apoptótica (KRITIKOU et al., 2003). A maioria das células da granulosa de folículos atréticos entram em apoptose e muitos fatores associados a esse processo foram demonstrados como cruciais para a regulação do crescimento e atresia folicular (MATSUDA et al., 2012). A falta de fatores-chave que promovem a sobrevivência celular ou a estimulação de ligantes que promovem a morte celular são as principais causas de apoptose em células da granulosa (MATSUDA-MINEHATA et al., 2006).

Estradiol, IGF-I, FSH, fator de crescimento epidermal (EGF), fator de crescimento fibroblástico básico (bFGF), interleucina-1 β (IL-1β) e interleucina-6 (IL-6) tem sido caracterizados como fatores anti-apoptóticos (HSU; HAMMOND, 1987; CHUN et al., 1995; HSU; HSUEH, 1997; HEINRICH et al., 1998; ROSENFELD et al., 2001; MAO et al., 2004). Esses fatores atuam através da rota de sinalização PI3K-AKT (*phosphatidylinositol 3-kinase)* (MATSUDA et al., 2012). Seus sinais atuam pela fosforilação da *forkhead* O (FOXO) a qual é excluída do núcleo e resulta no aumento da transcrição dos fatores anti-apoptóticos (BRUNET et al., 1999). Por outro lado, o sistema ligante-receptor (FAS ligante – FASLG), FAS (CD95), fator de necrose tumoral alfa (TNF-α e seu receptor TNFR), ligante relacionado a indução de apoptose (TRAIL e seus receptores DR4, DR5, DcR1 and DcR2), família de proteínas BCL2 (BCL2 domínio (BID), BCL2L11, BAX, BCL2 (BAK)) tem sido relacionados a atresia folicular (BAUD; KARIN, 2001; MATSUDA-MINEHATA et al., 2006; JAASKELAINEN et al., 2009; MATSUDA et al., 2012). Esses sinais induzem diferentes padrões pro-apoptóticos intracelulares que resultam na ativação da caspase 3 (CASP3) e subsequente fragmentação do DNA (MATSUDA et al., 2012).

O papel da rota de sinalização JAK/STAT na foliculogênese dos mamíferos ainda não está totalmente esclarecida (SOBINOFF et al., 2013). Durante a divergência folicular em bovinos, o aumento agudo da proteína STAT3 fosforilada em folículos coletados no dia 4 da onda folicular parece estar envolvido na morte celular e consequente atresia do folículo subordinado (GASPERIN et al., 2014b). A ativação da sinalização JAK/STAT foi identificada nesse processo pois a deleção da STAT3 resultou na diminuição da apoptose e atraso na regressão da glândula mamária após a lactação (CHAPMAN et al., 1999; KRITIKOU et al., 2003). Assim, STAT3 tem sido descrito como mediador essencial da involução da glândula mamária, o principal modelo para os estudos em apoptose, porém esse mecanismo também ainda não está claramente explicado (ABELL et al., 2005).

As gonadotrofinas, LH e FSH, exercem seus efeitos através dos seus receptores ligados à proteína G os quais aumentam a produção de AMPc e ativam a *protein kinase* A (PKA) (SEGER et al., 2001). Esses hormônios também podem ativar outros padrões de sinalização que incluem AKT (*protein kinase B*) e MAPK (*Mitogen-activated protein kinase*) (BABU et al., 2000; ZELEZNIK et al., 2003). Esses padrões também são considerados as principais vias de sinalização que mediam os efeitos do IGF (RYAN et al., 2008).

Muitos estudos demonstraram as funções da sinalização via AKT e MAPK nas células da granulosa, principalmente em modelos *in vitro,* e parecem ser importantes para a dominância folicular (EVANS; MARTIN, 2000). Alguns estudos também demonstram as funções da sinalização das AKT e MAPK em células da granulosa cultivadas *in vitro* (SEGER et al., 2001; RYAN et al., 2008). A sinalização PI3K-AKT foi descrita como sinal de transdução intracelular anti-apoptótico o qual é iniciado por hormônios e fatores de crescimento (MATSUDA et al., 2012).

A ativação STAT pode ter relação com os padrões de sinalização AKT e MAPK (RAWLINGS et al., 2004; ABELL; WATSON, 2005). A existência do padrão pró-apoptótico pelo qual STAT3 media uma mudança na composição da subunidade PI3K que posteriormente regula a sinalização PI3K-AKT foi proposta (ABELL et al., 2005). Esses autores sugerem que é por esse mecanismo que o STAT3 exerce sua função pró-apoptótica nas células epiteliais da glândula mamária. As interações melhor caracterizadas são entre STAT-MAPK e receptores tirosina quinase (RTK)/Ras/MAPK, e essa relação é considerada complexa envonvendo múltiplos níveis (RAWLINGS et al., 2004).

As ações da rota AKT são mais pronunciadas que a MAPK em células da granulosa (RYAN et al., 2008). Esses autores demonstraram em células da granulosa bovinas cultivadas que a inibição de AKT e MAPK resulta na diminuição das ações estimulatórias do FSH e IGF. Já em células da teca esta inibição resulta na diminuição das ações do LH *in vitro* e *in vivo* em ovinos. Altos níveis de AKT, pAKT, MAPK, e pMAPK na parede follicular de folículos dominantes também foram demonstradas em bovinos (EVANS; FORTUNE, 1997a) e ovinos (EVANS; MARTIN, 2000). Estudos prévios em nosso grupo de pesquisa demonstraram níveis similares de pMAPK3/1 em células da granulosa de folículos bovinos (GASPERIN et al., 2014b). Esses resultados sugerem que embora a fosforilação de MAPK3/1 seja associada com o aumento da estronegicidade do folículo dominante, a manutenção da abundância de pMAPK3/1 no folículo subordinado não resgata a sua esteroidogênese e saúde folicular.

Membros da família da interleucina (IL) 6 e fator inibidor de leucemia (LIF) compartilham a mesma subunidade de proteína do receptor de transmembrana IL6ST e também

são sinalizados através da STAT3 (HEINRICH et al., 1998). O ligante mais potente da rota da STAT3 é o LIF e esse possui um papel importante no processo apoptótico (KRITIKOU et al., 2003). Esses autores propuseram que STAT3 promove a regulação do pMAPK1/2 na involução da glândula mamária e que a perda da atividade do MAPK durante a involução é biologicamente significante e necessária para que o STAT3 medie a sua máxima função apoptótica. LIF também media seu efeito através da ativação de MAPK e AKT (BURDON et al., 2002), e a sinalização LIF/STAT3 coincide com a indução da apoptose nas células epiteliais (ABELL et al., 2005).

2.3. Hormônio Anti-mülleriano

Muitos fatores de sinalização atuam nas células da teca, granulosa e oócito contribuindo para a proliferação e diferenciação folicular (GLISTER et al., 2010). Particularmente nos estágios finais de desenvolvimento, esses fatores possuem a capacidade de modular a sensibilidade das células foliculares a gonadotrofinas. Nesse contexto, os membros da superfamília do fator de crescimento e diferenciação β (TGFβ), incluindo inibinas, ativinas, proteínas morfogenéticas ósseas (BMPs) e, mais recentemente, o hormônio anti-mülleriano (AMH) estão sendo estudados em várias espécies (KNIGHT; GLISTER, 2006). Esses estudos demonstram que ligantes, receptores, sinalizadores intermediários e proteínas de ligação associados à superfamília TGFβ são expressos no ovário, o que sugere um papel em múltiplos aspectos do desenvolvimento folicular, recrutamento, proliferação celular/atresia, esteroidogênese, maturação do oócito, ovulação e formação do corpo lúteo (KNIGHT; GLISTER, 2006; CANTY-LAIRD et al., 2010; GLISTER et al., 2010; RICO et al., 2011).

O Hormônio anti-mülleriano (AMH) é uma glicoproteína que pertence à superfamília TGFβ (CATE et al., 1986), com conhecida importância no desenvolvimento gonadal, onde é responsável pela regressão dos ductos de müller em fetos masculinos, recentemente tem sido estudada a sua relação com o desenvolvimento e função reprodutiva em ambos os sexos. O AMH é descrito como potencial candidato intra-ovariano com função de inibidor da ativação folicular primordial (DURLINGER et al., 1999), e a partir disso muitos estudos estão sendo desenvolvidos nessa área (MONNIAUX et al., 2012)).

Semelhante aos outros membros da superfamília TGFβ, o AMH atua via receptores serina/treonina kinase tipo I e II e posteriormente fosforilação das vias de sinalização SMADs. O receptor tipo II (AMHR2) é específico para AMH, enquanto que a maioria dos receptores tipo I (AMHR1) são compartilhados com as BMPs (REY et al., 2003).

Nas fêmeas, as células da granulosa são o único tipo celular capaz de produzir, as quais iniciam sua produção na ativação do crescimento folicular e terminam quando o folículo entra em atresia ou desenvolvimento final (MONNIAUX et al., 2012). Esses autores sugerem que a produção de AMH é restrita ao pool de folículos pré-antrais em crescimento e pequenos folículos antrais, bem como as concentrações plasmáticas de AMH são relacionadas ao tamanho desse pool. Entretanto, apesar de ser diferentemente regulado em folículos de diferentes classes (saudáveis e atrésicos) a participação do AMH e seus receptores durante a seleção folicular ainda é desconhecida. Entre as suas funções, o AMH inibe o recrutamento de folículos primordiais e diminui a responsividade dos folículos em crescimento ao FSH (DURLINGER et al., 2002a). Outros estudos sugerem que o AMH produzido por folículos secundários e terciários, inibe a ativação e diminui o crescimento de folículos primários (FORTUNE et al., 2011).

Por inibir a ativação de folículos primordiais, o AMH tem sido proposto como responsável por evitar o esgotamento da reserva ovariana (GIGLI et al., 2005), pois em camundongos knockout para esse gene houve um aumento das taxas de recrutamento de pequenos folículos antrais o que resultou em falência ovariana precoce (DURLINGER et al., 2002b).

Nos últimos dez anos, o AMH tem sido foco de vários estudos relacionados a tecnologias reprodutivas em humanos (SEIFER et al., 2002), bovinos (RICO et al., 2009), ovinos (LAHOZ et al., 2012) e caprinos (MONNIAUX et al., 2011). Pelo seu padrão de expressão, o AMH tem sido utilizado como marcador endócrino para o pool de folículos em crescimento, sendo reconhecido como o melhor marcador da reserva de folículos em humanos (VISSER et al., 2006), camundongos (KEVENAAR et al., 2006) e bovinos (RICO et al., 2009). Juntamente a isso, tem sido demonstrado que a baixa concentração plasmática de AMH é indicativo de envelhecimento ovariano (VAN ROOIJ et al., 2005), enquanto que a alta concentração está relacionada com síndrome do ovário policístico (PCOS) em mulheres. (PIGNY et al., 2003).

Mudanças na concentração de AMH no plasma de vacas e cabras foram observadas após a administração de FSH para superovulação e produção de embriões (RICO et al., 2009). Esses autores verificaram diferentes relações entre o AMH e a administração de FSH, onde em vacas o AMH aumenta no plasma durante o estro enquanto que diminui em cabras na mesma fase do ciclo estral. Na vaca, o aumento de AMH plasmático após tratamento com FSH é devido ao maior recrutamento de pequenos folículos antrais, sugerindo aumento do AMH pelo efeito estimulatório do FSH no crescimento folicular (RICO et al., 2009). Entretanto, o efeito do FSH

sobre a expressão e síntese de AMH, bem como sobre a expressão de seus receptores nas células da granulosa é desconhecido.

Em humanos, não há uma figura consistente dos níveis endócrinos de AMH durante o ciclo menstrual (MONNIAUX et al., 2012). Em vacas, há variação plasmática de AMH nas fases do ciclo estral além de haver diferenças individuais. Nesta espécie, os níveis endócrinos de AMH diminuem durante os dias seguintes ao estro em resposta a liberação pré e periovulatória de FSH (RICO et al., 2011). *In vitro,* foi demonstrado que as altas concentrações de FSH diminuem a produção de AMH pelas células da granulosa dos pequenos folículos antrais, os quais são altos produtores de AMH (MONNIAUX et al., 2012). Entretanto, esses dados devem ser melhor explorados *in vivo* uma vez que as células da granulosa de folículos pequenos produzem a maior quantidade de AMH justamente no período de maiores níveis de FSH.

Os efeitos do tamanho e atresia folicular na expressão de AMH foram demonstrados em bovinos. Folículos de três a sete milímetros, não estrogênicos, são altamente produtores de AMH, enquanto que folículos maiores de sete milímetros produzem baixas quantidades de AMH (RICO et al., 2009). Em células da granulosa de ratos e suínos cultivadas *in vitro*, o AMH atenua a atividade de aromatase (CYP19A1) e expressão de receptores de LH estimulada pelo FSH. A produção de AMH é caracterizada pela baixa atividade estrogênica (MONNIAUX et al., 2012), por diminuir a responsividade de folículos em crescimento ao FSH (DURLINGER et al., 2001), o AMH reprime a expressão de aromatase e receptores de LH estimuladas por FSH em células da granulosa de ratos e suínos, o que reforça a sua ação no desenvolvimento folicular. Esses dados demonstram um envolvimento do AMH na regulação da esteroidogênese e diferenciação folicular, sugerindo que o mesmo pode estar envolvido na seleção do folículo dominante em espécies monovulares, o que ainda não foi demonstrado.

Durante o desenvolvimento folicular terminal em bovinos, há uma relação inversa de AMH e CYP19A1 em células da granulosa, o que caracteriza um efeito oposto entre células imaturas e diferenciadas (RICO et al., 2011). O FSH pode orquestrar essa mudança, pois esse hormônio induz a expressão de CYP19A1 e é capaz de inibir a expressão de AMH e antagonizar os efeitos estimulatórios das BMPs nesse tipo celular.

A expressão de AMH tem relação inversa com a expressão de aromatase em células da granulosa de ovelhas em fase FSH dependente, indicando uma relação inibitória do AMH no processo FSH dependente (CAMPBELL et al., 2012). Foi proposto recentemente, que o estradiol pode mediar, em parte, os efeitos do FSH (GRYNBERG et al., 2012), e que durante o desenvolvimento dos folículos antrais as células da granulosa tornam-se mais esteroidogênicas

e mais sensitivas ao FSH (MONNIAUX et al., 2012). Nessas células em diferenciação, o FSH pode inibir a expressão de AMH e antagonizar os efeitos estimulatórios das BMPs, diminuindo as concentrações intrafoliculares de AMH. Isso sugere que as ações inibitórias do FSH na expressão de AMH podem ser mediadas pela alta concentração intrafolicular de estradiol, o que resulta no aumento da expressão de CYP19A1 nas células da granulosa dos maiores folículos antrais quando eles desenvolvem ao estágio pré-ovulatório.

