UNESP - UNIVERSIDADE ESTADUAL PAULISTA

Instituto de Biociências

Campus de Botucatu

REGULAÇÃO DA EXPRESSÃO DE FATORES SECRETADOS PELO OÓCITO (FSOs) E SEUS RECEPTORES DURANTE A MATURAÇÃO *IN VITRO* (MIV) BOVINA E AÇÕES NO CONTROLE DA EXPANSÃO DO CUMULUS

ESTER SIQUEIRA CAIXETA

TESE DE DOUTORADO EM CIÊNCIAS BIOLÓGICAS

BOTUCATU/SP
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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO DE AQUIS. E TRAT. DA INFORMAÇÃO
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CAMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ***ROSEMEIRE APARECIDA VICENTE***

Caixeta, Ester Siqueira.

Regulação da expressão de fatores secretados pelo oócito (FSOs) e seus receptores durante a maturação *in vitro* (MIV) bovina e ações no controle da expansão do cumulus / Ester Siqueira Caixeta. – Botucatu : [s.n.], 2012

Tese (doutorado) - Universidade Estadual Paulista, Instituto de Biociências Botucatu

Orientador: José Buratini Junior

Capes: 21005001

1. Farmacologia. 2. Biologia molecular. 3. Bovino.

Palavras-chave: Expansão do cumulus; Fatores EGF-*like*; Fatores secretados pelo oócito; Maturação *in vitro.*

UNESP - UNIVERSIDADE ESTADUAL PAULISTA

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ESTER SIQUEIRA CAIXETA

TESE DE DOUTORADO SUBMETIDA AO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS DO INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU, UNIVERSIDADE ESTADUAL PAULISTA – UNESP, PARA OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS BIOLÓGICAS – ÁREA: FARMACOLOGIA.

BOTUCATU/SP, 06 de FEVEREIRO de 2012

“Oh! Que me abençoes e me alargues as fronteiras, que seja comigo a tua mão e me preserves do mal, de modo que não me sobrevenha aflição!” I Crônicas 4:10

“Ao Deus que me revestiu de força e aperfeiçoou o meu caminho” (Salmos 18:32).

Aos meus amorosos pais Ivan e Elenice, que oraram por mim e estiveram sempre ao meu lado. Muito obrigada pelo carinho, incentivo e paciência constantes. Vocês serão sempre meu orgulho! Amo vocês!

Aos meus irmãos Dani e Bruna (cunhada), por serem tão especiais, e Mari, pelo quanto é importante para mim... A melhor conversa, a melhor risada, o mais precioso segredo... e sem dúvida, os melhores momentos.

Aos meus avós Sylvia (em memória), José, Cornélia e Isaltino, pelas orações e pelo exemplo de vida.

Ao Russo, que mesmo a distância soube se fazer presente sempre! Pelo companheirismo, carinho e força em todos os momentos. Você é essencial.

À tia Elen, mais que tia, uma irmã, pela disposição em me ajudar sempre, pelos conselhos, amizade e pelos momentos inesquecíveis que vivemos juntas, e ao Tio Marcelo, pela hospedagem em Botucatu e pela sua alegria contagiante.

AGRADECIMENTOS

Ao meu orientador, **Prof. Dr. José Buratini Junior**, agradeço pelos ensinamentos e atenção dispensada durante esses anos. Tenho por você grande admiração, gratidão e respeito.

À **UNESP – Instituto de Biociências**, pela oportunidade concedida para a realização deste doutorado.

Aos **Professores e Funcionários do Departamento de Fisiologia**, pela amizade e incentivo.

Aos **Funcionários da seção de pós-graduação**, Erivaldo, Luciene, Luciana e Davi, pela ajuda e colaboração durante todo o meu doutorado.

Ao **Prof. Ciro Moraes Barros**, do Departamento de Farmacologia (Unesp-Botucatu), por permitir o nosso acesso no seu laboratório sempre que foi necessário.

À grande amiga **Allice**, agradeço pelo alegre convívio durante parte deste período, pelo companheirismo, apoio e amizade. A nossa "casinha" contribuiu muito para o meu crescimento pessoal. Você ficará sempre no meu coração!

Ao **Prof. Cristopher A. Price**, do Centro de Pesquisa em Reprodução Animal, da Faculdade de Medicina Veterinária, (Universidade de Montreal, Canadá) pela constante ajuda no desenvolvimento deste trabalho.

Aos **amigos e colegas de doutorado**, Anthony, Cíntia, Diego, Mariana, Paula Fernanda, Paula Ripamonte e Rúbia ... a todos, muito obrigada!

À **Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP**, pela concessão da bolsa de doutorado e pelo suporte financeiro para a realização deste trabalho.

ÍNDICE

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS

Capítulo 1 e Resultados adicionais

ADAM = família desintegrina e metaloproteinases

ALK = receptor ativina semelhante a kinase

AMPc = adenosina monofosfato cíclico

AR = aldose redutase

AREG = ampiregulina

BMP15 = proteína morfogênica óssea 15

BMPRII = receptor de BMP do tipo II

BSA = albumina sérica bovina

BTC = betacelulina

COC = complexo cumulus-oócito

COX2 = ciclooxigenase 2

Cx = conexina

ECM = matriz extracelular

EGF = fator de crescimento epidermal

EGF-*like* = fatores de crescimento epidermal (EGF)-*like*

EGFR = receptores de EGF

EREG = epiregulina

ERK = quinases reguladas por sinais extracelulares

FGF = fatores de crescimento fibroblástico

FIV = fecundação *in vitro*

FSH = hormônio folículo estimulante

FSO = fatores secretados pelo oócito

G6PDH = glicose-6-fosfato desidrogenase

GDF9 = fator de crescimento e diferenciação 9

GFPT = L-glicosamina:D-frutose-6-fosfato acetil transferase (GFPT)

GLUT = facilitadores do transporte da glicose

GMPc = monofosfato cíclico de guanosina

HAS2 = hialurona sintetase 2

HB-EGF = fator de crescimento semelhante ao EGF ligado a heparina

HK = hexoquinase

IP3 = inositol trifosfato

IαI = fator derivado do soro inibidor inter-α-tripsina

LH = hormônio luteinizante

MIV = maturação *in vitro*

OGT = glicosilação O-*linked*

PDE3 = fosfodiesterase do tipo 3

PFK = fosfofrutoquinase

PGE2 = prostaglandina E2

PTGS2 = prostaglandina sintetase 2

PTX3 = pentraxina 3

RNA = ácido ribonucléico

RNAm = ácido ribonucléico mensageiro

SDH = sorbitol desidrogenase

TGFα = fator de crescimento transformante α

TGFβ = fatores de crescimento transformante β

TSG6 = proteína indutora do fator de necrose tumoral 6

TZP = processos citoplasmáticos trans-zonais

Capítulo 2 e Capítulo 3

ADAM = disintegrin and metalloproteinase family

ALK = activin receptor-like kinase

ANOVA = analysis of variance

AREG = amphiregulin

BMP15 = bone morphogenetic protein 15

BMPRII = BMP receptor II

BSA = bovine serum albumin

BTC = betacellulin

CO_2 = carbon dioxide

COC = cumulus–oocyte complexes

COX2 = cyclooxygenase 2

CYC-A = cyclophilin-A

DNA = deoxyribonucleic acid

EGF-like = epidermal growth factor-like

EREG = epiregulin

FGF = fibroblast growth factor

FSH = follicle-stimulating hormone

g = gravity

GAPDH = glyceraldehydes-3-phosphate dehydrogenase

GDF9 = growth and differentiation factor 9

h = hour

H2AFZ = histone H2AFZ

HAS2 = hyaluronan synthase 2

hCG = human chorionic gonadotropin

IVM = *in vitro* maturation

LH = luteinizing hormone

mRNA = messenger ribonucleic acid

NaCl = sodium chloride

OSF = oocyte secreted factors

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PTX3 = pentraxin 3

RNA = ribonucleic acid

RT-PCR = reverse transcription polymerase chain reaction

S.E.M. = error of the mean

TSG6 = tumor necrosis factor-stimulated gene-6 protein

RESUMO

CAIXETA, E.S. **Regulação da expressão de fatores secretados pelo oócito (FSOs) e seus receptores durante a maturação *in vitro* (MIV) bovina e ações no controle da expansão do cumulus.** Botucatu, 2012. Tese (Doutorado - Ciências Biológicas - Farmacologia) - Instituto de Biociências, IB, Universidade Estadual Paulista - UNESP.

O oócito participa ativamente dos mecanismos reguladores da maturação do complexo cumulus-oócito (COC) via secreção de fatores parácrinos. A proteína morfogênica óssea 15 (BMP15) e o fator de crescimento e diferenciação 9 (GDF9) têm concentrado a maior parte da atenção direcionada aos fatores secretados pelo oócito (FSO) e têm sido associados com a melhora na competência para o desenvolvimento do COC. Em adição, recentemente, detectamos a expressão de fatores de crescimento fibroblástico (FGFs) no oócito e seus receptores nas células do cumulus (FGF10 e seus receptores FGFR1B e 2B; FGF8 e 17 e seus receptores FGFR2C e 3C), sugerindo o envolvimento do sistema FGF na regulação da diferenciação das células do cumulus. O presente trabalho investigou a regulação da expressão do RNAm de FSOs (BMP15, GDF9, FGF8, FGF10 e FGF17) e seus receptores, bem como de membros da família de fatores de crescimento epidermal (EGF)-like [ampiregulina (AREG), epiregulina (EREG) e betacelulina (BTC)] durante a maturação *in vitro* (MIV) bovina estimulada pelo FSH. O FSH estimulou a expressão do FGFR2C, FGFR3C, FGFR1B, ALK6, AREG e EREG nas células do cumulus durante a MIV. A expressão do RNAm do FGF8 e FGF17, mas não da BMP15, GDF9 e FGF10 diminuiu no oócito durante a MIV. Em adição foram investigadas especificamente as ações da BMP15 e do FGF10 sobre a expansão do cumulus e expressão gênica de membros da família desintegrina e metaloproteinases (ADAM10 e ADAM17), membros da família dos fatores EGF-*like* (AREG, EREG e BTC) e de genes sabidamente envolvidos no controle da expansão do cumulus [ciclooxigenase 2 (COX2), hialurona sintetase 2 (HAS2), proteína indutora do fator de necrose tumoral 6 (TSG6) e pentraxina 3 (PTX3)]. A BMP15 e o FGF10 aumentaram a porcentagem de COCs completamente expandidos. Em adição, a BMP15 aumentou a expressão do RNAm da ADAM10, ADAM17, AREG, EREG, HAS2, COX2, PTX3 e TSG6. O FGF10 não alterou a expressão dos fatores EGF-*like*, mas aumentou a expressão do RNAm da COX2, PTX3 e TSG6. Em síntese, o FSH estimula expressão de receptores para importantes FSOs nas células do cumulus, assim como dos fatores EGF-like (AREG e EREG). A BMP15 e o FGF10 intensificam a expansão do cumulus durante a MIV por diferentes mecanismos. Enquanto o FGF10 estimulou rapidamente a COX2 e

subsequentemente a PTX3 e TGS6 sem alterar a expressão dos EGF-*like* e das ADAMs, o aumento na expressão da COX2, PTX3, TSG6 e HAS2 induzido pela BMP15 foi observado após ou em associação com o aumento na expressão da ADAM10, ADAM17, AREG e EREG. Estas observações sugerem que a BMP15 estimula a expressão dos genes indutores da expansão via membros da família EGF-like, enquanto o FGF10 parece atuar mais diretamente sobre a COX2.

Palavras Chave: Expansão do cumulus; Fatores EGF-*like*; Fatores secretados pelo oócito; Maturação *in vitro*.

ABSTRACT

CAIXETA, E.S. **Regulation of expression of oocyte secreted factors (OSFs) and their receptors during bovine *in vitro* maturation (IVM) and actions in the control of cumulus expansion.** Botucatu, 2012. Tese (Doutorado - Ciências Biológicas - Farmacologia) - Instituto de Biociências, IB, Universidade Estadual Paulista - UNESP.

