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REGULAÇÃO DA DIVERGÊNCIA FOLICULAR *IN VIVO*: UMA ABORDAGEM MOLECULAR

TESE DE DOUTORADO

Bernardo Garziera Gasperin

Santa Maria, RS, Brasil

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REGULAÇÃO DA DIVERGÊNCIA FOLICULAR *IN VIVO*: UMA ABORDAGEM MOLECULAR

Bernardo Garziera Gasperin

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária.**

Orientador: Prof. Paulo Bayard Dias Gonçalves

Santa Maria, RS, Brasil

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elaborada por
Bernardo Garziera Gasperin

como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

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RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

REGULAÇÃO DA DIVERGÊNCIA FOLICULAR *IN VIVO*: UMA ABORDAGEM MOLECULAR

AUTOR: BERNARDO GARZIERA GASPERIN
ORIENTADOR: PAULO BAYARD DIAS GONÇALVES
Data e Local da Defesa: Santa Maria, 17 de Agosto de 2012.

O controle local da seleção folicular em mamíferos ainda é pouco compreendido. O objetivo do presente estudo foi identificar fatores locais, receptores e rotas de sinalização envolvidas na seleção do folículo dominante e atresia dos subordinados em bovinos. Em um primeiro estudo, avaliou-se a regulação e função do FGF10 e do seu receptor FGFR2b durante a divergência folicular. A expressão de FGF10 e FGFR2b foi significativamente maior nas células da teca e granulosa, respectivamente, provenientes dos folículos subordinados. A injeção intrafolicular de FGF10 inibiu o crescimento folicular de maneira dose dependente e reduziu significativamente a síntese de estradiol. Nas células da granulosa, a injeção de FGF10 diminuiu a expressão de RNAm de CYP19A1 e ciclina D2, enquanto que uma tendência de aumento da expressão do receptor FGFR2b foi observada. Nas células da teca, um aumento significativo na expressão de FGF10 foi observado nos folículos tratados com FGF10. Em um segundo estudo, o padrão de expressão dos receptores de BMPs e das proteínas BMP15 e GDF9 foram avaliados em vacas ovariectomizadas em diferenes dias em relação ao inicio da onda folicular, comparando os dois maiores folículos antes (dia 2), durante (dia 3) ou após a divergência folícular (dia 4). No dia 2 da onda folicular, foi observada maior expressão do receptor BMPR-1A e tendências a maior expressão dos receptores BMPR-2 e -1B nos futuros folículos subordinados. No dia 3, quando os folículos dominantes e subordinados são identificados, a expressão de BMPR-1B e -2 foi maior nos folículos subordinados. No dia 4, o receptor BMPR1B (RNAm e proteína) foi significativamente mais expresso nas células da granulosa de folículos atrésicos. O aumento da expressão do BMPR1B durante a atresia folicular foi confirmado nas células da granulosa de folículos induzidos à atresia através do tratamento com FGF10 ou inibidor dos receptores de estradiol. A abundância de BMP15 e GDF9 no fluído folicular não diferiu entre folículos dominantes e subordinados. Em um terceiro estudo, buscou-se identificar rotas de sinalização diferentemente ativas nas células da granulosa durante a divergência. Os níveis de MAPK fosforilada foram significativamente superiores nos futuros folículos dominantes (dia 2), mas não diferiram entre os dois maiores folículos durante ou após a divergência. Folículos subordinados apresentaram maiores níveis de STAT3 fosforilada em relação aos seus respectivos dominantes em todos os pares de folículos coletados, sendo observado um aumento significativo em folículos atrésicos coletados no dia 4. Em conclusão, os resultados sugerem que a expressão reduzida de FGF10 e do receptor FGFR2b possibilitam o crescimento e diferenciação do folículo dominante, enquanto que o aumento da sinalização do FGF10 no folículo subordinado está associado com a atresia. O perfil de expressão dos receptores BMPR-2, -1B e -1A indica que os mesmos apresentam funções na regulação da divergência folicular em bovinos. A fosforilação da MAPK é um marcador inicial de dominância folicular, mas não é diferentemente regulada durante e após a divergência, enquanto que o padrão de ativação da STAT3 sugere que essa via está envolvida na morte das células da granulosa.

Palavras chave: FGF10. BMPRs. GDF9. BMP15. MAPK. STAT3.

ABSTRACT

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

REGULATION OF FOLLICULAR DEVIATION *IN VIVO*: A MOLECULAR APPROACH.

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Date and Place of Defense: Santa Maria, August 17th, 2012.

The role of local factors in follicular selection in mammals is not fully understood. The aim of the present study was to identify local factors, receptors and intracellular signaling pathways involved in bovine dominant follicle selection and subordinate follicles atresia. In the first study, the pattern of mRNA expression and function of FGF10 and its receptor FGFR2b was evaluated during bovine follicle deviation. FGF10 and FGFR2b were significantly more expressed in theca and granulosa cells retrieved from subordinate follicles, respectively. Intrafollicular FGF10 treatment in the larger follicle dose-dependently inhibited follicle growth and significantly reduced estradiol secretion. In granulosa cells, FGF10 treatment decreased CYP19A1 and cyclin D2 mRNA expression whereas FGFR2b tended to be more expressed after treatment. In theca cells, a significant increase in FGF10 expression was observed in FGF10-treated follicles. In a second study, BMPRs, BMP15 and GDF9 expression was evaluated in cows ovariectomized when the size of the largest and second largest follicle did not have a significant difference (D2), had slight difference (D3) or marked difference (D4). At day 2 of follicular wave, it was observed a significant increase in BMPR1A expression whereas BMPR-2 and -1B tended to be more expressed in future subordinate follicles. At day 3, when dominant and subordinate follicles are reliably identified, BMPR-2 and 1B were more expressed in subordinate follicles. At day 4, BMPR1B (mRNA and protein) was significantly more expressed in granulosa cells from atretic follicles. The increased BMPR1B expression during atresia was confirmed in granulosa cells from follicles induced to atresia with FGF10 or estradiol receptor antagonist treatment. Similar levels of BMP15 and GDF9 proteins were observed in follicular fluid from dominant and subordinate follicles. In a third study, we aimed to identify intracellular signaling pathways differentially activated in granulosa cells during deviation. Phosphorylated MAPK was more abundant in the future dominant follicle, but did not differ between follicles at the expected moment and after follicular deviation. Subordinate follicles phosphorylated STAT3 levels tended to increase at day 3 and were significantly greater at day 4 in comparison to dominant follicles. In conclusion, present results suggest that decreased FGF10 and FGFR2b expression allows dominant follicle growth and differentiation whereas increased FGF10 signaling in the subordinate follicle induces atresia. The patterns of BMPR-2, -1B and -1A indicate that these receptors play roles during follicle deviation. Phosphorylated MAPK abundance is an early marker of follicle dominance, but is not differentially regulated during and after deviation. The functional status of STAT3 suggests that this pathway is involved in granulosa cell death.

Keywords: FGF10. BMPRs. GDF9. BMP15. MAPK. STAT3.

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LISTA DE ABREVIATURAS E SIGLAS

FSH – hormônio folículo estimulante

LH – hormônio luteinizante

FSHR – receptor de FSH

LHCGR – receptor de LH

17α - 17α-pregnenolona

A4 – androsteneidiona

T4 – testosterona

IGF – fator de crescimento semelhante à insulina

PAPP-A – proteína plasmática associada à prenhez

IGFBP – proteína de ligação ao IGF

FGF – fator de crescimento fibroblástico

TGF-β – fator de crescimento transformante beta

BMP – proteína morfogenética óssea

GDF – fator de crescimento e diferenciação

BMPR – receptor de BMP

MAPK – proteína quinase ativada por mitógenos

STAT - transdutor de sinal e ativador de transcrição

IGFR – receptor de IGF

FGFR – receptor de FGF

RNAm – ácido ribonucleico mensageiro

TGFBR – receptor de TGF beta

ACVR – receptor de ativina

PKA – proteína quinase A

PI3K – fosfatidil inositol 3-quinase

EGF – fator de crescimento epidermal

PKC – proteína quinase C

mTOR - *mechanistic target of rapamycin*

AKT – proteína quinase B

GM – glândula mamária

SUMÁRIO

1. INTRODUÇÃO

O crescimento folicular na espécie bovina ocorre em ondas sendo primariamente controlado por fatores endócrinos, principalmente gonadotrofinas (FSH e LH), seus receptores (FSHR e LHCGR) e esteróides ovarianos. No início de cada onda folicular, em resposta a uma elevação nos níveis de FSH, um grupo de pequenos folículos antrais é recrutado, fenômeno denominado emergência folicular (ADAMS et al., 1992). Os pequenos folículos em crescimento secretam estradiol acarretando em diminuição nos níveis de FSH. Sob baixos níveis de FSH, apenas um folículo é selecionado para continuar o desenvolvimento, enquanto os demais entram em atresia durante o processo conhecido como divergência folicular (GINTHER et al., 1996). Uma vez que todos os folículos em crescimento estão sob o mesmo ambiente endócrino, a participação de fatores locais diferentemente expressos nos folículos parece ser determinante na diferenciação ou atresia folicular (FORTUNE et al., 2004). Entretanto, o controle local (autócrino e parácrino) do desenvolvimento folicular ainda é pouco compreendido em espécies monovulatórias. Nesse contexto, o bovino fornece um modelo *in vivo* bastante adequado para o estudo de eventos relacionados à seleção folicular, por ser uma espécie monovular e possibilitar o monitoramento e manipulação dos folículos em crescimento individualmente.

Dentre os fatores locais envolvidos na seleção folicular, o sistema IGF é o mais estabelecido. Níveis superiores de IGF livre intrafolicular potencializam o efeito do FSH sobre a esteroidogênese e proliferação celular (FORTUNE et al., 2001), permitindo que o futuro folículo dominante continue crescendo até que ocorra a diferenciação das células da granulosa, tornando-o responsivo ao LH. O futuro folículo dominante apresenta maiores níveis da enzima PAPP-A que realiza a proteólise das proteínas de ligação ao IGF (IGFBPs), disponibilizando maiores níveis de IGF livre (MONGET et al., 2002). Recentemente, outros fatores produzidos nas células foliculares e no oócito, como os FGFs e membros da família TGF-β têm despertado a atenção de diversos grupos de pesquisa.

Diversos membros da família FGF e seus receptores são expressos no ovário. De acordo com o padrão de expressão e função, alguns fatores como o FGF2 parecem ter ação positiva sobre o desenvolvimento do folículo dominante (BERISHA et al., 2000), enquanto outros como o FGF-7, -10, -17 e -18 (PARROTT & SKINNER, 1998; BURATINI et al., 2007; MACHADO et al., 2009; PORTELA et al., 2010) parecem estar envolvidos na atresia folicular. Especificamente, o FGF10 parece ter uma marcada atividade inibitória sobre a

síntese de estradiol nas células da granulosa *in vitro*, sendo mais expresso em folículos atrésicos provenientes de ovários de abatedouro (BURATINI et al., 2007). A maioria dos dados sobre a participação dos FGFs até então obtidos são provenientes de estudos *in vitro*. Portanto, experimentos funcionais *in vivo* ainda são necessários para melhor estabelecer a função fisiológica dos FGFs e seus receptores durante a foliculogênese.

Membros da família TGF-β produzidos pelo oócito, especialmente BMP15 e GDF9, juntamente com seus receptores, têm sido apontados como fatores cruciais na regulação da diferenciação folicular e determinação do número de folículos ovulatórios. Animais homozigotos para mutações inativadoras no gene da BMP15 ou GDF9 são estéreis devido ao bloqueio do desenvolvimento folicular no estágio de folículo primário, enquanto que animais heterozigotos apresentam maiores taxas ovulatórias (GALLOWAY et al., 2000; HANRAHAN et al., 2004). De forma similar ao observado em animais heterozigotos para as mutações, a imunização por curto período contra BMP15 e GDF9 promove aumento na taxa ovulatória em ovinos e bovinos (JUENGEL et al., 2004; JUENGEL et al., 2009). Uma mutação no receptor tipo I (BMPR1B), que altera a sinalização da BMP15, também foi identificada em ovelhas estando associada a um marcado incremento na taxa ovulatória (MULSANT et al., 2001). Além disso, demonstrou-se recentemente que o *knockout* dos receptores BMPR-1A e -1B em camundongos (EDSON et al., 2010) induz tumores ovarianos na maioria dos animais, sugerindo uma importante função na regulação do desenvolvimento e regressão dos folículos. Coletivamente, os dados demonstram que o sistema BMP está envolvido no desenvolvimento folicular pré-antral e regulando a diferenciação folicular antral. O melhor entendimento da função dos membros da família TGF-β durante a foliculogênese poderá ter impactos diretos no desenvolvimento de técnicas contraceptivas não hormonais e aumento da taxa ovulatória em humanos e animais.

Além do estudo da função de fatores locais, a identificação de rotas de sinalização ativas nas diferentes classes foliculares também fornece subsídios para o entendimento de processos fisiológicos e patológicos na dinâmica de desenvolvimento folicular e regressão de folículos atrésicos. O *knockout* das rotas utilizadas na sinalização dos receptores de BMPs (SMAD-1, -5 e -8) (PANGAS et al., 2008; MIDDLEBROOK et al., 2009), induzem tumores ovarianos na maioria dos animais. Utilizando modelos monovulatórios *in vivo*, a abundância da proteína MAPK ativa (fosforilada) foi identificada como um marcador precoce de dominância folicular em bovinos e ovinos (EVANS & MARTIN, 2000; RYAN et al., 2007). Por outro lado, um aumento da ativação da via STAT3 foi demonstrado durante a apoptose de células da granulosa provenientes de ratas hipofisectomizadas (RUSSELL & RICHARDS,

1999) em comparação com células de folículos pré-ovulatórios, sugerindo que a ativação dessa rota está envolvida na morte das células da granulosa. Para o estudo da atividade das diferentes rotas durante o processo de seleção folicular é imperativo o uso de técnicas que possibilitem avaliar a regulação da transcrição, tradução e eventos pós-traducionais.

O modelo bovino de divergência folicular vem sendo usado na identificação de fatores envolvidos na diferenciação e atresia dos folículos sob o mesmo ambiente endócrino, preservando a interação entre as células da teca, granulosa e oócito. Os objetivos do presente estudo foram determinar o padrão de expressão de fatores locais, seus receptores e rotas de sinalização envolvidas na seleção do folículo dominante e atresia dos folículos não selecionados. Para isso, utilizamos pela primeira vez uma abordagem *in vivo* que possibilita o estudo simultâneo de eventos transcricionais, traducionais e pós-traducionais nas células da granulosa coletadas antes, durante ou após a seleção do folículo dominante.

2. REVISÃO BIBLIOGRÁFICA

2.1. Controle da foliculogênese antral

O desenvolvimento folicular antral é regulado principalmente pelas gonadotrofinas FSH e LH, seus receptores e esteróides ovarianos. Durante o ciclo estral bovino, um aumento nos níveis de FSH estimula o crescimento de um grupo de pequenos folículos antrais, fenômeno denominado emergência folicular (ADAMS et al., 1992; GINTHER et al., 1996). Do grupo de folículos recrutados, apenas um é selecionado para continuar crescendo (folículo dominante) mesmo sob baixos níveis de FSH, enquanto os outros folículos (subordinados) entram em atresia, caracterizando a divergência folicular (GINTHER et al., 1996). Uma vez que todos os folículos estão sob o mesmo ambiente endócrino, fica evidente que os processos de seleção do folículo dominante e atresia dos subordinados são regulados localmente, por fatores produzidos no ovário (FORTUNE et al., 2004).

O controle autócrino/parácrino da foliculogênese desempenha um papel essencial na modulação do desenvolvimento folicular (EVANS & FORTUNE, 1997). Nesse contexto, uma das descobertas mais relevantes foi o sistema IGF, composto pelas proteínas IGF1 e IGF2, mitógenos moduladores da ação das gonadotrofinas nas células da granulosa e teca (BEG & GINTHER, 2006). Dois receptores (IGF1R e IGF2R) e seis proteínas ligantes aos IGFs (IGFBPs) também fazem parte do sistema (MONGET et al., 2002).

O IGF1 produzido pelas células da granulosa sob controle do FSH e estradiol atua em sinergismo com o FSH na estimulação da proliferação e esteroidogênese das células da granulosa *in vitro* (FORTUNE, 2001; MONGET et al., 2002). O IGF2 produzido pela teca é o principal ligante intrafolicular regulando o crescimento de folículos antrais bovinos via IGF1R (WEBB et al., 2004). O nível de IGF livre depende das IGFBPs, sendo que folículos estrógeno-ativos possuem baixos níveis das IGFBP-2, -4 e -5, enquanto que em folículos atrésicos elas se encontram elevadas, diminuindo a biodisponibilidade do IGF (WEBB et al., 2004). Níveis elevados de IGFs livres estimulam a síntese de andrógenos, bem como a atividade da aromatase (CYP19A1) e produção de inibina (BEG et al., 2002).

A regulação das IGFBPs ocorre por dois principais mecanismos. O FSH parece estimular a produção de proteases que as degradam, como a PAPP-A, e inibir fortemente a expressão de RNAm de IGFBP-2 nas células da granulosa bovina e IGFBP-4 e -5 nas células da granulosa de ratas (MONGET et al., 2002; HUNTER et al., 2004). Recentemente, outros

fatores produzidos pelas células ovarianas têm sido apontados como envolvidos na seleção do folículo dominante e atresia dos subordinados. Na figura 1 são ilustrados eventos relacionados à esteroidogênese e diferenciação do folículo em crescimento.

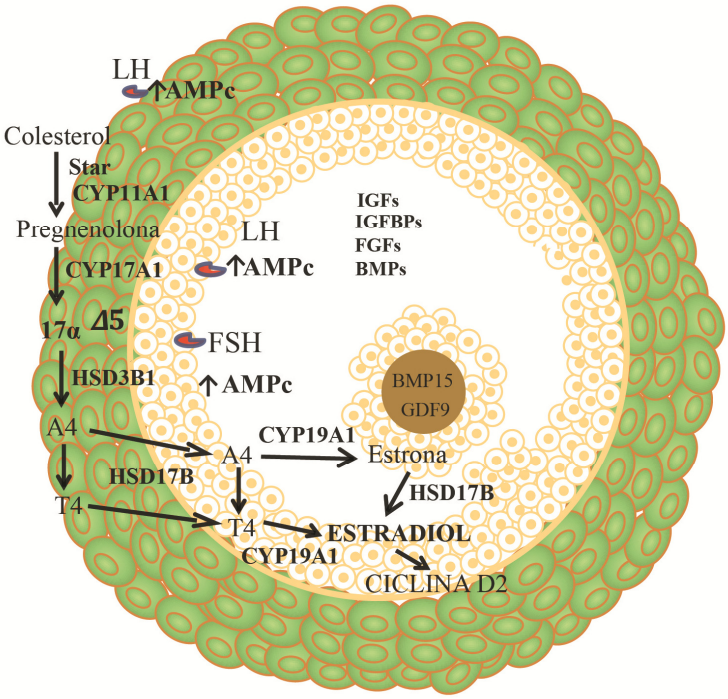

Figura 1 – Fatores envolvidos na esteroidogênese e diferenciação do folículo dominante. Sob estímulo do LH, as células da teca processam o colesterol, o qual é convertido a pregnenolona e posteriormente a 17α-pregnenolona (17α), a qual é convertida a andrógenos (A4: androsteneidiona e T4: testosterona). Os andrógenos sintetizados na teca atravessam a membrana basal e são convertidos a estradiol pelas células da granulosa. O estradiol induz a proliferação da granulosa estimulando a expressão de ciclina D2, fazendo com que as células entrem no ciclo celular, protegendo-as da apoptose. Durante a divergência, o folículo dominante sofre o processo de diferenciação, no qual corre um aumento significativo na expressão das enzimas CYP11A1, CYP17A1 e HSD3B nas células da teca, e de receptores de LH nas células da granulosa. Fatores locais produzidos pelas células foliculares como os IGFs, FGFs e BMPs são importantes reguladores dos processos de esteroidogênese e diferenciação, modulando os efeitos das gonadotrofinas.

2.2. Fatores de Crescimento Fibroblásticos

A família FGF é composta por mais de 20 fatores envolvidos no desenvolvimento embrionário, angiogênese, cicatrização e oncogênese (BASILICO & MOSCATELLI, 1992).

Essas proteínas são expressas em estágios iniciais e tardios do desenvolvimento e também em tecidos adultos, o que indica que desempenham papel importante como fatores de crescimento e diferenciação durante toda a vida (IGARASHI et al., 1998). Suas funções, dentre as quais a estimulação da proliferação celular, são mediadas através de seus receptores de alta afinidade FGFR-1 a -4 e por outros de baixa afinidade. Na última década, tem sido demonstrado o envolvimento da família dos FGFs na fisiologia ovariana. Do mesmo modo que os IGFs, os FGFs também parecem participar do desenvolvimento folicular pré-antral e antral (BERISHA et al., 2004).