No cultivo de células da granulosa de ovinos, a inibição da produção de estradiol pelas células da granulosa é modulada por IGF-I, o qual sabidamente induz maior responsividade ao FSH (CAMPBELL et al., 2012). Esses autores propoem que essa interação pode representar o mecanismo que explicaria o efeito estimulatório do IGF-I na população de pequenos folículos antrais. O aumento de IGF-I pode representar o mecanismo pelo qual o folículo se liberta da inibição intrafolicular de AMH na diferenciação induzida pelo FSH.

BMP4 e BMP6 *in vitro* podem suportar a expressão de AMH nas células da granulosa (RICO et al., 2011), o que sugere um papel das BMPs, originárias do oócito e da teca, no aumento e manutenção da expressão de AMH em pequenos folículos antrais e participação na sua regionalização durante o desenvolvimento folicular (RICO et al., 2011; MONNIAUX et al., 2012). Segundo esses autores, há outros fatores regulatórios de AMH, como o GH, insulina ou ácidos graxos não esterificados (NEFA), os quais atuam primariamente no crescimento folicular e/ou atresia e modificam a produção ovariana de AMH (MONNIAUX et al., 2012).

Segundo RICO et al., (2009), um possível efeito do AMH na regulação do desenvolvimento de folículos antrais ainda não foi bem estudado e mais estudos serão necessários para estabelecer se o AMH pode participar na regulação do desenvolvimento folicular final pela inibição da aromatase dependente de FSH em folículos antrais. Além disso, ainda não se sabe o papel regulatório do FSH na expressão de AMH nas células da granulosa *in vivo* e sua indução hormonal em folículos em crescimento (MONNIAUX et al., 2012). Estes autores indicam um efeito inibitório das altas concentrações de FSH na expressão de RNAm e proteína do AMH em bovinos.

A indução hormonal da produção de AMH nas células da granulosa de folículos em crescimento também ainda não é sabida (MONNIAUX et al., 2012). Portanto, um melhor entendimento dos fatores e mecanismos que regulam a produção de AMH é necessária para desvendar a regulação e estabelecer novas estratégias para o manejo da reserva de folículos em humanos, bem como para novas estratégias para melhorar a produção de embriões em animais domésticos.

3. ARTIGO 1

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

Enhanced LIF-STAT3 signalling in granulosa cells of atretic follicles in cattle

Gustavo Freitas Ilha, Monique T. Rovani, Bernardo G. Gasperin, Alfredo Quites Antoniazzi, Paulo Bayard Dias Gonçalves, Vilceu Bordignon, Raj Duggavathi

REPRODUCTION

1 **Enhanced LIF-STAT3 signalling in granulosa cells of atretic follicles in cattle**

2

3 *Short title:* LIF-STAT3 signalling in atretic follicles

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ABSTRACT

It is well established that the subordinate follicles of bovine follicular waves undergo atresia due to declining FSH concentrations; however, detailed mechanisms have not been deciphered. We used an FSH-induced co-dominance model to determine the status of the STAT3, AKT and MAPK pathways in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles. The DF was significantly larger than SF in saline treated cows; while, diameters of co-DF1 and co-DF2 from FSH group were similar. Higher cleaved CASP3 protein confirmed that the granulosa cells of SF but not of DF and co-DFs were apoptotic. Transcript abundance of *MAPK1/3* and *AKT1/2/3* was significantly higher in granulosa cells of SF than DF. However, there was a tendency for lower abundance of phosphorylated *MAPK3/1* and AKT in SF granulosa cells. Abundance of mRNA and phosphorylated protein of STAT3 was significantly higher in granulosa cells of SF than DF. Also, SF granulosa cells had higher levels of *LIFR* and *IL6ST* transcripts, the two receptors involved in STAT3 activation. All these molecular differences were absent in granulosa cells of co-DFs. Thus, we concluded that atresia of SF is associated with increased expression of *LIFR* and *IL6ST* along with highly activated STAT3 in granulosa cells.

3.1. Introduction

Even though bovine ovaries contain thousands of follicles, only approximately 1% of them ovulate and contribute to reproductive performance of a cow. As atresia is the most common outcome of follicular development, aberrant atresia will have significant impact on fertility. Inappropriate follicular atresia can lead to either follicular cyst or anovulation leading to infertility in cows.

Follicular development occurs in wave like patterns and is regulated by a complex interaction of gonadotropins with local intrafollicular regulatory molecules. During follicular wave, small follicles begin to grow together in response to a transient peak in circulating FSH concentrations [1, 2]. But only one follicle, selected to be dominant, continues to growth despite FSH levels reaching nadir, while, the others undergo atresia [3]. Follicular atresia is initiated with apoptosis of granulosa cells [4], which has been attributed to the depletion of survival factors including FSH. However, it is possible that follicular atresia has a stimulatory component such as pro-apoptotic signals, because apoptosis is localized to the atretic but not all other follicles.

Several studies have examined the role of protein kinase B (AKT) and mitogen-activated kinases (MAPK) in granulosa cells, mostly using *in vitro* culture models [5, 6]. Both gonadotropins have been shown to activate AKT and MAPK signaling in granulosa cells [7, 8] and these pathways are considered important for dominant follicle selection [9, 10]. Signal transducer and activator of transcription (STAT) is a family of proteins known to mediate signaling of cytokines and stimulate cell proliferation, differentiation, and apoptosis [11, 12]. The role of STAT signaling pathway in mammalian folliculogenesis remains relatively uncharacterized [13]. In this regard, our recent study showed increased phosphorylation of STAT3 in granulosa cells of atretic follicles collected on day 4 of the follicular wave and proposed that this pathway may be involved in granulosa cell apoptosis during atresia [10]. However, the identity of the signal that may activate STAT3 when the FSH concentrations are low remains to be established. Some factors such as tumor necrosis factor alpha and Fas ligand have been proposed to be involved in granulosa cell apoptosis. While Fas ligand has been shown in atretic follicles, its treatment alone does not induce granulosa cell apoptosis in vitro [14], suggesting other signalling pathway such as STAT3 pathway may contribute to follicular atresia. In this context, another ligand leukemia inhibitory factor (LIF) plays a central role in apoptosis during mammary gland involution through activating STAT3 by phosphorylation [15].

80 FSH-induced co-dominant follicle model in cattle is an excellent model for comparison
81 of the dominant (DF), subordinate (SF) and co-dominant follicles (co-DFs). Molecular
82 differences present between DF and SF, but absent in co-DFs will provide mechanistic basis of
83 follicular atresia during declining of FSH concentrations. The results of this study could
84 potentially contribute to developing novel therapeutic measures to enhance follicular
85 development during Assisted Reproductive Technologies (ARTs). Therefore, the aim of our
86 study was to test if STAT3, AKT and MAPK pathways are differentially regulated in granulosa
87 cells of DF and SF, and to examine if FSH treatment rescues such pathways in co-DFs.

88 **3.2. Materials and methods**

89 *3.2.1. Co-dominant follicle induction and granulosa cell collection*

90 All experimental procedures using cattle were reviewed and approved by the Federal
91 University of Santa Maria Animal Care and Use Committee (ACUC no. 23081.009594/2007-
92 41). Cyclic adult beef cows (n=20; 4 to 10 years old) were synchronized as described previously
93 [10]. Eight cows with synchronous estrus were then used to carry out FSH or saline treatment
94 during the first follicular wave. The ovaries of each cow were examined daily by transrectal
95 ultrasonography and the ovulation was considered day zero (Day 0). On the second day (Day
96 2), saline (CONTROL group, n=4) or a total dose of 100 mg FSH (FSH group, n=4, Folltropin-
97 V; Bioniche Animal Health, Belleville, Ontario, Canada) was administered i.m. every 12 hours
98 for 48 hours. The dose of FSH divided into two doses of 30 mg on day 2 and two doses of 20
99 mg on day 3. Ovaries were collected by colpotomy 12h after the last FSH/saline injection
100 (morning of Day 4). The two largest follicles were dissected out from the ovaries, granulosa
101 cells were harvested and follicular fluid was recovered. The largest and the second largest were
102 respectively classified as DF and SF in CONTROL cows, and co-DF1 and co-DF2 in FSH
103 cows.

104 *3.2.2. RNA extraction, reverse transcription and real-time PCR*

105 Granulosa cells mRNA and protein were extracted using AllPrep DNA/RNA/protein kit
106 (Qiagen) as described previously [10]. Quantitation and estimation of RNA purity was
107 performer using NanoDrop (Thermo Scientific - Waltham, USA; Abs 260/280 nm ratio)

108 spectrophotometer. Ratios above 1.8 were consider pure, and all samples were above this
109 threshold. To generate the cDNA, 500 ng RNA was first treat with 0.1 U DNAse (Invitrogen;
110 37°C – 5 min). After DNAse inactivation at 65°C for 10 min, samples were incubated in a final
111 volume of 20 µl with iScript cDNA Synthesis Kit (BioRad) following the manufacturer's
112 protocols.

113 Designing and validation of primers (Table 1), and qPCR were performed as described
114 previously [16]. Samples were run in duplicate and transcript levels for each gene were
115 expressed relative to the average abundance of *CYCLOPHILIN* and *GAPDH*. To test cross-
116 contamination with theca cells, polymerase chain reaction (PCR) for detection of the mRNAs
117 that encode *CYP17A1* in granulosa cells was performed in each sample and all samples were
118 free from contamination by theca cells (after 30 PCR cycles).

119 *3.2.3. Immunoblot analyses*

120 Granulosa cell protein samples obtained using AllPrep kit (Qiagen) were boiled at 95
121 °C for 5 min, subjected to 7.5% SDS gel and transferred onto nitrocellulose membranes. After
122 blocking for 2 h with 5% skimmed milk in Tris buffered saline (TBS) containing 0.1% tween-
123 20 (TBS-T), blots were incubated overnight at 4 °C with 1:1000 rabbit anti-human
124 phosphorylated STAT3 (#9131; Tyr 705; Cell Signaling Technology, Danvers, MA),
125 phosphorylated MAPK3/1 (#4376; Erk1/2; Thr 202/Tyr 204; Cell Signaling), phosphorylated
126 AKT (#2965; Thr 308; Cell Signaling), total STAT3 (#9132; Cell Signaling), total MAPK3/1
127 (#4695; Erk1/2; Cell Signaling), cleaved CASPASE3 (Asp175; #9661, Cell Signaling) or
128 1:5000 beta actin (ab8227; Abcam Inc.) with agitation, followed by three washes (10 min each)
129 with TBS-T. The blots were then incubated with 1:7500 goat anti-rabbit IgG-HRP (ab6721;
130 Abcam Inc., USA) for 2 h with agitation, followed by three washes (10 min each) with TBS-T.
131 Immunoreactivity was detected with Immun-Star WesternC Chemiluminescence Kit (BioRad,
132 CA, USA) according to the manufacturer's instructions and visualized using Chemidoc system
133 (BioRad, CA, USA). Quantification of bands of the western blots was performed using Image
134 Lab software (Bio-Rad Laboratory). ERK1 and 2 band densities were added together and were
135 represented as MAPK3/1. The abundance of phosphorylated proteins (pMAPK3/1, pAKT and
136 pSTAT3) was calculated relative to their respective total protein levels. STAR and CASPASE
137 total protein level was calculated based on beta actin as the loading control.

3.2.4. Statistical analysis

All data were tested for normal distribution using Shapiro-Wilk test, normalized when necessary and submitted to split plot ANOVA using SAS software (SAS Institute Inc., Cary, NC). To identify significant differences between groups were evaluated by the use of Bonferroni procedure on LS Means [17]. Results are presented as mean ± SEM. A $P < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. Developmental dynamics of the two largest follicles in CONTROL and FSH-treated cows

Follicular dynamics measured by ultrasonography in CONTROL and FSH groups are shown in Table 2. As expected, the diameter of DF was larger than SF in CONTROL cows ($P<0.05$) and there was no difference in diameters of co-DF1 and co-DF2 in FSH cows ($P>0.05$). The growth rate of DF, between day 3 and 4 after ovulation, was higher than that of SF in CONTROL cows ($P<0.05$), whereas, the growth rate of co-DF1 and co-DF2 during this period was similar in FSH treated cows ($P>0.05$). Furthermore, the diameter and growth rate of co-DFs of FSH treated cows were similar to the DF of CONTROL cows ($P>0.05$).

3.3.2. Health of granulosa cells of the two largest follicles from CONTROL and FSH-treated cows

Granulosa cells were harvested from the two largest follicles of each of the saline and FSH treated cows. To test if DF and co-DFs were physiologically more advanced than SF, we analyzed *CYP19A1* mRNA abundance in granulosa cells. Relative mRNA abundance of *CYP19A1* was, as expected, higher in granulosa cells of the DF than SF in saline treated cows ($P<0.05$; Fig. 1A). Granulosa cells of co-DFs in FSH treated cows had similar levels of the CYP19A1 transcript ($P>0.05$; Fig. 1A). Transcript levels of Cyclin D2 (*CCND2*), were higher in DF than SF ($P<0.05$) and similar between co-DFs ($P>0.05$; Fig. 1B). On the other hand, *CASP3* mRNA was more abundant in granulosa cells of SF than DF in CONTROL cows ($P<0.05$ and Fig. 1C) and low in both co-DFs ($P>0.05$ and Fig. 1C) of FSH treated cows. Also,

165 immunoblot assays revealed that the cleaved CASP3 protein was detected only in granulosa
166 cells SF but not in those of DF and co-DFs (Fig. 1D). Selection of the DF is associated with
167 increased expression of gonadotropin receptors, especially LH receptor (LHCGR) in granulosa
168 cells [3]. Thus, we evaluated the transcript abundance of gonadotropins receptors in granulosa
169 cells. Relative mRNA abundance *LHCGR* was higher in DF than SF in CONTROL cows
170 ($P<0.05$; Fig. 2A), but similar between granulosa cells of the co-DFs of FSH treated cows
171 ($P>0.05$ and Fig. 1A). Relative mRNA abundance of *FSHR* in granulosa cells did not differ
172 between follicle pairs in CONTROL or FSH cows (Fig. 2B).