The oocyte actively participates in the regulatory mechanisms of cumulus-oocyte complex (COC) maturation via secretion of paracrine factors. Bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9) have concentrated most of the attention directed to oocyte secreted factors (OSFs) and have been shown to enhance developmental competence of the COC. In addition, fibroblast growth factors (FGFs) have also been recognized as important OSFs. Recently, we detected the expression of FGFs in the oocytes and their receptors in cumulus cells (FGF10 and its receptors FGFR1B and 2B; FGF8 and 17 and their receptors FGFR2C and 3C), suggesting the involvement of the FGF system in the regulation of cumulus cells differentiation. The present study investigated the mRNA expression pattern for FSOs (BMP15, GDF9, FGF8, FGF10 and FGF17) and their receptors, as well as of epidermal growth factor (EGF)-like family members [ampiregulina (AREG), epiregulina (EREG) and betacelulina (BTC)] during bovine COC *in vitro* maturation (IVM) stimulated by FSH. The FSH stimulated mRNA expression of FGFR2C, FGFR3C, FGFR1B, ALK6, AREG and EREG in cumulus cells during IVM. Messenger RNA expression of FGF8 and FGF17, but not of BMP15, GDF9 and FGF10, decreased in the oocyte during IVM. In addition were specifically investigated the actions of BMP15 and FGF10 on cumulus expansion and gene expression of disintegrin and metalloproteinase family members (ADAM10 and ADAM17), of EGF-*like* family members (AREG, EREG and BTC) and the major expansion-inducing genes genes [cyclooxygenase 2 (COX2), hyaluronan synthase 2 (HAS2), pentraxin 3 (PTX3), tumor necrosis factor-stimulated gene-6 protein (TSG6)]. BMP15 and FGF10 increased the percentage of fully expanded cumulus-oocyte complexes. In addition, BMP15 increased mRNA expression of ADAM10, ADAM17, AREG, EREG, HAS2, COX2, PTX3 and TSG6. FGF10 did not alter the expression of EGF-like factors but enhanced mRNA expression of COX2, PTX3, and TSG6. In summary, FSH enhances the expression of receptors for important OSFs in cumulus cells and of EGF-*like* members (AREG and EREG). BMP15 and FGF10 improve the cumulus expansion during IVM but induced different gene expression patterns in cumulus cells. While FGF10 increased COX2 promptly and PTX3/TSG6 subsequently without altering AREG and EREG, the increases in

COX2, PTX3, TSG6, HAS2 expression induced by BMP15 were observed only after or in association with the increase in AREG and EREG expression. This observation suggests the BMP15 stimulates the expression of expansion-inducing genes via the EGF-like family members, while FGF10 appears to act upon more directly pathways.

Key words: Cumulus expansion, EGF-*like* factors, Oocyte secreted factors, *In vitro* maturation.

CAPÍTULO 1

1 INTRODUÇÃO E JUSTIFICATIVA

O ovário mamífero possui milhares de oócitos inclusos em folículos pré-antrais, entretanto, apenas uma pequena parcela destes folículos alcançam a ovulação, enquanto que os demais são eliminados pelo processo conhecido como atresia folicular. A natureza seletiva do desenvolvimento folicular tem como consequência um grande desperdício de células germinativas femininas, as quais poderiam ser aproveitadas pelo emprego de biotécnicas da reprodução como a superovulação seguida de transferência de embriões e a maturação e fecundação *in vitro* de oócitos obtidos por aspiração folicular. Sendo assim, há grande interesse no aprimoramento de tais biotécnicas, de forma a ampliar o potencial reprodutivo de fêmeas com genótipos de destaque ou de espécies ameaçadas, beneficiando tanto a produção de alimentos de origem animal, quanto a preservação da biodiversidade.

A eficiência da maturação oocitária é largamente superior em condições fisiológicas (*in vivo*) em comparação ao sistema *in vitro* (van de Leemput *et al.*, 1999; Rizos *et al.*, 2002). Nesse sentido, são necessárias informações mais completas e precisas sobre as interações entre as células somáticas e germinativas que formam o complexo cumulus-oócito (COC), bem como sobre as exigências para seu pleno funcionamento. Tal conhecimento contribuiria para o desenvolvimento de meios de cultivo que atendessem de maneira efetiva às exigências do processo de maturação *in vitro* (MIV), de forma a minimizar o impacto da perda da unidade folicular durante o cultivo. Não obstante, uma melhor eficiência da MIV beneficiaria biotécnicas de alta relevância sócio-econômica dependentes dela como a fecundação *in vitro* (FIV), a clonagem e a transgenia. Portanto, além de gerarem conhecimentos básicos valiosos, estudos aprofundados sobre a diferenciação dos oócitos e das células do cumulus podem contribuir para o aperfeiçoamento de biotécnicas da reprodução, o que demonstra sua importância prática.

A comunicação entre as células do cumulus e o oócito é bidirecional e essencial para a maturação nuclear e citoplasmática. Conseqüentemente, ela viabiliza a competência do oócito para a fecundação e geração de um embrião com alto potencial de desenvolvimento (Tanghe *et al*., 2002; Fair, 2003). O oócito participa ativamente dos mecanismos reguladores da maturação do COC via secreção de fatores parácrinos que agem em cooperação com os hormônios para controlar a diferenciação das células somáticas adjacentes e, indiretamente, dele próprio (Eppig, 2001; McNatty *et al.*, 2004, Gilchrist *et al.*, 2008). Dentre os fatores secretados pelo oócito (FSOs), a proteína morfogênica óssea 15 (BMP15) e o fator de crescimento e diferenciação 9 (GDF9) têm concentrado a maior parte das pesquisas e têm sido

associados com a melhora da competência para o desenvolvimento do COC (Juengel *et al*., 2004; Gilchrist *et al*., 2008).

Os fatores de crescimento fibroblástico (FGFs) constituem outro grupo de potenciais moléculas sinalizadoras derivadas do oócito. Recentemente foi demonstrada a expressão do FGF8, 10 e 17 em oócitos bovinos (Buratini *et al*., 2005; Buratini *et al*, 2007, Machado *et al*., 2009), possivelmente envolvidos na regulação da diferenciação das células do cumulus, já que elas expressam receptores para esses FGFs (Cho *et al.,* 2008; Zhang *et al*., 2010). O FGF10 é membro da subfamília FGF7, juntamente com o fator homônimo e com os FGFs 3 e 22, com quem compartilha características estruturais e afinidade pelos mesmos receptores (FGFR1B e FGFR2B; Itoh e Ornitz, 2004). Recentemente, foi demonstrado em bovinos que a adição de FGF10 durante a MIV de COCs estimula a expansão das células do cumulus e eleva as taxas de produção embrionária (Zhang *et al*., 2010). Em adição, já foi demonstrado que os FGFs estão envolvidos na regulação do metabolismo energético das células do cumulus em camundongos, uma vez que o FGF8 oocitário, em sinergismo com a BMP15, estimulou a expressão e atividade de enzimas glicolíticas nas células do cumulus (Sugiura *et al*., 2007).

Sendo assim, este trabalho teve como objetivo avaliar a participação e os mecanismos de ação da BMP15 e do FGF10 no controle da expansão das células do cumulus bovinas. Foi testada a hipótese de que estes fatores de crescimento estimulam a expressão de genes específicos na cascata que induz a expansão do cumulus. Em adição, foram avaliados os efeitos do FSH sobre a expressão dos genes envolvidos na expansão do cumulus e na sinalização de FSOs durante a MIV em bovinos.

2 REVISÃO DE LITERATURA

2.1 Interação entre o oócito e as células somáticas adjacentes

Os oócitos de mamíferos crescem e se desenvolvem em uma íntima relação de dependência com as células somáticas adjacentes (Eppig, 2001). É de conhecimento comum que as células somáticas dão suporte para o desenvolvimento do oócito, fornecendo nutrientes essenciais (Buccione *et al*., 1990a). Por muito tempo acreditou-se que o oócito tivesse um comportamento passivo na unidade folicular, apenas recebendo influências das células somáticas adjacentes. No entanto, nos últimos anos tornou-se evidente que o oócito é um regulador central da diferenciação e funções das células foliculares ao longo da foliculogênese. O oócito participa ativamente de processos fundamentais, tais como a oogênese, formação e ativação do *pool* de folículos primordiais, transição de folículo primário para secundário, transição de folículo pré-antral para antral, proliferação e diferenciação das células da granulosa, taxa de ovulação e fertilidade nas fêmeas mamíferas (Eppig, 2001; McNatty *et al*., 2004; Gilchrist *et al*., 2008; Su *et al*., 2009).

Durante a transição de folículos pré-antrais para o estágio antral, as células da granulosa são diferenciadas em duas populações anatômica e funcionalmente distintas: as células do cumulus, que estão diretamente associadas ao oócito, e as células da granulosa murais, que revestem internamente a parede folicular. Apesar da origem comum desses dois tipos celulares, existem diferenças quanto à produção de transcritos e proteínas (Latham *et al*., 1999). As células do cumulus são especializadas em oferecer suporte nutricional para o oócito, controlando seu crescimento e metabolismo (Haghighat e Van Winkle, 1990). Enquanto a função principal das células da granulosa murais é a produção de esteróides e a diferenciação em células luteínicas (Albertini *et al*., 2001).

Para a produção de um oócito apto a ser fecundado e competente para gerar um embrião viável, os compartimentos germinativo e somático do folículo precisam responder a sinais endócrinos, parácrinos e autócrinos, assim como aos sinais gerados através das junções *gap* que ligam as células foliculares ao oócito (Eppig, 2001). Uma das estratégias de comunicação entre o oócito e as células somáticas são os processos citoplasmáticos trans-zonais (TZP), extensões das células da granulosa/cumulus que penetram através da zona pelúcida e atingem a membrana do oócito, formando as junções do tipo *gap*. Tais junções permitem que o oócito permaneça fisica e metabolicamente ligado às células somáticas vizinhas durante a maior parte da foliculogênese (Albertini *et al*., 2001). Formadas por

proteínas da família das conexinas (Grazul-Bilska *et al*., 1997), as junções gap entre o oócito e células do cumulus têm como principal componente a conexina 37 (Cx37), enquanto aquelas que conectam as células da ganulosa murais e as células do cumulus são constituídas principalmente pela Cx43 (Simon *et al*., 1997; Gittens *et al*., 2003). De maneira geral, as propriedades das junções *gap* permitem o transporte direto e bidirecional de moléculas de pequeno peso molecular, tais como íons, metabólitos, aminoácidos e pequenas moléculas de sinalização intracelular (AMPc, GMPc e IP3) das células do cumulus para o oócito (Buccione *et al*., 1990a; Albertini *et al*., 2001).

A comunicação entre o oócito e as células somáticas foliculares também ocorre por sinalização parácrina (revisado por Gilchrist *et al*., 2008). Por meio de fatores de crescimento solúveis, o oócito modula o funcionamento e a diferenciação das células do cumulus e da granulosa de forma a controlar cuidadosamente o seu próprio microambiente e desenvolvimento (Eppig, 2001; Gilchrist *et al*., 2004; McNatty *et al*., 2004). Tem sido proposto que uma das principais funções dos FSOs durante a fase antral da foliculogênese é direcionar a diferenciação das células da granulosa em células do cumulus e manter seu distinto fenótipo e funções dentro do folículo (Eppig *et al*., 1997; Li *et al*., 2000). A manutenção deste fenótipo é dirigida através de uma ação bastante localizada dos FSOs dentro do folículo antral, na qual as células do cumulus são o alvo principal do oócito. Já as células da granulosa murais são menos influenciadas pelos FSOs, pois, ao contrário, elas seriam redirecionadas a funcionar como as células do cumulus (Hussein *et al*., 2005).

As consequências da sinalização dos FSOs nas células do cumulus são numerosas e incluem: estímulo ao crescimento (Gilchrist *et al*., 2006), prevenção da apoptose (Hussein *et al*., 2005), inibição da luteinização (Eppig *et al*., 1997; Li *et al*., 2000), regulação do metabolismo energético (Sugiura *et al*., 2005), biossíntese do colesterol (Su *et al*., 2008) e a regulação da expansão das células do cumulus (Buccione *et al*., 1990b). O oócito parece direcionar as células do cumulus a executar funções que ele próprio é incapaz de realizar, como por exemplo, a glicólise (Biggers *et al*., 1967; Sutton-McDowall *et al*., 2010). Assim, a capacidade do oócito em controlar tais funções nas células do cumulus provavelmente seja uma importante característica para a aquisição de sua competência para o desenvolvimento (Gilchrist *et al*., 2011).