Diversos FGFs e seus receptores (FGFRs) têm sido descritos em folículos ovarianos, sugerindo funções no controle parácrino do desenvolvimento folicular (PARROTT & SKINNER, 1998; BURATINI et al., 2007; PORTELA et al., 2010). O padrão de expressão do FGF2 sugere que esse fator está envolvido na proliferação vascular durante o crescimento do folículo dominante (BERISHA et al., 2000). Além disso, alguns FGFs são conhecidos como moduladores de esteroidogênese (BAIRD & HSUEH, 1986). Tratamento com FGF7 reduz a atividade da enzima CYP19A1 *in vitro* (PARROTT & SKINNER, 1998), e uma ação semelhante foi atribuída ao FGF17, o qual é significativamente mais expresso nas células da granulosa e teca de folículos atrésicos (MACHADO et al., 2009). Recentemente, Portela et al. (2010) demonstraram que o FGF18 proveniente das células da teca está envolvido na atresia folicular.

2.2.1. Fator de Crescimento Fibroblástico 10

O FGF10 foi isolado originalmente do mesênquima pulmonar de rato e identificado como essencial para a regulação de eventos morfogênicos (YAMASAKI et al., 1996). Atribui-se ao FGF10 o papel de fator quimiotático na formação do pulmão, o que pode ser confirmado pela ausência completa de pulmões em camundongos *knockout* para o gene desse fator (MIN et al., 1998; SEKINE et al., 1999). Os padrões temporal e espacial de expressão do FGF10 parecem diferir da maioria dos outros membros da família FGF, portanto, o seu significado fisiológico ainda precisa ser melhor elucidado (YAMASAKI et al., 1996). O FGF10 é semelhante ao FGF7 em relação à estrutura e às propriedades funcionais. Ambos são mitógenos para as células epiteliais e apresentam alta afinidade pelo receptor FGFR-2b, altamente expresso no epitélio pulmonar de embriões nos estágios inicias de desenvolvimento (PETERS et al., 1992; IGARASHI et al., 1998; OHUCHI et al., 2000). Essa similaridade sugere que o FGF7 e o FGF10 atuam de forma sinérgica ou redundante (IGARASHI et al.,

1998). Quanto à participação no controle da fisiologia reprodutiva, estudos em útero neonatal ovino detectaram a expressão gênica do FGF10 e do FGF7, participando da regulação da morfogênese endometrial (CHEN et al., 2000).

Em humanos, a expressão de FGF10 foi demonstrada nas células da teca e estroma ovariano (TANIGUCHI et al., 2008). No ovário bovino, a expressão do RNAm do FGF10 foi detectada em oócitos, células da teca de folículos antrais e tecido luteal (BURATINI et al., 2007; CASTILHO et al., 2008). O principal receptor de FGF10 (FGFR-2b) é expresso no oócito (ZHANG et al., 2010), células do cumulus (CHO et al., 2008), na granulosa e teca interna (BERISHA et al., 2004), sendo sua expressão positivamente regulada por FSH na granulosa (BURATINI et al., 2007). Esses dados sugerem o envolvimento do FGF10 na sinalização parácrina oriunda do oócito e células da teca, tendo como alvo as células da granulosa. Além disso, os níveis de RNAm do FGF10 nas células da teca diminuem com os níveis intrafoliculares de estradiol, indicando uma regulação durante a foliculogênese (BURATINI et al., 2007).

Estudos *in vitro* demonstram que a adição de FGF10 em cultivo de células da granulosa bovina acarreta em diminuição dose-dependente na produção de estradiol (BURATINI et al., 2007). A partir dos dados existentes, sugere-se um modelo no qual o FGF10, na fase antral inicial, atuaria como regulador da proliferação e inibidor da diferenciação celular. À medida que ocorre o desenvolvimento folicular, a diminuição progressiva na expressão do gene do FGF10 nas células da teca do folículo dominante, possibilitaria a diferenciação celular e o aumento na síntese de estradiol.

2.3. Proteínas Morfogenéticas Ósseas (BMPs)

O grupo das proteínas morfogenéticas ósseas é composto por cerca de 20 ligantes (BMPs) e sete receptores serina/treonina quinases (BMPRs) divididos em tipo I e tipo II. Essas proteínas, do mesmo modo que os fatores de crescimento e diferenciação (GDFs) pertencem à superfamília TGF-β (KNIGHT & GLISTER, 2006). Os fatores pertencentes à família TGF-β se ligam, inicialmente, a receptores tipo II, os quais fosforilam os domínios quinase de receptores tipo I, que por sua vez conduzem a sinalização fosforilando proteínas denominadas SMADs reguladas por receptores (R-SMADs). Além das R-SMADs, *common-partner* SMADs (Co-SMADs) e SMADs inibitórias (I-SMADs) também estão envolvidas na regulação da sinalização intracelular (ITOH et al., 2000). As SMADs formam complexos

nucleares que isoladamente, ou em combinação com outros fatores, regulam a transcrição de genes alvo.

2.3.1. Mutações e imunização ativa

O interesse no estudo da função do sistema BMP na fisiologia ovariana em mamíferos teve início na última década. Mutações inativadoras nas proteínas BMP15 (GALLOWAY et al., 2000) e GDF9 (HANRAHAN et al., 2004) foram identificadas em ovelhas, estando associadas à infertilidade, quando em homozigose, ou aumento da taxa ovulatória, nos indivíduos heterozigotos. Isso se deve ao fato dessas proteínas exercerem funções essenciais durante o desenvolvimento folicular pré-antral (HANRAHAN et al., 2004; MCNATTY et al., 2007) e ao mesmo tempo desempenharem funções na regulação da diferenciação em folículos antrais (MCNATTY et al., 2005). Postula-se que, com um alelo do gene inativado, a reduzida atividade desses fatores de crescimento induz a diferenciação precoce dos folículos em desenvolvimento (com menor número de células da granulosa). Em acordo com essa hipótese, células da granulosa de ovelhas heterozigotas para a mutação inativadora da BMP15 apresentam maior responsividade ao LH (MCNATTY et al., 2009).

Estudos funcionais *in vivo* comprovam a relevância do sistema BMP nas diferentes fases do desenvolvimento folicular. Juengel et al. (2009) realizaram imunização ativa por curto período contra as proteínas GDF9 e BMP15 em vacas e obtiveram superovulação em alguns animais, sugerindo que esses fatores estão envolvidos na seleção do folículo dominante e determinação da taxa ovulatória. Resultados semelhantes foram obtidos em ovelhas, nas quais um incremento significativo na taxa ovulatória foi observado após curto período de imunização contra BMP15 ou GDF9. Essa imunização não causou efeitos negativos aparentes na fecundação dos oócitos, no desenvolvimento embrionário e capacidade das ovelhas manterem uma gestação a termo (JUENGEL et al., 2004). Por outro lado, a imunização contra essas proteínas por períodos prolongados causou um bloqueio no desenvolvimento folicular em ovelhas (MCNATTY et al., 2007). Coletivamente, esses resultados demonstram um grande potencial de utilização da regulação das proteínas BMP15 e GDF9 como ferramenta para incrementar a taxa ovulatória ou como contraceptivo em animais domésticos e humanos.

Nas células da granulosa, o efeito sinérgico entre BMP15 e GDF9 é mediado pelo receptor BMPR2 (EDWARDS et al., 2008). A ativação do BMPR2 leva a fosforilação do receptor tipo I, sendo os BMPR-1B e 1A responsáveis pela sinalização da BMP15 e o TGFBR1 (ALK5), do GDF9. Uma mutação no receptor tipo I BMPR1B (ALK6), que além da

BMP15 também participa da sinalização da BMP2 e 4, foi identificada em ovelhas estando associada a um marcado incremento na taxa ovulatória (MULSANT et al., 2001; SOUZA et al., 2001). Essa mutação difere das mutações inativadoras nas proteínas ligantes pelo fato de ter um efeito aditivo, sendo que indivíduos homozigotos para a mutação apresentam as maiores taxas ovulatórias.

2.3.2. Mecanismos de ação e regulação da expressão

Apesar dos fenótipos observados em animais portadores de mutações demonstrarem que as proteínas BMP15, GDF9 e o receptor BMPR1B estão entre os principais fatores locais envolvidos na determinação da taxa ovulatória, o exato mecanismo de ação desse sistema durante a foliculogênese antral é desconhecido. Estudos *in vitro* demonstram diversas funções, muitas vezes opostas, sobre a proliferação, diferenciação e esteroidogênese das células da granulosa. A adição de GDF9 em cultivo de células da teca bovina estimula a proliferação celular e diminui a esteroidogênese estimulada por LH ou IGF, através da inibição da CYP11A1 e de receptores de LH (LHCGR) (SPICER et al., 2008). Um efeito negativo sobre a síntese de estradiol também foi observado após adição de GDF9 em cultivo de células da granulosa tratadas com FSH e IGF (SPICER et al., 2006). A ação da BMP15 e do GDF9 parece ser sinérgica e variável de acordo com a espécie de origem da proteína (MCNATTY et al., 2005). Em ratas, o GDF9 estimula a proliferação das células da granulosa, efeito não observado nas espécies ovina e bovina. No caso da BMP15, o estímulo à proliferação foi observado nas células da granulosa de ratas, ruminantes e mulheres (MCNATTY et al., 2005). Esses dados sugerem cuidado na extrapolação de resultados entre diferentes espécies.

A regulação da expressão de algumas BMPs e de seus receptores em folículos antrais foi avaliada na rata (ERICKSON & SHIMASAKI, 2003), na porca (PARADIS et al., 2009) e, recentemente, na vaca (GLISTER et al., 2010). Erickson & Shimasaki (2003) observaram elevada expressão dos receptores BMPR1A, BMPR1B e BMPR2 em folículos atrésicos de ratas, sugerindo a participação da sinalização através desses receptores nesse processo. Além dos receptores, altos níveis de expressão de BMP2 e BMP6 foram observados na granulosa de folículos atrésicos, enquanto níveis indetectáveis de BMP4 na granulosa e BMP7 na teca foram observados nessa classe de folículos. Portanto, o sistema BMP parece desempenhar ações parácrinas estimulatórias e inibitórias no controle local da foliculogênese.

Jayawardana et al. (2006) utilizando folículos bovinos classificados como pré-seleção (diâmetro médio 7,7 mm) e pós seleção (diâmetro médio de 15 mm), demonstraram que a expressão de BMPR2 e BMPR1A foi significativamente maior na granulosa de folículos pós-seleção. É importante ressaltar que a utilização de folículos com diâmetro médio de 15 mm (pré-ovulatórios) pode não ser o modelo mais adequado para se estudar seleção folicular. Os mesmos autores demonstraram um efeito positivo do estradiol sobre a expressão de BMPR2 e BMPR1A nas células da granulosa, sendo essa expressão aumentada quando houve combinação com FSH. O FSH isoladamente diminuiu a expressão destes genes. Esses resultados contrariam os dados obtidos após imunização ativa os quais sugerem que uma diminuição na sinalização da BMP15 e GDF9 estaria associada com diferenciação do folículo dominante e aumento da taxa ovulatória.

Outras BMPs foram estudadas no ovário bovino. Glister et al. (2004) demonstraram a expressão das BMPs 4, e 7 nas células da teca e da BMP6 no oócito e células da granulosa. Os mesmos autores, utilizando cultivo de células da granulosa demonstraram a participação dessas proteínas no controle da esteroidogênese e proliferação celular. Kayani et al. (2009) demonstraram que os receptores BMPR1A, BMPR1B, BMPR2, ACVR1A, ACVR1B, ACVR2A e ACVR2B são expressos nos dois tipos celulares. Entretanto, Glister et al. (2010) utilizando folículos provenientes de abatedouro, classificados de acordo com o diâmetro, demonstraram haver pouca regulação da expressão dos receptores de BMPs ao longo do crescimento folicular. Entretanto, os autores não levaram em conta o *status* de saúde folicular (dominantes *vs*. subordinados).

Caixeta et al. (2009) na busca por marcadores de competência em oócitos bovinos observaram um padrão elevado de expressão de BMP15 e GDF9, porém sem regulação ao longo do desenvolvimento folicular antral. Spicer et al. (2008) demonstraram expressão de GDF9 nas células da granulosa e oócito, mas não nas células da teca. Células da granulosa de folículos grandes expressam menos RNAm de GDF9 do que as provenientes de folículos pequenos. O padrão de expressão do receptor BMPR1A nas células da teca também foi maior em células provenientes de folículos pequenos.

A participação do sistema BMP na regulação parácrina da foliculogênese é incontestável. Alterações no padrão fisiológico de expressão desse sistema estão associadas a distúrbios reprodutivos como falência ovariana precoce em mulheres (DIXIT et al., 2006). Teixeira et al. (2002) identificaram uma diminuição na expressão de GDF9 em oócitos de mulheres com a síndrome do ovário policístico. Experimentos utilizando camundongas *knockout* para os receptores BMPR1A e BMPR1B demonstram que estes receptores

apresentam funções distintas durante a foliculogênese, mas atuam de forma redundante como supressores de tumores ovarianos (EDSON et al., 2010). Os mesmos autores demonstraram que alterações no padrão normal de expressão desses receptores induzem a formação de tumores de células da granulosa.

Diferenças marcantes na expressão e função das BMPs e seus receptores têm sido descritas nas diferentes espécies. Entretanto, o padrão de expressão e a função dessas proteínas durante a divergência, em um modelo que possibilite avaliar a expressão em folículos dominantes e subordinados momentos antes, durante e após a seleção folicular, ainda não foram descritos.

2.4. Rotas de sinalização ativas durante a divergência folicular

A via de sinalização intracelular ativada por FSH e LH é a via da PKA, que resulta em fosforilação e ativação ou supressão de fatores de transcrição que regulam genes alvo nas células da granulosa e luteais (GONZALEZ-ROBAYNA et al., 2000). Essas gonadotrofinas também ativam as vias da MAPK e PI3K (SU et al., 2002; WAYNE et al., 2007). Vários estudos demonstraram funções dessas vias no ciclo celular e diferenciação de células da granulosa. As vias MAPK3/1 e PI3K foram avaliadas em folículos bovinos, sendo positivamente correlacionadas com desenvolvimento folicular (RYAN et al., 2007). Fatores como a insulina, leptina, IGF1 e EGF-like growth factors modulam a sinalização das gonadotrofinas e suas ações são mediadas por diferentes rotas de sinalização. Diversas vias de sinalização como PKA (SAYASITH et al., 2008), MAPK (SILVA et al., 2006), PI3K (MANI et al., 2010), PKC (ROY et al., 2009) e mTOR (HOU et al., 2010), foram demonstradas em cultivos de células somáticas ovarianas bovinas. A análise da funcionalidade dessas vias através da quantificação das formas fosforiladas em relação às formas totais durante a divergência folicular pode indicar ligantes e receptores envolvidos na diferenciação do folículo dominante e na regressão de folículos atrésicos.

Uma das vias de sinalização mais estudadas nas células foliculares é a MAPK. Entretanto, sua função durante o desenvolvimento folicular não está completamente esclarecida. Em cultivo de células da granulosa, o tratamento com inibidor dessa via aumenta a expressão da enzima CYP19A1 estimulada por FSH (SILVA et al., 2006). Entretanto, as vias MAPK e Akt são estimuladas por FSH e IGF nas células da granulosa bovina *in vitro* (RYAN et al., 2008). Além disso, o tratamento com inibidor de MAPK *in vivo* bloqueia o desenvolvimento folicular e síntese de estradiol em ovelhas (RYAN et al., 2008) e a MAPK

fosforilada foi identificada como um marcador precoce de dominância folicular em bovinos e ovinos (EVANS & MARTIN, 2000; RYAN et al., 2007).

A proteína STAT3 é ativada por diversos ligantes como interleucinas e hormônios. O envolvimento dessa via na proliferação celular, diferenciação e prevenção de apoptose já foi demonstrado. Por outro lado, um aumento da ativação desta via foi demonstrado durante a apoptose de células da granulosa coletadas de ratas hipofisectomizadas (RUSSELL & RICHARDS, 1999) em comparação com células de folículos pré-ovulatórios. A fosforilação da STAT3 está envolvida na indução da apoptose durante a involução da glândula mamária (CHAPMAN et al., 1999), inibindo a via da PI3K (ABELL et al., 2005). Recentemente, foi demonstrado que a STAT3 regula a morte celular mediada por lisossomos, a qual está envolvida na involução da glândula mamária (KREUZALER et al., 2011). O envolvimento dessa via de sinalização na divergência folicular ainda não foi estudado.

3. ARTIGO 1

TRABALHO PUBLICADO:

FGF10 INHIBITS DOMINANT FOLLICLE GROWTH AND ESTRADIOL SECRETION *IN VIVO* IN CATTLE

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FGF10 inhibits dominant follicle growth and estradiol secretion *in vivo* in cattle

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Short Title: FGF10 in bovine follicular growth.

Footnotes

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3.1. Abstract

Fibroblast growth factors (FGFs) are involved in paracrine control of follicle development. It was previously demonstrated that FGF10 decreases estradiol secretion in granulosa cell culture and that theca cell *FGF10* mRNA expression is decreased in healthy follicles from abattoir ovaries. The main objectives of the present study were to evaluate *FGF10* and *FGFR2b* mRNA expression during follicular development *in vivo*, to evaluate the effect of FGF10 on follicle growth using *Bos taurus taurus* cows as a model and to gain more insight into the mechanisms through which FGF10 inhibits steroidogenesis. Messenger RNA encoding both *FGF10* and *FGFR2b* (main FGF10 receptor) were significantly more expressed in subordinate follicles than in dominant follicles. The intrafollicular injection of FGF10 into the largest growing follicle at 7-8mm in diameter interrupted the dominant follicle growth in a dose dependent manner (11±0.4, 8.3±1 and 5.9±0.3mm for 0, 0.1 and 1µg/mL FGF10, at 72h after treatment; $P<0.05$). In a third experiment, follicles were obtained 24h after FGF10 (1µg/mL) or PBS treatment through ovariectomy. In theca cells, FGF10 treatment did not affect mRNA encoding steroidogenic enzymes, *LHCGR* and *IGFBPs*, but significantly upregulated *FGF10* mRNA expression. The expression of *CYP19A1* mRNA in granulosa cells was downregulated by FGF10 treatment, which was accompanied by a fifty-fold decrease in estradiol production, and decreased *cyclin D2* mRNA. These results shown that *FGF10* and its receptor *FGFR2b* are more expressed in subordinate follicles and provide solid *in vivo* evidence that FGF10 acts as an important regulator of follicular growth in cattle.

3.2. Introduction

Follicular development is mainly orchestrated by gonadotropins (FSH and LH), their receptors and ovarian steroids. During the bovine estrous cycle, an initial rise in FSH

stimulates the growth of a cohort of small antral follicles (Adams *et al.* 1992, Ginther *et al.* 1996). From this group of growing follicles, only one is selected (dominant follicle) for continued growth even during the nadir of FSH secretion, while all other follicles (subordinates) enter atresia in an event known as follicle deviation (Ginther *et al.* 1996). As all follicles are under the same endocrine environment, the process of follicle development and atresia involves many locally differentially produced factors (Fortune *et al.* 2004). There is a complex autocrine and paracrine system that is not well understood, the IGF system being the most characterized during follicle development (Stewart *et al.* 1996, Ginther *et al.* 2004, Sudo *et al.* 2007).

Several fibroblast growth factors (FGFs) and their receptors (FGFRs) have been detected in ovarian follicles, suggesting roles in the paracrine control of follicle development (Parrott & Skinner 1998, Buratini *et al.* 2007, Portela *et al.* 2010). The pattern of *FGF2* expression suggests that this factor is involved in vascular proliferation during bovine dominant follicle growth (Berisha *et al.* 2000). Moreover, some FGFs are involved in steroidogenesis control. Treatment with FGF7 reduced basal and FSH-stimulated levels of granulosa cell CYP19A1 activity *in vitro* (Parrott & Skinner 1998). A similar anti-steroidogenic effect was attributed to FGF17, which increases significantly in granulosa and theca cells from atretic follicles (Machado *et al.* 2009) and is negatively regulated by FSH and IGF1. Recently, Portela *et al.* (2010) demonstrated that FGF18 from theca cells is involved in follicle atresia.

Fibroblast growth factor 10 (FGF10) was first described in rat embryos and lungs from adult rats (Yamasaki *et al.* 1996). Mice lacking the *Fgf10* gene showed total absence of lung formation, indicating that FGF10 is crucial during organogenesis (Min *et al.* 1998). In the ovary, the expression of *FGF10* was detected in human theca and stromal cells (Taniguchi *et al.* 2008) and in bovine oocytes, theca cells from antral follicles and luteal cells (Buratini *et*

al. 2007, Castilho *et al.* 2008). In bovine theca cells, *FGF10* mRNA abundance decreases in healthy follicles (Buratini *et al.* 2007), and recombinant FGF10 inhibits estradiol secretion from cultured bovine granulosa cells (Buratini *et al.* 2007, Portela *et al.* 2008). The main FGF10 receptor (FGFR2b) is expressed in bovine cumulus cells (Cho *et al.* 2008), oocytes (Zhang *et al.* 2010), granulosa and theca interna cells (Berisha *et al.* 2004). Its expression in granulosa cells is positively and negatively regulated by FSH and IGF1, respectively (Buratini *et al.* 2007). Collectively, these data suggest a role for FGF10 in the regulation of antral folliculogenesis in cattle.