173 *3.3.3. Phosphorylation status of STAT3, MAPK3/1 and AKT in granulosa cells of the two* 174 *largest follicles in CONTROL and FSH treated cows*

175 We first measured the mRNA and protein levels of STAT3, MAPK3/1 and AKT in
176 granulosa cells of the two largest follicles from CONTROL and FSH treated cows. Relative
177 levels of *MAPK1* and *MAPK3* mRNA were higher in granulosa cells of SF than DF and co-DFs
178 ($P<0.05$; Fig. 3A and B). In contrast to the mRNA profile, the abundance of phosphorylated
179 MAPK3/1 proteins tended to be lower in granulosa cells of SF than DFs ($P<0.1$; Fig. 3C).
180 Relative mRNA levels of *AKT1*, *AKT2* and *AKT3* were higher in SF than DF and co-DFs
181 ($P<0.05$; Fig. 4A, B and C, respectively). Similar to MAPK3/1 proteins, the abundance of
182 phosphorylated AKT tended to be lower in granulosa cells of SF than DFs ($P<0.1$; Fig. 4D).
183 Relative abundance of *STAT3* mRNA was higher in granulosa cells of SF than in DF and co-
184 DFs ($P<0.05$; Fig. 5A). In line with the mRNA pattern, the relative abundance of the
185 phosphorylated isoform of STAT3 protein was higher in granulosa cells of SF compared to DF
186 and co-DFs ($P<0.05$; Fig. 5B).

187 *3.3.4. Transcript abundance of the receptors of known STAT3 activating ligands in granulosa* 188 *cells of the two largest follicles in CONTROL and FSH treated cows*

189 It is well established that leukaemia inhibitory factor (LIF) acting through its receptor
190 (LIFR) stimulates STAT3 [18]. Also, LIFR forms heterodimer with a glycoprotein, IL6ST to
191 form a high affinity receptor through which LIF signaling is triggered [18]. In line with the
192 activation of STAT3, *LIFR* and *IL6ST* mRNA levels were higher in granulosa cells of SF than
193 DF and co-DFs ($P<0.05$; Fig. 6A and B, respectively). On the other hand, mRNA levels of

194 interleukin-6 receptor (*IL6R*), which also forms heterodimer with IL6ST for STAT3 activation,
195 were higher ($P<0.05$) in DF than SFs, and similar between co-DFs ($P>0.05$; Fig. 6C).

196 **3.4. Discussion**

197 Understanding the molecular mechanisms involved in atresia of follicles in response to
198 declining FSH concentrations is important for developing novel methods to enhance follicular
199 development during ARTs. Subordinate follicles undergo atresia due to declining FSH and can
200 be rescued by exogenous FSH [19, 20]. Our recent study demonstrated that STAT3 signalling
201 pathway is activated in granulosa cells of SF undergoing atresia [10]. In this study, we used
202 FSH-induced co-dominant follicle model to investigate signalling pathways in granulosa cells
203 of SF and examined if altered pathways of SF are rescued in FSH-induced co-DF.
204 Administration of small doses of FSH at the expected time of follicular deviation prevents the
205 decline in FSH levels and thus, one or more SFs escape atresia to become co-DFs [19, 21].
206 Indeed, in FSH treated cows of the present study, the two largest follicles continued growing at
207 a similar growth rate and they did not differ in size, in accordance with previous reports [19].
208 There were no significant differences in *FSHR* mRNA abundance in granulosa cells from DF,
209 SF and co-DFs. This is in line with the observations of the previous studies [10, 22]. Taken
210 together, these data indicate that presence FSHR alone does not rescue the SF from atresia. The
211 *LHCGR* mRNA levels in granulosa cells were higher in DF and co-DFs than SF. These data are
212 similar to previous reports [22, 23] and together indicated that FSH-induced LHCGR prevents
213 the DF from undergoing atresia in response to declining FSH concentrations. The lower
214 *LHCGR* expression in SFs was associated with a remarkable increase in the mRNA abundance
215 of *CASP3* and cleaved CASP3 protein. FSH treatment inhibited CASP3 expression and
216 cleavage in granulosa cells of co-DFs. These observations suggest that follicular atresia in low
217 FSH environment involves induction of CASP3 expression and cleavage leading to granulosa
218 cell apoptosis.

219 STATs are a family of transcription factors that mediate signalling of cytokines and
220 stimulate cell proliferation, migration and apoptosis [11, 12]. The receptors of cytokines such
221 as LIF and IL-6 share a common transmembrane protein receptor, IL6ST (a.k.a. gp130) to
222 activate STATs [18]. After receptor activation, STATs become activated through tyrosine
223 phosphorylation and regulate transcription [24]. We recently showed an increase in phospho-
224 STAT3 levels in granulosa cells of SFs collected on day 4 of the follicular wave [10]. In the
225 present study, phospho-STAT3 protein levels and mRNA levels were high observed in the

226 granulosa cells from SF, confirming our previous observations [10]. The increased phospho-
227 STAT3 abundance in granulosa cells of SF suggests that this pathway is potentially activated
228 by factors involved in inhibition of granulosa cell estradiol synthesis, proliferation and/or
229 differentiation.

230 A switch from survival to death signalling induces physiological apoptosis. Deletion of
231 STAT3 resulted in reduced levels of apoptosis and delayed mammary gland involution [15, 25].
232 Therefore, our current and previous [10] data support the hypothesis that STAT3 signalling
233 pathway is involved in granulosa cell apoptosis during atresia and that its suppression is one of
234 the mechanisms of FSH-regulated development of the DF and co-DF.

235 The activation STAT signalling has been shown to have crosstalk with AKT and MAPK
236 signaling pathways [11, 26]. It was proposed that STAT3 exerts its pro-apoptotic function in
237 mammary epithelial cells by inhibiting (PI3K)-AKT signaling through an unknown pro-
238 apoptotic pathway [27]. Our study showed that in granulosa cells there was high mRNA
239 expression and low protein abundance of AKT and MAPK pathways at SF in relationship with
240 DF and co-DFs. These data indicate that activated STAT3 may hinder translation of AKT and
241 MAPK proteins resulting in inhibition of these pathways. Nevertheless, more studies are
242 necessary to explain the STAT3/AKT and STAT3/MAPK interaction at follicular granulosa
243 cells.

244 Inhibition of AKT and MAPK pathways abrogates the stimulatory actions of FSH and
245 insulin-like growth factor (IGF) on cultured bovine granulosa cells [6]. We found that despite
246 high expression of MAPK1/3 and AKT1/2/3, the phosphorylated isoforms of these proteins
247 were lower in granulosa cells of SF than DFs. Higher levels of pAKT and pMAPK proteins
248 were shown in the follicle wall of DFs in cattle [28] and sheep [9]. Our results that the co-DFs
249 in FSH treated cows had similar amounts of pMAPK and pAKT proteins suggest that FSH
250 maintains AKT and MAPK protein activity in growing co-DFs.

251 The deprivation of key survival factors or stimulation by death ligands is the main cause
252 of apoptosis and both contribute to granulosa cell apoptosis [29]. Estradiol, IGF-I, FSH,
253 epidermal growth factor (EGF), and IL-6 have been characterized as anti-apoptotic factors [18,
254 30-34]. These factors act by activating AKT signalling pathway [4]. On the other hand, the
255 death ligand-receptor systems such as Fas ligand (FASLG), Fas (CD95), tumor necrosis factor-
256 alpha (TNF-α and its receptor TNFR), BCL2 family proteins have been implicated in follicular
257 atresia [4, 29, 35, 36]. These signals induce different intracellular pro-apoptotic pathways,
258 which result in cleavage of CASP3 leading to apoptosis [4]. Our recent work has highlighted
259 the potential role of the STAT3 pathway during the follicular atresia [10].

260 LIF is a STAT3 activating ligand that has been shown to play a role in the apoptotic
261 process during the mammary gland involution [15]. That study proposed that during mammary
262 gland involution, STAT3 signaling inhibits MAPK1/2 activity, which is necessary for STAT3-
263 mediated apoptosis. LIF/STAT3 signaling coincides with induction of apoptosis in mammary
264 epithelial cells [27]. Our results demonstrated a positive association of higher level of mRNA
265 levels of *LIFR*, *IL6ST* and *STAT3* with increased STAT3 activity in SF granulosa cells. On the
266 other hand, *IL6R* mRNA abundance was lower in SF granulosa cells. As both LIFR and IL6R
267 require dimerization with IL6ST for their signaling, the expression pattern of these three
268 receptors suggests that there is an overall increase in LIFR signalling while IL6R signalling is
269 reduced. These results suggest that LIF may be the primary ligand activating STAT3 pathway
270 in granulosa cells undergoing apoptosis. Our study is the first to suggest that LIF/STAT3
271 signaling may play a role in granulosa cell apoptosis. Further mechanistic studies are required
272 to identify the relationship between these three signaling pathways MAPK, AKT and STAT3
273 in ovarian granulosa cells, as well as to identify the origin of LIF in atretic follicles.
274 In summary, our results showed that atresia of SF was associated with increased STAT3
275 phosphorylation that could be attributed to an increased expression of *LIFR* and *IL6ST* in
276 granulosa cells. Such increased STAT3 signalling was inhibited by the FSH treatment in
277 granulosa cells of co-DFs. Temporal relationship between phosphorylated STAT3 and cleaved
278 CASP3 in granulosa cells of SFs indicates that an increase in STAT3 but not MAPK or AKT
279 signaling may be involved in apoptosis during follicular atresia. We conclude that FSH-
280 treatment rescued granulosa cells of co-DFs through activation of MAPK and AKT, and
281 inhibition of STAT3 pathways. Further studies are required to delineate the mechanisms by
282 which declining FSH concentrations result in increased LIF-STAT3 signalling in granulosa
283 cells of SFs. It would also be interesting to study the potential contribution of STAT3 in ovarian
284 dysfunction in lactating dairy cows.
285

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393

Table 1 - Primers used in the expression analysis of *Bos taurus taurus* genes.

Gene		Primer sequence (5'-3')	Conc. (nM)
CYCLOPHILIN	F	GGTCATCGGTCTCTTTGGAA	200
	R	TCCTTGATCACACGATGGAA	200
GAPDH	F	ACCCAGAAGACTGTGGATGG	200
	R	CAACAGACACGTTGGGAGTG	200
CYP19A1	F	GTGTCCGAAGTTGTGCCTATT	200
	R	GGAACCTGCAGTGGGAAATGA	200
LHCGR	F	GCACAGCAAGGAGACCAAATAA	200
	R	TTGGGTAAGCAGAAACCATAGTCA	200
FSHR	F	AGCCCCTTGTCACAACTCTATGTC	200
	R	GTTCCTCACCGTGAGGTAGATGT	200
CYP17A1	F	CCATCAGAGAAGTGCTCCGAAT	200
	R	GCCAATGCTGGAGTCAATGA	200
STAT3	F	CTGCAGCAGAAGGTTAGCTACAAA	200
	R	TTCTAAACAGCTCCACGATTCTCTC	200
CCND2	F	TGCCCCAGTGCTCCTACTTC	200
	R	CGGGTACATGGCAAACTTGA	200
CASP3	F	TGCAGAAGTCTGACTGGAAAACCCAAAC	200
	R	TCATCCTCAGCACCACTGTCTGTCTC	200
AKT1	F	GATTCTTCGCCAGCATCGTG	300
	R	GGCCGTGAACTCCTCATCAA	200
AKT2	F	GCCGAATAGGAGAACTGGGG	200
	R	CACGTCTGAGGTCGACACAA	200
AKT3	F	GTGGCGCACACTTTAACTGA	200
	R	ACCCGCTCTCTCGACAAATG	200
MAPK1	F	TATTCGAGCACCGACCATCG	200
	R	TGGAAGGTTTGAGGTCACGG	200
MAPK3	F	ACCCAAGGAACGACTGAAGG	200
	R	GCTGGGCACACAGTCCATTT	200
LIFR	F	AAACTGCCGGCATCTAAGGT	200
	R	TCATGAGGTTGCTGGGACAG	200
IL6ST	F	AGAGTGGGACCACCTTCCTA	200
	R	TCTGTGTAGGCTGCCATTCG	200
IL6R	F	GACCAGAGGACAATGCCACA	200
	R	TCACTCTACTGAGGGGGCTC	200

F, Forward primer; R, Reverse primer; Conc., primer concentration used for gene amplification.

Table 2 - Follicular dynamics determined by transrectal ultrasonography in cows treated with saline (CONTROL, n=4) or FSH (FSH, n=3) every 12 h for 48 h (30-30 mg, 20-20 mg, respectively) stating at day 2 after ovulation. Profiles of diameter (day 3 and day 4) and growth rate (GR, day 3 to day 4) for the dominant (DF) and the subordinate (SF) follicles in CONTROL and the co-dominant follicles (co-DF1 and co-DF2) in FSH cows are shown.

Group	Follicle	Day 3	Day 4	GR
CONTROL	DF	6.3±1.5	9.5 ± 1.3	3.2 ± 0.8
	SF	6.0±1.1	6.1 ± 0.6*	0.1 ± 0.6*
FSH	co-DF1	5.3±0.6	8.6 ± 0.9	3.4 ± 0.3
	co-DF2	4.7±0.7	7.5 ± 0.7	2.8 ± 0.7

* indicates significant difference from DF ($P<0.05$).

Figure Legends

Figure 1: *CYP19A1* (A), *CCND2* (B) and *CASP3* (C) mRNA expression and Cleaved caspase 3 protein abundance (D) in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles. * indicates statistical significance ($P<0.05$).

Figure 2. *FSHR* (A) and *LHCGR* (B) mRNA expression in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles.* indicates statistical significance ($P<0.05$).

Figure 3: *MAPK1* (A) and *MAPK3* (B) mRNA expression and phosphorylated *MAPK3/1* abundance (C) in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles. * indicates statistical significance ($P<0.05$).

Figure 4: *AKT1* (A), *AKT2* (B) and *AKT3* (C) mRNA levels and phosphorylated *AKT* abundance (D) in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles. * indicate statistical significance ($P<0.05$).

Figure 5: *STAT3* mRNA expression (A) and phosphorylated *STAT3* abundance (B) in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles. * indicates statistical significance ($P<0.05$).