A identificação dos FSOs e a elucidação de seus alvos e mecanismos de ação são de extrema importância para a compreensão de como o oócito controla o desenvolvimento das células somáticas (Su *et al*., 2009). Muitas pesquisas têm focado os membros da superfamília de fatores de crescimento transformante β (TGFβ), especialmente o GDF9 e a BMP15 como

constituintes chave dos FSOs (Gilchrist *et al*., 2004, Juengel *et al*., 2004, Shimasaki *et al*., 2004). Tem sido demonstrado que o GDF9 e a BMP15 são importantes reguladores da proliferação, esteroidogênese, apoptose e expansão das células do cumulus e favorecem a competência do oócito para a fecundação e desenvolvimento embrionário (Eppig, 2001; Gilchrist *et al*., 2004; Juengel *et al*., 2004; Hussein *et al*., 2006; Yeo *et al*., 2008; Hussein *et al*., 2011). Em adição, a expressão alterada ou bloqueada desses fatores causa danos severos à função ovariana e à fertilidade (McNatty *et al*., 2004; Gilchrist *et al*., 2004).

Além dos TGFβ, outros FSOs têm sido reconhecidos como participantes do controle parácrino da diferenciação das células do cumulus, dentre os quais destaca-se a família dos FGFs. A expressão do FGF8, 10 e 17 nos oócitos de roedores e bovinos (Valve *et al*, 1997; Buratini *et al*., 2005; Buratini *et al*., 2007, Machado *et al*. 2009), bem como de seus receptores FGFR1B, FGFR2B, FGFR2C e FGFR3C nas células do cumulus (Cho *et al*., 2008, Zhang *et al*., 2010), sugere o envolvimento dos FGFs na sinalização parácrina entre estes dois tipos celulares. No decorrer desta revisão os FSOs, BMP15 e FGF10, serão abordados mais detalhadamente.

A melhor compreensão da comunicação bidirecional entre o oócito e as células somáticas foliculares pode ser utilizada para beneficiar as metodologias da MIV e, consequentemente, melhorar a produção embrionária após a fecundação e cultivo *in vitro*.

2.2 Importância das células foliculares na maturação oocitária

A MIV é uma técnica de reprodução assistida em que oócitos imaturos coletados de folículos antrais são maturados *in vitro* para a posterior fecundação e produção embrionária (Hardy *et al*., 2000). Apesar dos avanços no processo de produção *in vitro* de embriões bovinos, os resultados da MIV são inferiores quando comparados à maturação *in vivo* (Rizos *et al*., 2002). Mesmo com esta limitação, o uso das técnicas de maturação e produção embrionária *in vitro* no setor da bovinocultura tem aumentado grandemente (Thibier, 2006), o que justifica o incentivo em melhorar a eficiência da MIV. Este incentivo se aplica também à reprodução humana. Na espécie humana uma grande vantagem da MIV em relação à técnica de FIV convencional é a redução do uso de hormônios estimulatórios, diminuindo os riscos de efeitos secundários adversos e os custos para as pacientes (Jurema e Nogueira, 2006). Porém, o sucesso da MIV em humanos é significantemente menor comparado com a FIV após protocolos hormonais que levam à maturação *in vivo* dos oócitos (Banwell e Tompson, 2008).

Este fator restringe o uso da MIV em humanos e justifica os estudos para o aprimoramento desta técnica (Gilchrist *et al*., 2008).

Tem sido postulado que a baixa competência de oócitos maturados *in vitro* decorre principalmente da remoção dos COCs do ambiente intra-folicular. Tal procedimento compromete a comunicação entre o oócito e as células somáticas, além de impossibilitar a ação de diversos fatores reguladores da maturação nuclear e citoplasmática que estão presentes no fluido folicular (Lonergan *et al*., 1994; Coticchio *et al*., 2004; Krisher, 2004). A maturação do oócito *in vivo* é um processo complexo, controlado pelas gonadotrofinas e peptídeos intra-foliculares. A ação de peptídeos intra-foliculares no processo de maturação oocitária tem sido amplamente reportada (Aktas *et al*., 1995; Tsafriri *et al*., 1996). É sabido que o ambiente folicular é responsável por manter o oócito em prófase I (estágio de vesícula germinativa) durante a foliculogênese, assim como pela retomada da meiose no oócito durante a maturação. Dentre os fatores envolvidos na regulação meiótica, destaca-se o AMP cíclico (AMPc), o qual pode ser sintetizado pelo próprio oócito ou também fornecido pelas células da granulosa murais e pelas células do cumulus através das junções *gap*. Altos níveis intra-oocitários de AMPc mantem o oócito em vesícula germinativa através a supressão da atividade do fator promotor da maturação (MPF; Aktas *et al*., 1995; Tsafriri *et al*., 1996; Bilodeau-Goeseels, 2011). Em adição, as células somáticas do folículo também fornecem GMP cíclico (GMPc) para o oócito, o qual inibe a fosfodiesterase do tipo 3 (PDE3), uma enzima que degrada o AMPc (Tsafriri *et al*., 1996).

Além da influência dos peptídeos intra-foliculares, as células da teca e da granulosa são alvos das gonadotrofinas e mediam suas ações no controle da maturação do oócito (Feuerstein *et al*., 2007). O pico pré-ovulatório de LH induz mudanças no folículo ovariano que desencadeiam a maturação do oócito, expansão das células do cumulus, ruptura da parede folicular e liberação do COC (revisado por Richards *et al*., 2002). Apesar da reconhecida importância do LH, informações mais detalhadas sobre o exato mecanismo molecular envolvido na maturação ainda se fazem necessárias. Recentemente demonstrou-se que o pico pré-ovulatório de LH induz um rápido e transitório aumento na produção dos membros da família de fatores de crescimento epidermal (EGF)-*like* [ampiregulina (AREG), epiregulina (EREG) e betacelulina (BTC)] nas células somáticas do folículo (Park *et al*., 2004). Os fatores de crescimento EGF-*like* ativam os receptores de EGF (EGFR) presentes nas células da granulosa e células do cumulus, desencadeando a fosforilação da ERK1/2, essencial para transmitir os sinais intracelulares que ativam a retomada da maturação meiótica do oócito e expansão das células do cumulus (Sela-Abramovich *et al*., 2005; Fan *et al*., 2009).

2.3 Fatores EGF-*like*

A família de proteínas EGF-*like* inclui, entre outros, o fator de crescimento epidermal (EGF), o fator de crescimento transformante α (TGFα), o fator de crescimento semelhante ao EGF ligado a heparina (HB-EGF), a AREG, a EREG, a BTC e as neuregulinas (revisado por Conti *et al*., 2006). Inicialmente, o EGF foi descrito como um potente indutor da expansão das células do cumulus em ratos (Dekel e Sherizly, 1985). Posteriormente, outros estudos demonstraram efeito positivo do EGF na MIV de COCs em ratos (Harper e Bracket, 1993; Lorenzo *et al*., 1996), camundongos (Das *et al*., 1992) e bovinos (Lonergan *et al*., 1996; Rieger *et al*., 1998). Sakaguchi *et al*. (2000) observaram uma aceleração da retomada da meiose em COCs bovinos cultivados na presença de EGF, o que não ocorreu em oócitos desnudos, sugerindo que o EGF participa do controle da maturação nuclear de forma indireta via células do cumulus. Desta forma, supõe-se que a expressão do EGFR nas células do cumulus seja requerida para que o EGF desempenhe suas funções (Conti *et al*., 2006). De fato, a expressão do EGFR foi detectada por imunofluorescência e RT-PCR em células do cumulus de bovinos (Zhao *et al*., 2005) caprinos (Gall *et al*., 2004), suínos (Chen *et al*., 2008), humanos (Maruo *et al*., 1993) e ratos (Chabot *et al*., 1986).

Além do EGF, outros membros da família EGF-*like*, como a AREG, EREG e BTC, têm sido estudados no contexto da maturação do COC. Como citado anteriormente, é conhecido que o pico de LH desencadeia a maturação do oócito e a expansão do cumulus (Richards *et al*., 2002). No entanto, este efeito do LH parece ser indireto, conforme indica o padrão de expressão de seu receptor (LHR). O LHR está expresso nas células da teca e nas células da granulosa murais, porém é ausente ou praticamente ausente nas células do cumulus e oócitos de folículos pré-ovulatórios de bovinos e camundongos (Peng *et al*., 1991; van Tol *et al*., 1996; Nogueira *et al*. 2007). Um grande avanço na compreensão desta questão ocorreu quando Park e colaboradores (2004) mostraram que o pico de LH induz a expressão dos fatores EGF-*like* (AREG, EREG e BTC) nas células da granulosa murais em decorrência da sinalização pelo LHR. Sendo assim, foi proposto um modelo de ação parácrina e autócrina dos fatores EGF-*like* como mediadores das ações do LH (Hsieh e Conti, 2005; Figura 1). Neste modelo, o LH interage com seu receptor nas células da granulosa murais de folículos pré-ovulatórios e ativa a cascata de sinalização AMPc/PKA (Freimann *et al*., 2004), estimulando a expressão do RNAm da AREG, EREG e BTC. Estes fatores são sintetizados como precursores transmembranicos e necessitam ser clivados pelos membros da família desintegrina e metaloproteinases (ADAMs) para serem liberados da superfície celular em sua

forma ativa (Ben-Ami *et al*., 2006). Os membros da família de proteases ADAMs são sugeridos como os principais reguladores da liberação proteolítica dos fatores EGF-*like* em formas solúveis. Em especial, a ADAM17 é comumente responsabilizada pela liberação da AREG, EREG, HB-EGF e TGFα em várias linhagens celulares distintas (Hsieh e Conti, 2005; Yamashita *et al*., 2009). No entanto, outros estudos indicam que mais de uma ADAM participa da regulação do processamento proteolítico dos ligantes específicos do EGFR, como as ADAM 9, 10 e 12 (Hsieh e Conti, 2005).

Em continuidade ao modelo proposto, os fatores EGF-*like*, liberados como moléculas solúveis, ativam os EGFR nas células da granulosa e do cumulus e estimulam através da via de sinalização MAPK/ERK a retomada da meiose, maturação do oócito e expressão de genes promotores expansão das células do cumulus (Ashkenazi *et al*., 2005; Conti *et al*., 2006). A indução da retomada da meiose induzida pela ERK1/2 não está completamente caracterizada, mas, provavelmente, envolve a fosforilação da Cx43 estimulando o fechamento das junções *gap* com subsequente perda do efeito inibitório do AMPc e GMPc foliculares (Gilchrist *et al*., 2011). Já a expansão das células do cumulus, também estimulada pelos fatores EGF-*like*, está associada à indução da expressão da ciclooxigenase 2 (COX2), da hialurona sintetase 2 (HAS2), da proteína indutora do fator de necrose tumoral 6 (TSG6) e da pentraxina 3 (PTX3), considerados genes críticos para o remodelamento da matriz extracelular nos COCs e para a ruptura do folículo *in vivo* (Park *et al*., 2004; Ashkenazi *et al*., 2005; Shimada *et al*., 2006).