The fact that some FGFs regulate steroidogenesis has long been recognized (Baird & Hsueh 1986). However, the participation of more recently described FGFs in ovarian reproductive events needs further clarification. The addition of FGF10 to bovine granulosa cells culture decreases estradiol production in a dose-dependent manner (Buratini *et al.* 2007, Portela *et al.* 2008). Nevertheless, the mechanism through which FGF10 decreases estradiol secretion was not assessed in the culture systems. The aforementioned studies were predominantly *in vitro*, therefore *in vivo* functional studies are necessary to establish the physiological role of FGFs in follicle development. The main objectives of the present study were to compare *FGF10* and *FGFR2b* mRNA expression between the two largest follicles before and after follicular deviation during the first follicle wave in *Bos taurus taurus* cows, and to test the effect of an intrafollicular injection of FGF10 in the dominant follicle on follicular growth, steroidogenesis and cell differentiation *in vivo*.

3.3. Results

FGF10 and FGFR2b mRNA expression near follicle deviation

Abundance of mRNA encoding *FGF10* and its receptor, *FGFR2b*, was measured in the largest and second largest follicles of cows before (largest follicle <8.5mm) and after

(largest follicle >8.5mm) deviation during the first follicle wave. A total of 24 follicles were collected from 12 cows (out of 18 synchronized cows). The largest and second largest follicles before expected time of deviation were 7.4±0.2 and 6.6±0.3mm diameter, respectively ($P<0.05$) and those after deviation were 10.1±0.7 and 6.8±0.5 ($P<0.05$; Figure 1A). Follicles obtained before deviation were classified as largest (LF) or second largest follicle (SLF) and those obtained after deviation were classified as dominant (DF) or subordinate follicle (SF) based on follicular diameter. Estradiol levels were significantly higher ($P<0.05$) in the largest follicle before (190.1±54.9 *vs.* 80.3±48.4ng/mL for LF and SLF, respectively) and after deviation (249.4±39.7 *vs.* 3.98±3.1ng/mL for DF and SF, respectively; Figure 1B).

For the gene expression analyses in theca cells, samples from 4 cows were discarded due to contamination by granulosa cells in one of the samples from the pair, leaving 4 pairs of follicles collected before and 4 pairs collected after deviation. *FGF10* mRNA abundance did not differ between LF and SLF before deviation, but was more abundant in theca cells from SF compared to DF after deviation ($P<0.01$; Figure 1C).

From a total of 24 granulosa samples obtained from 12 pairs of follicles, 4 samples were positive for *CYP17A1* mRNA expression, indicating the presence of theca cells in the granulosa sample. After removing these 4 cows from the analysis, cells from 3 pairs of follicles collected before and 5 pairs collected after deviation were used to evaluate *FGFR2b* mRNA expression. The expression of *FGFR2b* was significantly higher in granulosa cells from SF compared to DF after deviation ($P<0.01$; Figure 1D). The abundance of mRNA encoding *FGF10* in theca cells and *FGFR2b* in granulosa cells were negatively correlated to estradiol concentration in follicular fluid ($P<0.01$; Figure 1E and 1F, respectively).

Follicular growth after intrafollicular injection of FGF10

To test the hypothesis that FGF10 acts as an inhibitor of follicular growth, follicles were injected with PBS (control group) or FGF10 at a final intrafollicular concentration of 0.1 or 1µg/mL, and follicular growth was monitored daily. The intrafollicular injection of FGF10 interrupted dominant follicle growth compared to control group and a dose-response effect was observed (Figure 2). All follicles from control group continued to grow and spontaneously ovulated 96-120h after PBS intrafollicular injection, validating the follicular wave synchronization and intrafollicular injection protocols. When treated with 0.1µg/mL FGF10, two follicles regressed while the other two follicles ovulated 96-120h after FGF10 treatment. All follicles that received an intrafollicular injection of FGF10 in a final concentration of 1µg/mL regressed 24h after injection (decreased in comparison to diameter at treatment) and were significantly smaller than control follicles from 48 to 72h after intrafollicular injection and failed to ovulate.

Changes in follicular environment after in vivo FGF10 treatment

To gain insight into the mechanisms through which FGF10 prevents follicular growth, we assessed the changes in mRNA expression of key genes known to be involved in steroidogenesis and follicular cells differentiation. Growth of follicles treated with FGF10 (in a final intrafollicular concentration of 1µg/mL) was blocked at 24h post-injection (-0.2±0.1 *vs*. 1.1±0.4mm/24h in FGF10 and control groups, respectively; Figure 3). Follicular fluid estradiol concentrations were lower in FGF10-treated follicles (5.6±3.8 *vs* 264.9±115.5ng/mL estradiol in FGF10 and PBS groups, respectively; $P<0.01$; Figure 3). Treatment with FGF10 significantly decreased abundance of mRNA encoding *CYP19A1* and *cyclin D2* in granulosa cells ($P<0.05$; Table 1), while *FGFR2b* mRNA tended to be upregulated after FGF10 treatment. Expression of *STAR*, *HSD17B1*, *HSD3B1*, *FSHR*, *LHCGR*, *IGFBP-2* and *-5*, and *X-linked Inhibitor of Apoptosis Protein* (*XIAP*) was not affected by FGF10 ($P>0.05$; Table 1).

In theca cells, there was no difference between control and FGF10 treated follicles in mRNA encoding steroidogenic enzymes (*STAR*, *CYP11A1*, *CYP17A1*, and *HSD3B1*), *LHCGR, cyclin D2* and *IGFBP-2* and *-3* (P>0.05; Table 2). However, theca cell *FGF10* mRNA expression was upregulated after FGF10 treatment (P<0.05).

3.4. Discussion

The involvement of theca cell-derived factors in the regulation of follicle growth and steroidogenesis is still poorly understood. Our significant findings are: (1) both *FGF10* and *FGFR2b* are upregulated in the subordinate follicle; (2) a single intrafollicular injection of FGF10 interrupts dominant follicle growth in a dose-dependent manner and (3) FGF10 negatively regulates granulosa *CYP19A1* and *cyclin D2* mRNA expression and decreases estradiol concentration in follicular fluid.

In bovine granulosa cells obtained from abattoir ovaries, Berisha *et al*. (2004) observed that *FGFR2b* mRNA expression was positively associated to follicular fluid estradiol level. However, in our *in vivo* model the abundance of *FGFR2b* mRNA was consistently higher in the smaller follicles in all the pairs of samples. These discrepant results may be related to follicular health and differentiation status, since Berisha *et al*. (2004) did not include atretic follicles (as assessed by progesterone follicular fluid levels) in their study. In our follicular deviation model, we accessed the difference between dominant and non-dominant follicles (healthy *vs*. atretic follicles) near follicular deviation. Additionally, it was previously demonstrated that IGF1 negatively regulates *FGFR2b* mRNA expression in granulosa cells (Buratini *et al.* 2007). It is well established that an important difference between dominant and subordinate follicles is the intrafollicular level of free IGF1 (Ginther *et al.* 2004, Sudo *et al.* 2007). Thus, the increased free IGF1 levels may be involved in the downregulation of *FGFR2b* mRNA in dominant follicles granulosa cells.

Fibroblast growth factor 7 (also known as KGF) is structurally similar to FGF10 and also binds to FGFR2b. When added to bovine granulosa cells culture, FGF7 and FGF10 have negative effects on CYP19A1 activity (Parrott & Skinner 1998) and estradiol synthesis (Buratini *et al.* 2007), respectively. *FGF10* mRNA is not readily detectable (after 30 PCR cycles) in granulosa cells (Buratini *et al.* 2007), indicating that FGF10 synthesized in theca cells and/or oocyte is regulating granulosa cells functions during follicle deviation.

Recently, it has been shown that intrafollicular injection of cocaine- and amphetamine-regulated transcript (CART), a granulosa cell-derived factor, significantly reduced estradiol synthesis in bovine pre-ovulatory follicles (Lv *et al.* 2009). In the present study we addressed the function of a theca cell and oocyte-derived factor during antral follicle growth. All the follicles that received an intrafollicular injection of FGF10 at a final intrafollicular concentration of 1µg/mL ceased growing 24h after injection and 'lost' the dominant status. This is unlikely to be a cytotoxic effect, as abundance of mRNA encoding a number of genes was not affected in either granulosa or theca cells by this treatment. Similar concentrations of FGF10 have been previously used *in vitro* without affecting cell viability (Steinberg *et al.* 2005, Benjamin *et al.* 2007, Buratini *et al.* 2007). The *in vivo* model was suitable to study the role of FGF10 in follicular growth as follicles injected with PBS maintained their growth, reached ovulatory size and spontaneously ovulated.

To investigate the action of FGF10 on follicular cell mRNA expression, we chose the dose of 1µg/mL as it was effective in suppressing follicular growth. The ovariectomy was performed 24h after intrafollicular injection to avoid the collection of follicles in advanced atresia. In granulosa cells *CYP19A1* mRNA expression was downregulated by FGF10 treatment which was accompanied by a decrease in estradiol production. These results are in agreement with previous reports of decreased estradiol production in cultured granulosa cells after FGF10 treatment (Buratini *et al.* 2007, Portela *et al.* 2008). The intracellular pathway

195 responsible for the negative effect of FGF10 on *CYP19A1* expression and estradiol synthesis
196 remains unknown.

197 The lack of significant effect on the expression of gonadotropin receptors indicate that
198 FGF10 function is downstream of FSH and LH signaling and is not related to granulosa cell
199 differentiation. Markers of follicular atresia such as *IGFBP-2* and *-5* (Stewart *et al.* 1996) and
200 the suppressor of apoptosis *XIAP*, an important survival factor in the control of follicular
201 atresia (Wang *et al.* 2003), were not affected by FGF10. This is interesting and implies a
202 specific role for FGF10 in inhibiting estradiol secretion. Recently, Portela *et al.* (2010)
203 demonstrated that FGF18 from theca cells is involved in follicle atresia. Nevertheless, FGF18
204 seems to have a broad range of anti-steroidogenic effects on granulosa cells, as it
205 downregulates *CYP19A1*, *HSD3B1*, *FSHR*, *STAR* and *HSD17B1 in vitro* (Portela *et al.* 2010).
206 In the present study, progesterone concentration in follicular fluid was not assessed but we did
207 not identify significant effects of FGF10 on *HSD3B1* or *CYP11A1* mRNA in either granulosa
208 or theca cells. The different actions of FGF10 and FGF18 are likely related to the activation of
209 different receptors; whilst FGF10 preferably activates FGFR1b and FGFR2b, FGF18
210 activates FGFR3c and FGFR4 (Zhang *et al.* 2006).

211 The intrafollicular FGF10 treatment also decreased *cyclin D2* mRNA abundance in
212 granulosa cells. Cyclin D2 regulates granulosa cell proliferation (Robker & Richards 1998) by
213 controlling G1 to S transition and is regulated in part by estradiol (Quirk *et al.* 2006). Thus,
214 suppression of follicle growth by FGF10 may be a result of its effect on *CYP19A1* and
215 estradiol synthesis and cell proliferation. In the absence of estradiol stimulus to cell cycle
216 progression, granulosa cells become very susceptible to apoptosis (Quirk *et al.* 2006) and
217 follicles enter atresia. Nevertheless, FGF10 *in vitro* did not affect cell proliferation (Buratini
218 *et al.* 2007) in contrast to the downregulation of *cyclin D2* expression promoted by
219 intrafollicular injection of FGF10.

Theca cells are not the only source of FGF10 (Buratini *et al.* 2007). We cannot rule out that oocyte derived FGF10 is also involved in regulation of follicle growth. Also, cumulus cells and oocytes express *FGFR-1b* and *-2b* (Cho *et al.* 2008, Zhang *et al.* 2010). Thus, FGF10 intrafollicular injection may interfere with oocyte-granulosa cells interactions, culminating in follicle atresia. However, the pattern of *FGF10* and *FGFRs* mRNA expression in oocytes during follicle growth is unknown and it is very difficult to get enough samples to assess *in vivo* FGF10 effects in cumulus-oocyte complexes.

In theca cells, there was no difference between control and FGF10 treated follicles in mRNA encoding steroidogenic enzymes (*CYP17A1*, *CYP11A1*, *STAR*, *HSD3B1*), *cyclin D2*, *LHCGR* and *IGFBP-2* and *-3,* binding proteins known as negative modulators of IGF1 actions in theca cell proliferation and androstenedione and progesterone production (Spicer *et al.* 1997). These results suggest that theca cells from FGF10-treated follicles were still producing precursors for estradiol synthesis and further indicate that granulosa cell *CYP19A1* is the main target of FGF10. Other evidence that theca cell factors regulate follicle development is the fact that intrafollicular treatment with FGF10 significantly increased theca cell *FGF10* mRNA expression, suggesting an interesting autocrine positive feedback loop. The fact that granulosa cell *FGFR2b* mRNA expression tended to be upregulated after FGF10 treatment is consistent with this hypothesis. We also evaluated *FGFR2b* mRNA expression in theca cells in control and treated follicles. However, this receptor is weakly expressed in theca cell (data not shown).

In conclusion, results presented in this manuscript provide the first *in vivo* evidence that FGF10 acts as an important regulator of follicular growth, being differentially expressed in dominant and subordinate follicles from cows. Moreover, FGF10 treatment selectively downregulates *CYP19A1* and estradiol synthesis, indicating that FGF10 inhibits subordinate follicle development. Taken together, the present results suggest that reduced FGF10

signaling in dominant follicles accounts for continued follicle growth, whereas enhanced FGF10 signaling in subordinate follicle favors atresia through the inhibition of estradiol production and *cyclin D2* expression.

3.5. Material and methods

FGF10 and FGFR2b mRNA expression near follicle deviation

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee (ACUC nº 23081.009594/2007-41). Eighteen cyclic adult (5-9 years old) beef cows (Hereford and Aberdeen-Angus), with an average body condition score of 3 (1–5, emaciated to obese), were synchronized with two intramuscular injections of 125µg sodium cloprostenol (PGF2α analogue; Intervet Schering Plough, Brazil), 12h apart. Twelve cows were observed in estrus within 3–5 days after PGF2α and were used in the experiment. Ovaries were then examined once a day by transrectal ultrasonography, using an 8-MHz linear-array transducer (Pie Medical AquilaVet, Maastricht, The Netherlands), and all follicles larger than 5mm were drawn using 3 virtual slices of the ovary allowing a three-dimensional localization and monitoring of individual follicles during the first follicular wave after ovulation. The cows were ovariectomized at days 2, 3 or 4 of the follicular wave to recover the two largest follicles from each cow. Follicle deviation occurs when the largest follicle reaches 8.5mm (Ginther *et al*., 1996; Ginther *et al*., 2000; Ferreira *et al*., 2011a; Ferreira *et al*., 2011b), therefore follicle waves were classed as before or after deviation when the largest follicle was smaller or larger than 8.5mm, respectively.

The ovariectomy was performed via colpotomy in the standing position (Drost *et al.* 1992) under caudal epidural anesthesia using 80mg lidocaine chlorhydrate (4mL lidocaine 2%). Ovaries were washed with saline and follicular fluid samples were recovered for estradiol assay. Granulosa cells were harvested from follicles through repeated flushing with

PBS and theca cells were obtained through dissection of the follicle wall. Samples were frozen until RNA extraction for PCR analysis.

Intrafollicular injection

This experiment was performed to assess the effects of FGF10 on growth and ovulation of the follicular-phase follicle, using an intrafollicular injection method validate in our laboratory (Ferreira *et al.* 2007, Ferreira *et al.* 2011b). Follicle waves of *Bos taurus taurus* cyclic adult (5-9 years old) beef cows (Hereford and Aberdeen-Angus), with an average body condition score of 3, were synchronized by the placement of a progesterone releasing intravaginal device (1g progesterone, DIB – Intervet Schering Plough, Brazil), an intramuscular (IM) injection of 2mg estradiol benzoate (Genix, Anápolis, Brazil) to induce follicular regression and a new follicular wave. Two IM injections of 250µg sodium cloprostenol (12h apart; Intervet Schering Plough, Brazil) were also administered. Four days later, the progesterone devices were removed and ovaries were monitored daily for at least 3 days before treatment to ensure that new follicles were growing and that no aged follicles were present in the ovaries. Only cows without a corpus luteum in the ultrasound image were included in the study to avoid progesterone inhibitory effect during final follicular growth and ovulation. When the largest follicle of the growing cohort reaches a diameter between 7 to 8mm it is reliably identifiable as the future dominant follicle (Ferreira *et al*., 2011a; Ferreira *et al.* 2011b), and was injected with PBS (Control group; n=4) or human recombinant FGF10 (rhFGF10, Peprotech, USA) diluted in PBS. A total of 11 out of 18 cows responded to the synchronization protocol and were successfully injected. The injection volume was calculated based on the volume of follicular fluid estimated by the linear regression equation $V = -685.1 + 120.7D$, where V corresponds to the estimated follicular volume and D to the diameter of the follicle to be injected (Ferreira *et al.* 2007). The administered volume corresponded to approximately one tenth of total follicular fluid volume and the concentration

294 of FGF10 was 10 fold higher than the desired final intrafollicular concentration *i.e.* 0.1µg/mL
295 (n=4) or 1µg/mL (n=3). The average follicular diameter (± standard error) and diameter range
296 at treatment were 7.5±0.1, ranging from 7.1 to 8.1mm in PBS group, 7.2±0.1, ranging from
297 7.0 to 7.4mm in FGF10 0.1µg/mL group and 7.6±0.1 ranging from 7.1 to 7.9mm in FGF10
298 1µg/mL group. Two hours after the injections, follicles were evaluated to ensure that no
299 follicle damage occurred during procedure (a reduction in diameter larger than 1mm within 2h
300 of injection is evidence of follicle leakage). Animals were monitored daily by ultrasound to
301 evaluate effects on follicular dynamics and ovulation.

302 Based on follicular dynamics, the third experiment was performed with cows
303 synchronized as above. In this experiment, nine out of 14 cows responded to the protocol and
304 were intrafollicular injected and one cow was discarded due to follicular rupture after
305 injection. The average of follicular diameter (± standard error) and diameter range at the
306 moment of follicular injection were 7.8±0.2, ranging from 7.1 to 8.2mm in PBS group and
307 7.9±0.2 ranging from 7.8 to 8.2mm in FGF10 1µg/mL group. PBS (n=4) and FGF10
308 (1µg/mL; n=4)-treated follicles were obtained through ovariectomy via colpotomy 24h after
309 intrafollicular injection.

310 ***RNA extraction, reverse transcription and real-time PCR***

311 All materials were obtained from Invitrogen Life Technologies (São Paulo, Brazil)
312 except where otherwise stated. For theca cells, total RNA was extracted with Trizol® reagent
313 according to manufacturer's instructions. Total RNA was extracted from granulosa cells using
314 the RNeasy® Mini Kit (Qiagen Biotecnologia, São Paulo, Brazil). Quantitation and
315 estimation of RNA purity was performed using NanoDrop (Thermo Scientific - Waltham,
316 USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.8 were considered pure, and
317 samples below this threshold were discarded. To generate the cDNA, 1µg RNA was first
318 treated with 0.1U DNase and then incubated in a final volume of 20µl with dNTP (0.5mM

final each; Omniscript kit, Qiagen), 1µM oligo dT, RNAse out inhibitor (10U; Invitrogen), Omniscript reverse transcriptase (RT; 4U; Qiagen), and 1x buffer RT (2µl; Qiagen). The reaction was performed in four steps: step one: 37°C – 5min and step two: 65°C – 10min (DNA digestion); step three: 37°C – 60min and step four: 93°C – 3min (reverse transcription). The cDNA generated was stored at -20°C.

To test cross-contamination, PCR detection of the mRNAs that encode *CYP19A1* in theca and *CYP17A1* in granulosa cells was performed in each sample. All granulosa cells samples expressing *CYP17A1* mRNA and theca cells expressing *CYP19A1*after 30 PCR cycles were considered to be contaminated as previously described by Buratini *et al*. (2005). In the first experiment, when one of the granulosa or theca samples in a pair of follicles was contaminated, the pair was removed from the analysis; this approach of keeping the samples in pairs was adopted to allow including the "cow" effect in the statistical model.

Real-time polymerase chain reaction (PCR) was conducted in a Step One Plus instrument (Applied Biosystems, Foster City, CA) with Platinum SYBR Green qPCR SuperMix and bovine-specific primers (Table 3) taken from the literature or designed using Primer Express Software (Applied Biosystems). The thermal cycling parameters were: 3min at 95ºC, 40 cycles of 15s at 95ºC, 30s at 60ºC, and 30s at 72ºC. The product identity was verified through melting-curve analyses. Samples were run in duplicate and were expressed relative to *GAPDH* as housekeeping gene. The relative quantification of gene expression across treatments was evaluated using the ΔΔCT method (Livak & Schmittgen 2001). The fold change in relative mRNA concentrations was calculated using the formula 2–ΔΔCT.