Figure 6. *LIFR* (A), *IL6ST* (B) and *IL6R* (C) mRNA expression in granulosa cells of dominant (DF), subordinate (SF) and co-dominant (co-DF) follicles. * indicate statistical significance ($P<0.05$).

Figure 1.

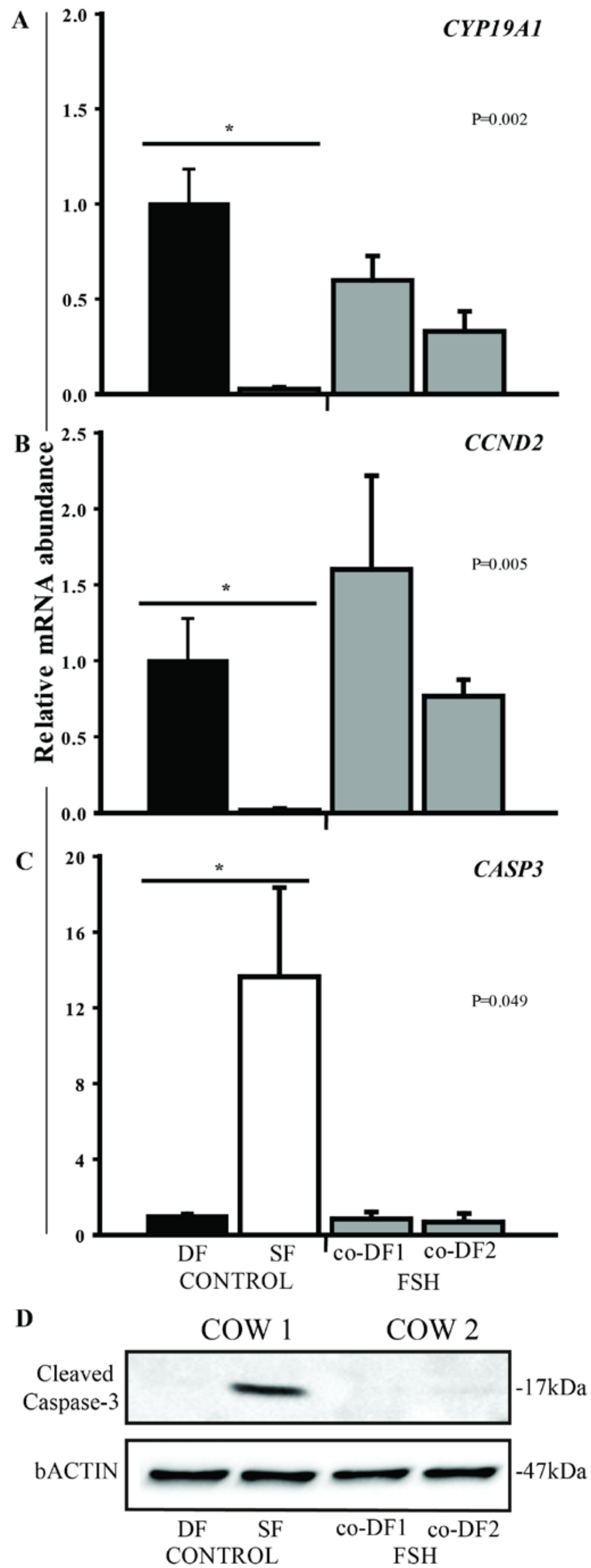


Figure 2.

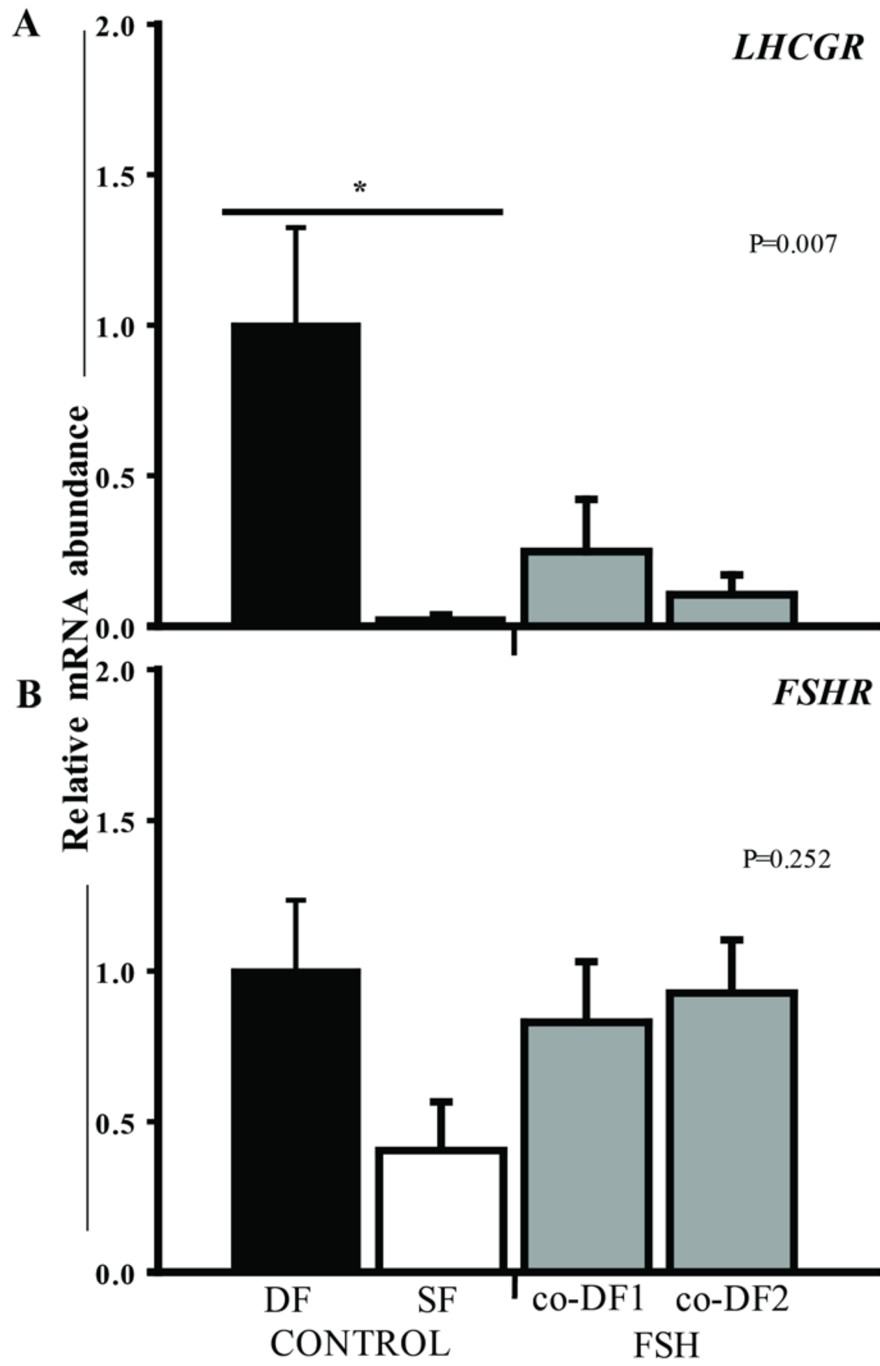


Figure 3.

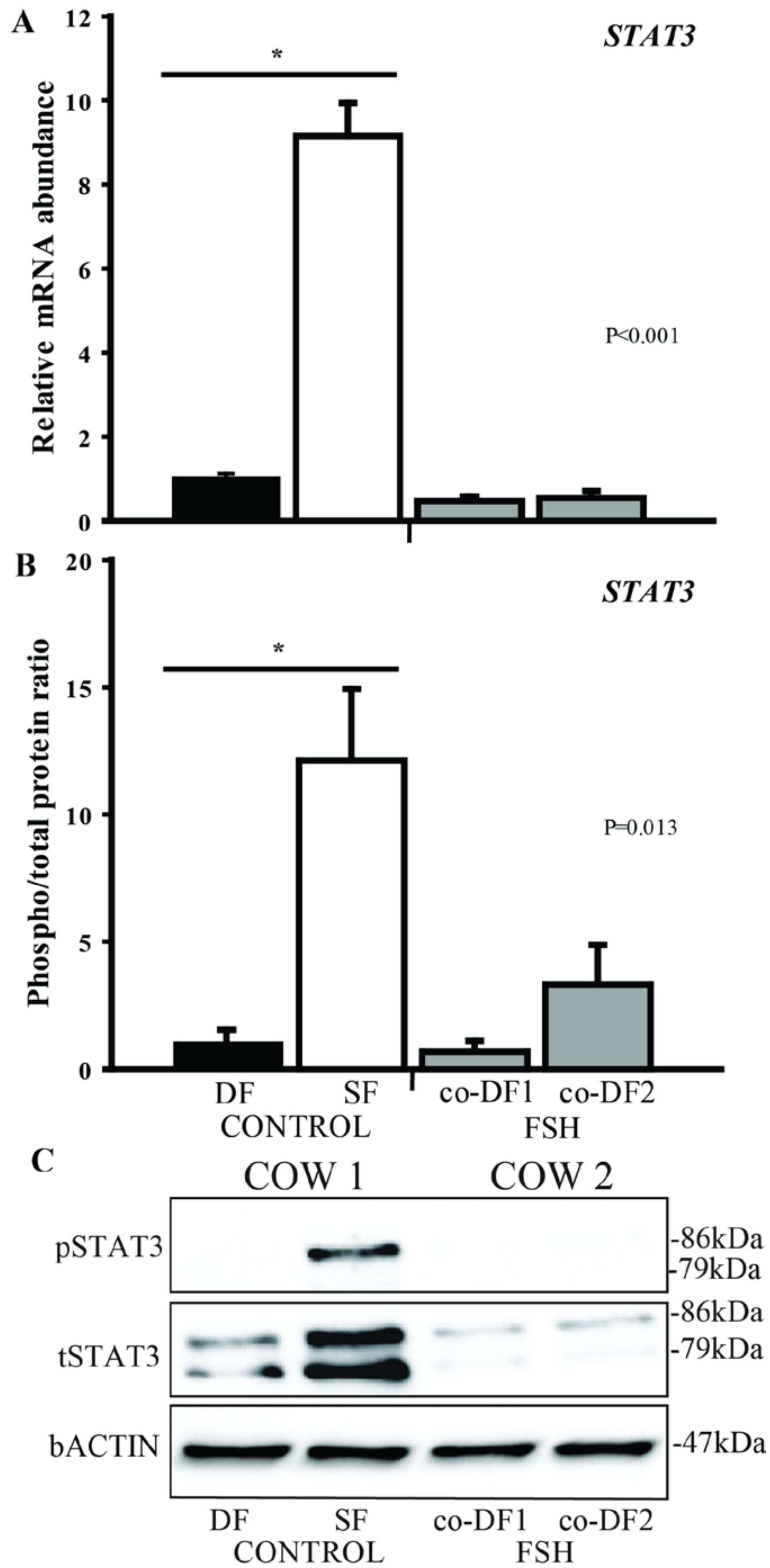


Figure 4.

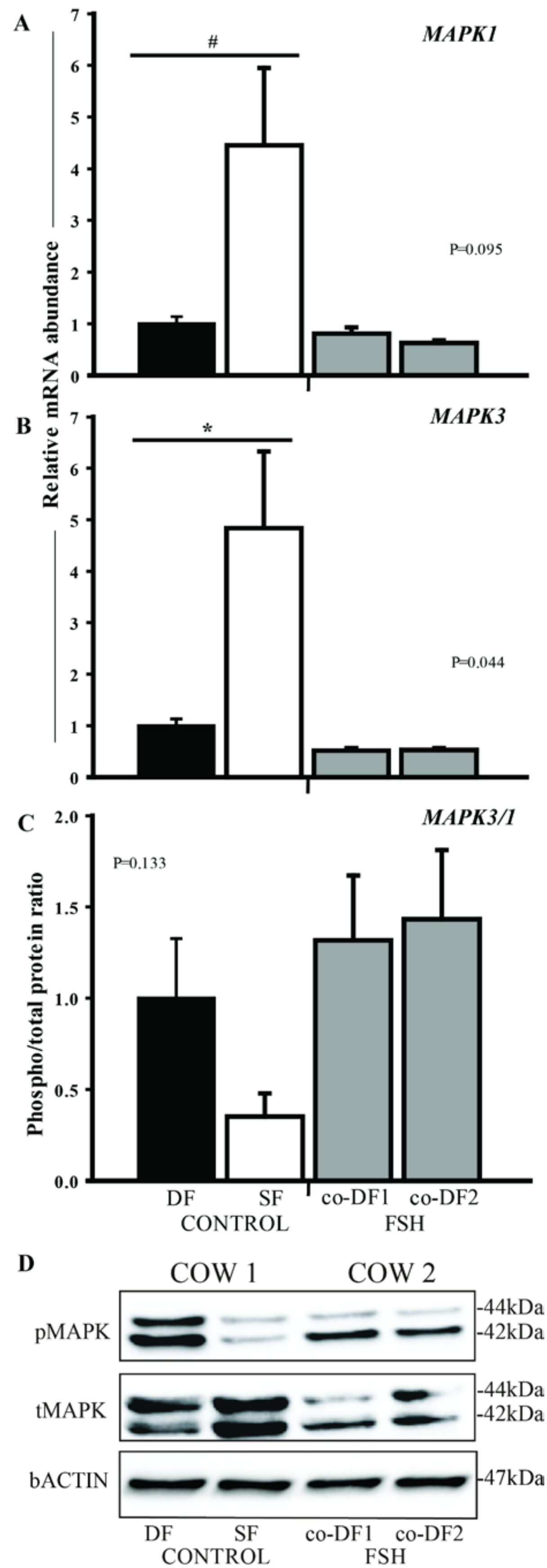


Figure 5.