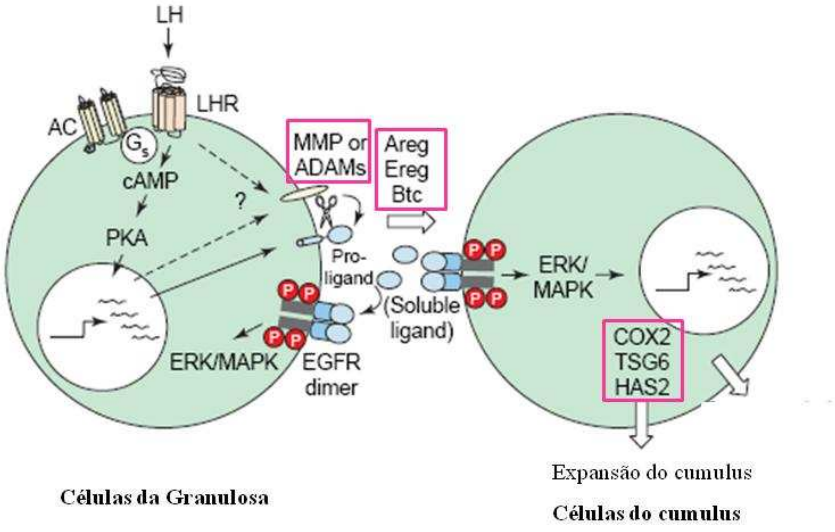

Figura 1 – Modelo da regulação *in vivo* do sistema EFG-*like* pelo LH nas células da granulosa e nas células do cumulus, estimulando a retomada da meiose no oócito e a expansão do cumulus (adaptado de Hsieh e Conti, 2005).

Estudos sugerem que os fatores EGF-*like* sejam também mediadores das ações do FSH *in vitro*. Freimann e colaboradores (2004) mostraram que o FSH estimula a expressão da AREG e EREG em células da granulosa humanas após cultivo por 24 horas. Interessantemente, COCs de camundongos cultivados com FSH e expostos a anticorpo anti-EGFR ou a bloqueador do EGFR (AG1478) tiveram a retomada da meiose completamente inibida, corroborando a hipótese de que as ações do FSH são mediadas pelos fatores EGF-*like* (Downs e Chen, 2008). Este mesmo trabalho demonstrou ainda que o FSH induz acúmulo da proteína AREG nas células do cumulus. Contudo, não existem informações sobre o padrão temporal de expressão gênica dos fatores EGF-*like* ou sobre sua regulação pelo FSH nas células do cumulus durante a MIV de COCs bovinos.

2.3.1 Genes alvo dos fatores EGF-like na indução da expansão das células do cumulus

O processo de expansão do cumulus envolve a secreção de uma matriz rica em ácido hialurônico pelas células do cumulus e a expressão de uma variedade de proteínas necessárias para a síntese e estabilidade da matriz (Buccione *et al*., 1990b; Hess *et al*., 1999; Yamashita *et al*., 2009). A HAS2, um gene alvo dos fatores EGF-*like*, é considerada a principal enzima responsável por controlar a síntese de ácido hialurônico durante a expansão do cumulus em mamíferos (Schoenfelder e Einspanier, 2003). O ácido hialurônico pode ser formado a partir de compostos como as hexosaminas, as glicosaminas e a glicose (Chen *et al*., 1990). A estrutura e estabilidade deste grande e linear mucopolissacarídeo são garantidas pela interação com diferentes proteínas produzidas pelo próprio cumulus ou originárias do soro (Chen *et al*., 1992). Tem sido sugerido que a síntese de ácido hialurônico e a mucificação do cumulus contribui para a penetração e fecundação dos oócitos pelos espermatozóides através da participação nos processos de capacitação espermática e/ou reação acrossomal (Slotte *et al*., 1993; Gutnisky *et al*., 2007).

A COX2 (ou prostaglandina sintetase 2; PTGS2), também considerada um gene alvo dos fatores EGF-*like*, é uma enzima responsável por controlar a síntese de prostaglandinas (PG), como a PGE2. A COX2 é produzida pelas células do cumulus e pelas células da granulosa murais e é necessária para a completa expansão do cumulus e para o processo de ovulação (Lim *et al*., 1997). Em ovários de mamíferos duas isoformas da enzima COX já foram identificadas: COX1, que é expressa de forma constitutiva nas células da teca; e a COX2, cuja expressão é induzida pelo LH nas células da granulosa murais e nas células do

cumulus (Ochsner *et al*., 2003). Durante o período pré-ovulatório, a produção folicular de PGs derivadas da COX2 é crítica para a expansão do cumulus e maturação do oócito (Hizaki *et al*., 1999). De fato, estudos prévios mostram uma função da PGE2 no estímulo da expansão do cumulus *in vitro* em camundongos e bovinos (Eppig, 1981; Calder *et al*., 2001). Em adição, a PGE2 foi capaz de estimular a progressão do ciclo celular durante a maturação oocitária *in vitro* e o desenvolvimento embrionário inicial em bovinos (Nuttinck *et al*., 2011). Em camundongos a diminuição da atividade da COX2 compromete a expansão do cumulus, a completa maturação do oócito e a ovulação (Lim *et al*., 1997; Davis *et al*., 1999).

Adicionalmente, o silenciamento da COX2 ou o comprometimento da sinalização das prostaglandinas prejudicou a expressão da TSG6 em camundongos e suínos (Oshsner *et al*., 2003; Takahashi *et al*., 2006; Yamashita *et al*., 2011). A TSG6 é uma proteína de ligação ao ácido hialurônico que estabiliza a estrutura da hialurona na matriz do cumulus expandido (Richards, 2005). A TSG6 não somente se liga à hialurona como também forma um complexo estável com as cadeias pesadas do fator derivado do soro inibidor inter-α-tripsina (IαI) durante a expansão do COC. Assim, as cadeias pesadas tornam-se covalentemente ligadas à hialurona na matriz do cumulus e essas ligações contribuem para a estabilidade da matriz (Chen *et al*., 1996; Fulop *et al*., 2003; Figura 2). No ovário, o RNAm da TSG6 é rapidamente induzido nas células da granulosa e nas células do cumulus de folículos pré-ovulatórios após o pico de LH (Ochsner *et al*., 2003). Camundongos com deficiência de TSG6 são incapazes de estruturar uma matriz extracelular rica em hialurona, provocando falha na expansão do cumulus e, consequentemente, infertilidade (Fulop *et al*., 2003).

A PTX3, outro gene alvo dos fatores EGF-*like*, também é essencial para a estabilidade da matriz do cumulus. Esta proteína tem afinidade de ligação à TSG6, e esta interação parece contribuir na estruturação da matriz do cumulus (Richards, 2005). Em camundongos e humanos, o RNAm da PTX3 é estimulado nas células do cumulus nos momentos que precedem a ovulação (Varani *et al*., 2002; Scarchilli *et al*., 2007). Camundongos com deficiência de PTX3 apresentaram instabilidade na matriz expandida e infertilidade (Varani *et al*., 2002; Scarchilli *et al*., 2007).

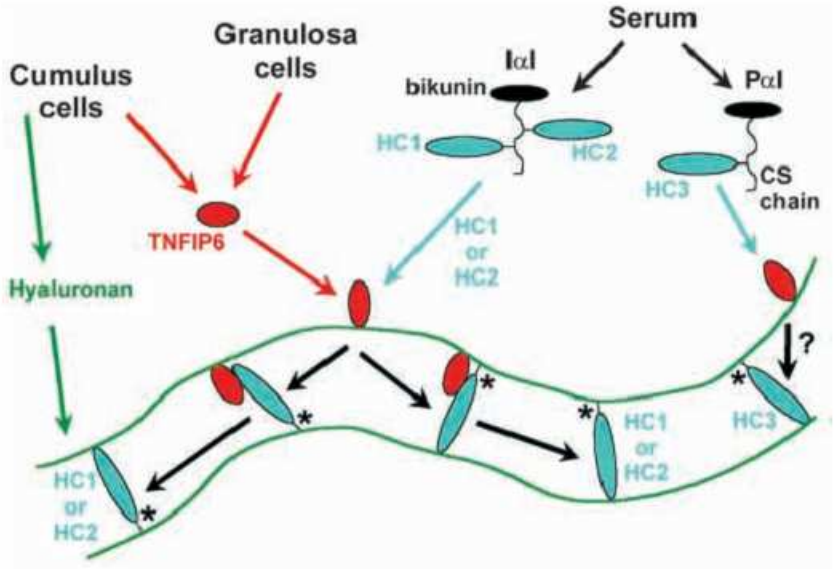

Figura 2 – Modelo esquemático para a função proposta da TSG6 na estabilização da matriz extracelular do cumulus. A TSG6 forma um complexo com as cadeias pesadas do fator derivado do soro inibidor inter-α-tripsina (HC-TSG6) que contribui para a estabilização da matriz como resultado de uma forte afinidade da TSG6 em se ligar ao ácido hialurônico (Fulop *et al*., 2003).

2.4 Fatores de crescimento secretados pelos oócitos

2.4.1 FGF10

Em mamíferos, os FGFs compõem uma família de pelo menos 22 membros (Zhang *et al*., 2006). Tais fatores apresentam padrões temporais e espaciais de expressão específicos e estão envolvidos na regulação de diversos processos biológicos como atividade mitogênica, quimiotática e angiogênica em diversos tipos de células e tecidos (Basilico e Moscatelli, 1992). Essas moléculas estão expressas em estágios iniciais e tardios do desenvolvimento embrionário e também em tecidos adultos, o que indica que elas atuam como fatores de crescimento e diferenciação celular durante toda a vida (Igarashi *et al*., 1998).

Cinco genes distintos codificam receptores de alta afinidade que interagem com os membros da família FGF (FGFR1-5; Sleeman *et al*., 2001; Kim *et al*., 2001). Os genes FGFR1 a 4 codificam receptores do tipo tirosina-quinase localizados na membrana plasmática. Arranjos transcricionais alternativos (“alternative splicing”) do FGFR1, FGFR2 e FGFR3 possibilitam a formação das isoformas funcionais (“B” e “C”), que apresentam diferentes graus de afinidade pelos diversos FGFs (Chellaiah *et al*., 1994; Ornitz *et al*., 1996).

Os FGFs são classificados em sete subfamílias de acordo com análises filogenéticas: sub-famílias do FGF1, FGF4, FGF7, FGF8, FGF9, iFGF e hFGF, (Itoh e Ornitz, 2004). O FGF10, também conhecido como KGF-II (fator de crescimento dos queratinócitos II), pertence à subfamília do FGF7 (FGF3, 7, 10 e 22), cujos membros interagem preferencialmente com dois receptores, o FGFR2B e o FGFR1B (Itoh e Ornitz, 2004; Zhang *et al*., 2006). O FGF10 foi originalmente isolado do mesênquima pulmonar de ratos e é essencial para regulação da organogênese (Igarashi *et al*., 1998), o que pode ser confirmado pela ausência completa de pulmões em camundongos nocaute para o FGF10 (Min *et al*., 1998; Sekine *et al*., 1999).

No que se refere à fisiologia reprodutiva, destaca-se a detecção da expressão do FGF10, juntamente com a do FGF7, no útero neonatal ovino. Acredita-se que ambos participem da regulação da morfogênese endometrial, onde o FGF10 atuaria como fator quimiotático direcionador do crescimento e ramificação glandular, e o FGF7 estimularia a proliferação de células epiteliais (Taylor *et al*., 2001).

A expressão do FGF10 foi recentemente detectada em oócitos e células da teca de folículos antrais bovinos (Buratini *et al*., 2007). Apesar da similaridade estrutural entre o FGF7 e o FGF10, seus padrões de expressão diferem no folículo, já que o FGF7 não é expresso em oócitos (Buratini *et al*., 2007). A expressão do FGF10 mostrou-se diminuída em folículos saudáveis estrogênicos e o tratamento com FGF10 inibiu a produção de estradiol de células da granulosa (Buratini *et al*., 2007). A detecção de receptores para o FGF10 nas células da granulosa murais (Berisha *et al*., 2004) e do cumulus (Cho *et al*., 2008; Zhang *et al*., 2010) condiz com a ação parácrina do FGF10, oriunda do oócito e das células da teca, com alvo nas células da granulosa murais e do cumulus.

Recentemente, Zhang e colaboradores (2010) demonstraram que a suplementação do meio de maturação com FGF10 aumenta a porcentagem de oócitos com extrusão do primeiro corpúsculo polar após a maturação, a expansão das células do cumulus e as taxas de desenvolvimento embrionário. Porém, os mecanismos que viabilizam tais efeitos estimulatórios do FGF10 ainda não foram elucidados. É provável que o FGF10 produzido pelo oócito atue nas células do cumulus de forma a melhorar a sua capacidade de controlar a maturação oocitária e o subsequente desenvolvimento embrionário inicial (Zhang *et al*., 2010).