Estradiol assay

Follicular fluid samples from follicles were collected and individually stored at -196°C. Estradiol concentration was determined using the multispecies Estradiol ELISA kit (Cayman Chemical, Ann Arbor, USA). The dilution of follicular fluid samples ranged

between 1:10 to 1:1000. The intra- and inter-assay coefficient of variation were 9.3% and 12.9%, respectively.

Statistical analysis

The regression analysis and correlation between follicular fluid estradiol levels and *FGF10* or *FGFR2b* mRNA expression were calculated using JMP software. The assessment of treatment effects on follicular dynamics was performed as repeated measures data and analyzed using the MIXED procedure with a repeated measure statement and using the compound symmetry as covariance structure. Main effects of treatment group, day and their interaction were determined. Differences between follicular sizes at a specific time point were compared between groups using estimates. All analyses from follicular dynamics were performed using SAS software package (SAS Institute Inc., Cary, NC). Continuous data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. The differences between the two largest follicles were accessed by paired Student's T test using cow as subject. Other continuous data were submitted to ANOVA using JMP software (SAS Institute Inc., Cary, NC). Results are presented as means±standard error of the mean (s.e.m). A $P<0.05$ was considered statistically significant.

3.6. Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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505 **Figure legends**
506

507 **Figure 1**: Regulation of *FGF10* and *FGFR2b* mRNA abundance in first follicular wave. After
508 estrus detection, follicular dynamics and ovariectomy, the two largest follicles from 12 cows
509 were collected before or after the expected time of follicular deviation. Panels A and B show
510 follicular diameter and estradiol levels, respectively, from 12 pairs of follicles collected
511 before (largest follicle (LF) and second largest follicle (SLF); n=6 pairs) or after deviation
512 (dominant follicle (DF) and subordinate follicle (SF); n=6 pairs). Cross-contamination of
513 theca and granulosa cells was assessed and when one of the granulosa or theca samples in a
514 pair of follicles was contaminated, the pair was removed from the analysis. In panel C, 4 pairs
515 of follicles collected before and 4 collected after deviation were used to assess *FGF10* mRNA
516 expression in theca cells. Panel D shows *FGFR2b* mRNA expression in granulosa cells from
517 3 pairs of follicles collected before and 5 collected after deviation. Correlations between
518 follicular fluid estradiol levels and theca cell *FGF10* mRNA (including the 16 follicles used
519 in Panel C) and granulosa cell *FGFR2b* mRNA (including the 16 follicles used in Panel D)
520 are shown in panels E and F, respectively. Asterisks indicate significant differences between
521 pairs of follicles accessed by paired Student's T test using cow as subject ($P<0.05$).
522

523 **Figure 2**: *In vivo* effect of FGF10 treatment on bovine follicular growth. A new follicular
524 wave was induced and when the largest follicle reached a diameter between 7 to 8mm, PBS
525 (control; n=4) or FGF10 in doses of 0.1 (n=4) or 1µg/mL (n=3) was intrafollicularly injected
526 in a single follicle per cow. Main effects of treatment group, day and their interaction were
527 determined using the MIXED procedure with a repeated measure statement. Differences
528 between follicular sizes at a specific time point were compared between groups using
529 estimates and different letters indicate statistical significance ($P<0.05$).
530

531 **Figure 3**: Follicular growth (mm/24h) and follicular fluid estradiol levels after FGF10
532 treatment. A new follicular wave was induced and when the largest follicle reached a diameter
533 between 7 to 8mm, PBS (control; n=4) or FGF10 (1µg/mL; n=4) was intrafollicularly injected
534 in a single follicle per cow. Cows were ovariectomized 24h after intrafollicular injection.
535 Different letters indicate statistical significance.
536

Table 1 – Effect of FGF10 treatment on granulosa cell mRNA expression.

Gene	PBS	FGF10	Fold change in FGF10 group	"P" value
HSD17B1	0.42±0.07	0.34±0.33	0.8	0.81
STAR	0.04±0.01	0.21±0.13	4.86	0.46
HSD3B1	0.58±0.22	0.49±0.3	0.84	0.81
CYP19A1	79.08±50.08	1.18±0.71	0.01	0.02
FGFR2b	6.48±3.07	33.78±19.89	5.21	0.07
Cyclin D2	14.56±0.97	3.13±2.17	0.21	0.01
FSHR	0.42±0.11	0.45±0.28	1.08	0.91
LHCGR	5.18±4.8	0.3±0.06	0.06	0.41
IGFBP2	25.72±22.15	29.1±2.65	1.13	0.88
IGFBP5	1.18±0.65	2.87±1.78	2.43	0.36
XIAP	7.76±4.13	29.77±15.35	3.84	0.23

Table 1: A single 7 to 8mm follicle was injected with PBS (n=4) or FGF10 (1µg/mL; n=4) and the cows were ovariectomized 24h after intrafollicular injection. Data are presented as the average gene expression (arbitrary units) ± s.e.m. in PBS and FGF10 treated follicles. Fold change was calculated by dividing average relative gene expression of both groups by expression in PBS (control) group.

544 **Table 2** – Effect of FGF10 treatment on theca cells mRNA expression.

Gene	PBS	FGF10	Fold change in FGF10 group	"P" value
STAR	0.26±0.25	0.05±0.03	0.2	0.98
CYP11A1	1.96±1.75	0.94±0.52	0.48	0.82
CYP17A1	820.3±731.32	58.06±25.67	0.07	0.22
HSD3B1	1.94±0.98	2.73±1.86	1.41	0.89
IGFBP2	1.63±0.44	1.63±0.26	1.0	0.99
IGFBP3	0.5±0.13	0.46±0.11	0.91	0.8
FGF10	1.24±0.47	10.36±6.12	8.34	0.04
LHCGR	0.9±0.38	4.14±3.8	4.62	0.7
Cyclin D2	1.41±0.53	1.69±0.45	1.2	0.7

545 Table 2: A single 7 to 8mm follicle was injected with PBS (n=4) or FGF10 (1µg/mL; n=4)
546 and the cows were ovariectomized 24h after intrafollicular injection. Data are presented as the
547 average gene expression (arbitrary units) ± s.e.m. in PBS and FGF10 treated follicles. Fold
548 change was calculated by dividing average relative gene expression of both groups by
549 expression in PBS (control) group.

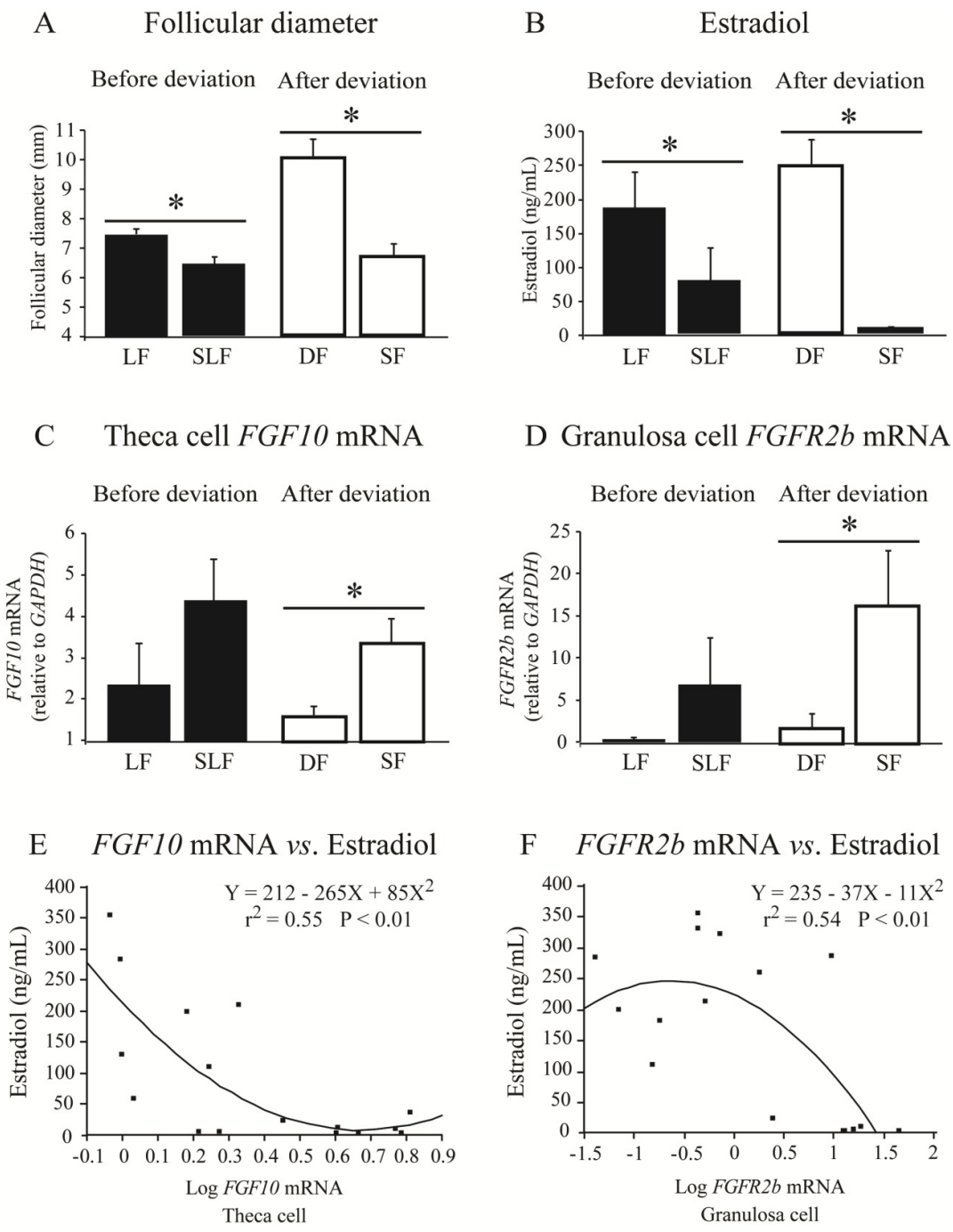
550

Table 3 - Primers used in the expression analysis of *Bos taurus taurus* genes.

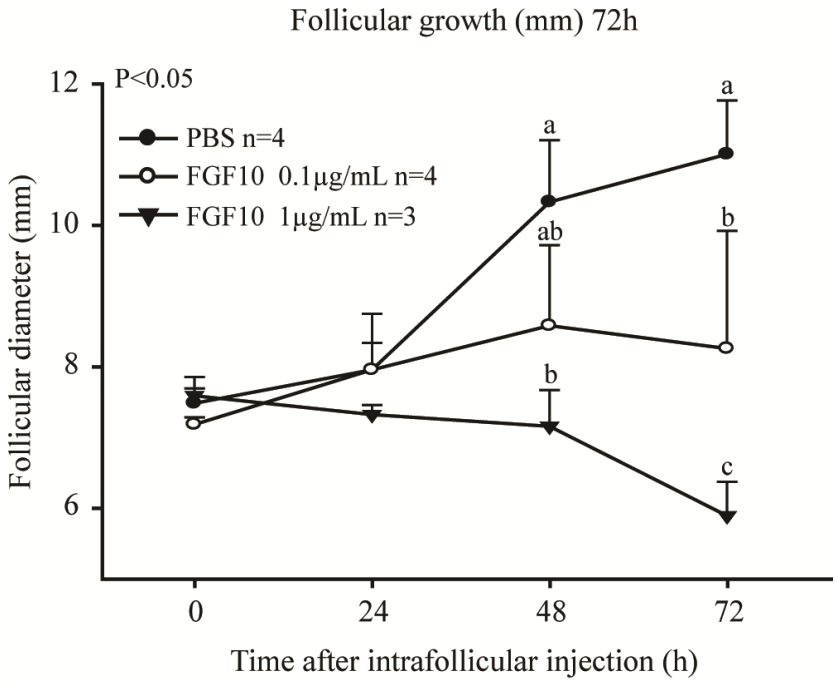
Gene		Sequence	Conc. (nM)	Reference/accession nº
GAPDH	F	GATTGTCAGCAATGCCTCCT	200	NM_001034034.1
	R	GGTCATAAGTCCCTCCACGA	200	
CYP19A1	F	GTGTCCGAAGTTGTGCCTATT	300	(Luo & Wiltbank 2006)
	R	GGAACCTGCAGTGGGAAATGA	300	
CYP17A1	F	CCATCAGAGAAGTGCTCCGAAT	200	(Lagaly *et al.* 2008)
	R	GCCAATGCTGGAGTCAATGA	200	
LHCGR	F	GCACAGCAAGGAGACCAAATAA	200	NM_174381.1
	R	TTGGGTAAGCAGAAACCATAGTCA	200	
HSD17B1	F	TGTGGTACTCATTACCGGCTGTT	200	NM_001102365.1
	R	CAGCGTGGCATACACTTTGAA	200	
HSD3B1	F	GCCCAACTCCTACAGGGAGAT	200	(Orisaka *et al.* 2006)
	R	TTCAGAGCCCACCCATTAGCT	200	
CYP11A1	F	CTTGCACCTTTCTGGCTAGG	200	(Orisaka *et al.* 2006)
	R	AAGGGGAAGAGGTAGGGTGA	200	
STAR	F	CCCAGCAGAAGGGTGTCATC	200	(Buratini *et al.* 2005)
	R	TGCGAGAGGACCTGGTTGAT	200	
FSHR	F	AGCCCCTTGTCACAACTCTATGTC	200	(Luo & Wiltbank 2006)
	R	GTTCCTCACCGTGAGGTAGATGT	200	
XIAP	F	GAAGCACGGATCATTACATTTGG	200	(Boelhauve *et al.* 2005)
	R	CCTTCACCTAAAGCATAAAATCCAG	200	
Cyclin D2	F	TGCCCCAGTGCTCCTACTTC	200	(Mihm *et al.* 2008)
	R	CGGGTACATGGCAAACTTGA	200	
IGFBP2	F	GACGGGAACGTGAACTTGATG	200	(Voge *et al.* 2004)
	R	TCCTTCATGCCGGACTTGA	200	
IGFBP3	F	AAAGAGATGTTTGAAATGCCTAGTTTT	200	(Voge *et al.* 2004)
	R	TCAAACTCGGTTTCACTGACTACTG	200	
IGFBP5	F	GTTTGCTGAACGAAAAGAAGCTA	200	(Voge *et al.* 2004)
	R	CGAGTAGGTCTCCTCTGCCATCT	200	
FGF10	F	AAGGAGATGTCCGCTGGAGAAAGCTA	300	NM_001206326.1
	R	ACTGTACGGGCAGTTCTCCTTCTT	300	
FGFR2b	F	TGTGGTTGGAGGTGATGT	300	(Cho *et al.* 2008)
	R	CGAGTGCTTCAGAACCTTG	300	

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

Figure 1


A Follicular diameter
Before deviation
After deviation
Follicular diameter (mm)
LF
SLF
DF
SF
B Estradiol
Estradiol (ng/mL)
C Theca cell FGF10 mRNA
FGF10 mRNA (relative to GAPDH)
D Granulosa cell FGFR2b mRNA
FGFR2b mRNA (relative to GAPDH)
E FGF10 mRNA vs. Estradiol
Y = 212 - 265X + 85X2
r2 = 0.55 P < 0.01
Log FGF10 mRNA
Theca cell
F FGFR2b mRNA vs. Estradiol
Y = 235 - 37X - 11X2
r2 = 0.54 P < 0.01
Log FGFR2b mRNA
Granulosa cell

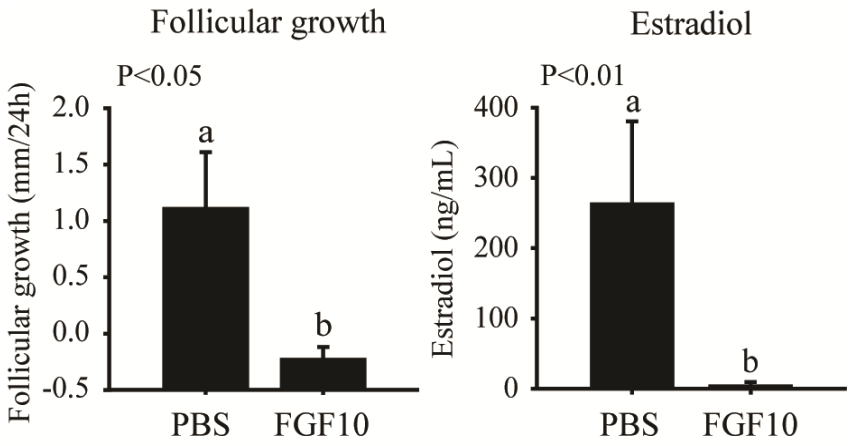
559 **Figure 2**



560

561

562 **Figure 3**



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564

4. ARTIGO 2

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

BONE MORPHOGENETIC PROTEIN RECEPTOR 1B IS UPREGULATED DURING BOVINE FOLLICLE ATRESIA.

Bernardo G. Gasperin, Rogério Ferreira, Monique T. Rovani, Vilceu Bordignon, Raj Duggavathi, José Buratini and Paulo Bayard D. Gonçalves.

JOURNAL OF ENDOCRINOLOGY, 2012.

Bone morphogenetic protein receptor 1B is upregulated during bovine follicle atresia.

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Short Title: BMPR1B during follicle atresia.

Footnotes

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4.1. Abstract

Bone morphogenetic proteins are known to be involved in determining ovulation quota in mammals. The mechanisms through which these proteins determine follicle fate are unknown. In the present study, we used a monovulatory model to evaluate the regulation of BMP15, GDF9 and their receptors (BMPRs and TGFBR1) during dominant follicle (DF) selection. Before follicular deviation (day 2 of follicular wave), *BMPR1A* mRNA was significantly more expressed in the second largest follicles. At the expected moment of follicular deviation (day 3), *BMPR2* and *BMPR1B* were significantly more expressed in subordinate follicles (SF). *BMPR1B* mRNA and protein were significantly more abundant in atretic (as assessed by cleaved caspase 3) SFs retrieved at day 4. The upregulation of *BMPR1B* was confirmed after estradiol receptor antagonist and FGF10-induced atresia whereas *BMPR2* tended to be more expressed after FGF10 treatment. BMP15 and GDF9 concentrations in follicular fluid did not differ between DFs and SFs at day 4. In conclusion, the abundance of BMP15 and GDF9 precursors in follicular fluid does not differ in fully differentiated and atretic follicles, however the expression pattern of BMPRs suggests an inhibitory effect on follicle differentiation and development. The results further suggest that BMPR1B has a negative effect on final follicular growth and differentiation

Keywords: follicle deviation, atresia, BMPR, BMP15, GDF9.

4.2. Introduction

Bone morphogenetic proteins (BMPs) family is composed by approximately 20 ligands and 7 serine-threonine receptors divided in type I and type II receptors. These proteins and the growth differentiation factors (GDFs) are members of transforming growth factors beta superfamily (TGF-β) (Knight & Glister 2006). Naturally occurring mutations in BMP15

(Galloway *et al.* 2000) and GDF9 (Hanrahan *et al.* 2004) are associated with infertility in homozygous and high ovulation rate in heterozygous ewes. It is postulated that when one of the alleles is inactive the reduced bioactivity of the growth factors leads to the development of follicles that differentiate earlier and have fewer granulosa cells then normal follicles (Juengel *et al.* 2004a, Juengel *et al.* 2009).

The effect of reduced bioactivity of BMP15 and GDF9 was also demonstrated using immunization against these proteins in ewes (Juengel *et al.* 2004b) and cows (Juengel *et al.* 2009). In both species, short term immunization induced superovulation, without a negative effect in oocyte fertilization, embryo development and gestation in ewes (Juengel *et al.* 2004b). It was also demonstrated that long-term immunization induced a block in follicle development in ewes and cows (McNatty *et al.* 2007, Juengel *et al.* 2009). Collectively, these data not only revealed that BMP15 and GDF9 are crucial for early follicle development but also suggest a potential role of these proteins in the regulation of dominant follicle selection and differentiation in monovular species. In this concept, understanding BMP system functions during follicle selection would have a huge impact in both contraception and fertility/superovulation in farm species and human assisted reproduction.

Functional BMP15 and GDF9 (mature forms) are produced after cleavage from precursors proteins (Juengel *et al.* 2004a). In mice, BMP15 mature form is produced after hCG stimulation, suggesting that BMP15 signaling is involved in ovulation-related events in this specie (Yoshino *et al.* 2006). Nevertheless, the pro-regions of GDF9 and BMP15 may have biological activities once some TGF-β member's pro-regions are involved in folding and dimerization of mature proteins (McIntosh *et al.* 2011). Previous studies evaluated BMP15 and GDF9 in follicular fluid from women (Wu *et al.* 2007, Gode *et al.* 2011) but the regulation of these factors during dominant follicle selection is still unknown.