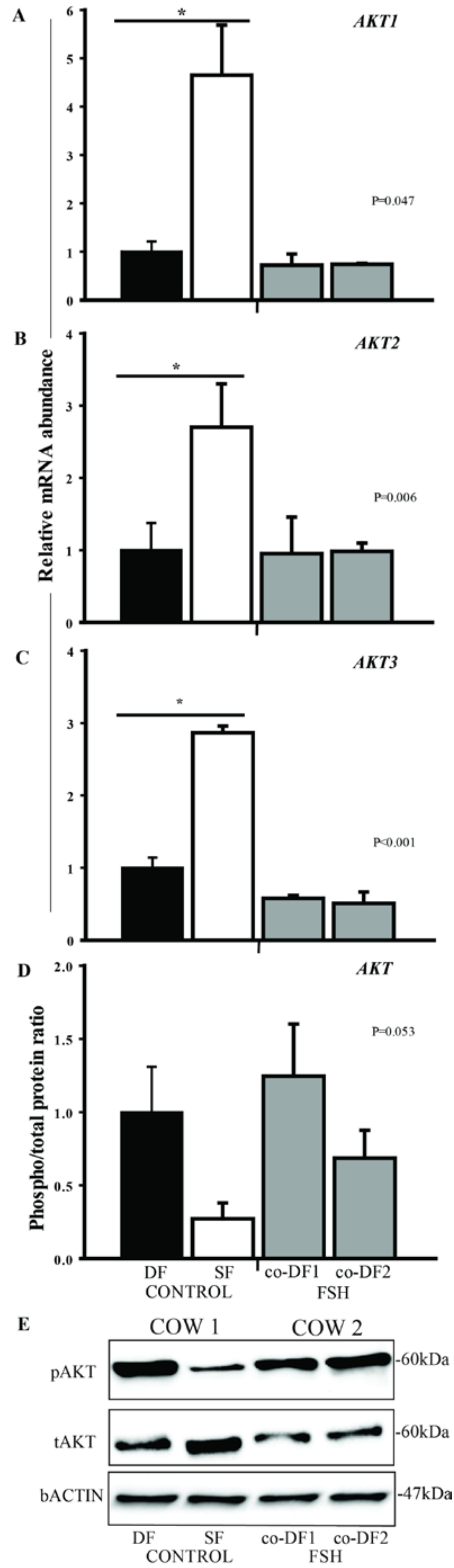
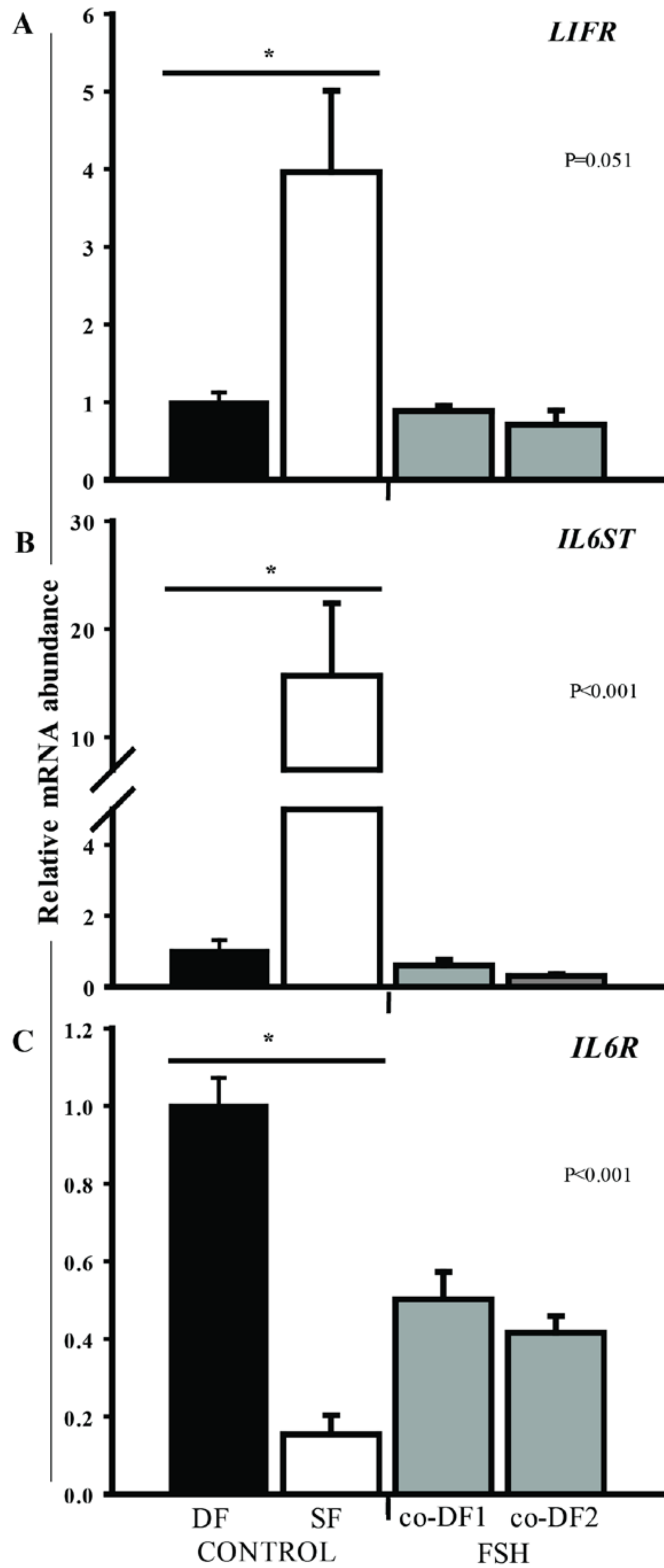


Figure 6.



4. ARTIGO 2

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO:

Anti-mullerian hormone and AMHR2 expression around follicle deviation in cattle and their regulation by FSH *in vivo*

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DOMESTIC ANIMAL ENDOCRINOLOGY, 2014

1 **Anti-mullerian hormone and AMHR2 expression around follicle deviation in**
2 **cattle and their regulation by FSH *in vivo***
3
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26
27 **Key words**: follicular deviation, co-dominant, granulosa, FSH, AMH
28

Abstract

Anti-mullerian hormone (AMH) has been pointed as an important marker of ovarian reserve and seems to be useful in predicting the response to ovarian stimulation in several species. The objective of this study was to gain insight into AMH and its receptor (AMHR2) regulation in granulosa cells and their regulation by FSH *in vivo*. In *Experiment 1*, granulosa cells were retrieved on days 2 (before), 3 (at the expected moment) or 4 (after deviation) of follicular wave to recover the two largest follicles. In *Experiment 2*, four doses of FSH (30, 30, 20 and 20 mg) or saline were administered (i.m.) twice a day starting on day 2 of the first follicular wave of the cycle. Granulosa cells from the two largest follicles from each group were collected 12 h after the last treatment. Follicular status (healthy *vs*. atretic) was confirmed by *CYP19A1, LHCGR, CCND2 and CASP3* mRNA expression and cleaved CASP3 protein abundance in granulosa cells. In *Experiment 1*, *AMH* mRNA abundance was similar in F1 and F2 before deviation (Day 2). On the other hand, *AMH* mRNA levels were higher in DF than SF at the expected time (Day 3) and after (Day 4) deviation ($P<0.05$). There was no difference in *AMHR2* mRNA level during the deviation process ($P>0.05$). However, after deviation (day 4) *AMHR2* mRNA tended to be more abundant in DFs compared to SFs ($P<0.1$). In the *Experiment 2*, *AMH* mRNA level was higher in the second largest follicle from FSH group. In accordance to these results, AMH protein was significantly more abundant in granulosa cells from FSH-stimulated follicles ($P<0.05$). *AMHR2* mRNA abundance was higher in healthy follicles compared to atretic follicles (SF from control group; $P<0.05$). Our results suggest that AMH expression is regulated during follicular deviation, being stimulated by FSH, whereas *AMHR2* is downregulated during advanced atresia.

51 **4.1. Introduction**

52 Follicular development is coordinated by both endocrine and paracrine signals, being
53 the endocrine control well known and stablished. However, the local control of ovarian function
54 and the interaction between systemic hormones and local factors is still need to be investigated.
55 Local factors produced by theca, granulosa cells and oocytes are important regulators of cell
56 proliferation and differentiation determining, at least in part, follicular fate. In this context,
57 Transforming Growth Factor β (TGFβ) super family members functions have been
58 demonstrated in several species (1).
59 Anti-Mullerian hormone (AMH) has been extensively studied during male reproductive
60 tract development (2). In females, AMH is the unique TGFβ member strictly expressed in
61 granulosa cells. Despite its high expression in granulosa cells, little is known about the precise
62 function of this factor during follicle development (3). AMH function in the ovary involves
63 follicle activation inhibition (4) and some studies have shown the importance of this hormone
64 as a predictor of assisted reproductive technologies outcome (ARTs). Furthermore, AMH is
65 considered the best endocrine marker of small antral follicles population in humans (5) as well
66 as ruminants (6-8).
67 In mice, AMH suppression resulted in a premature depletion of primordial follicles
68 reserve (9). AMH has been suggested to play an essential role in regulating FSH sensitivity in
69 follicular cells by inhibiting FSH effects in mouse (10), human (11) and sheep (12) granulosa
70 cells. Thus, when AMH expression decreases, the follicle becomes more sensitive to FSH and
71 can be recruited to enter the pool of follicles that may become dominant (13). However,
72 mechanisms that regulate AMH production by granulosa cells are poorly understood (6).
73 The expression of AMH is dependent on the stage of follicular development (14).
74 Granulosa cells from growing follicles, specially large preantral and small antral healthy
75 growing follicles, express the highest amounts of AMH in mammal ovaries (15). However,
76 AMH production by large antral follicles and the importance of this factor and its receptor
77 around follicular deviation and the effect of FSH on AMH expression during follicular growth
78 has not been thoroughly investigated. The objective of this study was to investigate the
79 regulation of AMH and its receptor (AMHR2) around follicular deviation and the effects of
80 FSH on their expression.
81

82 **4.2. Materials and methods**

83 *4.2.1. Animal husbandry*

84 All experimental procedures using cattle were approved by the Institutional Committee
85 for Ethics in Animal Experiments at the Federal University of Santa Maria, RS, Brazil. Adult
86 cyclic *Bos taurus taurus* beef cows were used in this study.

87 *4.2.2. Experiment 1: levels of mRNA for AMH and AMHR2 in granulosa cells during the*
88 *follicular deviation period*

89 This experiment was performed according to previous studies (16,17). Normal cyclic
90 adult multiparous and non-lactating beef cows (n=32; 4 to 7 years old) were synchronized with
91 two injections of sodium cloprostenol (Ciosin; Intervet/Schering-Plough; 250 µg; im) 12 h apart
92 and observed for estrus within 3–5 days after treatment. Ovaries were examined by daily
93 transrectal ultrasonography. The day of follicular emergence was retrospectively identified as
94 the day on which the diameter of the largest follicle was between 4 and 5 mm (18). Both ovaries
95 were collected from each group of cows on days 2 (n=3 cows), 3 (n=3 cows) and 4 (n=4 cows)
96 of the follicular wave. Ovariectomies were performed via colpotomy under caudal epidural
97 anesthesia using 80 mg lidocaine chlorhydrate (19). The two largest follicles were dissected
98 and granulosa cells were harvested through repeated flushing with PBS. This approach allowed
99 us to investigate transcripts abundance of *AMH* and *AMHR2* genes in granulosa cells in the
100 largest (F1) and second largest (F2) follicles before deviation (Day 2), in the dominant (DF)
101 and subordinate (SF) at the expected time (Day 3) and after follicular deviation (Day 4).

102 *4.2.3. Experiment 2: Induction of co-dominant follicles by FSH stimulation and sample*
103 *collection*

104 Adult multiparous and non-lactating beef cows (n=20; 4 to 7 years old) with regular
105 estrous cycles were synchronized with two injections of sodium cloprostenol (Ciosin;
106 Intervet/Schering-Plough; 250 µg; im). After estrus detection, seven cows were examined daily
107 by transrectal ultrasonography and the ovulation was considered as day zero (Day 0). On day
108 two (Day 2), saline (CONTROL group, n=4) or 100mg of FSH (FSH group, n=3, Folltropin-V;

109 Bioniche Animal Health, Belleville, Ontario, Canada) was injected i.m. 12 hours apart in four
110 injections (30-30mg – day 2, 20-20mg – day 3). Cows were examined by transrectal
111 ultrasonography to follow follicular growth. Ovaries were collected by colpotomy 12h after the
112 last FSH/saline injection (Day 4). The two largest follicles were dissected from the ovaries and
113 granulosa cells and follicular fluid were harvested. Follicles obtained from the CONTROL
114 group were classified as largest (DF) and subordinate follicles (SF) and follicles from FSH
115 group were classified as largest co-dominant follicles (co-DF1) and second largest co-dominant
116 follicles (co-DF2).

117 *4.2.4. RNA extraction, reverse transcription and real-time PCR*

118 Granulosa cells mRNA and protein were extracted using AllPrep DNA/RNA/protein kit
119 (Qiagen). Quantitation and estimation of RNA purity was performed using NanoDrop (Thermo
120 Scientific - Waltham, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.8 were
121 considered pure, and all samples were above this threshold. To generate the cDNA, 500 ng
122 RNA was first treated with 0.1 U DNAse (Invitrogen; 37°C – 5 min). After DNAse inactivation
123 at 65°C for 10 min, samples were incubated in a final volume of 20 µl with iScript cDNA
124 Synthesis Kit (BioRad) following the manufacturer's protocols.
125 To test cross-contamination with theca cells, polymerase chain reaction (PCR) for
126 detection of mRNAs that encode *CYP17A1* in granulosa cells was performed in each sample
127 and all samples were confirmed to be free from contamination by theca cells (after 30 PCR
128 cycles). Real-time quantitative PCR (qPCR) reactions were conducted in CFX 384 real-time
129 PCR detection system (BioRad) using FastStart Universal SYBR Green Master (with Rox;
130 Roche Diagnostics, Canada) and bovine-specific primers for *AMH* (Initiator sense:
131 ACACCGGCAAGCTCCTCAT and anti-sense: CACCATGTTTGGGACGTGG) and *AMHR2*
132 (Initiator sense: AGGGCTCCCTGTGCCACTA and anti-sense:
133 GATCTCGGTGGGCGATACCT) designed using Primer Express Software v3.0 (Applied
134 Biosystems, USA) and synthesized by Invitrogen (Canada). Standard two-step qPCR was
135 performed with initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at
136 95 ºC for 15 sec and annealing/extension at 58 ºC for 30 sec.
137 To optimize the qPCR assay, serial dilutions of a cDNA template were used to generate a
138 standard curve by plotting the log of the starting quantity of the dilution factor against the CT
139 value obtained during amplification of each dilution. Reactions with a coefficient of

140 determination (R2) higher than 0.98 and efficiency between 95 to 105% were considered
141 optimized. The relative standard curve method was used to assess the amount of a particular
142 transcript in the samples as first described (20). Briefly, for each gene, standard cDNAs were
143 amplified along with sample cDNAs in the same PCR run. The target mRNA quantity in each
144 sample was determined from the relative standard curve (using sample Ct values) and expressed
145 in arbitrary units corresponding to the dilution factors of the standard RNA preparation.
146 Samples were run in duplicate and were expressed relative to *Cyclophilin* (Initiator sense:
147 GGTCATCGGTCTCTTTGGAA and anti-sense: TCCTTGATCACACGATGGAA) and
148 *GAPDH* (Initiator sense: ACCCAGAAGACTGTGGATGG and anti-sense:
149 CAACAGACACGTTGGGAGTG) as internal control genes.