2.4.2 Fatores de crescimento transformantes β (TGFβ)

Os membros da superfamília dos TGFβ são considerados importantes peptídeos reguladores intraovarianos (Findlay *et al*., 2002; Knight e Glister 2003; Juengel e McNatty, 2005). Dentre os membros desta superfamília, a BMP15 e o GDF9 têm concentrado a maior parte da atenção direcionada aos FSOs (Juengel *et al*., 2004; Gilchrist *et al*., 2008). Tais fatores parecem ser cruciais para o desenvolvimento folicular e ovulação, além de participarem da regulação parácrina da maturação oocitária e da diferenciação das células da granulosa e do cumulus (Juengel e McNatty, 2005; Su *et al*., 2009). Tanto a BMP15 quanto o GDF9 exercem seus efeitos biológicos através da ligação a um receptor do tipo I, referido como um receptor ativina semelhante a kinase (ALK) e ao receptor de BMP do tipo II (BMPRII; Moore *et al*., 2003; Mazerbourg *et al*., 2004). A BMP15 age via BMPRII e ALK6 (também chamado receptor de BMP tipo 1B; BMPRIB) e ativa a fosforilação intracelular da SMAD1/5/8 (Moore *et al*., 2003). Em contraste, o GDF9 se liga ao BMPRII e ALK5 (também chamado receptor TGFβ tipo I), levando à ativação das SMADs2/3 (Mazerbourg *et al*., 2004). As SMADs fosforiladas se translocam para o núcleo onde interagem com reguladores transcricionais e induzem a expressão de genes alvo (Gilchrist *et al*., 2008).

Embora a expressão oocitária de BMP15 e GDF9 seja necessária para a fertilidade nas fêmeas (Dong *et al*., 1996; Hanrahan *et al*., 2004), existem notáveis variações entre as espécies quanto ao requerimento destes fatores. Enquanto em ovinos a expressão de ambos os fatores é fundamental para a foliculogênese normal (Hanrahan *et al*., 2004), em camundongos, uma deleção específica da BMP15 não provoca defeitos na foliculogênese. No entanto, esses camundongos exibem defeitos no processo de ovulação, bem como na qualidade dos oócitos, diminuindo assim as taxas de fecundação (Yan *et al*., 2001). Além disso, a expressão em heterozigose da BMP15 e do GDF9 causa um aumento na taxa de ovulação e na fertilidade em ovelhas (Hanrahan *et al*., 2004; Juengel e McNatty, 2005), embora não afete a fertilidade em roedores (Dong *et al*., 1996; Yan *et al*., 2001).

Em adição, a BMP15 e o GDF9 aumentam a competência para o desenvolvimento dos COCs (Juengel *et al*., 2004; Gilchrist *et al*., 2008). A administração de BMP15 ou GDF9 durante a MIV dos COCs estimulou a expansão do cumulus em camundongos e bovinos (Elvin *et al*., 1999; Leyens *et al*., 2004; Dragovic *et al*., 2005; Yoshino *et al*., 2006) e a produção de blastocistos em bovinos (Hussein *et al*., 2006, 2011). Além disso, a adição de GDF9 no meio de maturação aumentou significantemente a taxa de sobrevivência após a transferência de blastocistos em camundongos (Yeo *et al*., 2008).

Conforme já mencionado, Sugiura e colaboladores (2007) demostraram ações sinérgicas entre a BMP15 e o FGF8 no controle do metabolismo energético das células do cumulus em camundongos. Contudo, ainda são necessários estudos sobre a interação entre FGFs e a BMP15 no controle de outros processos pertinentes à maturação do COC. Portanto, a avaliação dos efeitos da associação do FGF10 com a BMP15 sobre a expansão das células do cumulus e controle transcricional de genes envolvidos em tal processo foi inserida neste trabalho.

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CAPÍTULO 2

BONE MORPHOGENETIC PROTEIN 15 (BMP15) AND FIBROBLAST GROWTH FACTOR 10 (FGF10) ENHANCE CUMULUS EXPANSION AND DIFFERENTLY REGULATE GENE EXPRESSION IN BOVINE CUMULUS CELLS

O presente artigo foi submetido para publicação no periódico *Fertility and Sterility* e encontra-se de acordo com as normas de submissão exigidas pelo periódico, exceto a apresentação das referências bibliográficas.

Running Title: BMP15 & FGF10 enhance cumulus expansion

Bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 10 (FGF10) enhance cumulus expansion and differently regulate gene expression in bovine cumulus cells

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FUNDING

This work was supported by the “Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)” and the “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)”, Brazil.

Capsule: Using a bovine model of *in vitro* oocyte maturation, we demonstrate that BMP15 enhances cumulus expansion by increasing expression of cumulus EGF-like ligands, whereas FGF10 does so by acting upon COX2.

ABSTRACT

Oocyte secreted factors (OSFs) regulate differentiation of cumulus cells and thus are of pivotal relevance for fertility. Bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 10 (FGF10) are OSFs and enhance oocyte competence by unknown mechanisms. We tested the hypothesis that BMP15 and FGF10, alone or in synergism, enhance cumulus expansion in cattle, a valuable model for the investigation of human reproductive biology. BMP15 and FGF10 alone increased the percentage of fully expanded cumulus-oocyte complexes, but in combination did not further augment it. We then assessed the effects of BMP15 and FGF10 on mRNA expression of a variety of genes that regulate cumulus expansion (e.g., *AREG*, *EREG*, *BTC*, *EGFR*, *ADAM10*, *ADAM17*, *COX2*, *HAS2*, *PTX3* and *TSG6*), as well as of themselves and their receptors in cumulus cells. The expression patterns of cumulus regulating genes were assessed during *in vitro* maturation (IVM), and with the exception of *BTC* mRNA, which levels decreased with time in culture, abundance of all transcripts was up-regulated during IVM. BMP15 increased mRNA expression of *ADAM10*, *ADAM17*, *AREG*, *EREG*, *HAS2* and *FGFR1B* at 12 hours of culture, and of *COX2*, *PTX3*, *TSG6*, *BMPRII* and *ALK6* at 22 hours of culture. FGF10 did not alter the expression of EGF-like factors, OSFs or receptors, but enhanced mRNA expression of *COX2* at 4 hours, *PTX3* at 12 hours, and *TSG6* at 22 hours. This study provides evidence that BMP15 and FGF10 enhance cumulus expansion differently, the first acting upon *ADAM10*, *ADAM17*, *AREG* and *EREG*, and the second on downstream genes, particularly *COX2*.

Key words: BMP15 / cumulus expansion / FGF10 / EGF-like ligands / COX2

INTRODUCTION

A potentially key component of assisted reproduction is *in vitro* maturation (IVM) of cumulus–oocyte complexes (COC), but this technique has poor success rates in humans compared with other species such as cattle (Gilchrist *et al*., 2008). Clearly, more detailed information is required on the cascade of molecular events that lead to resumption of meiosis and expansion of the COC. The main trigger of COC expansion is LH (reviewed by Richards *et al*., 2002). The effects of LH on the COC are mediated by epidermal growth factor (EGF)-like family members, amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC), which are secreted by mural granulosa cells in response to LH and act upon cumulus cells, where they stimulate their own synthesis (Park *et al*., 2004; Ashkenazi *et al*., 2005; Conti *et al*., 2006). FSH can also stimulate directly the synthesis of EGF-like growth factors in cumulus cells (Downs and Chen, 2008). EGF-like growth factors are synthesized as transmembrane precursors and must undergo proteolytic cleavage ("shedding") by members of the disintegrin and metalloproteinase (ADAM) family (Ben-Ami *et al*., 2006a). These soluble molecules then activate the EGF receptor (EGFR) on cumulus cells and stimulate the expression of genes necessary for cumulus expansion, including hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (COX2), tumor necrosis factor-stimulated gene-6 protein (TSG6) and pentraxin 3 (PTX3; Ashkenazi *et al*., 2005; Conti *et al*., 2006; Shimada *et al*., 2006; Su *et al*., 2010).

The periovulatory cascade described above has been determined from studies in rodents, and some caution should be exercised when extrapolating to other species. Most obviously, the time from the LH surge to ovulation is shorter in rodents (12-14 h) in comparison with monovulatory species such as cattle (28-30 h), horses, humans and primates (38-42 h; Stouffer, 2002; Sirois *et al*., 2004; Mihm et al., 2011), which is directly related to species-specific intervals between the LH surge and the induction of *COX2* expression. The more rapid induction of *COX2* in mural granulosa cells from rodents compared to primates and cattle (Sirois *et al*., 2004) is likely due to the more rapid increase in *AREG* and *EREG* expression (Freimann *et al*., 2004; Park *et al*., 2004; Ben-Ami *et al*., 2006ab; Fru *et al*., 2007; Xu *et al*., 2011; Portela *et al*., 2011). These data suggest that the cow is a better model than the mouse for human ovulation. More strikingly, however, is a major difference in *BTC* expression between primates and rodents; LH/hCG increased *BTC* mRNA in mouse granulosa cells but decreased *BTC* mRNA levels in monkeys (Park *et al*., 2004; Fru *et al*., 2007).

Oocyte secreted factors (OSFs) regulate cumulus cell differentiation and function including sensitivity to gonadotropins, cumulus expansion, energetic metabolism,

steroidogenesis and vulnerability to apoptosis (Buccione *et al*., 1990; Eppig *et al*., 1997, 1998; Li *et al*., 2000; Hussein *et al*., 2005; Sugiura *et al*., 2005). Developmental competence of the COC is also impacted by OSFs, the most well-known of which are bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9) (Juengel *et al*., 2004; Gilchrist *et al*., 2008). BMP15 treatment during IVM stimulated cumulus expansion in mice (Yoshino *et al*., 2006) and blastocyst production in cattle (Hussein *et al*., 2006, 2011). Fibroblast growth factors (FGFs) have also been recognized as important OSFs; FGF8 is a major oocyte-specific factor in mice (Valve *et al*., 1997), and BMP15 and FGF8 interact to enhance glycolysis in mouse cumulus cells (Sugiura *et al*., 2007). However, in cattle FGF8 is expressed in granulosa cells as well as oocytes (Buratini et al., 2005). Another FGF of interest is FGF10, which was localized to the bovine oocyte, and its receptors (FGFR1B and FGFR2B) to cumulus cells (Buratini *et al*., 2007; Cho *et al*., 2008). Supplementation of the IVM medium with FGF10 enhanced cumulus expansion and blastocyst formation in cattle (Zhang *et al*., 2010).

In this study we first tested the hypothesis that BMP15 alone or in synergism with FGF10 would enhance cumulus expansion in cattle. Then, to shed light on the mechanisms by which these OSFs enhance cumulus expansion, we tested the effects of BMP15, FGF10 and the combination of both on expression of specific genes in the cascade that leads to cumulus expansion (e.g., *AREG*, *EREG*, *BTC*, *EGFR*, *ADAM10*, *ADAM17*, *COX2*, *HAS2*, *PTX3* and *TSG6*). In addition, we also assessed the effects of BMP15 and FGF10 on the expression of both OSFs in the oocyte and their receptors in cumulus cells.

MATERIALS AND METHODS

Unless specified, all chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

In vitro maturation

Ovaries of adult cows (predominantly Nellore, Bos indicus) were obtained at an abattoir local to the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline solution (0.9% NaCl) containing antibiotics (penicillin G; 100 IU/ml and streptomycin; 100 ug/ml) at 35-37°C. COCs were aspirated from 3 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 ml conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogenous cytoplasm and compact multilayer of cumulus cells were used (Grade 1 and 2). COCs were washed and

transferred in groups of 20 to a 100 µl drop of maturation medium, TCM199 containing Earle's salts supplemented with 1 ug/ml FSH (pFSH, Folltropin-V® Bioniche, ON, CA), 10 UI/ml LH (pLH, Lutropin-V®, Bioniche, ON, CA), 22 ug/ml sodium pyruvate, 75 ug/ml amicacin and 4 mg/ml BSA. Drops were covered with mineral oil and incubated at 38.5°C in 5% CO_2 in humidified air.