The cooperative effect of BMP15 and GDF9 was shown to be mediated by the type II receptor BMPR2 (Edwards *et al.* 2008). After binding to the ligands, the type II receptor phosphorylates a type I receptor, being BMPR1B and TGFBR1 the main type I receptors for BMP15 and GDF9, respectively (Mazerbourg *et al.* 2004, Pulkki *et al.* 2012). A mutation in BMPR1B (known to mediate BMP-2, -4 and -15 signaling) is also associated with superovulation in ewes (Mulsant *et al.* 2001, Souza *et al.* 2001). The mutation in BMPR1B differs from the mutations in the ligands, once both heterozygous and homozygous animals have increased ovulation rates.

The regulation of BMPs and their receptors during antral follicle development was evaluated in rats (Erickson & Shimasaki 2003), pigs (Paradis *et al.* 2009) and cattle (Glister *et al.* 2010). Erickson et al. (2003) observed elevated mRNA expression of *BMPR1A*, *BMPR1B* and *BMPR2* in rat atretic follicles, suggesting a role for BMP system during these events. Regarding monovulatory specie, Glister et al. (2010) did not observe regulation of BMP receptors during antral follicle development in cattle. However, the authors evaluated mRNA expression in follicles obtained in abattoir classified according to follicle diameter. The regulation of BMP receptors during follicle development and atresia in monovulatory species is still unknown.

Several pieces of evidence suggest an important role for the BMP system in follicular differentiation and ovulation. For instance, deregulation of BMP15 signaling is associated to premature ovarian failure (Dixit *et al.* 2006). Also, aberrant expression of GDF9 was observed in women with polycystic ovarian syndrome (Teixeira Filho *et al.* 2002). Furthermore, gene knockout of BMPR1A and/or BMPR1B demonstrated that these receptors act redundantly to suppress ovarian tumors (Edson *et al.* 2010). Based on these findings, we hypothesized that the BMP system is a critical component for the regulation of follicle deviation in monovulatory species. Follicle deviation is characterized by the continued growth

of only one follicle (dominant), even during the nadir of FSH secretion, while all other follicles (subordinates) undergo atresia (Beg & Ginther 2006). The objective of the present study was to evaluate the regulation of BMP15, GDF9 and their receptors during follicle deviation in cattle.

4.3. Materials and Methods

Experiment 1: BMP receptors mRNA expression near deviation

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee. Adult cyclic *Bos taurus taurus* beef cows (Hereford and Red Angus; 400-500 kg body weight), with body condition score of 3 (1–5, emaciated to obese) were used in this study. Thirty-two cows were given two doses of a prostaglandin F2α (PGF2α) analogue (cloprostenol, 250 µg; Schering-Plough Animal Health, Brazil) intramuscularly (im), 11 days apart. Fifteen animals observed in estrus within 3–5 days after the second PGF2α administration were ovariectomized during the first follicular wave of the estrous cycle. The day of the follicular emergence (on average, Day 1 of the cycle) was designated as Day 0 of the wave and was retrospectively identified as the last day on which the dominant follicle was 4 to 5 mm in diameter (Evans & Fortune 1997). Ovaries were then examined once a day by transrectal ultrasonography, using an 8 MHz linear-array transducer (Aquila Vet scanner, Pie Medical, Netherlands) and all follicles larger than 5 mm were drawn using 3 to 5 virtual slices of the ovary allowing a three-dimensional localization of follicles and monitoring individual follicles during follicular wave (Jaiswal *et al.* 2004). Cows were randomly assigned to be ovariectomized by colpotomy on days 2 (n=4), 3 (n=4) or 4 (n=7) of the follicular wave to recover the largest and second largest follicle from each cow. This approach allowed to investigate BMPRs when the size of the largest and second largest follicle did not have a significant difference (day 2 of the follicular wave), had

slight difference (day 3) or marked difference (day 4), i.e. before, during and after dominant follicle selection, respectively.

Experiment 2: Effect of estradiol receptor blockade on BMPR expression

Based on results of the first experiment, we evaluated *BMPRs* mRNA expression after the blockade of estradiol receptors. Ten *Bos taurus taurus* adult cyclic cows were synchronized by the placement of a progesterone releasing intravaginal device (1 g progesterone, DIB – Intervet Schering Plough, Brazil), an intramuscular (IM) injection of 2 mg estradiol benzoate (Genix, Anápolis, Brazil) to induce follicular regression and a new follicular wave. Two IM injections of 250 µg sodium cloprostenol (12 h apart; Intervet Schering Plough, Brazil) were also administered. Four days later, the progesterone devices were removed and ovaries were monitored daily for at least 3 days before treatment to ensure that new follicles were growing and that no aged follicles were present in the ovaries. Only cows without a corpus luteum in the ultrasound image were included in the study to avoid progesterone inhibitory effect during final follicular growth and ovulation. When the largest follicle of the growing cohort reaches a diameter between 7 to 8 mm it is reliably identifiable as the future dominant follicle (Ferreira et al., 2011), and was injected with fulvestrant (selective estrogen receptor antagonist) in a final concentration of 100 µM or saline. Cows were ovariectomized (n=3/group) 12 h after intrafollicular injection. Intrafollicular injections were performed as previously described (Ferreira *et al.* 2007).

Experiment 3: Effect of FGF10 intrafollicular injection on BMPRs mRNA expression

We previously demonstrated that FGF10 inhibits dominant follicle growth decreasing *CYP19A1* and *cyclin D2* mRNA expression and estradiol secretion (Gasperin *et al.* 2012). To demonstrate a possible interaction between FGF10 and BMP system during follicle selection and to confirm BMPRs upregulation during follicle atresia, twelve *Bos taurus taurus* adult

cyclic cows were submitted to an hormonal protocol as described in experiment 2. When the largest follicle of the growing cohort reached the diameter of 7-8 mm, it was performed an intrafollicular injection of PBS (control) or FGF10 at a final intrafollicular concentration of 1 µg/mL. Cows were ovariectomized (n=4/group) via colpotomy 24 h after intrafollicular injection.

Experiment 4: BMPR1B, BMP15 and GDF9 protein abundance in dominant and subordinate follicles.

Granulosa cells recovered from dominant (n=5) and subordinate follicles (n=6) at day 4 of follicular wave were stored in RNAlater (Qiagen Inc., Mississauga, ON) and kept in NL2. Protein was extracted using Allprep DNA/RNA/Protein mini kit (Qiagen) and diluted in a laemmli-related buffer. Follicular fluid samples were collected to evaluate the concentration of BMP15 and GDF9 abundance in dominant and subordinate follicles at day 4 of follicular wave.

RNA extraction, reverse transcription and real-time PCR

Granulosa cells RNA and protein were extracted using AllPrep DNA/RNA/protein kit (Qiagen). Quantitation and estimation of RNA purity was performed using NanoDrop (Thermo Scientific - Waltham, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.8 were considered pure, and samples below this threshold were discarded. To generate the cDNA, 500 ng RNA was first treated with 0.1 U DNase (Invitrogen; 37°C – 5 min). After DNase inactivation at 65 °C for 10 min, samples were incubated in a final volume of 20 µl with iScript cDNA Synthesis Kit (BioRad). The cDNA synthesis was performed in three steps: 25 °C – 5 min, 42 °C – 30 min and 85 °C – 5 min.

To test cross-contamination with theca cells, PCR detection of the mRNAs that encode *CYP17A1* in granulosa cells was performed in each sample and all samples were free of

contamination after 30 PCR cycles. Real-time polymerase chain reactions (PCR) were conducted in a CFX384 real-time PCR detection system (BioRad, Hercules, CA) using FastStart Universal SYBR Green Master (with Rox; Roche Diagnostics, Canada) and bovine-specific primers (Table 1) taken from the literature or designed using Primer Express Software (Applied Biosystems). Standard two-step qPCR was performed with initial denaturation at 95 ºC for 5 min followed by 40 cycles of denaturation at 95 ºC for 15 sec and annealing/extension at 58 ºC for 30 sec. The product identity was verified through melting-curve analyses.

To optimize the quantitative PCR (qPCR) assay, serial dilutions of a cDNA template were used to generate a standard curve by plotting the log of the starting quantity of the dilution factor against the Ct value obtained during amplification of each dilution. Reactions with a coefficient of determination (R2) higher than 0.98 and efficiency between 95 to 105% were considered optimized. The relative standard curve method was used to assess the amount of a particular transcript in the samples as previously described (Cikos et al. 2007). Briefly, for each gene, standard cDNAs were amplified along with sample cDNAs in the same PCR run. The target mRNA quantity in each sample was determined from the relative standard curve (using sample Ct values) and expressed in arbitrary units corresponding to the dilution factors of the standard RNA preparation. Samples were diluted 1:20 and 2 µl of cDNA were used in each well. Samples were run in duplicate and results are expressed relative to *cyclophilin* or *GAPDH* as housekeeping genes. The selection of housekeeping genes was based on Ct variance (as reflected by the standard deviation) between groups in each experiment.

Western blot

After boiling the samples at 95 ºC for 5 min, granulosa cell protein samples were subjected to 10 % SDS gel and electrotransferred onto nitrocellulose membranes. After

194 blocking for 2 h with 5 % skimmed milk in PBS containing 0.1 % tween-20 (PBS-T), blots
195 were incubated overnight at 4 ºC with 1:1000 rabbit anti-human BMPR1B (GTX102453;
196 GeneTex., CA, USA), 1:1000 rabbit anti-human cleaved caspase-3 (Asp175; #9661, Cell
197 Signaling Technology, Danvers, MA) or 1:5000 rabbit anti-human beta actin (ab8227; Abcam
198 Inc., USA) with agitation, followed by three washes (10 min each) with PBS-T. The blots
199 were then incubated with 1:5000 goat anti-rabbit IgG-HRP (ab6721; Abcam Inc., USA) for 2
200 h with agitation, followed by three washes (10 min each) with PBS-T.

201 Total protein concentration in follicular fluid (FF) samples was evaluated through
202 Bradford Assay. Samples (70 µg of FF protein) were diluted in laemmli buffer, boiled at 95
203 ºC for 5 min, subjected to 12 % SDS gel and electrotransfered onto nitrocellulose membranes.
204 Western blot analysis were performed as above described using 1:500 rabbit anti-human
205 GDF9 (GTX108410; GeneTex., CA, USA) or 1:1000 rabbit anti-human BMP15
206 (GTX110245; GeneTex., CA, USA) and 1:5000 goat anti-rabbit IgG-HRP (ab6721; Abcam
207 Inc., USA) as secondary antibody.

208 In both experiments, immunoreactivity was detected with Immun-Star WesternC
209 Chemiluminescence Kit according to the manufacturer's instructions and visualized using
210 Chemidoc analyser (BioRad, CA, USA). Quantification of bands of the western blots was
211 performed using Image Lab software (Bio-Rad Laboratory).

212 ***Immunofluorescence assessment***

213 Ovaries from one cow on day 4 of follicular wave were collected by colpotomy and
214 the two largest follicles were isolated. The follicles were fixed in 4% paraformaldehyde for 6
215 h and paraffin embedded. Histological sections with 5 µm in thickness and slides preparations
216 were made to perform immunofluorescence analysis. Slides were deparaffinized using Xylene
217 for 15 min., rehydrated through a graded alcohol series (one times for 5 min. in each 100%,
218 90%, 80%, 70% and 50% dilution), and rinsed for 15 min. in ddH2O. Endogenous peroxidase

activity was then blocked for 20 min in 0.3% H2O2 and washed three times in PBS1X for 5 min. After washing, the slides were carefully blotted using a PAP pen (Vector Laboratory, Burlingame, CA) around the tissue. A blocking solution (PBS1X with 3% of Bovine Serum Albumin and 0.2% Twen-20) was used to block non-specific sites during 2 h at room temperature in a humidify chamber. After washed three times in PBS1X during 5 min., the same blocking solution was used to incubate the primary BMPR1B antibody (GTX102453; GeneTex., CA, USA) in a humidified chamber overnight at 5oC. After this incubation, samples were washed three times in a PBS1X containing 0.2% Tween-20 for 5 min. before being incubated for 1 h at room temperature to a goat anti-rabbit IgG antibody conjugated with AlexaFluor 488 (1:500; Invitrogen). Then, slides were washed in three times in a PBS1X containing 0.2% Tween-20 for 5 min. Finally, to enable nuclear staining visualization, samples were incubated with 300 nM of 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS1X for 5 min. at room temperature. Slides were mounted with a space between the coverslip, filled with 50 µl drop of Aqueous Mounting Medium (Fluoromount; Sigma) and sealed with nail polish.

Estradiol assay

Follicular fluid samples from follicles (from experiments 2 and 3) were collected and individually stored at -196 ºC. Estradiol concentration was determined using the multispecies Estradiol ELISA kit (Cayman Chemical, Ann Arbor, USA). Intra-assay coefficient of variation was 9.33.

Statistical analysis

All continuous data were tested for normal distribution using Shapiro-Wilk test, normalized when necessary and submitted to ANOVA using JMP software (SAS Institute Inc., Cary, NC). The differences between the two largest follicles in each day were accessed

243 by paired Student's T test using cow as subject. Results are presented as mean ± standard
244 error of the mean (SEM). A $P<0.05$ was considered statistically significant.

245 **4.4. Results**

246 Follicles obtained before deviation were classified as largest (F1) or second largest
247 follicle (F2) and were 7.1±0.3 and 6.3±0.2 mm diameter, respectively ($P>0.05$). Follicles
248 obtained at the expected moment or after deviation were classified as dominant (DF) or
249 subordinate follicle (SF). DF and SF diameters were significantly different ($P<0.05$) at day 3
250 (8.4±0.4 vs. 7±0.6) and day 4 (9.6±0.2 and 6.7±0.2).

251 Aiming to validate our in vivo model, we evaluated mRNA expression of genes
252 known to be involved in follicle development. At day 2, *CYP19A1* mRNA tended to be more
253 expressed in F1 and significant differences were observed between DF and SF at days 3 and 4
254 (Figure 1A). *LHCGR* mRNA expression tended to increase in DFs at the expected moment of
255 follicle deviation (day 3) and a dramatic increase was observed in day 4 DFs (Figure 1B;
256 $P<0.05$). Dominant follicle selection occurred in the absence of *FSHR* mRNA expression
257 regulation but at day 4 SFs expressed lower levels of *FSHR* compared to DFs (Figure 1C;
258 $P<0.05$).

259 Before deviation (day 2) *BMPR2* and *BMPR1B* tended to be more expressed, whereas
260 *BMPR1A* mRNA was significantly more expressed in the second largest follicles (Figure 2-A,
261 -C and -D). On day 3, when dominant and subordinate follicles were identified, *BMPR1B* and
262 *BMPR2* were significantly more expressed in subordinate follicles (Figure 2-A and D;
263 $P<0.05$). *BMPR1B* mRNA (Figure 2D) and protein (Figure 5A) were significantly more
264 abundant in the atretic (as assessed by cleaved caspase 3) subordinate follicles retrieved on
265 day 4 of follicular wave ($P<0.05$). BMPR1B was localized in both granulosa and theca cell
266 layers from dominant and subordinate follicles (Figure 5B). The upregulation of *BMPR1B*

was confirmed after estradiol receptor antagonist and FGF10-induced follicle atresia (Figures 3B and 4B, respectvely). The expression of *TGFBR1* mRNA did not differ between the two largest follicles throughout deviation (Figure 2B).

To measure the abundance of BMP15 and GDF9 proteins, follicular fluid samples were collected at day 4 of follicular wave. Both BMP15 and GDF9 were detected in bovine follicular fluid but protein abundance did not differ between dominant and subordinate follicles ($P>0.05$; Figure 6).

4.5. Discussion

There is evidence suggesting an important role for the BMP system during follicle development. These proteins and their receptors have been extensively studied but their functions and regulation in granulosa cells during follicle development are still poorly understood. In the present study we observed that BMPRs, mainly BMPR1B, are upregulated in subordinate follicles whereas concentrations of BMP15 and GDF9 in dominant and subordinate follicles are not significantly regulated at day 4.

The participation of BMPs signaling during bovine follicle selection was previously indicated by the effects of active immunization against BMP15 and GDF9 in cows, which induced superovulation in 60% of animals (Juengel *et al.* 2009). Based on results from in vivo experiments in bovine and ovine models, we formulated the hypothesis that BMP signaling is differentially regulated in dominant and subordinate follicles. On day 2 of follicular wave, when the two largest follicles present in the ovary are healthy, *BMPR2* and *BMPR1B* tended to be more expressed, whereas *BMPR1A* mRNA was significantly more expressed in the second largest follicles. When dominant and subordinate follicles were identified (day 3), *BMPR1B* and *BMPR2* mRNA expression were significantly higher in subordinate follicles. High expression of *BMPRs* were previously demonstrated in granulosa cells of rat atretic

follicles (Erickson & Shimasaki 2003). In the present study, opposite expression patterns of *BMPR1B* and *LHCGR* were observed. Recently, Crawford et al. (2011) demonstrated that oocytes from sheep homozygous for a mutation in BMPR1B (Booroola ewes) express lower levels of BMP15 and have granulosa cells that acquire LH responsiveness earlier than those from wild-type ewes. The basis for the increased LH responsiveness in ewes with the natural mutation seems to be suppressed BMPR1B signaling (Mulsant *et al.* 2001). So, it is reasonable to infer that BMPR1B upregulation in subordinate follicles is involved in the inhibition of LHCGR expression during follicle deviation. These observations are in agreement with the hypothesis that BMP15 signaling must decrease to allow follicular differentiation and ovulation. As reported in the ewe (Juengel *et al.* 2004a), it seems that BMPRs signaling has a negative effect on follicle development and/or differentiation of bovine follicles.

In BMP15 heterozygous mutant ewes, increased responsiveness to hCG (as assessed by cAMP production) but not FSH was observed in granulosa cells (McNatty *et al.* 2009). The fact that bovine dominant follicle selection occurs in the absence of *FSHR* mRNA regulation (Evans & Fortune 1997, Luo *et al.* 2011) further suggests that the superovulation observed after BMP15 and GDF9 immunization (Juengel *et al.* 2009) is more likely related to regulation in *LHCGR* than *FSHR* expression.

Edson *et al.* (2010) proposed that BMPR-1A and -1B act redundantly to prevent ovarian tumors. In the present study, we demonstrate that these receptors are upregulated in regressing follicles during follicle deviation, an event in which follicles that were not selected regress and disappear from the ovaries. In a rodent knockout model, the blockade in BMPR-1A and -1B signaling induced ovarian tumor in more than 80% of animals (Edson *et al.* 2010). The fact that double (SMADs 1 and 5) or triple (SMADs 1, 5 and 8) conditional knockouts induce infertility and metastatic granulosa cell tumors further suggests that BMP

316 signaling is involved in tumor suppression (Pangas *et al.* 2008). Based on these data, we can
317 speculate that BMPR-1A and -1B signaling pathways are involved in the dynamic changes
318 that happen during normal follicle development and regression.

319 It was previously demonstrated that GDF9 modulates gonadotropin actions in rat
320 granulosa cells, inhibiting FSH-induced estradiol secretion and LHCGR expression (Vitt *et al.*
321 2000). A link between estradiol and BMP system has also been suggested in human breast
322 cancer cell line (Takahashi *et al.* 2008). However, the regulation of BMP system by estradiol
323 in vivo has never been described in granulosa cells. To test the hypothesis that BMPRs are
324 regulated by estradiol, we intrafollicularly injected fulvestrant, an inhibitor of ER-mediated
325 transcriptional activity through disruption of ER dimerization and nuclear localization
326 (Osborne *et al.* 2004), and collected ovaries 12 h after treatment. The fact that *BMPR1B* but
327 not *BMPR1A* and *BMPR2* mRNA expression was significantly higher in granulosa cell from
328 fulvestrant-treated follicles, is in accordance with the pattern of BMPRs expression observed
329 during deviation, in which *BMPR1B* expression is significantly upregulated in subordinate
330 follicles compared to healthy growing follicles. Furthermore, BMPs suppressed estradiol-
331 induced mitosis whereas estradiol downregulated *BMPR-1B* and *-1A* mRNA expression in
332 breast cancer cell line (Takahashi *et al.* 2008). Therefore, we propose a model in which
333 BMPR1B expression must be downregulated to allow follicle development and, in the
334 absence of estradiol signaling, BMPR1B upregulation leads to follicle atresia.

335 Recently, we identified FGF10 as an important regulator of follicle development,
336 being more expressed in subordinate follicles and inducing atresia when intrafollicularly
337 injected (Gasperin *et al.* 2012). FGF10 treatment induced a significant increase in *BMPR1B*
338 mRNA while *BMPR2* tended to be more expressed after treatment. It is not possible to
339 conclude if the increase in BMPRs mRNA is a cause or a consequence of FGF10 actions on
340 granulosa cells. However, we evaluated the effect of FGF10 on several genes related to

steroidogenesis and granulosa differentiation and only observed significant differences in granulosa cells *CYP19A1* and *cyclin D2* mRNA expression, suggesting that BMPR1B is acutely regulated and a potential marker during atresia.