150 *4.2.5. Immunoblot analyses*

151 Granulosa cell protein samples obtained using AllPrep kit (Qiagen) were boiled at 95
152 °C for 5 min, subjected to 7.5% SDS gel and transferred on to nitrocellulose membranes. After
153 blocking for 2 h with 5% skimmed milk in Tris buffered saline (TBS) containing 0.1% tween-
154 20 (TBS-T), blots were incubated overnight at 4 °C with 1:1000 goat anti-human AMH (sc-
155 6886; MIS C-20, Santa Cruz Biotechnology Inc.) or rabbit anti-human 1:5000 beta actin
156 (ab8227; Abcam Inc.) with agitation, followed by three washes (10 min each) with TBS-T. The
157 blot of AMH antibody was then incubated with 1:5000 donkey anti-goat IgG-HRP (sc-2033,
158 Santa Cruz Biotechnology Inc.) and the beta actin blot with goat anti-rabbit IgG-HRP (ab6721;
159 Abcam Inc., USA) for 2 h with agitation, followed by three washes (10 min each) with TBS-T.
160 Immunoreactivity was detected with Immun-Star WesternC Chemiluminescence Kit (BioRad,
161 CA, USA) according to the manufacturer's instructions and visualized using Chemidoc system
162 (BioRad, CA, USA). Quantification of protein bands of the western blots was performed using
163 the Image Lab software (Bio-Rad Laboratory). The abundance of protein was calculated based
164 on beta actin as the loading control.

165 *4.2.6. AMH concentration in follicular fluid*

166 Follicular fluid samples from each follicle were used to measure AMH protein (ng/mL)
167 using a quantitative three-step sandwich type immunoassay (Bovine AMH Elisa kit -Ansh
168 Labs). In the first step, serially diluted Calibrators and unknown samples are added to AMH

169 antibody coated micro titer wells and incubated. After the first incubation and washing, the
170 wells were incubated with biotinylated AMH antibody solution. After the second incubation
171 and washing, the wells were incubated with streptavidin horseradish peroxidase conjugate
172 (SHRP) solution. After the third incubation and washing step, the wells were incubated with
173 substrate solution followed by an acidic stopping solution. The degree of enzymatic turnover
174 of the substrate was determined by dual wavelength absorbance measurement at 450 nm as
175 primary test filter and 630 nm as reference filter in a plate reader (1 ng/mL AMH = 7.14 pM).
176 The assay analytical sensitivity was calculated by the interpolation of mean plus two standard
177 deviation of 14 replicates of each calibrator.

178 *4.2.7. Statistical analyses*

179 All continuous data were tested for normal distribution using Shapiro–Wilk test and
180 normalized when necessary. Variations in transcript levels were analysed by ANOVA and
181 multiple comparisons between days or groups were performed by least square means using the
182 JMP Software. Main effects of treatment group and day, and their interaction were determined.
183 Differences between the two largest follicles were accessed by paired Student's t-test using cow
184 as subject. Results are presented as means ± S.E.M. $P \leq 0.05$ was considered statistically
185 significant.
186

187 **4.3. Results**

188 *4.3.1. Follicular deviation model validation*

189 Follicles obtained before deviation (day 2) were classified as largest (F1) or second
190 largest follicles (F2) and were 7.4 ± 0.16 mm and 6.6 ± 0.44 mm diameter, respectively ($P >$
191 0.05). Follicles obtained at the expected time (day 3) or after (day 4) deviation were classified
192 as dominant (DF) or subordinate follicle (SF) and the diameters were significantly different at
193 day 3 (7.9 ± 0.44 vs 6.9 ± 0.16, $P<0.05$) and day 4 (9.55 ± 0.32 vs 6.75 ± 0.21, $P<0.05$),
194 respectively. Aiming to validate our in vivo model, we first assessed mRNA levels of aromatase
195 (*CYP19A1*) and LH receptor (*LHCGR*) genes in granulosa cells from the largest and second
196 largest follicles on days 2 (n = 4), 3 (n = 4) or 4 (n = 6) of the follicular wave. Subordinate

197 follicles expressed lower levels of *CYP19A1* and *LHCGR* at days 3 and 4 of follicular deviation
198 (data not shown). These results confirm that the ovaries obtained on days 2, 3, and 4 of the first
199 follicular wave were before, at the expected time, and after follicular deviation, respectively.
200 Immunoblot assays revealed that the relative abundance of the cleaved CASP3 (17kDa) was
201 higher (8 fold; $P<0.05$) in granulosa cells of day 4 SF confirming the apoptotic state of the cells.
202 In line with this, *CCND2* mRNA abundance, an important regulator of granulosa cell cycle,
203 was higher ($P<0.05$) in granulosa cells of DFs than SFs, indicating that granulosa cells from
204 DFs were proliferating.

205 *4.3.2. AMH and AMHR2 dynamics at follicular deviation*

206 Abundance of mRNA encoding *AMH* and *AMHR2* receptor was measured in granulosa cells
207 from the largest and second largest follicles around deviation of the first follicle wave (Figure
208 1A and B). While *AMH* mRNA level did not differ between F1 and F2 before deviation (Day
209 2), DF had higher levels of *AMH* mRNA compared to SF at the expected moment (Day 3) and
210 after deviation (Day 4; $P<0.05$). Interestingly, mRNA abundance of *AMH* was lower in all
211 follicles obtained on days 3 and 4 when compared to follicles obtained on day 2, being this
212 decreased more pronounced in atretic follicles. *AMHR2* mRNA levels did not differ between
213 F1 and F2 and DF and SF on days 2 and 3, respectively. However, *AMHR2* mRNA expression
214 tended to be higher in DF compared to SF after deviation (day 4-$P<0.1$). Notwithstanding,
215 AMH concentration in follicular fluid of D4 follicles was higher in SFs compared to DFs
216 (2348.67 ± 255.2 and 659.1 ± 261.6 ng/mL, respectively, $P<0.001$, data not show).

217 *4.3.3. FSH induced follicles co-dominant model*

218 Based on the findings from *Experiment 1* we evaluated whether FSH treatment would
219 induce AMH expression in FSH-induced co-dominant follicles. Follicles from control group
220 were classified as largest (DF) and subordinate follicles (SF) and follicles collected from FSH
221 group were classified as largest co-dominant (co-DF1) and second largest co-dominant follicles
222 (co-DF2). As expected, the diameters of DF and SF differed (9.5 ± 1.3 and 6.1 ± 0.6 mm,
223 respectively; $P<0.05$). However, diameters of co-DF1 and co-DF2 did not differ in FSH group
224 (8.6 ± 0.9 and 7.5 ± 0.7 mm). To test if DFs and co-DFs were physiologically more advanced than
225 SFs, we analyzed *CYP19A1* mRNA abundance in granulosa cells from individual follicles.

226 Relative mRNA abundance of *CYP19A1* was higher in the DF than SF and similar between co-
227 DFs from each pair (P<0.05; data not shown). *LHCGR* mRNA levels were higher in DFs than
228 SFs (P<0.05). However, mRNA abundance of *LHCGR* in granulosa cells of the co-DF1 did not
229 differ from co-DF2. mRNA abundance of *CCND2* was higher in DFs and co-DFs, however it
230 was almost indetectable in SFs (P<0.05) indicating cells proliferation only in DF and co-DFs
231 (21). mRNA for *CASP3* was more abundant in SFs than DFs and co-DFs (P<0.05) and
232 immunoblot assays revealed that cleaved CASP3 (17kD) protein was only present in SFs. These
233 data suggest that granulosa cells from SF were entering apoptosis, leading to follicular atresia
234 (22,23).

235 *4.3.4. AMH and AMHR2 mRNA expression and protein at FSH induced follicles*

236 Abundance of mRNA encoding *AMH* and *AMHR2* receptor was measured in granulosa
237 cells from DFs, SFs and co-DFs (Figure 2A and B). *AMH* mRNA levels in granulosa cells were
238 similar between follicles within groups (Figure 2A). However FSH supplemented follicles had
239 more *AMH* mRNA than control follicles (P<0.05). On the other hand, *AMHR2* mRNA was
240 higher in DFs than SFs (P<0.05) and similar between co-DFs (P>0.05, Figure 2A). These data
241 are in accordance to what was observed when we assessed AMH protein in granulosa cells
242 (Figure 3B and C). AMH protein bands were more intense in FSH-supplemented follicles
243 compared to control follicles (P<0.05). No differences were observed in total AMH protein in
244 follicular fluid obtained from DF, SF and co-DFs (Figure 3A). However, AMH concentration
245 was more abundant in SFs than DFs (1500.53 ± 273.83 vs 420.02 ± 103.74, P<0.05) and similar
246 between co-DFs (data not shown).

247 **4.4. Discussion**

248 It is well established that AMH is mainly expressed by granulosa cells from large preantral
249 and small antral healthy growing follicles (15). However, AMH production by large antral
250 follicles, the importance of this factor for follicular deviation, and the effect of FSH on AMH
251 expression during follicular growth have not been thoroughly investigated. In this study, two
252 experimental models *in vivo* were used to investigate the regulation of AMH and its receptor
253 AMHR2 during follicular deviation and the effects of FSH on their expression in granulosa
254 cells. Our results show for the first time that: 1) AMH expression differs between dominant and
255 subordinate follicles during and after follicular deviation; 2) FSH injection increased AMH

256 abundance at both mRNA and protein levels in the second largest follicles (co-DF2) compared
257 to subordinate follicles from control cows; and 3) *AMHR2* is downregulated in subordinate
258 atretic follicles.

259 Before evaluating AMH/AMHR2 regulation, both in vivo models were validated. In the
260 follicular deviation model, we confirmed that deviation occurs on day 3, when the DF reaches
261 a diameter significantly higher than the SF. Indeed, the first significant difference in diameters
262 of DF and SF was seen on day 3. On a previous study we have demonstrated that follicular
263 deviation occurs without significant changes in *FSHR* mRNA abundance in granulosa cells
264 from DFs and SFs (24). However, *LHCGR* mRNA levels increased in granulosa cells with
265 development of the DF but no changes were observed in SF (24). Significant lower levels of
266 *FSHR* and *LHCGR* mRNA in SFs on day 4 was associated with a remarkable increase in the
267 abundance of cleaved caspase 3, with suggest that granulosa cells were in the process of
268 apoptosis (25).

269 In the second *in vivo* model based on FSH treatment, we observed that the two largest
270 follicles continued growing at a similar growth rate and did not differ in size, with is in
271 accordance with previous reports (26). There were no significant differences in *FSHR* mRNA
272 abundance in granulosa cells from DFs, SFs and co-DFs. This is also in line with observations
273 from previous reports (24,27). Levels of *LHCGR* mRNA in granulosa cells were higher in DFs
274 and co-DFs than in SFs. These data are similar to previous reports showing higher expression
275 of *LHCGR* in granulosa cells from healthy growing follicles during follicle deviation (27,28)
276 and suggest that FSH-induced LHCGR is important for the selection of the DF. Lower levels
277 of *LHCGR* mRNA in SFs were associated with remarkable increase in mRNA abundance of
278 *CASP3* and cleaved CASP3 protein, suggesting that their granulosa cells were undergoing
279 apoptosis leading to follicular atresia (22,23,25).

280 In agreement with previous studies, AMH expression was observed in granulosa cells
281 of large antral follicles (6,14,29-31). It is well established that the expression of AMH is
282 depends on the stage of follicular development (14), and that granulosa cells of growing
283 follicles, largest preantral and small antral healthy growing follicles express the highest
284 amounts of AMH in mammal ovaries (15). Our results corroborate with these reports by
285 showing *AMH* expression during follicular deviation *in vivo*, and the expression of *AMHR2* in
286 granulosa cells with suggests the existence of a possible autocrine regulation by AMH in bovine
287 antral follicles.

288 Studies using the rat as a model have shown that AMH expression decreases during the
289 final phase of follicular development (31). Similarly, AMH mRNA is undetectable in granulosa

290 cells from human pre-ovulatory follicles and the abrupt decline in AMH expression coincides
291 with the selection of the dominant follicles (32-34). Moreover, bovine follicles obtained from
292 abattoir-derived ovaries and classified as early atretic according to histological findings, have
293 lower *AMH* mRNA in granulosa cells from compared to healthy follicles (6). Our results are in
294 agreement with those findings (6,14) and demonstrate that differences in *AMH* mRNA levels
295 are already observed near follicular deviation. Interestingly, *AMH* mRNA abundance was lower
296 in all follicles obtained on days 3 and 4 when compared to follicles obtained on day 2,
297 irrespective of follicle status. These results suggest that AMH expression may be regulated by
298 FSH, as both decrease from follicle emergence to ovulation. The fact that AMH decrease is
299 more pronounced in atretic follicles is also in accordance to this hypothesis as FSHR mRNA
300 levels are decreased in these follicles (14,24).

301 AMH has been suggested to play an essential role in the FSH sensitivity by inhibition
302 of FSH effects in mouse (10) and human (11) granulosa cells. On the other hand, FSH has been
303 shown to decrease AMH production in bovine granulosa cells *in vitro* (14,35). In contrast, our
304 results suggest that granulosa cells from FSH-induced codominant follicles *in vivo* have more
305 *AMH* mRNA and protein compared to those from control follicles. In addition, the effects of
306 AMH are dependent on its receptor and *AMHR2* mRNA were higher in DFs than SFs but similar
307 between co-DFs.