To assess the temporal expression patterns of the targeted cumulus expansion inducing genes (*AREG*, *EREG*, *BTC*, *ADAM10*, *ADAM17*, *COX2*, *HAS2*, *PTX3* and *TSG6*) during IVM, a time course experiment was designed. COCs were cultured under the conditions described above for 1, 4, 8, 12, 16 and 22 hours and cumulus cells were recovered for gene expression analysis from pools of 20 COCs (n=4/time point). Immature COCs were used to represent 0 hours.

To test the effects of OSFs on cumulus expansion and gene expression, maturation medium was supplemented with grading doses of recombinant human BMP15 (R&D Systems, Minneapolis, MN, USA; 0, 10, 50 and 100 ng/ml; n=4) or recombinant human FGF10 (R&D Systems; 0, 0.5, 10 and 50 ng/ml; n=4). To test the synergism between BMP15 and FGF10 in the regulation of cumulus expansion and gene expression, the maturation medium was supplemented separately with BMP15 (100 ng/ml; n=4), FGF10 (10 ng/ml; n=4) and BMP15 (100 ng/ml) plus FGF10 (10 ng/ml; n=4). These BMP15 and FGF10 doses were chosen as they were the lowest to enhance cumulus expansion in the previous dose response experiments. All treatments were tested at 4, 12 and 22 hours of culture; time points were chosen based on the time course experiment. Different times of culture were assessed in different experiments and thus treatments were compared within each time point but not between time points.

The effects of graded doses of BMP15, FGF10 and of their combination on cumulus expansion were tested at 22 hours of culture. Cumulus expansion was visually assessed according to a subjective scoring system. Grades 1 to 3 were attributed to increasing degrees of expansion (1-poor expansion, characterized by few morphological changes compared with before maturation; 2-partial expansion, characterized by fair expansion but notable clusters lacking expansion; 3-complete or nearly complete expansion; Zang *et al*., 2010).

Gene expression analysis

Cumulus cells and oocytes (n=20/group) were mechanically separated by repeated pipetting in PBS. Denuded oocytes were recovered and washed three times in PBS. Cumulus cells were transferred to a 1.5 ml tubes, centrifuged twice for 5 min at 700g, the supernatant

was discarded and 350 µl of the RNA extraction lysis buffer was added to the cell pellets. The cell suspension and oocytes were stored at -80°C until RNA extraction.

The cumulus cells from time course experiment and, both the oocytes and cumulus cells, from BMP15 and/or FGF10 experiments had its total RNA extracted using the RNeasy® kit (Qiagen, Mississauga, ON, CA) as recommended by the manufacturer. After purification, RNA samples were eluted in 30 µl of RNAse free water. The total RNA concentration in cumulus cells samples was measured by spectrophotometer using a NanoDrop ND® 1000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (100 ng/reaction for cumulus cells samples; entire RNA sample for oocyte samples) was incubated with DNAse I (1 U/µg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using Oligo-dT primers and according with the protocol provided by the Omniscript or Sensiscript kits (Qiagen, Mississauga, ON, CA) for cumulus and oocyte samples, respectively. The reagents were incubated at 37ºC for 60 min and then at 93º for 3 min for enzyme inactivation.

Relative real time RT-PCR analysis was performed with an ABI 7500 thermocycler using Power Sybr Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil). The final volume of the PCR mix was 25 µl and PCR cycling conditions were: 95°C for 10 min (1 cycle), denaturing at 95°C for 10 sec followed by annealing for 1 min (40 cycles). The primers sequences, amplicons sizes and annealing temperatures for each target gene are shown in Table I. Reactions were optimized to provide maximum amplification efficiency for each gene. Each sample was run in duplicates, and the specificity of the PCR products was assessed by melting curve analyses (except for FGF17) and amplicon size determined by electrophoresis in 2% agarose gels.

To select the most stable housekeeping gene, Cyclophilin-A (*CYC-A*), Glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and Histone H2AFZ (*H2AFZ*) amplification profiles were compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/genorm; Vandesompele *et al*., 2002); the most stable housekeeping gene was *CYC-A* for oocytes and cumulus cells.

The relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as calibrator (Pfaffl, 2001). Mean efficiency values for each gene were calculated from the amplification profile of individual samples with LinRegPCR software (Ramakers *et al*., 2003).

Statistical Analysis

Cumulus expansion data were transformed to arcsine, and gene expression data were transformed to logarithms when not normally distributed. The effects of time on gene expression and of treatments with BMP15 and/or FGF10 on cumulus cell expansion and gene expression were tested by analysis of variance (ANOVA), and means were compared with the Tukey-Kramer HSD test. The analyses were performed with JMP software (SAS Institute, Cary, NC, USA) and the results are presented as mean ± standard error of the mean (SEM). Differences were considered significant when $P<0.05$.

RESULTS

Effects of BMP15 and FGF10 on cumulus expansion

COCs were exposed to graded doses of BMP15 and FGF10 and cumulus expansion was recorded. The percentage of grade 3 COCs (complete or nearly complete cumulus expansion) was increased by BMP15 at 100 ng/ml (Fig. 1A) and by FGF10 at 10 ng/ml (Fig. 1B). A combination of BMP15 with FGF10 increased the percentage of fully expanded COCs cumulus expansion compared to the control, but did not further enhance cumulus expansion compared to either BMP15 or FGF10 alone (Fig. 1C).

Effect of time on cumulus-expansion related gene expression

Before testing the effects of BMP15 and FGF10 on the abundance of mRNA encoding key genes in the regulation of cumulus expansion, the temporal expression patterns of such genes throughout IVM were assessed. Abundance of mRNA encoding all target genes except *BTC* increased significantly during IVM; *BTC* decreased significantly with time (Fig. 2). Levels of *AREG*, *EREG*, *HAS2*, *PTX3* and *TSG6* mRNA in cultured cells were significantly higher after 1 hour IVM compared with immature control cumulus cells, whereas abundance of mRNA encoding *ADAM17*, *ADAM10* and *COX2* increased at 4 hours (*ADAM17* and *COX2*) or 12 hours (*ADAM10*) of culture. The expression of most target genes peaked between 8 and 12 hours after start of IVM and decreased by 22 hours of IVM, except those for *PTX3* and *TSG6* which peaked at 12 hours and remained increased until the end of IVM (at 22 hours; Fig 2).

Effects of BMP15 and FGF10 on cumulus-expansion related gene expression

To gain insight into the mechanisms by which BMP15 and FGF10 enhance cumulus expansion, we examined whether BMP15 and FGF10 regulate expression of key genes involved in the ovulatory cascade. BMP15 at the dose effective to enhance expansion (100 ng/ml) increased mRNA expression of *ADAM10*, *ADAM17*, *AREG*, *EREG* and *HAS2* mRNA levels at 12 hours of culture, most of which remained elevated by BMP15 at 22 hours of culture (Fig. 3 and 4). There were significant effects of BMP15 on *COX2*, *PTX3* and *TSG6* mRNA levels at 22 hours of culture but not before (Fig. 4), whereas BMP15 had no effect on *BTC* and *EGFR* mRNA abundance (Fig. 3).

FGF10 did not affect abundance of mRNA encoding *ADAM10*, *ADAM17*, *EGF*-like factors or *EGFR* (Fig. 5), but stimulated, at the dose effective to enhance expansion (10 ng/ml), *COX2* mRNA levels by 4 hours of culture, which was also observed at 12 and 22 of culture. FGF10 increased *PTX3* mRNA levels at 12 and 22 hours, and *TSG6* mRNA levels only at 22 hours (Fig. 6). BMP15 and FGF10 in combination did not alter abundance of mRNA of any of the genes measured compared to each growth factor alone (data not shown).

Effects of BMP15 and FGF10 on mRNA encoding BMP/FGF signaling molecules

BMP15 at the dose that enhanced cumulus expansion (100 ng/ml) stimulated abundance of mRNA encoding its own receptors (*AKL6* and *BMPRII*) in cumulus cells at 22 hours of maturation, and had a biphasic effect on *FGFR1B* mRNA abundance, which was decreased at 4 hours and increased at 12 and 22 hours of culture (Fig. 7). In contrast, expression of *FGFR2B* mRNA was not altered by BMP15 treatment (data not shown). FGF10 did not change mRNA abundance of FGF10 and BMP15 receptors, and the combination of BMP15 with FGF10 did not alter expression of the receptors differently than BMP15 alone (data not shown). Neither FGF10 nor BMP15 altered *FGF10* or *BMP15* mRNA expression in oocytes (data not shown).

DISCUSSION

In the face of increasing evidence of crucial roles for OSFs in the regulation of oocyte competence and cumulus differentiation (Gilchrist *et al*., 2011), we report for the first time that BMP15 enhances cumulus expansion in a non-rodent model. We also present novel mechanistic information based on gene expression data indicating that BMP15 and FGF10 act at different loci to enhance cumulus expansion; whilst BMP15 appears to stimulate expression of genes at the beginning of the cascade leading to expansion (*ADAM10*, *ADAM17*, *AREG*

and *EREG*), FGF10 seems to act upon downstream genes that contribute more directly to expansion (*COX2*, *PTX3* and *TSG6*).

The positive effect of BMP15 on cumulus expansion agrees with previous studies in mice (Yoshino *et al.*, 2006). In cattle, BMP15 has been reported to benefit oocyte competence and early embryo development by still unknown mechanisms (Hussein *et al*., 2006, 2011), but its effects on cumulus expansion had not been assessed. The enhanced of cumulus expansion might be involved in the mechanisms by which BMP15 increases blastocyst rates (Hussein *et al*., 2006, 2011), as increased cumulus expansion has been previously associated with improved embryo development (Furnus *et al*., 1998). In the present study cumulus expansion was also enhanced by FGF10, confirming a recent report (Zhang *et al*., 2010). However, in the present study, the lowest dose of FGF10 that enhanced cumulus expansion was 10 ng/ml, whilst in the earlier report it was 0.5 ng/ml (Zhang *et al*., 2010). This discrepancy is possibly due to different suppliers and biological activity of FGF10 preparations, but might also be related to the concentration of FSH in the IVM medium as FSH can increase the expression of FGF10 receptors in granulosa cells (Buratini *et al*., 2007); we used 1 µg/ml of FSH in the IVM medium, whereas Zhang *et al*. (2010) used 25 µg/ml FSH.

Before assessing the effects of BMP15 and FGF10 on gene expression, the temporal expression patterns of the target genes during IVM were determined. To our knowledge, there are no previous reports describing the temporal patterns of expression of *ADAM10*, *ADAM17* and *EGF*-like factors in cumulus cells during IVM in the cow, primates or humans. A transitory increase in *ADAM10* and *ADAM17* mRNA abundance in cumulus cells from COCs submitted to IVM with FSH was observed in the present study, similar to that reported for *ADAM17* in the pig (Yamashita *et al*., 2007). *AREG* and *EREG* mRNA abundance was also transiently elevated for a ~ 12 h period in a manner similar to that observed during IVM in the pig (Yamashita *et al.*, 2007). *COX2* is considered to be an EGF-like target gene, and correspondingly abundance of *COX2* mRNA was stimulated for a longer period than those of the EGF-like factors. This is in agreement with previous findings in the bovine COC (Calder *et al*., 2001), but contrasts with data in the mouse, where *COX2* mRNA levels peak and decline very rapidly in cultured cumulus cells (Joyce *et al*., 2001).

Other available data are derived from studies with granulosa rather than cumulus cells, and generally show patterns similar to that in cumulus cells. Abundance of *AREG* and *EREG* mRNA levels increased 6 – 12 h following LH challenge in humans, primates and cattle (Freimann *et al*., 2004; Ben-Ami *et al*., 2006b; Fru *et al*., 2007; Xu *et al*., 2011; Portela *et al*., 2011), in contrast to the very rapid induction and subsequent decline (within 4 h) observed in

mice (Park *et al*., 2004; Carletti and Christenson, 2009). These data demonstrate that the species difference in the timing of *COX2* gene expression between mice and larger species (Sirois *et al*., 2004) is likely due at least in part to differences in the regulation of *AREG* and *EREG* expression.