The fact that *BMPR1B* mRNA upregulation precedes follicle atresia suggests that the decrease in estradiol synthesis observed during follicle regression may be mediated by BMPR1B signaling. Nevertheless, we cannot rule out a participation of BMPR1B in granulosa cell apoptosis. In chondrogenic cells, the inhibition of BMPR1B signaling (through transfection with a dominant negative form of BMPR1B) induced an increase in the anti-apoptotic protein Bcl-2 and a decrease in the apoptosis effector cleaved caspase 3 (Itoh *et al.* 2008). Furthermore, in human osteosarcoma cell line, BMP2 induces apoptosis through BMPR1B, increasing activity of effector caspases 3, 6 and 7 (Haÿ *et al.* 2004). Using constitutively active forms of BMPRs, which have increased kinase activity and signal in the absence of ligand or type II receptor, it was demonstrated that BMPR-1B but not -1A is involved in cell death during embryogenesis (Zou *et al.* 1997). These data are in agreement with the pattern of BMPRs expression around deviation, which suggests the participation of BMPR1B during atresia whereas *BMPR1A* is not differentially expressed in healthy *vs.* atretic follicles. The fact that *BMPR1A* was upregulated at day 2 (but not at days 3 and 4) suggests an involvement in inhibition of follicle differentiation but a minor function during follicular atresia.

Based on the phenotype of ewes heterozygous for BMP15 or GDF9 inactivating mutations, which have multiple ovulations, we formulated the hypothesis that levels of these proteins are reduced in dominant follicles. However, we found similar levels of both proteins in dominant and subordinate follicles after deviation. Follicular fluid levels of BMP15 and GDF9 were previously evaluated in the women, being mature GDF9 levels positively correlated with oocyte nuclear maturation and embryo quality (Gode *et al.* 2011). Wu *et al.*

(2007) demonstrated that high levels of BMP15 precursor in women follicular fluid is associated to high quality oocytes and embryonic development. Furthermore, the same authors demonstrated a positive correlation between BMP15 and follicular fluid estradiol levels. We did not measure estradiol in follicular fluid but based on *CYP19A1* mRNA expression and on the dramatic difference in estradiol between bovine dominant and subordinate follicles at day 4 of follicular wave (Ferreira *et al.* 2011), the present results does not support a positive association between BMP15 and GDF9 proteins and estradiol.

In conclusion, mRNA encoding BMP receptors are upregulated in subordinate follicles and after FGF10 and fulvestrant-induced atresia. The abundance of BMP15 and GDF9 precursors in follicular fluid does not differ in fully differentiated and atretic follicles. These results further suggest that BMPRs signaling, especially BMPR1B, has a negative effect on final follicular growth and differentiation.

4.6. Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

4.7. Funding

This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). CNPq supported B G Gasperin with a scholarship.

4.8. Acknowledgements

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Figure legends

Figure 1. Expression of follicle development markers in granulosa cells. Samples were recovered from the two largest follicles from each cow collected at days 2 (n=4 pairs), 3 (n=4 pairs) or 4 (n=7 pairs) of the first follicular wave of a cycle. Asterisk (*) indicates statistical difference between largest and second largest follicle accessed by paired Student's T test using cow as subject. * $p<0.05$; # $p<0.1$.

Figure 2. Expression of BMPR2 (A), TGFBR1 (B), BMPR1A (C) and BMPR1B (D) mRNA in granulosa cell during follicular development. Granulosa cells samples were recovered from the two largest follicles from each cow collected at days 2 (n=4 pairs), 3 (n=4 pairs) or 4 (n=7 pairs) of the first follicular wave of a cycle. Asterisk (*) indicates statistical difference between largest and second largest follicle accessed by paired Student's T test using cow as subject. * $p<0.05$; # $p<0.1$.

Figure 3. Expression of bone morphogenetic proteins receptors mRNA in granulosa cells 12 h after intrafollicular selective estrogen receptor antagonist treatment. Granulosa cells were recovered from saline and fulvestrant treated follicles 12 h (n=3/group) after intrafollicular injection (mean±s.e.m.). Figure 3D shows estradiol levels in follicular fluid from injected follicles. Bars with no common letter are different (a±b; $P<0.05$).

Figure 4. Expression of bone morphogenetic proteins receptors mRNA in granulosa cells 24 h after intrafollicular FGF10 treatment. Granulosa cells were recovered from PBS and FGF10 (1 µg/mL) treated follicles 24 h (n=4/group) after intrafollicular injection (mean±s.e.m.).

555 Figure 4D shows estradiol levels in follicular fluid from injected follicles. Bars with no
556 common letter are different ($a \neq b$, $P<0.05$).
557
558 **Figure 5.** (A) Granulosa cells BMPR1B protein abundance in dominant (F1; n=5) and
559 subordinate follicles (F2 and F3; n=6) recovered at day 4 of follicular wave. BMPR1B protein
560 abundance is represented based on beta actin. The atretic status of subordinate follicles was
561 confirmed by the presence of cleaved caspase 3. (B) BMPR1B localization in the granulosa
562 and theca cells from the two largest follicles collected on day 4 of follicular wave as detected
563 by immunofluorescence.
564
565 **Figure 6**. Follicular fluid BMP15 and GDF9 abundance in dominant (DF; n=4) and
566 subordinate follicles (SF; n=4) recovered at day 4 of follicular wave.
567

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

Gene		Sequence	Conc. (nM)	Reference/accession nº
GAPDH	F	GATTGTCAGCAATGCCTCCT	200	NM_001034034.1
	R	GGTCATAAGTCCCTCCACGA	200	
CYCLOPHILIN	F	GGTCATCGGTCTCTTTGGAA	200	(Santos *et al.* 2011)
	R	TCCTTGATCACACGATGGAA	200	
CYP19A1	F	GTGTCCGAAGTTGTGCCTATT	300	(Luo & Wiltbank 2006)
	R	GGAACCTGCAGTGGGAAATGA	300	
CYP17A1	F	CCATCAGAGAAGTGCTCCGAAT	200	(Lagaly *et al.* 2008)
	R	GCCAATGCTGGAGTCAATGA	200	
LHCGR	F	GCACAGCAAGGAGACCAAATAA	200	NM_174381.1
	R	TTGGGTAAGCAGAAACCATAGTCA	200	
FSHR	F	AGCCCCTTGTCACAACTCTATGTC	200	(Luo & Wiltbank 2006)
	R	GTTCCTCACCGTGAGGTAGATGT	200	
BMPR2	F	CCACTGGCCTCACTCCAAGT	200	XM_002685492
	R	CCCGACTGGCTGTGAAACAT	200	
TGFBR1	F	ACCACTGCAATAAAATAGAACTTCCA	200	NM_174621.2
	R	TGACAGCTGCCAGTTCAACAG	200	
BMPR1A	F	TGGATTGCCCTTACTGGTTCAGCGA	200	NM_001076800.1
	R	CCACGCCATTTACCCATCCACA	200	
BMPR1B	F	AAAGTGGCGTGGCGAAAAGGTAGCT	200	NM_001105328.1
	R	CCCGTCCCTTTGATATCTGCAGCAA	200	

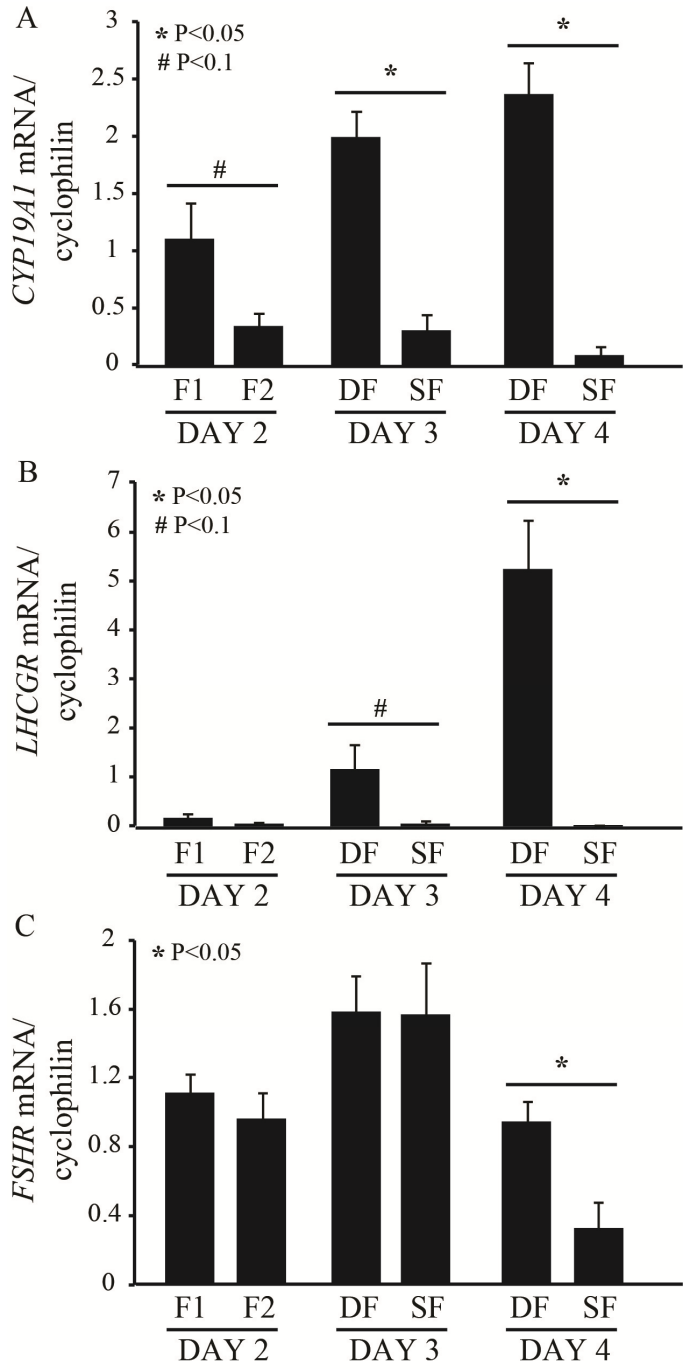
569 F, Forward primer; R, Reverse primer; Conc., primer concentration used for gene

570 amplification.

571

572

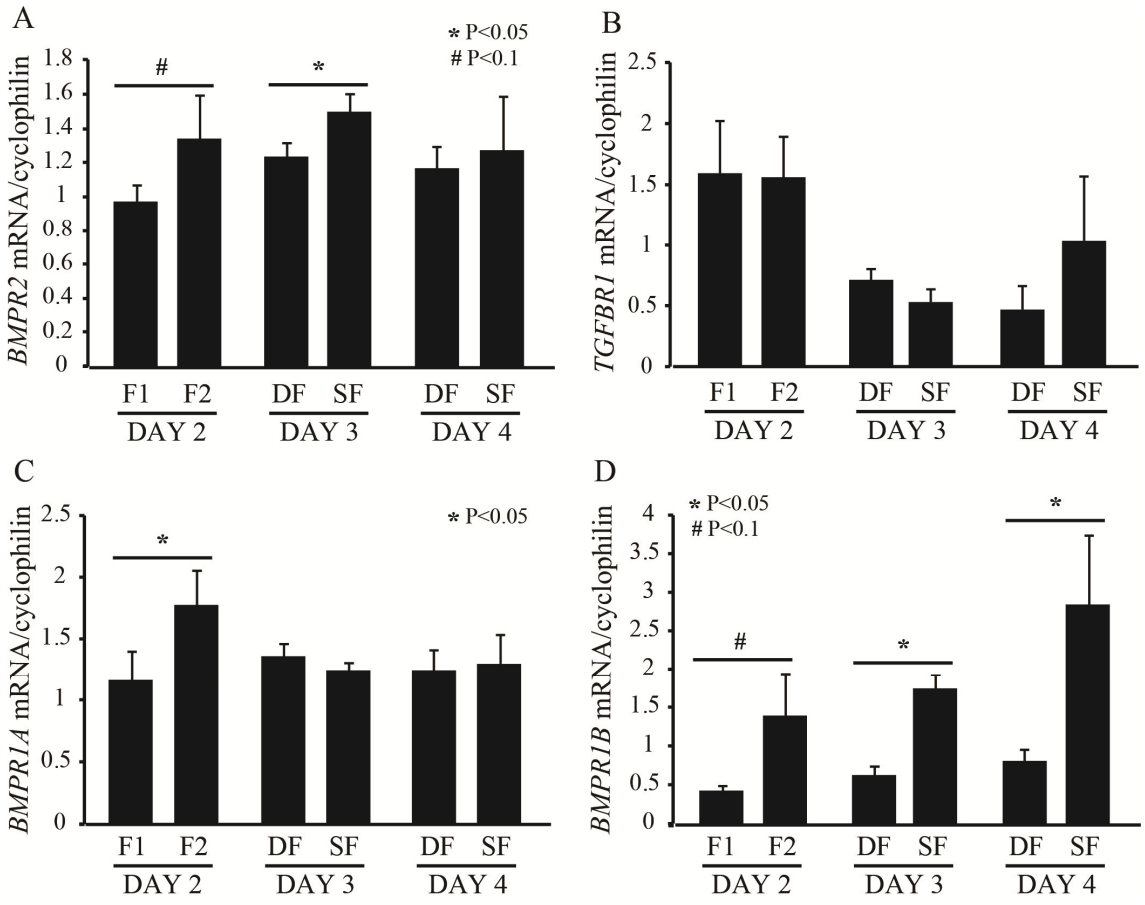
573 **Figure 1**



574

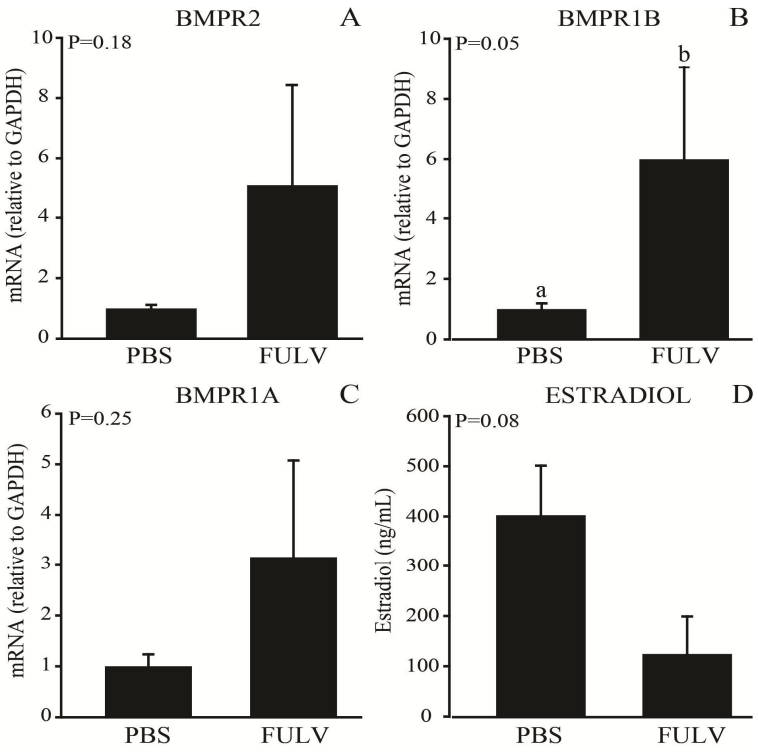
575

576 **Figure 2**



577

578 **Figure 3**



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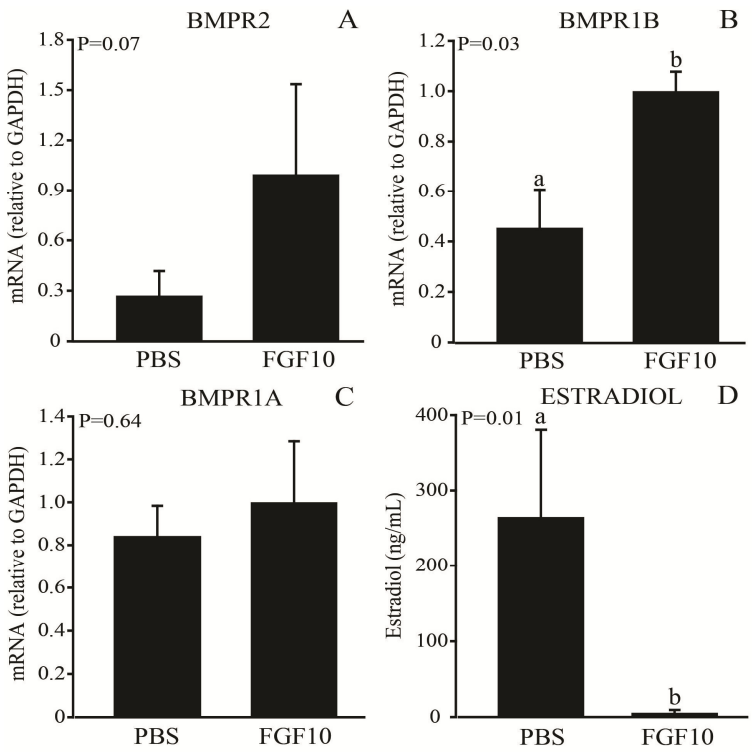
580

581 **Figure 4**



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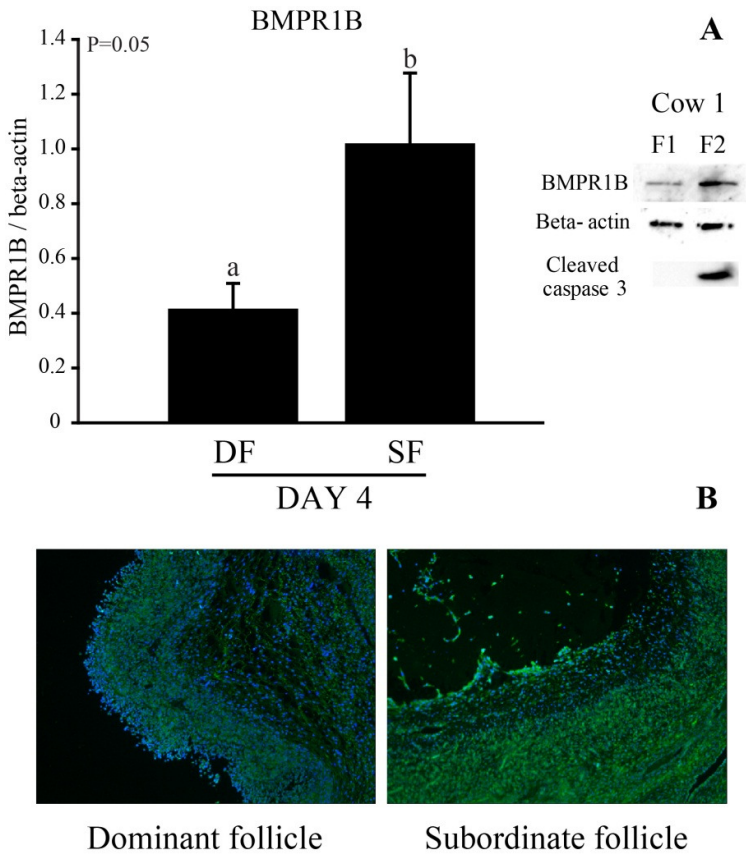
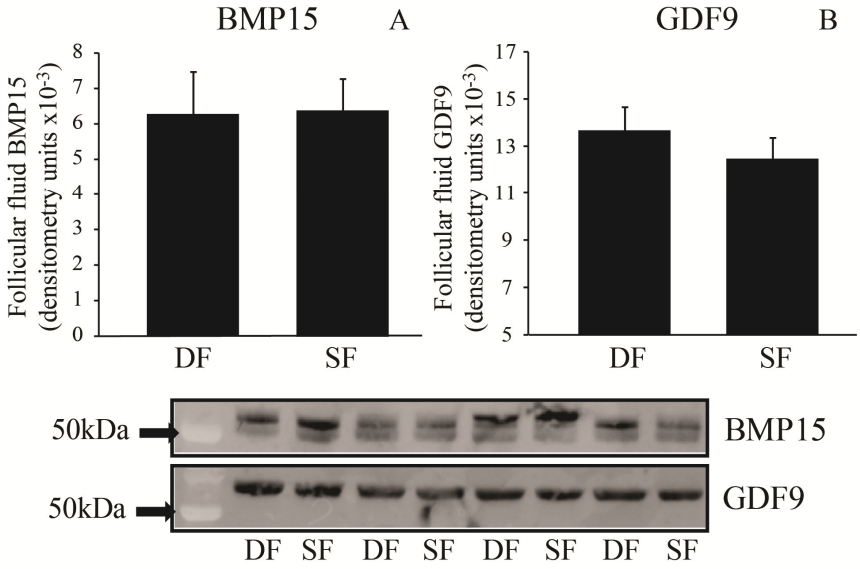
583

584 **Figure 5**



585

586 **Figure 6**



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5. ARTIGO 3

TRABALHO A SER ENVIADO PARA PUBLICAÇÃO:

FUNCTIONAL STATUS OF STAT3 AND MAPK INTRACELLULAR SIGNALING PATHWAYS DURING BOVINE FOLLICULAR DEVIATION

Bernardo G. Gasperin, Vilceu Bordignon, Raj Duggavathi, João F. Oliveira and Paulo Bayard D. Gonçalves.