308 An inverse relationship between AMH (*AMH* mRNA expression in granulosa cells or
309 intrafollicular AMH concentrations) and oestrogen production (*CYP19A1* mRNA expression in
310 granulosa cells or oestradiol intrafollicular concentrations) has been reported recently in
311 follicles from goat (7), human (36) and bovine (6). In these species, granulosa cell AMH
312 expression *in vivo* decreased as the follicle approached terminal development, while the
313 *CYP19A1* expression increased. Our results support this inverse relationship by confirming that
314 when comparing small and large healthy follicles *AMH* and *CYP19A1* mRNA levels varied in
315 opposite directions. Thus, AMH and CYP19A1 can be proposed as functional markers of
316 immature and fully differentiated granulosa cells, respectively (6).

317 There is evidence that AMH can play a modulatory role in the control of gonadotropin-
318 responsive and gonadotropin-dependent stages during follicle development (12,34). These data
319 were first proposed in mouse when AMH was shown to be correlated with decreased
320 responsiveness to FSH in growing follicles (10). In cultured human lutein-granulosa cells,
321 AMH inhibited FSH-dependent *CYP19A1* mRNA and protein and estradiol production (11),
322 and repressed *CYP19A1* and *LHCGR* expression in FSH-stimulated rat and porcine granulosa
323 cells (15). In contrast with these previous data our study shows that FSH-induced healthy

324 follicles have similar *CYP19A1*, *LHCGR* and *AMH* mRNA levels. These data suggest that AMH
325 does not inhibit FSH-stimulated genes and estradiol related genes in granulosa cells *in vivo*.
326 In humans, a significant positive correlation was shown between mRNA expression in
327 granulosa cells and AMH protein levels in the corresponding follicular fluid (34). In bovine,
328 variations in AMH intrafollicular concentrations are not always parallel to those in *AMH*
329 mRNA levels in granulosa cells (6). However, both AMH concentrations in the follicular fluid
330 and *AMH* mRNA expression in granulosa cells decrease during the development of healthy
331 antral follicles up to the preovulatory stage and they are clearly affected by atresia (14). Atresia
332 is accompanied by a parallel decrease in *AMH* mRNA expression in granulosa cells (15). These
333 authors showed a discrepancy in *AMH* mRNA levels and intrafollicular AMH concentrations,
334 which decreased and increased with atresia, respectively. In our study AMH concentration was
335 more abundant in SFs than DFs but did not differ between co-DFs. These results suggest that
336 AMH is regulated at both transcriptional and posttranscriptional levels and the efficiency of its
337 absorption by follicular blood vessels may also play a role in regulating AMH accumulation in
338 follicular antrum (15).
339 In summary, we have shown that AMH signaling is present and likely regulated in
340 granulosa cells around follicular deviation. We propose a possible regulation of AMH
341 expression in bovine antral follicles.
342

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350

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459

460 **Figure legends**

461 **Figure 1**: Levels of mRNA for *AMH* (A) and *AMHR2* (B) receptor in granulosa cells
462 around follicular deviation. “Fol” indicates the effect of follicle (F1 *vs* F2 and DF *vs* SF).
463 Different letters indicates statistical difference between groups ($P < 0.05$).

464 **Figure 2.** *AMH* (A) and *AMHR2* (B) mRNA levels in granulosa cells of DF and SF and
465 co-DFs. Different letters indicate statistical difference ($P<0.05$) between follicles accessed by
466 paired Student’s T test using cow as subject.

467 **Figure 3**: Total AMH protein in follicular fluid (A) and AMH protein abundance in granulosa
468 cells (B) of DF and SF and co-DFs. Different letters indicate statistical difference ($P<0.05$)
469 between follicles accessed by paired Student’s T test using cow as subject.

Figure 1

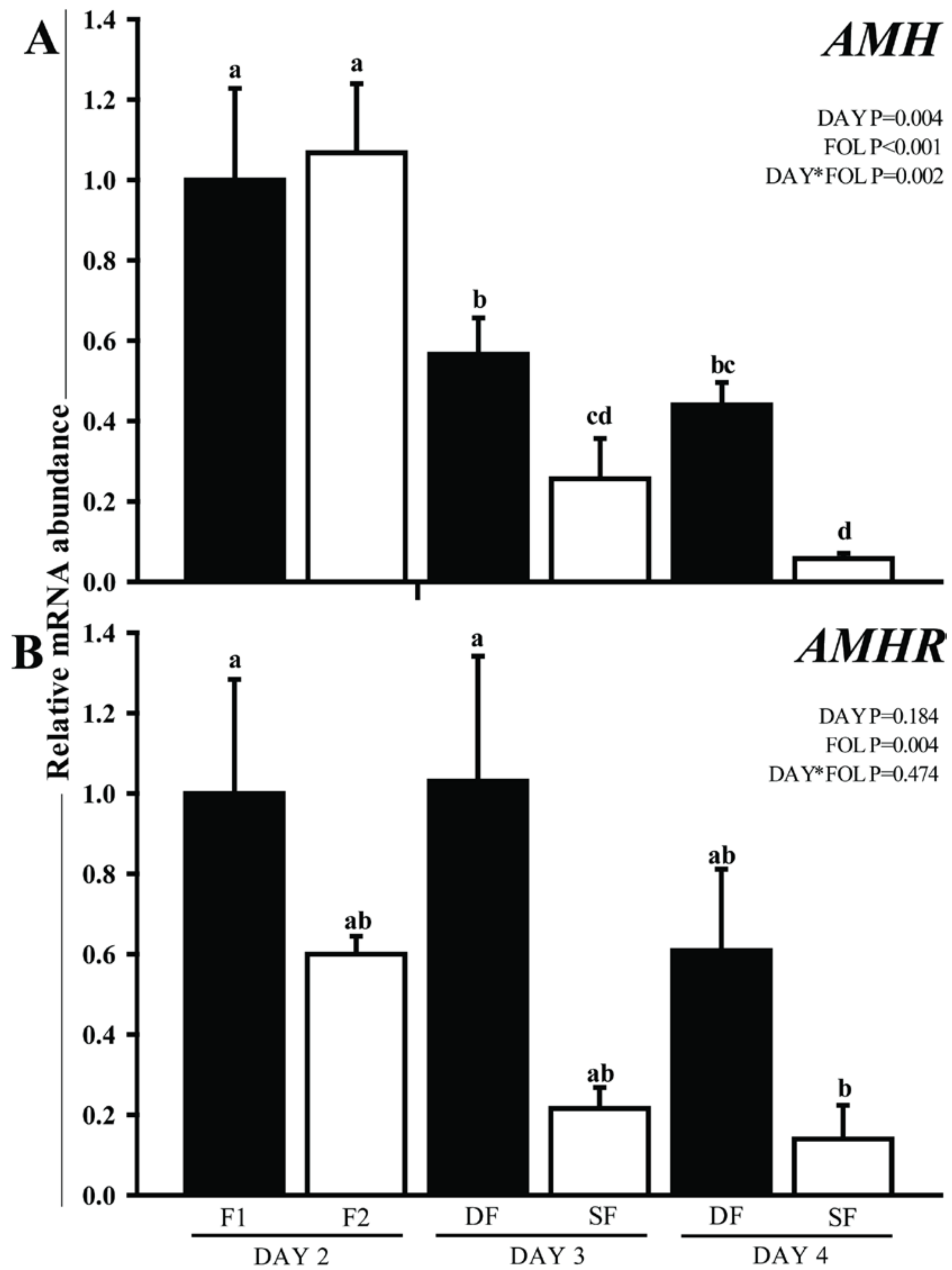


Figure 2

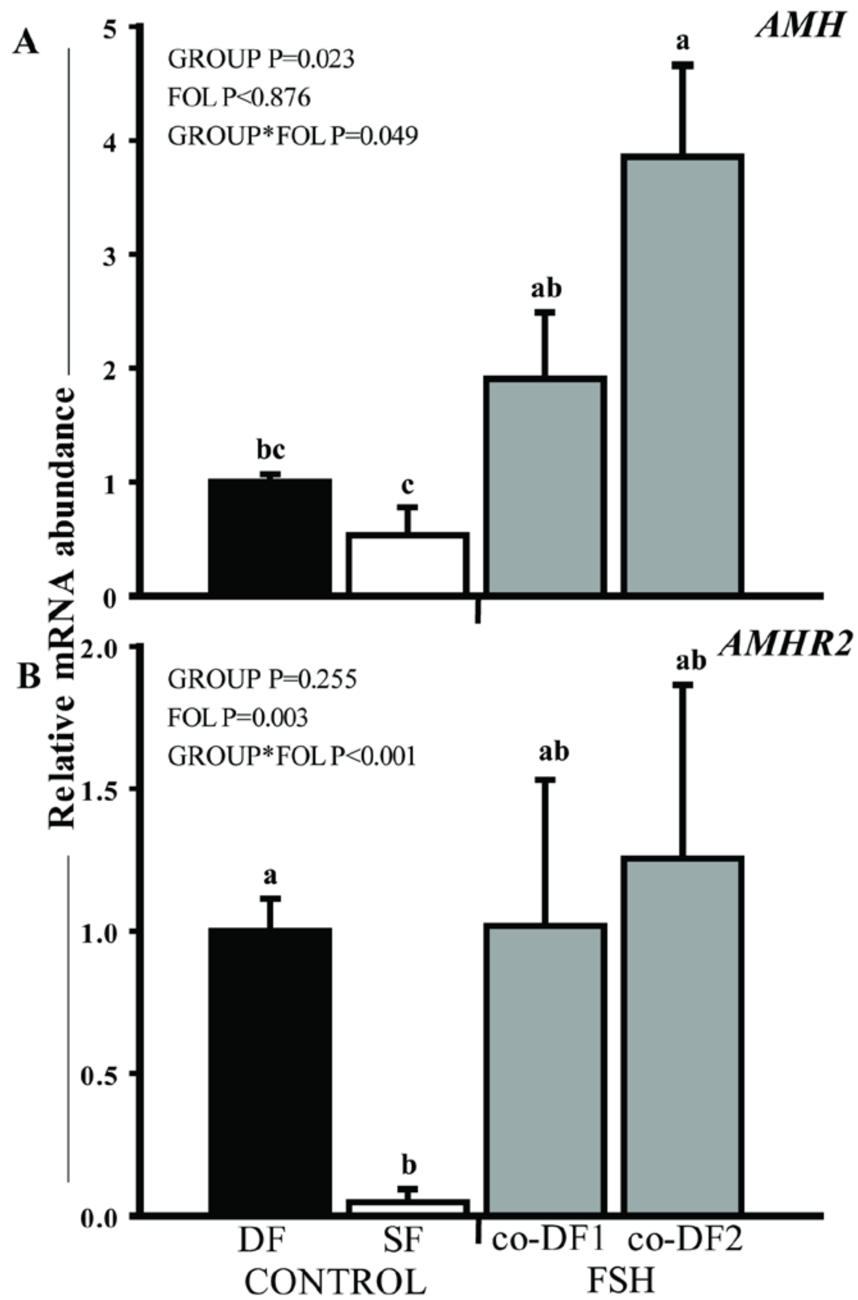
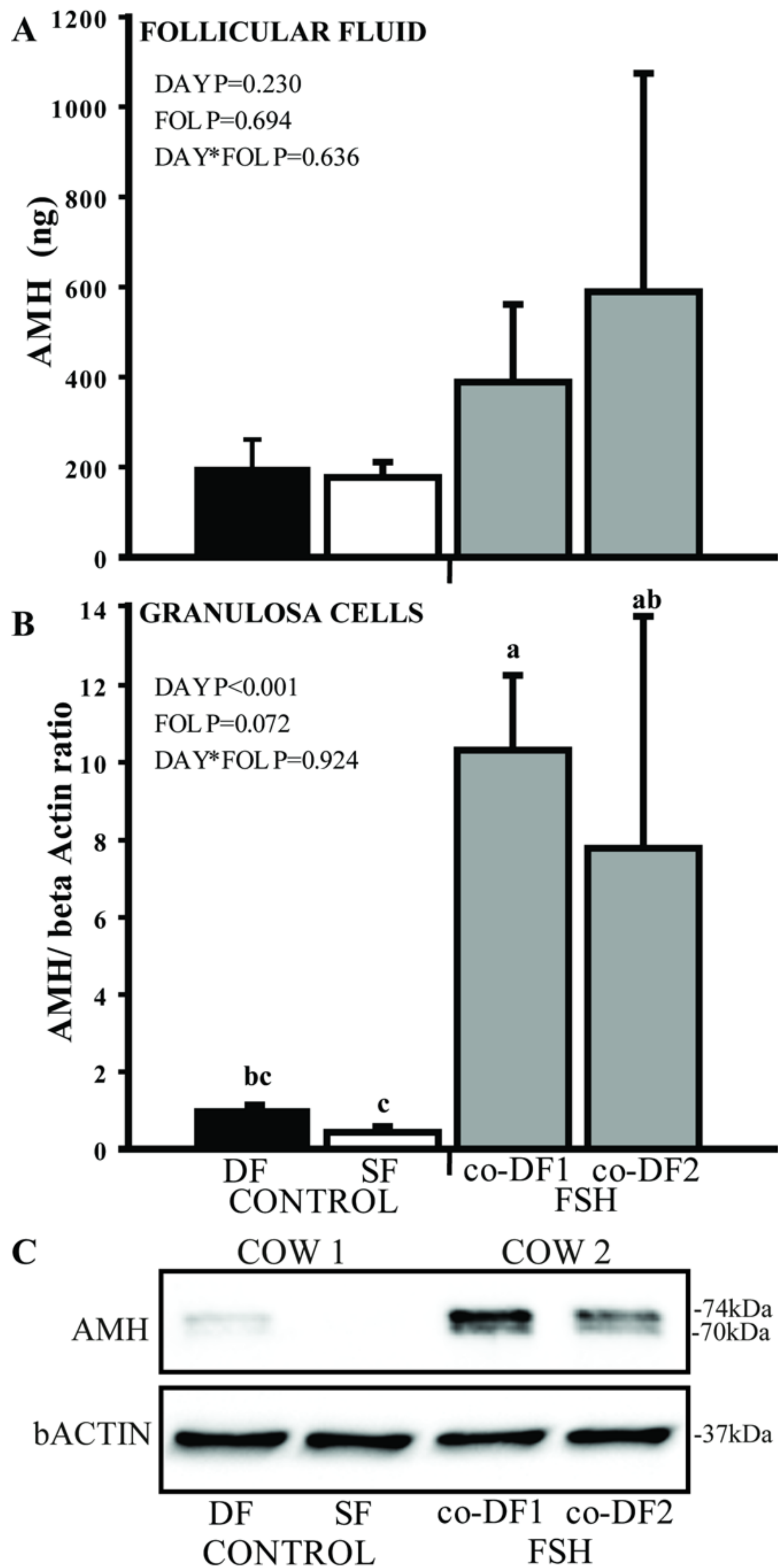


Figure 3.