Another major difference between mice and cattle/primates is the pattern of expression of *BTC*. *BTC* mRNA levels decreased in bovine cumulus cells with time in culture in the presence of FSH (present study), as it does in pig cumulus cells (Procházka *et al*., 2011) and in mural granulosa cells from non-human primates following gonadotropic stimulation *in vivo* (Fru *et al*., 2007). However, in mice gonadotropins increase *BTC* expression in granulosa cells (Park *et al*., 2004). LH treatment was reported to have no impact on *BTC* mRNA levels in human granulosa cells as indicated by microarray analysis (Ben-Ami *et al*., 2006a). Together, these observations highlight species-specific mechanisms controlling upstream events in the ovulatory cascade, and reinforce the usefulness of the bovine model for the investigation of human ovulation.

In the present study, *HAS2* expression levels peaked between 4 and 8 hours and decreased thereafter, similarly to previous observations during bovine IVM (Schoenfelder and Einspanier, 2003). In non-human primates, *HAS2* mRNA abundance in the periovulatory follicle was increased 12 hours after *in vivo* treatment with hCG and declined to control levels at 24 hours post-treatment, although no time points earlier than 12 hours were assessed (Xu *et al*. 2011). Abundance of mRNA encoding *PTX3* and *TSG6* mRNA levels peaked later than those for *COX2* e *HAS2*, and remained highly stimulated until 22 hours of IVM. The temporal expression pattern of *TSG6* mRNA agrees with a previous study of hCG stimulated bovine granulosa cells (Sayasith *et al*., 2008). An increase in *TSG6* mRNA expression 12 hours after hCG treatment was previously reported in non-human primates (Xu *et al*. 2011). Again, in mice, the temporal changes in *HAS2*, *PTX3* and *TSG6* mRNA expression following *in vivo* gonadotropic stimulation are much quicker and of shorter duration than those in cattle and primates (Fulop *et al*., 1997; Ochsner *et al*., 2003), consistent with the shorter duration of the period from the LH surge to ovulation in rodents in comparison with cows and primates (Sirois *et al*., 2004).

In association with its increase in cumulus expansion, BMP15 also stimulated the expression of genes critical for this process. To our knowledge this is the first report of the stimulatory action of BMP15 on the expression of *ADAM10* and *ADAM17*. The stimulatory effects of BMP15 on *AREG*, *EREG*, *COX2*, *HAS2*, *PTX3* and *TSG6* observed in the present study are in agreement with previous reports in the mouse (Yoshino *et al*., 2006; Li *et al*.,

2009). However, while *BTC* mRNA expression was stimulated by BMP15 in the mouse (Yoshino *et al*., 2006), this was not observed in the present study, likely because of the marked species differences in *BTC* expression. Moreover, this finding indicates that BTC is not as important as AREG and EREG for cumulus expansion in cattle. BMP15 did not alter mRNA expression of *EGFR* in the present study, in agreement with a previous study in mice, in which BMP15 only stimulated EGFR mRNA and protein in cumulus cells when in combination with GDF9 (Su *et al*., 2010).

The stimulatory effect of BMP15 on the expression of *ADAM10*, *ADAM17*, *AREG* and *EREG* was first seen at 12 hours of culture, whereas the expression of downstream regulatory genes (*COX2*, *PTX3* and *TSG6*) was up-regulated later on at 22 hours of culture. In contrast, in the mouse, BMP15 induced *COX2* more acutely, in association with increased expression of *AREG*, *EREG* and *BTC* (Yoshino *et al*., 2006). These observations point again to differences between species in the regulation of cumulus gene expression and, as mentioned above, may reflect species-differences in the timing of ovulation after the LH surge. Since EGF-like factors stimulate the expression of *COX2*, *HAS2*, *PTX3* and *TSG6* (Ashkenazi *et al*., 2005; Conti *et al*., 2006; Shimada *et al*., 2006; Su *et al*., 2010), the chronology of the effects observed in the present study suggests that BMP15 acts at the beginning of the cascade that leads to cumulus expansion by enhancing the expression of genes required for the production and release of active forms of EGF-like factors. The temporal association between BMP15 induced increases in the expression of *EGF*-like factors and *HAS2* suggest that *HAS2* transcription is more acutely regulated by AREG and EREG compared with *COX2*, *PTX3* and *TSG6*, although we cannot rule out a direct effect of BMP15 on *HAS2*.

In contrast to BMP15, FGF10 did not alter the expression of *ADAM10*, *ADAM17* or *EGF*-like factors, but did affect the expression of downstream genes. *COX2* expression was the first gene stimulated by FGF10, which occurred from 4 hours of culture. Next was *PTX3*, which was increased by FGF10 at 12 hours, followed by *TSG6* expression, which was up-regulated at 22 hours of culture. TSG6 was reported to be a target of prostaglandin (PG) action in mice and pigs, and either silencing of COX2 or disruption of PG signaling severely impaired *TSG6* expression (Oshsner *et al*., 2003; Takahashi *et al*., 2006; Yamashita *et al*., 2011). Therefore, the effects of FGF10 on *TSG6* expression are likely downstream of COX2. Embryo quality have been associated with higher levels of *COX2* expression in cumulus cells (Mckenzie *et al*., 2004; Gilchrist *et al*., 2011), therefore, the present data suggest that the improvement in embryo development induced by FGF10 (Zhang *et al*., 2010) might be due to increased expression of *COX2*.

The hypothesis that BMP15 and FGF10 would synergize to enhance cumulus expansion was not confirmed by the present data. This hypothesis was motivated by the cooperation of BMP15 and FGF8 to promote glycolysis in mouse cumulus cells (Sugiura *et al*., 2007). This difference is possibly due to the specific receptors activated by FGF8 and FGF10; FGF8 activates FGFR3C, FGFR4 and FGFR2C whereas FGF10 activates FGFR1B and FGFR2B (Zhang *et al*., 2006).

In the present study we also assessed whether BMP15 and FGF10 regulate the expression of themselves, of each other and of their receptors. FGF10 did not alter mRNA levels of either ligands or receptors. This is in contrast with a recent study in which FGF10 stimulated the expression of *BMP15* in the oocyte during IVM in cattle (Zhang *et al*., 2010). The reasons for this difference are not clear but might be related to the different culture systems, particularly to the biological activity of different FGF10 preparations and concentrations of FSH in the medium as discussed above. Although BMP15 did not alter mRNA expression of either OSF, it did stimulate the expression of *FGFR1B* at 12 and 22 hours of culture and of its own receptors (*ALK6* and *BMPRII*) at 22 hours of culture.

In summary, this study presents novel findings from functional and gene expression studies that allow a better comprehension of the actions of OSFs in the regulation of cumulus expansion. We have provided evidence that BMP15 enhances cumulus expansion in the cow and that BMP15 and FGF10 regulate the expression of genes that induce cumulus expansion at different steps in the ovulatory cascade: whereas BMP15 appears to act upon *ADAM10*, *ADAM17*, *AREG* and *EREG*, FGF10 appears to act more directly on downstream genes of the cumulus expansion cascade, particularly *COX2*.

ACKNOWLEDGEMENTS

We thank Drs R Bueno da Silva, A C S Castilho and F M Dalanezi for their technical assistance.

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FIGURE LEGENDS

Figure 1. - Effects of BMP15 (A), FGF10 (B) and the combination of both (C) on cumulus expansion. COCs were cultured with increasing doses of BMP15 and FGF10, and with combination of BMP15 (100 ng/ml) and FGF10 (10 ng/ml) at doses that stimulated cumulus expansion. After culture for 22 hours, the degree of expansion was classified from grades 1 to 3. The proportion of fully expanded COCs (grade 3) was increased by BMP15 at 100 ng/ml and FGF10 at 10 ng/ml, but the combination of both did not further stimulate expansion. Different letters within grades indicate significant differences ($P<0.05$). Data were derived from four independent replicates for each treatment.

Figure 2. – Effects of time on *AREG*, *EREG*, *BTC*, *ADAM10*, *ADAM17*, *COX2*, *HAS2*, *PTX3* and *TSG6* mRNA abundance in cumulus cells during IVM. COCs were cultured for 1, 4, 8, 12, 16 and 22 hours. Groups of immature COCs were used to represent 0 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different ($P<0.05$). Data were derived from four independent replicates.

Figure 3. - Effects of grading doses of BMP15 on *ADAM10/17*, *AREG*, *EREG*, *BTC* and *EGFR* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of BMP15 for 4, 12 and 22 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different ($P<0.05$). Data were derived from four independent replicates for each time point.

Figure 4. - Effects of grading doses of BMP15 on *COX2*, *HAS2*, *PTX3* and *TSG6* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of BMP15 for 4, 12 and 22 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different ($P<0.05$). Data were derived from four independent replicates for each time point.

Figure 5. - Effects of grading doses of FGF10 on *ADAM10/17*, *AREG*, *EREG*, *BTC* and *EGFR* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of FGF10 for 4, 12 and 22 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05). Data were derived from four independent replicates for each time point.

Figure 6. - Effects of grading doses of FGF10 on *COX2*, *HAS2*, *PTX3* and *TSG6* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of FGF10 for 4, 12 and 22 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05). Data were derived from four independent replicates for each time point.

Figure 7. - Effects of grading doses of BMP15 on *FGFR1B*, ALK6 and *BMPRII* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of BMP15 for 4, 12 and 22 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05). Data were derived from four independent replicates for each time point.

Table 1. Information of specific primers used for amplification in real time PCR.

Genes	Primer Sequence	Fragment size (bp)	Annealing Temperature (°C)
CYC-A	F 5'-GCCATGGAGCGCTTTGG-3' R 5'-CCACAGTCAGCAATGGTGATCT-3'	65	60
GAPDH	F 5'-GGCGTGAACCACGAGAAGTATAA-3' R 5'-CCCTCCACGATGCCAAAGT-3'	119	62
H2AFZ	F 5'-GAGGAGCTGAACAAGCTGTTG-3' R 5'-TTGTGGTGGCTCTCAGTCTTC-3'	74	60
AREG	F 5'-CTTTCGTCTCTGCCATGACCTT-3' R 5'-CGTTCTTCAGCGACACCTTCA-3'	100	60
EREG	F 5'-ACTGCACAGCATTAGTTCAAACTGA-3' R 5'-TGTCCATGCAAACAGTAGCCATT-3'	100	60
BTC	F 5'-GCCCCAAGCAGTACAAGCAT–3' R 5'-GCCCCAGCATAGCCTTCATC–3'	100	59
EGFR	F 5'-AAAGTTTGCCAAGGGACAAG-3' R 5'-AAAGCACATTTCCTCGGATG-3'	253	53
COX2	F 5'-AAGCCTAGCACTTTCGGTGGAGAA–3' R 5'-TCCAGAGTGGGAAGAGCTTGCATT–3'	168	60
HAS2	F 5'-ACACAGACAGGCTGAGGACAACTT–3' R 5'-AAGCAGCTGTGATTCCAAGGAGGA–3'	133	60
PTX3	F 5'-CCTCAGCTATCGGTCCATAA–3' R 5'-ATTGAAGCCTGTGAGGTCTGC–3'	294	54
TSG6	F 5'-GCAAAGGAGTGTGGTGGTGTGTTT–3' R 5'-ACTGAGGTGAATGCGCTGACCATA–3'	135	60
ADAM10	F 5'-ACCCCCCAAAGTCTCTCACA–3' R 5'-AATCATGCGGAGATCCAAAGTT–3'	210	60
ADAM17	F 5'-TGGGATGTGAAGATGTTGCTAGA–3' R 5'-ATCCAAGTGTTCCCATATCAAAATC-3'	105	60
FGFR1B	F 5'-ACGTCCTGGTGACGGAGG-3' R 5'-CCGGTGCCATCCATTTGA-3'	126	60
FGFR2B	F 5'-TGTGGTTGGAGGTGATGT-3' R 5'-CGAGTGCTTCAGAACCTTG-3'	141	58
ALK6	F 5'-CAAACCAGCAATTGCCCATCGAGA–3' R 5'-AAGCCCAGGTCAGCTATACAGCAA–3'	87	60
BMPRII	F 5'-CCCACTCTTCGGCACCCTGG-3' R 5'-CCCCGCAGTTATTTCCCCCG-3'	100	59
FGF10	F 5'- AAGGAGATGTCCGCTGGAGAAAGCT-3' R 5'- ACTGTACGGGCAGTTCTCCTTCTT3'	104	60
BMP15	F 5'-GTCAGCAGCCAAGAGGTAGTG–3' R 5'-CCCGAGGACATACTCCCTTAC–3'	360	59