MOLECULAR REPRODUCTION AND DEVELOPMENT, 2012.

Functional status of STAT3 and MAPK intracellular signaling pathways during bovine follicular deviation

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Short title: STAT3 and MAPK during bovine deviation.

Keywords: deviation, granulosa, STAT3, MAPK, apoptosis.

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Abbreviations: F1 and F2, largest and second largest follicle before deviation, respectively; DFs, dominant follicles; SFs, subordinate follicles. ANOVA, analysis of variance.

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5.1. Abstract

Follicle development is coordinated by gonadotropins, steroids and growth factors, which activate multiple signaling pathways such as mitogen activated protein kinase (MAPK). Phosphorylated MAPK (pMAPK) level was pointed as an early marker of follicle dominance whereas phosphorylated STAT3 (pSTAT3) is increased in granulosa cells from hypophysectomized rats. The aim of this study was to test the hypothesis that MAPK and STAT3 pathways are regulated in granulosa cells during follicle deviation. Cyclic beef cows were synchronized and ovariectomized at days 2, 3 or 4 of the follicular wave to recover the two largest follicles. Follicular diameter did not differ at day 2 but significantly increased in dominant follicles (DFs) at days 3 and 4 of follicular wave. *CYP19A1* mRNA expression was higher in the largest follicle from each pair regardless the day of follicular wave. Cleaved caspase 3 levels were significantly higher in day 4 subordinate follicles (SFs), further validating the model. Before deviation, pMAPK levels were significantly upregulated in the future DF, but did not differ between follicles at the expected moment and after deviation. *STAT3* mRNA and total protein (tSTAT3) increased in SFs collected at day 4. Levels of pSTAT3 tended to increase in SFs collected at day 3 and a dramatic increase in pSTAT3 was observed in day 4 SFs. In conclusion, pMAPK is increased in the future DF but does not seem to be a good marker of follicle dominance. The abundance of pSTAT3 in granulosa cells suggests that this pathway is involved in granulosa cell death.

5.2. Introduction

The emergence of ovarian follicular waves is preceded by a transient peak in circulating concentration follicle stimulating hormone (FSH). FSH signals through its membrane receptors in granulosa cells, by activating primarily protein kinase A (PKA)

pathway (Gonzalez-Robayna et al. 2000), stimulating the growth of several follicles. Growing follicles secrete estradiol and inhibin, which lead to decreasing FSH levels and resulting in the selection of the dominant follicle (DF) that continues to grow despite decreasing FSH levels. It is hypothesized that the DF thrives in this milieu because of paracrine factors, which increase FSH-responsiveness of the DF until it becomes luteinizing hormone (LH) dependent (Beg and Ginther 2006). Remaining subordinate follicles (SFs) of the wave regress and enter atresia through apoptosis (Evans et al. 2004). This process, wherein one follicle continues to grow and other follicles begin to regress, is denominated by follicle deviation and is expected to occur at day 3 of follicular wave (day 0 = day of wave emergence) (Beg and Ginther 2006; Evans and Fortune 1997).

Growth factors such as insulin, leptin, insulin-like growth factor 1 (IGF1) and epidermal growth factor-like factors modulate gonadotropin signaling through various intracellular signaling pathways. As demonstrated in conditional knockout mice, mitogen-activated protein kinases (MAPKs), that mediate signal transduction by extracellular stimuli, are essential for follicular granulosa cell function and fertility (Fan et al. 2009). The role of MAPK signaling pathway during follicle development is not fully understood. In cultured bovine granulosa cells, MAPK inhibitor significantly upregulated FSH-stimulated *CYP19A1* mRNA expression (Silva et al. 2006). Contrastingly, another study (Ryan et al. 2008) showed that FSH and IGF1, the known activators of granulosa cell function, activated MAPK pathway. In the same study, inhibition of MAPK in vivo blocked follicle development and estradiol synthesis in ewes (Ryan et al. 2008). Furthermore, the level of pMAPK has been proposed as an early marker of the follicular dominance in cattle (Ryan et al. 2007). Despite its possible involvement in both dominant follicle selection and follicle atresia, the analysis of the functional status of MAPK during bovine deviation has not been assessed.

Signal transducer and activator of transcription 3 (STAT3) is involved in cell proliferation, differentiation and apoptosis (Richard and Stephens 2011). On one hand, STAT3-pathway is activated by several ligands such as interleukins and leptin and seems to be involved in apoptosis prevention (Boelhauve et al. 2005; Liu et al. 2010). On the other hand, activated STAT3 (phosphorylated at Tyr705) was shown to induce apoptosis in lung carcinoma cells through downregulation of Bcl-2 (Ozenne et al. 2012). Furthermore, STAT3 phosphorylation is involved in triggering the apoptosis during a physiological mechanism of mammary gland involution in mice (Chapman et al. 1999), reducing the phosphoinositide-3-OH kinase (PI(3)K) pathway (Abell et al. 2005). Recently, it has been shown that STAT3 regulates lysosomal-mediated cell death, which is involved in mammary gland involution (Kreuzaler et al. 2011).

It was previously demonstrated that pSTAT3 is increased in granulosa cells from hypophysectomized rats (Russell and Richards 1999), but not in granulosa cells collected from preovulatory follicles. Besides the involvement of STAT3 pathway in proliferative, differentiation and apoptotic events, the analysis of functional status of this intracellular signaling pathway during follicle deviation has never been assessed. The aim of this study was to test the hypothesis that MAPK and STAT3 pathways are differentially activated in granulosa cells during bovine follicle deviation.

5.3. Results

Follicular dynamics was monitored daily from the day of estrus. Follicles obtained before deviation (day 2) were classified as largest (F1) and second largest (F2) follicles. The diameters of F1 and F2 on day 2 did not differ significantly (7.1±0.3 and 6.3±0.2 mm diameter, respectively; $P>0.05$; Figure 1A). Follicles obtained on the expected day of deviation or later were classified as dominant (DF) or subordinate (SF) follicles. The

diameters of DF and SF were significantly different ($P<0.05$) at day 3 (8.4±0.4 vs. 7±0.6) and day 4 (9.6±0.2 and 6.7±0.2; Figure 1A).

To confirm that F1 and DF were physiologically more advanced than F2 and SF, we analyzed *CYP19A1* mRNA abundance in granulosa cells purified from individual follicles. After ovariectomies, we performed simultaneous extraction of RNA and protein from each sample. *CYP19A1* mRNA expression was higher in the largest follicle from each pair regardless the day of follicular wave, validating the follicular dynamics (Figure 1B). Histology assessment demonstrated that day 4 DFs had several granulosa cell layers whereas subordinate follicles had reduced numbers of granulosa cell (Figure 1C).

Caspase-3 activation (resulting from its cleavage) is required in the normal execution of granulosa cell death, being a conserved feature of granulosa cell demise (Matikainen et al. 2001). Cleaved caspase 3 protein levels were significantly higher (8 fold) in granulosa cells of day 4 SF (Figure 2), demonstrating that SF by day 4 had entered atresia.

Phosphorylated and total MAPK proteins (Erk1 and Erk2) were found in granulosa cell from both dominant and subordinate follicles (Figure 3). On day 2 of follicular wave pMAPK was more abundant in the larger follicles ($P<0.05$), but no significant differences were observed at the expected moment (day 3) or after deviation (day 4).

STAT3 mRNA (Figure 4A) and total (tSTAT3; Figure 4B) protein increased in granulosa cell from subordinate follicles collected at day 4. pSTAT3 levels tended to increase in subordinate follicles collected at day 3 and a dramatic increase in STAT3 phosphorylation was observed in day 4 subordinate follicles (Figure 4C).

5.4. Discussion

In the present study we used an in vivo model which allows the study of events occurring before, at the expected time and after follicular deviation at transcriptional,

translational and post-translational levels. Most importantly, molecular phenotype at all three levels was evaluated within each granulosa cell sample using cutting edge technique that allowed for extraction of both RNA and protein from a single sample. Our findings were: (1) pMAPK was more abundant in the future dominant follicle before deviation and no significant differences are observed at the expected time and after deviation; (2) pSTAT3 levels tends to increase in subordinate follicles collected at day 3 and significantly increases in day 4 atretic subordinate follicles.

Several studies have demonstrated distinct functions for MAPK signaling in granulosa cells. Based on *CYP19A1* mRNA expression in dominant follicles and the presence of cleaved caspase 3 in day 4 subordinate follicles, we observed that MAPK pathway is activated during follicle development and atresia, in contrast to a previous study in ewes in which pMAPK was detected in follicular wall (theca and granulosa cells) from dominant but not subordinate follicles (Evans and Martin 2000). In bovine granulosa cells in vitro, both MAPK and Akt (protein kinase B) signaling pathways were upregulated by IGF and FSH (Ryan et al. 2008), factors known to stimulate follicle development, and pMAPK has been pointed as a marker of follicle dominance (Ryan et al. 2007). In fact, in our in vivo model, pMAPK was increased in the future dominant follicle before deviation, but was not consistently associated with follicle dominance at the expected moment and after follicular deviation. It was previously demonstrated that eCG withdrawal induces a decrease in MAPK phosphorylation, preceding the apoptotic process in rat granulosa cells (Gebauer et al. 1999; Peter and Dhanasekaran 2003). It is well established that the future dominant follicle is more sensitive to FSH due to locally produced factors, especially greater levels of free IGF1 (Mani et al. 2010). Thus, the decreased sensitivity to gonadotropin in the subordinate follicle may account for the lower MAPK activity, inducing the apoptotic process. The absence of significant differences at day 3 and 4 of follicular wave suggests that throughout follicle deviation MAPK is active in

proliferating granulosa cells but is also activated during granulosa cell death, as previously demonstrated (Gebauer et al. 1999).

Treatment with MAPK inhibitor (PD98059) decreased FSH-induced estradiol secretion in vivo and in vitro (Ryan et al. 2008). On the contrary, disruption of Erk1/2 in mouse granulosa cells induced an abnormal increase in estradiol secretion (Fan et al. 2009). In the present study, MAPK was not consistently associated with follicular health as previously suggested. In addition, the abundance of pMAPK protein and *CYP19A1* mRNA during deviation does not support the concept of a tonic inhibition of *CYP19A1* expression (Silva et al. 2006). Collectively, results from present and previous studies suggest that MAPK pathway is activated by factors involved in both follicular development/differentiation and atresia/apoptosis.

In swine, STAT3 has been shown to be expressed in oocytes, granulosa and theca cells, being phosphorylated by epidermal growth factor in granulosa cells (Wen et al. 2006). Another important activator of STAT3 pathway in granulosa cells is leptin and a biphasic effect of leptin on progesterone production was demonstrated in primary culture of swine granulosa (Ruiz-Cortés et al. 2003). In bovine, leptin at physiological levels antagonizes insulin-induced steroidogenesis (Spicer and Francisco 1997).

In the present study, increased pSTAT3 protein levels were observed in the granulosa cells of all non-dominant follicles. Nevertheless, an acute increase in pSTAT3 levels was observed only in subordinate follicles collected on day 3 or 4 of follicular wave. It was previously demonstrated that pSTAT3 strongly activates *STAT3* gene (Cheon et al. 2011) and this may explain why pSTAT3 levels tended to increase at day 3, while *STAT3* mRNA and tSTAT3 were only upregulated at day 4, suggesting a positive feedback loop. Furthermore, unphosphorylated STAT3 seems to act as a transcription factor, regulating the expression of a set of genes that is mostly distinct from those activated in response to pSTAT3 (Yang et al.

2005). Thus, functional experiments are necessary to elucidate the roles of pSTAT3 and possibly unphosphorylated STAT3 on granulosa cell differentiation and apoptosis.

The functional status of STAT3 during deviation suggests that this pathway is activated by factors involved in inhibition of estradiol secretion and/or differentiation. Furthermore, the dramatic increase in pSTAT3 in day 4 subordinate follicles suggests that activation of STAT3 pathway may be involved in triggering granulosa cell apoptosis. In lung adenocarcinoma cell line, accumulation of pSTAT3 induced downregulation of the antiapoptotic protein Bcl-2 (Ozenne et al. 2012). Thus, one possible mechanism of the pro-apoptotic effect of pSTAT3 is modulating Bcl-2. Based on the observed pattern of pSTAT3 abundance in the present study, it is possible that the increase in STAT3 activation is involved in granulosa cell death and that its suppression in dominant follicles may accounts for continued follicle growth and differentiation. Recently, it was demonstrated that STAT3 activation (phosphorylation at Tyr 705) is involved in mammary gland lysosomal-mediated cell death (Kreuzaler et al. 2011). Lysosomal function destabilization has also been shown to be a mechanism involved in bovine granulosa cell death (Alonso-Pozos et al. 2003). Thus, we cannot rule out the participation of STAT3 in inducing programmed cell death without involvement of the classical apoptotic programme.

In conclusion, pMAPK is increased in the future dominant follicle but does not seem to be the hallmark of the DF throughout follicle deviation. The abundance of pSTAT3 in granulosa cells suggests that this pathway is involved in granulosa cell death, as previously demonstrated during mammary gland involution.

5.5. Materials and methods

Estrus synchronization and granulosa cell collection

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee. Cyclic adult beef cows (n=32; 4 to 7 years old) were synchronized with two injections of sodium cloprostenol (Ciosin; Intervet/Schering-Plough; 250 µg; im) 12 h apart and observed for estrus within 3–5 days after the treatment. Ovaries were examined by daily transrectal ultrasonography. The day of the follicular emergence was retrospectively identified as the day on which the diameter of the dominant follicle (DF) was between 4 and 5 mm (Evans and Fortune 1997). Both ovaries were collected from each cow by colpotomy on days 2 (n=4 cows), 3 (n=4 cows) or 4 (n=6 cows) of the follicular wave to recover the two largest follicles from each cow.

RNA extraction, reverse transcription and real-time PCR

Granulosa cells RNA and protein were extracted using AllPrep DNA/RNA/protein kit (Qiagen). Quantitation and estimation of RNA purity was performed using NanoDrop (Thermo Scientific - Waltham, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.8 were considered pure, and all samples were above this threshold. To generate the cDNA, 500 ng RNA was first treated with 0.1 U DNAse (Invitrogen; 37°C – 5 min). After DNAse inactivation at 65°C for 10 min, samples were incubated in a final volume of 20 µl with iScript cDNA Synthesis Kit (BioRad) following the manufacturer's protocols.

To test cross-contamination with theca cells, polymerase chain reaction (PCR) for detection of the mRNAs that encode *CYP17A1* in granulosa cells was performed in each sample and all samples were free from contamination by theca cells (after 30 PCR cycles). Real-time quantitative PCR (qPCR) reactions were conducted in CFX 384 real-time PCR detection system (Bio-Rad, Hercules, CA) using FastStart Universal SYBR Green Master (with Rox; Roche Diagnostics, Canada) and bovine-specific primers (Table 1) taken from the literature. Standard two-step qPCR was performed with initial denaturation at 95 °C for 5 min

followed by 40 cycles of denaturation at 95 ºC for 15 sec and annealing/extension at 58 ºC for 30 sec. The product identity was verified through melting-curve analyses.

To optimize the qPCR assay, serial dilutions of a cDNA template were used to generate a standard curve by plotting the log of the starting quantity of the dilution factor against the CT value obtained during amplification of each dilution. Reactions with a coefficient of determination (R2) higher than 0.98 and efficiency between 95 to 105% were considered optimized. The relative standard curve method was used to assess the amount of a particular transcript in the samples as described by Cikos et al. (2007). Briefly, for each gene, standard cDNAs were amplified along with sample cDNAs in the same PCR run. The target mRNA quantity in each sample was determined from the relative standard curve (using sample Ct values) and expressed in arbitrary units corresponding to the dilution factors of the standard RNA preparation. Samples were run in duplicate and were expressed relative to cyclophilin as housekeeping gene.

Immunoblotting

Granulosa cell protein samples obtained using AllPrep kit were boiled at 95 °C for 3 min, subjected to 10% SDS gel and electrotransfered onto nitrocellulose membranes. After blocking for 2 h with 5% skimmed milk in Tris buffered saline (TBS) containing 0.1% tween-20 (TBS-T), blots were incubated overnight at 4 °C with 1:1000 rabbit anti-human phosphorylated STAT3 (#9131; Tyr 705; Cell Signaling), phosphorylated MAPK (#4376; Erk1/2; Thr 202/Tyr 204; Cell Signaling), total STAT3 (#9132; Cell Signaling), total MAPK (#4695; Cell Signaling), cleaved caspase-3 (Asp175; #9661, Cell Signaling Technology, Danvers, MA) or 1:5000 beta actin (ab8227; Abcam Inc.) with agitation, followed by three washes (10 min each) with TBS-T. The blots were then incubated with 1:7500 goat anti-rabbit IgG-HRP (ab6721; Abcam Inc., USA) for 2 h with agitation, followed by three washes (10 min each) with TBS-T. Immunoreactivity was detected with Immun-Star WesternC

Chemiluminescence Kit (BioRad, CA, USA) according to the manufacturer's instructions and visualized using Chemidoc system (BioRad, CA, USA). Quantification of bands of the western blots was performed using Image Lab software (Bio-Rad Laboratory). ERK1 and 2 band densities were added together and were represented as MAPK. The abundance of phosphorylated proteins (pMAPK and pSTAT3) was calculated relative to their respective total protein levels. Total protein (STAT3 and cleaved caspase 3) levels were calculated based on beta actin as the loading control.

Histology of dominant and subordinate follicles

Ovaries from one cow on day 4 of follicular wave were collected by colpotomy and the two largest follicles were isolated. The follicles were fixed in 4% paraformaldehyde for 6 h and paraffin embedded. Histological sections (5 µm thickness) were deparaffinized using CitriSolv (Fisher Scientific, Pittsburgh, PA) for 10 min., rehydrated through a graded alcohol series (one times for 3 min. in each 100%, 100%, 95%, 80%, dilution), and rinsed in ddH2O for 5 min. For PAS (Periodic Acid-Schiff) staining, the sections were oxidized in Periodic acid (0.5%, 5 min) and stained with Schiff's reagent (Fisher Scientific), followed by immersion in running tap water. Nuclei were counterstained with hematoxylin (10 min) and sections were dehydrated and mounted.

Statistical analysis

All continuous data were tested for normal distribution using Shapiro-Wilk test, normalized when necessary and submitted to ANOVA using JMP software (SAS Institute Inc., Cary, NC). The differences between the two largest follicles in each day were accessed by paired Student's T test using cow as subject. Results are presented as mean ± standard error of the mean (SEM). A $P<0.05$ was considered statistically significant.

5.6. Acknowledgements

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362 **Figure legends**

363 **Figure 1**: Follicular diameter determined by daily transrectal ultrasonography (A) and
364 *CYP19A1* mRNA expression (B) in the two largest follicles from each cow collected at day 2
365 (n=4), day 3 (n=4) or day 4 (n=6) of follicular wave. Different letters indicate statistical
366 significance. Asterisk (*) indicates statistical difference between largest and second largest
367 follicle accessed by paired Student's T test using cow as subject. * $p<0.05$; # $p<0.1$. Panel C
368 shows periodic acid-Schiff (PAS) stained follicular wall sections from the two largest follicles
369 from one cow ovariectomized on day 4 of follicular wave. The arrows indicate the basal
370 membrane (BM) between granulosa cell (GC) and theca cell (TC) layers.
371
372 **Figure 2:** Cleaved caspase 3 protein abundance in dominant (n=3) and subordinate follicles
373 (n=4) collected at day 4 of follicular wave. Total protein levels were calculated based on beta
374 actin as the loading control. Different letters indicate statistical significance.
375
376 **Figure 3**: Phosphorylated MAPK abundance in the two largest follicles collected before (Day
377 2; n=4 cows), at the expected moment (Day 3; n=4 cows) or after (Day 4; n=6 cows) follicular
378 deviation. The abundance of phosphorylated proteins was calculated based on the
379 correspondent total protein level. Asterisk (*) indicates statistical difference between largest
380 and second largest follicle accessed by paired Student's T test using cow as subject; * $p<0.05$.
381
382 **Figure 4**: *STAT3* mRNA (A), total STAT3 (B) and phosphorylated STAT3 abundance (C) in
383 the two largest follicles collected before (Day 2; n=4 cows), at the expected moment (Day 3;
384 n=4 cows) or after (Day 4; n=6 cows) follicular deviation. Cows were synchronized and
385 granulosa cells from the two largest follicles from each cow were recovered and submitted to
386 simultaneous extraction of mRNA and protein. The abundance of phosphorylated proteins

was calculated based on the correspondent total protein level. Total protein levels were calculated based on beta actin as the loading control. Asterisk (*) indicates statistical difference between largest and second largest follicle accessed by paired Student's T test using cow as subject. * $p<0.05$; # $p<0.1$.