5. DISCUSSÃO

No período de seleção e divergência folicular, os fatores intrafoliculares parecem desempenhar funções inibitórias ou estimulatórias relevantes, permitindo o estabelecimento da dominância. Na busca pelo conhecimento dos fatores locais que atuam no desenvolvimento folicular, nossa equipe vem estudando os mecanismos envolvidos no crescimento, assim como na regulação da seleção e divergência folicular em espécie monovulatória. Neste contexto, os objetivos do presente trabalho foram em um primeiro estudo (Artigo 1) estabelecer um modelo de codominância folicular *in vivo* e nesse estudar se os padrões de sinalização STAT3, AKT e MAPK são regulados nas células da granulosa nos folículos induzidos por FSH (co-DFs) em comparação ao DFs e SFs. Em um segundo estudo (Artigo 2), utilizamos dois modelos *in vivo* para avaliar a participação do AMH e seu receptor (AMHR2) em células da granulosa durante a divergência folicular e a influência do FSH no perfil de AMH em células da granulosa de folículos codominantes.

O FSH é um modulador chave para manter o folículo estrógeno-ativo. Entretanto, durante a divergência folicular, os níveis de FSH diminuem fazendo com que a maioria dos folículos entre em atresia (RIVERA; FORTUNE, 2001a). Durante essa fase, apenas o folículo dominante continua seu crescimento sendo auxiliado por mecanismos locais que o permite crescer mesmo em baixos níveis de FSH. No presente trabalho, desenvolvemos um modelo de codominância a partir da suplementação de FSH, possibilitando a coleta de folículos em um mesmo ambiente endógeno para o estudo dos eventos que ocorrem durante a divergência folicular.

O modelo de folículos codominantes induzidos por FSH permitiu compararmos o folículo dominante (DF), o folículo subordinado (SF) e os folículos codominantes (co-DFs). Baseado nesse modelo foi possível atribuir as características morfológicas e moleculares do segundo co-DF à sinalização pelo FSH bem como utilizar o modelo para o estudo de mecanismos mediados pelo FSH na regulação do desenvolvimento folicular durante as ondas foliculares. Estudos prévios utilizaram o modelo folicular de codominância através do uso exógeno de FSH (RIVERA; FORTUNE, 2001b; LOPEZ et al., 2005; BARROS et al., 2012; CHASOMBAT et al., 2013; GLICK et al., 2013) ou pelo uso de fêmeas selecionadas por partos múltiplos (ECHTERNKAMP et al., 2004; ECHTERNKAMP et al., 2009).

Até o presente momento, o efeito do FSH na transcrição e tradução das principais vias de sinalização celular no folículo; STAT3, AKT e MAPK ainda não foi descrito. Com isso

utilizamos o modelo supracitado e nele estudamos esses padrões de sinalização para buscarmos melhor esclarecer os processos envolvidos na divergência folicular.

Em um modelo de divergência folicular, nosso grupo de pesquisa demonstrou um aumento agudo na proteína STAT3 fosforilada em folículos coletados no dia 4 da onda folicular, que sugere que STAT3 possa estar envolvido na morte celular e consequente atresia do folículo subordinado (GASPERIN et al., 2014b). No presente trabalho, os níveis de STAT3 fosforilada e RNAm foram aumentados em células da granulosa de SF, confirmando esses resultados (GASPERIN et al.). O aumento da abundância da proteína STAT3 fosforilada sugere que esse padrão de sinalização é potencialmente ativado por fatores envolvidos na inibição da proliferação, diferenciação e síntese de estradiol nas células da granulosa. Com isso, mantemos a hipótese de que o padrão de sinalização STAT3 está envolvido na morte das células da granulosa e que essa supressão é através dos mecanismos regulados por FSH em DFs e co-DFs.

A ativação STAT pode ter relação com os padrões de sinalização AKT e MAPK (RAWLINGS et al., 2004; ABELL; WATSON, 2005). A existência do padrão pró-apoptótico pelo qual STAT3 media uma mudança na composição da subunidade PI3K que posteriormente regula a sinalização PI3K-AKT foi proposta (ABELL et al., 2005). Esses autores sugerem que é por esse mecanismo que o STAT3 exerce sua função pró-apoptótica nas células epiteliais da glândula mamária. Nosso estudo demonstrou que em células da granulosa há maior expressão de RNAm e baixa abundância proteica de AKT e MAPK em SF em relação a DF e co-DFs. Esses dados indicam pode estar ocorrendo uma regulação no processo de tradução, no entanto mais estudos são necessários para explicar a interação entre STAT3/AKT a STAT3/MAPK em células da granulosa.

As ações do padrão AKT são mais pronunciadas que o padrão MAPK em células da granulosa (RYAN et al., 2008). Esses autores demonstraram que a inibição de ambos resulta na inibição das ações estimulatórias do FSH e IGF em células da granulosa bovinas cultivadas, LH nas células da teca *in vitro*, e *in vivo* possuem efeito negativo na produção de estradiol no líquido folicular e no crescimento folicular em ovelhas. Altos níveis de AKT, pAKT, MAPK, e pMAPK na parede folicular de folículos dominantes também foram demonstradas em bovinos (EVANS; FORTUNE, 1997a) e ovinos (EVANS; MARTIN, 2000). Nossos resultados em co-DFs demonstraram quantidades similares das proteínas MAPK e AKT fostoriladas sugerindo que o FSH mantém a atividade desses padrões no crescimento de co-DFs. Com isso, esses resultados sugerem que, em nível molecular, o declínio das concentrações de FSH reduziu a atividade dos padrões MAPK e AKT em SFs, levando as células da granulosa a atresia. Por outro lado, evidenciamos a importância da sinalização da STAT3 durante a atresia folicular.

Membros da família da interleucina (IL) 6 e fator inibidor de leucemia (LIF) compartilham a mesma subunidade de proteína do receptor de transmembrana IL6ST e também são sinalizados através da STAT3 (HEINRICH et al., 1998). O ligante mais potente da rota da STAT3 é o LIF e este possui um papel importante no processo apoptótico (KRITIKOU et al., 2003). Esses autores propuseram que STAT3 promove a regulação do pMAPK1/2 na involução da glândula mamária e que a perda da atividade do MAPK durante a involução é biologicamente significante e necessária para que o STAT3 medie a sua máxima função apoptótica. LIF também media seu efeito através da ativação de MAPK e AKT (BURDON et al., 2002) e a sinalização LIF/STAT3 coincide com a indução da apoptose nas células epiteliais (ABELL et al., 2005). Nossos resultados demonstraram uma relação positiva dos altos os níveis de RNAm de *LIFR*, *IL6ST* e *STAT3* em associação com o aumento da atividade proteica da STAT3 nas células da granulosa de SF. Por outro lado, a abundância de *ILR6* foi menor em associação com a diminuição da atividade de MAPK e AKT. LIFR e IL6R necessitam da dimerização com *IL6ST* para sua sinalização, o padrão de expressão desses três receptores sugerem que há um aumento na sinalização do LIFR enquanto a sinalização do IL6R é reduzida. Esses resultados sugerem que LIF pode estar participando da sinalização da rota da STAT3 nas células da granulosa levando a apoptose. Nosso estudo é o primeiro a sugerir que a sinalização LIF/STAT3 pode possuir um papel no processo de apoptose nas células da granulosa. Porém, mais estudos são necessários para identificar a relação entre esses três padrões de sinalização MAPK, AKT e STAT3 nas células da granulosa e identificar a origem do LIF em folículos atrésicos.

Nosso segundo artigo estudou o AMH, foco de vários estudos em tecnologias de reprodução assistida, esse hormônio tem sido utilizado como o marcador endócrino ideal para o pool de folículos em crescimento, sendo reconhecido como o melhor indicador da reserva de folículos em várias espécies. Um possível efeito do papel do AMH na regulação do desenvolvimento de folículos antrais e seleção do folículo dominante ainda não foi estudado. Portanto, estudos são necessários para estabelecer se o AMH pode participar na regulação do desenvolvimento folicular final.

Os maiores níveis de AMH em ovários de mamíferos estão restritos a células da granulosa de folículos pré-antrais secundários em fase final de desenvolvimento e em folículos antrais saudáveis e em crescimento (MONNIAUX et al., 2012). Entretanto, a produção de AMH por folículos antrais grandes, a importância desse fator na divergência folicular e o efeito do FSH na expressão de AMH durante o crescimento folicular ainda não tinha sido estudada. Neste estudo, dois modelos *in vivo* foram utilizados para investigar a regulação do AMH e seu

receptor (AMHR2) durante a divergência folicular e os efeitos da suplementação de FSH na produção de AMH pelas células da granulosa.

De acordo com estudos prévios, observamos a expressão de AMH em células da granulosa de grandes folículos antrais (BEZARD et al., 1987; BAARENDS et al., 1995; VISSER et al., 2006; RICO et al., 2009; RICO et al., 2011). Além disso, nossos resultados são pioneiros em demonstrar o padrão de expressão do AMH e AMHR2 *in vivo* durante a divergência folicular em uma espécie monovulatória.

Estudos em ratos demonstraram que a expressão de AMH é maior em folículos pré-antrais e pequenos folículos antrais e esta expressão diminui no crescimento folicular final (BAARENDS et al., 1995). Da mesma forma, a expressão de AMH é indetectável em células da granulosa de folículos pré-ovulatórios de mulheres, e esta diminuição coincide com a seleção do folículo dominante (WEENEN et al., 2004; GRONDAHL et al., 2011; JEPPESEN et al., 2013). Por outro lado, em ovários de bovinos obtidos após abate, a expressão de AMH em células da granulosa de folículos em atresia inicial diminuíram comparados com folículos saudáveis (RICO et al., 2009). Nossos resultados estão de acordo com estudos prévios (RICO et al., 2009; RICO et al., 2011) e demonstram *in vivo* a diferente expressão de AMH entre DFs e SFs durante a divergência folicular. A abundância de RNAm para AMH foi menor em todos os folículos obtidos nos dias 3 e 4 quando comparados a folículos obtidos no dia 2. Estes resultados corroboram com estudos prévios e demonstram que esta diminuição na expressão é mais pronunciada em folículos atrésicos (RICO et al., 2011).

O AMH atua na sensitividade do folículo ao FSH pela inibição dos efeitos deste hormônio em células da granulosa de camundongos (DURLINGER et al., 2001) e humanos (GROSSMAN et al., 2008). Por outro lado, o FSH diminuiu a produção de AMH em células da granulosa bovinas cultivadas *in vitro* (RICO et al., 2011; SCHEETZ et al., 2012). A regulação da expressão de AMH por FSH foi estudada em células da granulosa *in vitro* e o FSH diminuiu os níveis de RNAm para AMH em células de folículos entre 5 e 10 mm de diâmetro (RICO et al., 2011). Em contraste, nossos resultados *in vivo* sugerem que células da granulosa de folículos codominantes induzidos por FSH possuem maior expressão de RNAm e proteína de AMH do que folículos controle. Adicionalmente, os efeitos do AMH são dependentes do seu receptor e a expressão de AMHR2 foi maior em DFs em relação a SFs e similar entre os co-DFs.

Em espécie monovular, o AMH é altamente expresso pelas células da granulosa de pequenos folículos antrais e pré-antrais em ovinos (DURLINGER et al., 2002b). Em espécie monovulatória, ou não, esses estágios de desenvolvimento folicular são responsivos a

gonadotrofinas, portanto isso pode ser significante à proporção de que pequenos folículos antrais, os quais expressam altos níveis de AMH, foram aumentados pelo tratamento com FSH (CAMPBELL et al., 2012). Nossos resultados demonstram uma diminuição nos níveis de RNAm para AMH no decorrer do processo da divergência folicular. Com isso, podemos sugerir que as altas quantidades de AMH em co-DFs após as a indução por FSH exógeno podem ser explicadas pela alta sensibilidade por esse hormônio após prévia diminuição na produção de AMH (VISSER; THEMMEN, 2014).

Em células da granulosa cultivadas *in vitro*, o AMH inibiu a produção de RNAm para CYP19A1 e consequentemente a produção de estradiol dependentes de FSH em humanos (GROSSMAN et al., 2008), ratos e suínos (MONNIAUX et al., 2012). Em contraste com esses estudos, nosso modelo *in vivo* demonstrou em ovários bovinos que a indução de folículos por FSH exógeno possui níveis similares de expressão dos genes CYP19A1 e AMH. Esses dados sugerem que o AMH não inibe a expressão de genes relacionados com a síntese de estradiol a nível de células da granulosa *in vivo*.

Em humanos, há uma relação positiva entre os níveis de RNAm para AMH e os níveis de proteína no líquido folicular correspondente (JEPPESEN et al., 2013). Já em bovinos, variações na concentração intrafolicular de AMH não correspondem com níveis de RNAm nas células da granulosa (RICO et al., 2009). Nossos resultados confirmam dados obtidos em estudos prévios (RICO et al., 2009), em que as concentrações de AMH no líquido intrafolicular são mais abundantes em SFs em relação a DFs, mas são similares em co-DFs.

CONCLUSÃO

O presente estudo demonstra que a atresia em folículos subordinados está associada ao aumento sinalização da rota STAT3, em contraste com o desenvolvimento ativo de folículos dominantes e codominantes. A relação temporal entre as proteínas STAT3 fosforilada e CASP3 clivada em folículos subordinados indicam que o aumento na sinalização da rota da STAT3, e não da MAPK ou AKT, pode estar envolvida na apoptose durante a atresia folicular. Com isto concluímos neste primeiro estudo que o FSH atua nas células da granulosa de folículos dominantes através da ativação das rotas da MAPK e AKT, e inibição da STAT3.

No segundo estudo, demonstramos que há diferença de expressão de AMH entre folículos saudáveis e atrésicos durante a divergência folicular e que essa expressão diminui de acordo com a evolução do processo de divergência. Em sequência, demonstramos que a suplementação de FSH exógeno resultou no aumento do AMH em células da granulosa de folículos codominantes. Com isso, concluímos que a expressão de AMH é regulada durante a divergência folicular em bovinos e que o perfil de AMH é induzido por FSH nas células da granulosa de bovinos *in vivo*.

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