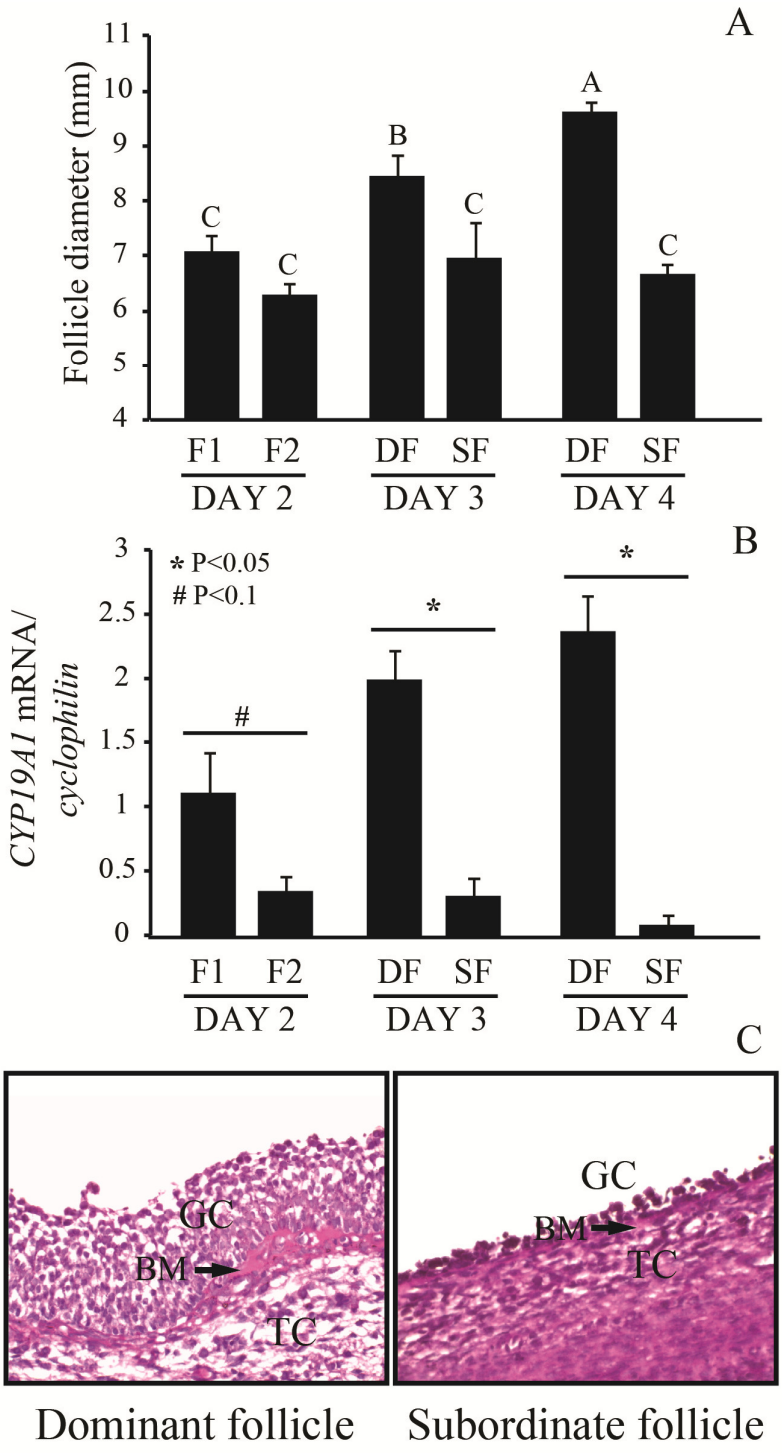
392 **Table 1** - Primers used in the expression analysis of *Bos taurus taurus* genes.

Gene		Sequence	Conc. (nM)	Reference/accession nº
CYCLOPHILIN	F	GGTCATCGGTCTCTTTGGAA	200	(Santos et al. 2011)
	R	TCCTTGATCACACGATGGAA	200	
CYP19A1	F	GTGTCCGAAGTTGTGCCTATT	300	(Luo and Wiltbank 2006)
	R	GGAACCTGCAGTGGGAAATGA	300	
CYP17A1	F	CCATCAGAGAAGTGCTCCGAAT	200	(Lagaly et al. 2008)
	R	GCCAATGCTGGAGTCAATGA	200	
STAT3	F	CTGCAGCAGAAGGTTAGCTACAAA	200	(Boelhauve et al. 2005)
	R	TTCTAAACAGCTCCACGATTCTCTC	200	

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

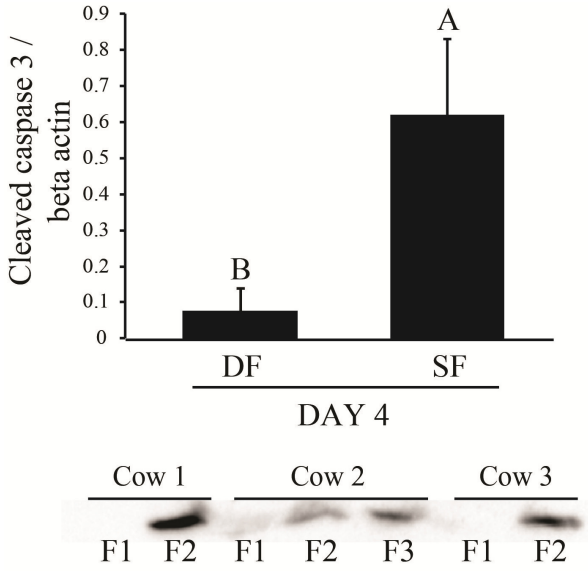
395

396 **Figure 1**



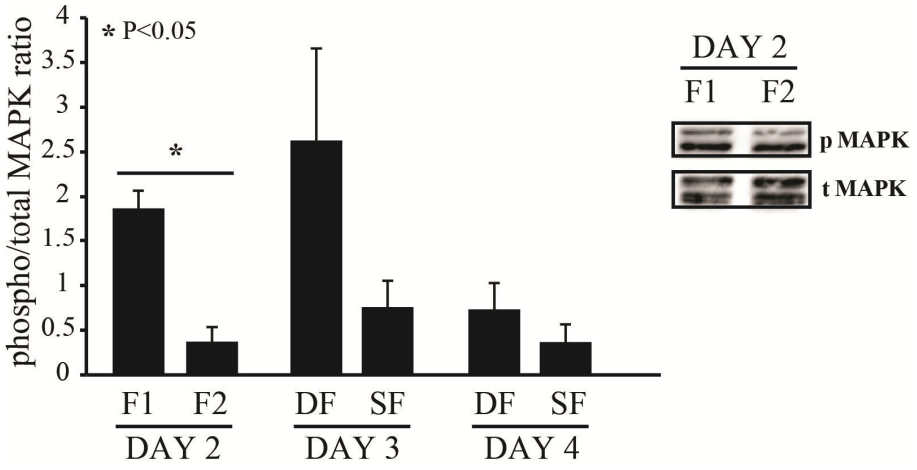
397

398 **Figure 2**



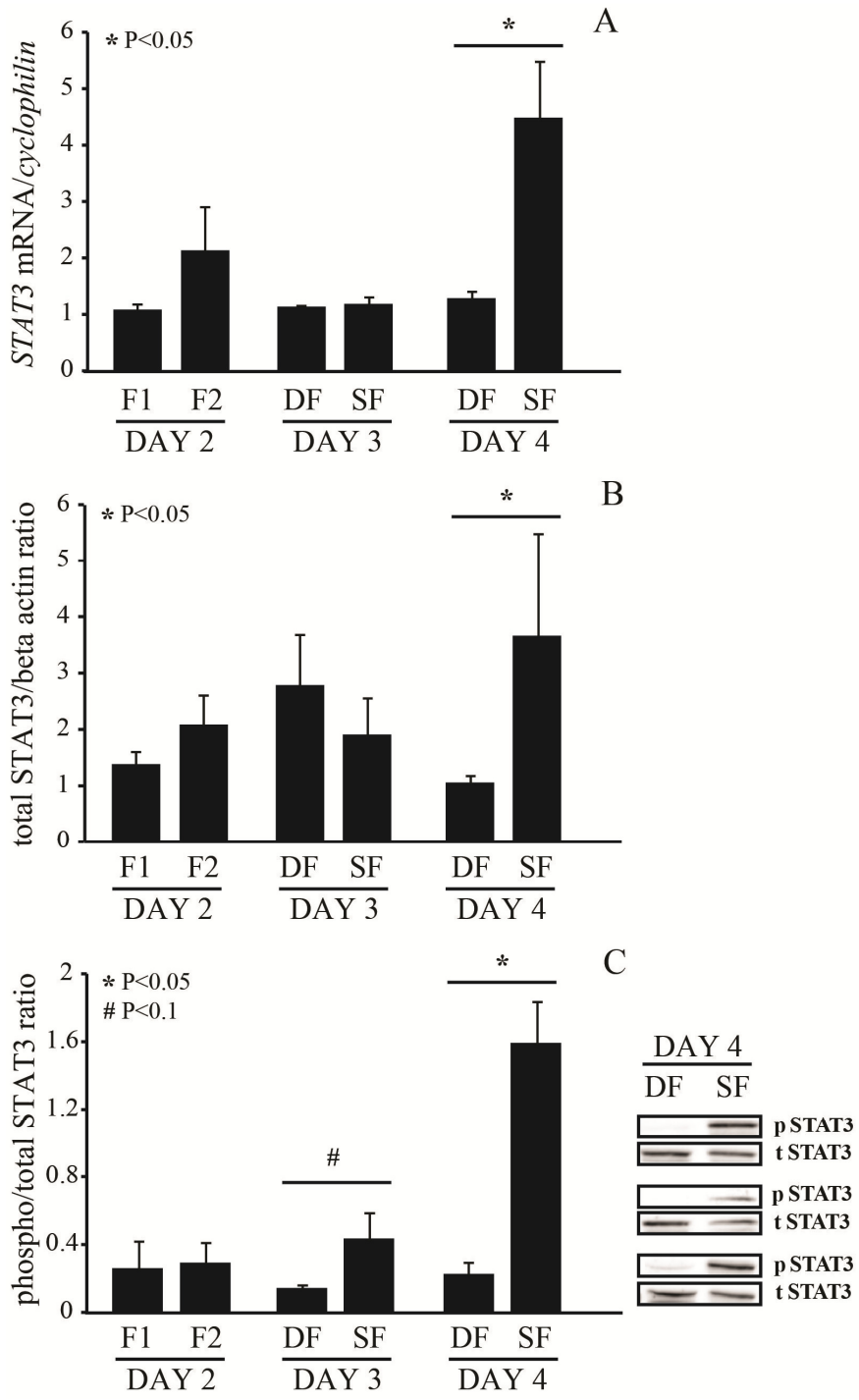
399

400 **Figure 3**



401

402 **Figure 4**



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404

6. DISCUSSÃO

O controle endócrino da foliculogênese antral está bem estabelecido, graças aos estudos pioneiros aliando dinâmica de crescimento folicular através de ultrassonografia e dosagens hormonais. Entretanto, alguns eventos fisiológicos como a seleção do folículo dominante, à semelhança dos processos iniciais da ovulação, envolve a regulação de fatores locais produzidos pelas células foliculares e oócito (Figura 1). Na busca por um melhor entendimento da regulação de fatores locais e rotas de sinalização envolvidas na seleção do folículo dominante e atresia dos subordinados, nosso grupo vêm utilizando o modelo de sincronização de ondas foliculares seguida de ovariectomia em momentos estratégicos para estudos de eventos que ocorrem antes, no momento e após a divergência folicular. Além do modelo de caracterização, o modelo de injeção intrafolicular nos permite manipular o microambiente folicular para estudar a função de fatores e receptores *in vivo*, mantendo a interação entre os diferentes tipos celulares, sob o mesmo ambiente endócrino.

No primeiro estudo, investigamos a expressão e a função do FGF10 durante o crescimento folicular. Dados de um estudo anterior demonstram maior expressão do FGF10 nas células da teca de folículos em estágios iniciais de atresia, provenientes de ovários de abatedouro, e uma ação negativa do FGF10 sobre a síntese de estradiol *in vitro* (BURATINI et al., 2007). Entretanto, o perfil de expressão *in vivo* e o mecanismo da inibição do FGF10 sobre a síntese de estradiol eram até então desconhecidos. Os dados do presente estudo demonstram uma maior expressão de FGF10 e do FGFR2b nas células da teca e granulosa, respectivamente, em folículos subordinados após a divergência. Além disso, observamos que um dos efeitos agudos do FGF10 sobre as células da granulosa é a diminuição na expressão de CYP19A1, o que explica o decréscimo na síntese de estradiol, em semelhança ao observado após adição de FGF7 em cultivo de granulosa (PARROTT & SKINNER, 1998). Curiosamente, observamos uma tendência ao aumento da expressão do receptor FGFR2b na granulosa e aumento significativo na expressão de FGF10 na teca após tratamento com FGF10, sugerindo um mecanismo de retroalimentação positiva e confirmando que esses fatores são mais expressos durante a atresia. Entretanto, não observamos efeitos sobre as demais enzimas esteroidogênicas, receptores de gonadotrofinas e genes relacionados à apoptose. A diminuição da expressão de ciclina D2 possivelmente seja um efeito indireto, uma vez que esse gene é regulado positivamente pelo estradiol e, na ausência do estímulo do estradiol sobre a proliferação celular, as células da granulosa se tornam mais sensíveis à

apoptose (QUIRK et al., 2006). Portanto, os resultados sugerem uma ação mais específica do FGF10 sobre a síntese de estradiol em comparação ao FGF18, o qual também parece estar relacionado à atresia folicular, porém regulando negativamente diversas enzimas esteroidogênicas (PORTELA et al., 2010).

O fato de que a diminuição na sinalização da BMP15 ou GDF9 é capaz de induzir superovulação, em decorrência de mutações inativadoras em heterozigose (GALLOWAY et al., 2000; HANRAHAN et al., 2004), imunizações contra as proteínas (JUENGEL et al., 2004; JUENGEL et al., 2009) ou mutação no receptor BMPR1B (MULSANT et al., 2001), nos levou a hipótese de que esses fatores são diferentemente expressos em folículos dominantes e subordinados. A expressão do BMPR1A foi significativamente maior apenas nos futuros folículos subordinado (dia 2). Os receptores BMPR-2 e -1B foram significativamente mais expressos nos folículos subordinados no dia 3, enquanto que no dia 4 apenas o BMPR1B foi significativamente mais expresso nos folículos atrésicos. Coletivamente, os dados sugerem um envolvimento desses receptores na inibição da diferenciação das células da granulosa e/ou indução de apoptose. O receptor BMPR1B foi significativamente mais expresso 12 h após o bloqueio dos receptores de estradiol e 24 h após o tratamento com FGF10, confirmando uma maior expressão durante a atresia folicular. Quanto aos ligantes, não foi observada diferença significativa nos níveis de BMP15 ou GDF9 no fluído folicular de folículos dominantes e subordinados. Baseados nesses dados, pode-se inferir que os folículos de animais portadores de mutações no receptor BMPR1B, além de se tornarem responsivos ao LH precocemente (MCNATTY et al., 2009), podem também ser mais resistentes à atresia.

Na busca por rotas de sinalização diferentemente ativas durante a divergência, observamos que a fosforilação da MAPK é um marcador inicial de dominância, conforme anteriormente sugerido (RYAN et al., 2007). Entretanto, a ausência de alteração na expressão durante e após a divergência sugerem que esta rota é ativada por fatores envolvidos com desenvolvimento folicular e também durante a regressão dos folículos não selecionados. Em acordo com essa hipótese, Gebauer et al. (1999) observaram uma diminuição na atividade da MAPK em células da granulosa de ratas imediatamente antes do início do processo apoptótico e, posteriormente, um aumento durante as fases finais da cascata apoptótica. Em um segundo momento, observamos que a ativação da rota STAT3 é restrita às células da granulosa de folículos atrésicos. De forma similar, esta rota parece inativa nas células da GM durante a lactação, apresentando elevada atividade durante a involução, processo também mediado por

apoptose (ABELL et al., 2005). O *knockout* da rota STAT3 em camundongos causa um atraso na involução da GM (CHAPMAN et al., 1999) e o bloqueio dessa rota *in vitro* torna as células da GM mais resistentes à morte mediada por lisossomos (KREUZALER et al., 2011), mecanismo também envolvido na morte das células da granulosa (ALONSO-POZOS et al., 2003). Coletivamente, os dados de literatura durante a involução da GM e os do presente estudo fornecem evidencias do envolvimento da ativação da rota STAT3 durante a morte das células da granulosa. Entretanto, estudos funcionais são necessários para comprovar essa hipótese.

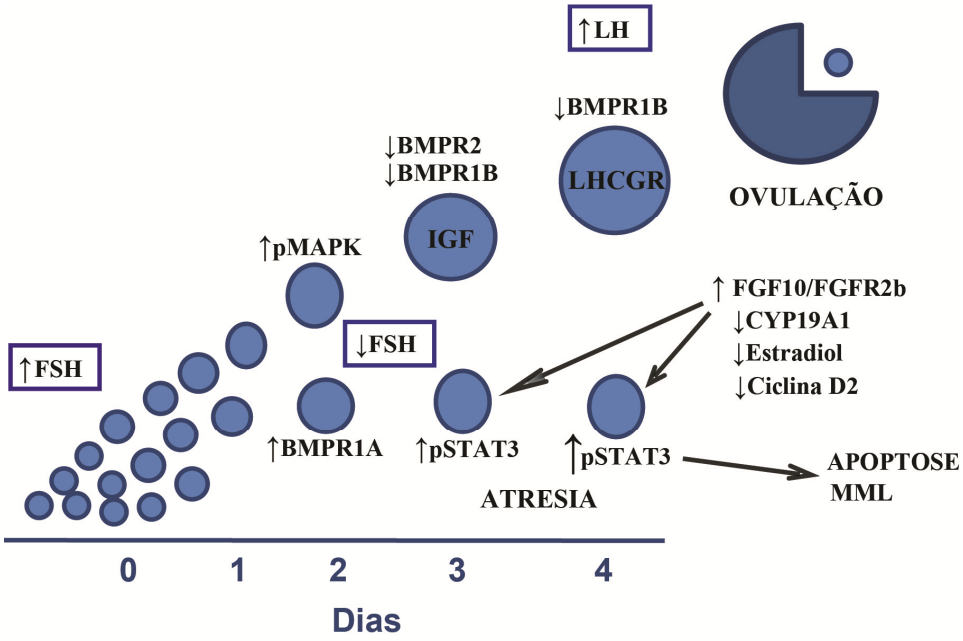

Figura 1 – Após a emergência de uma onda folicular, durante o período de declínio dos níveis de FSH, apenas um folículo continua a se desenvolver, sofrendo diferenciação e passando a depender principalmente de LH. Antes da divergência, as células da granulosa do futuro folículo dominante apresentam maior atividade da rota MAPK, em comparação ao maior folículo subordinado. Maiores níveis de IGF1 livre e menor expressão dos receptores BMPR-1B e -2 são características do folículo dominante no momento da divergência. Maior expressão de FGF10/FGFR2b no folículo subordinado bloqueiam o desenvolvimento através de inibição da expressão de CYP19A1, síntese de estradiol e expressão de ciclina D2. No folículo subordinado, a atividade da rota STAT3 começa a aumentar no momento da divergência permanecendo elevada durante a atresia, possivelmente induzindo a morte das células da granulosa por apoptose ou através de morte mediada por lisossomos (MML).

7. CONCLUSÃO

No presente estudo, os padrões de expressão de fatores locais, seus receptores e rotas de sinalização foram caracterizados em folículos antes, durante e após a divergência folicular. O fato do FGF10 e de seu receptor FGFR2b serem mais expressos em folículos subordinados após a divergência, nas células da teca e granulosa, respectivamente, sugere uma ação inibitória sobre o desenvolvimento folicular. Em acordo com essa hipótese, a injeção intrafolicular de FGF10 bloqueou o desenvolvimento do folículo dominante, diminuindo especificamente a síntese de estradiol e a expressão da enzima CYP19A1 e de ciclina D2. O padrão de expressão dos receptores de BMPs, especialmente do BMPR1B, sugerem um envolvimento na inibição da diferenciação e indução da atresia em folículos subordinados, enquanto os ligantes BMP15 e GDF9 parecem não ser diferentemente expressos em folículos dominantes e subordinados. A rota de sinalização MAPK é um marcador precoce de dominância folicular, porém não é diferentemente ativa durante e após a divergência, sugerindo uma ativação da mesma durante o desenvolvimento do dominante e atresia dos subordinados. O fato da rota STAT3 estar significativamente mais ativa em folículos subordinados durante e após a divergência sugerem fortemente que essa via de sinalização está envolvida na morte das células da granulosa, em semelhança ao observado durante a involução da glândula mamária no modelo roedor.

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