F= forward primer; R= reverse primer

Figure 1

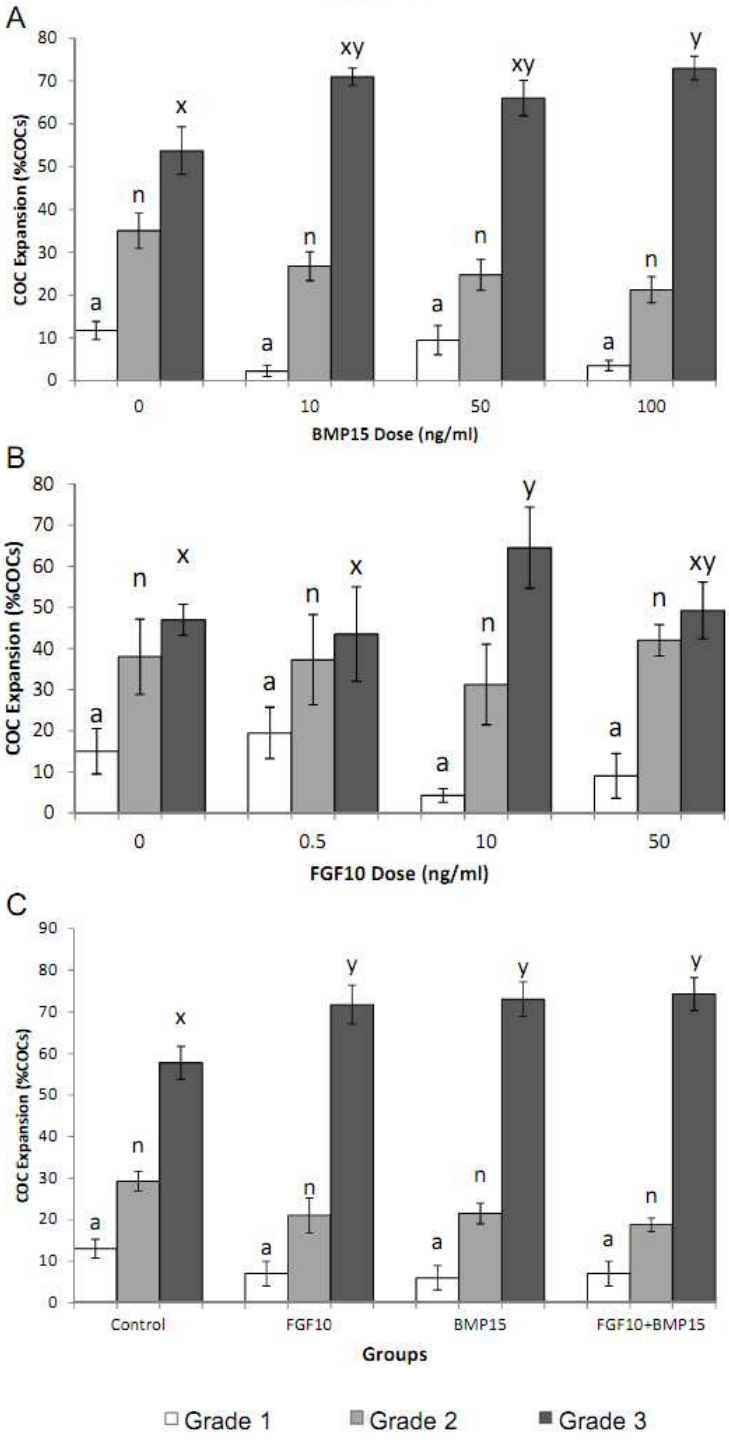

A
COC Expansion (%COCs)
BMP15 Dose (ng/ml)
0
10
50
100
B
COC Expansion (%COCs)
FGF10 Dose (ng/ml)
0
0.5
10
50
C
COC Expansion (%COCs)
Control
FGF10
BMP15
FGF10+BMP15
Groups
Grade 1
Grade 2
Grade 3

Figure 2

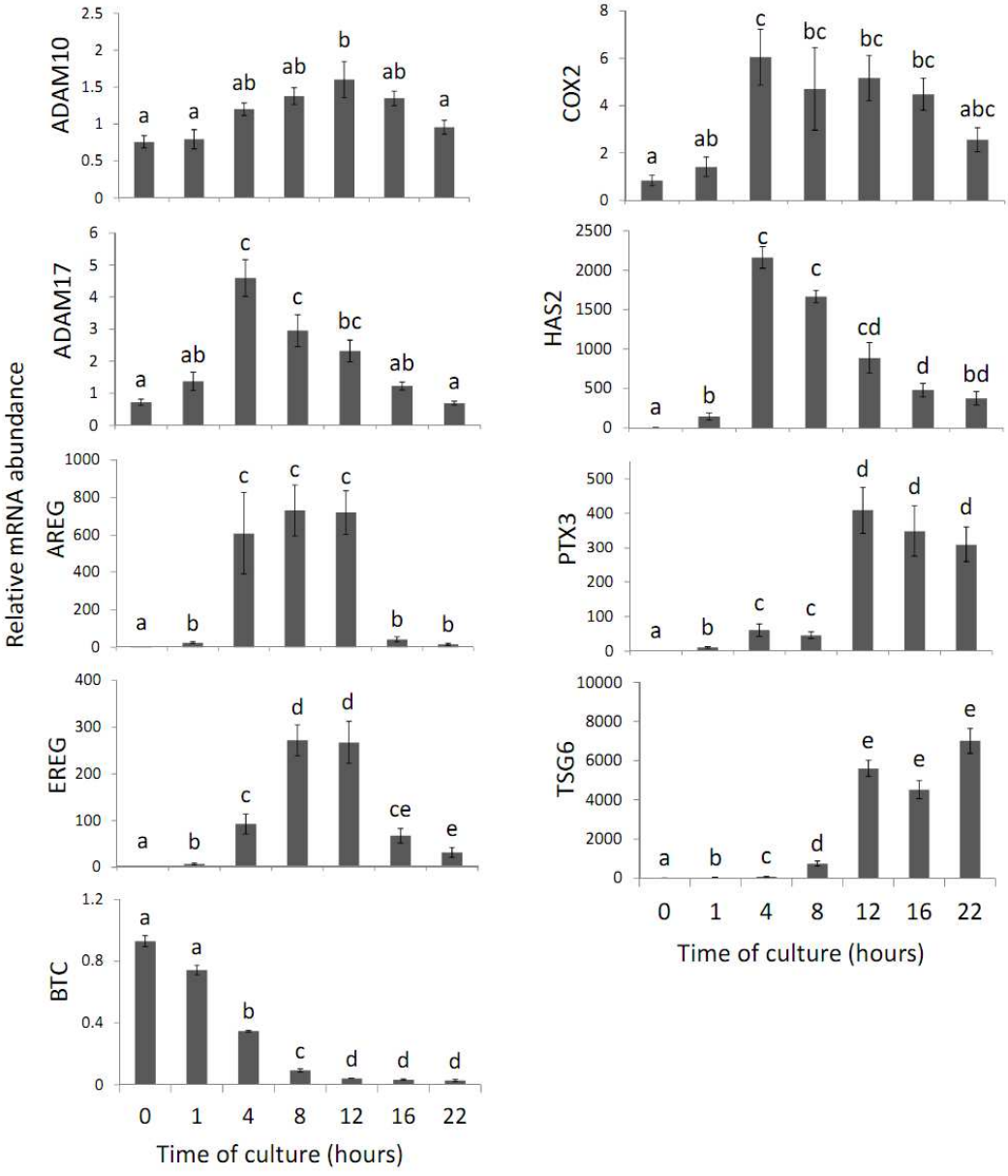

ADAM10
COX2
ADAM17
HAS2
AREG
PTX3
EREG
TSG6
BTC
Relative mRNA abundance
Time of culture (hours)
0 1 4 8 12 16 22

Figure 3

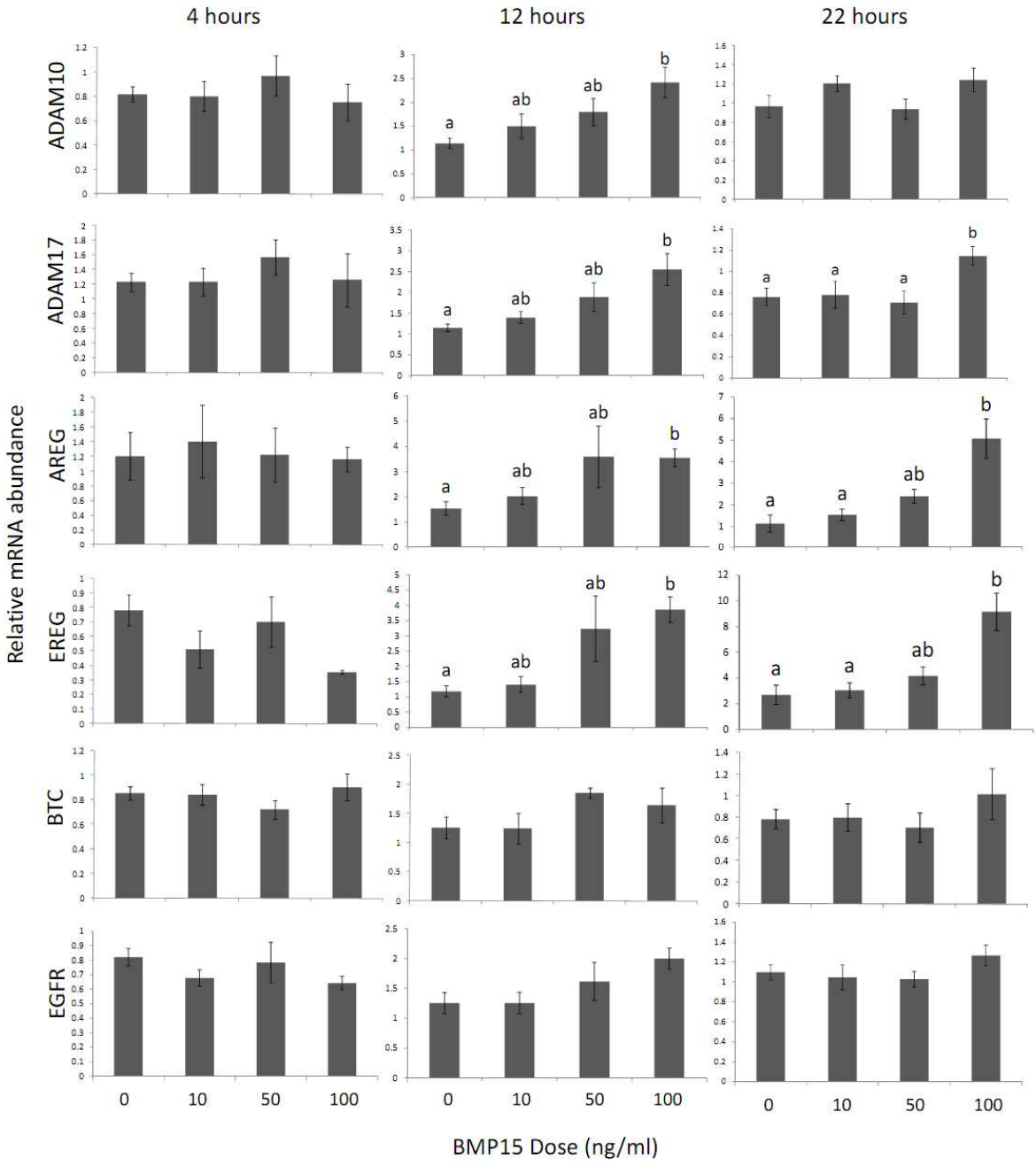

4 hours
12 hours
22 hours
ADAM10
ADAM17
AREG
EREG
BTC
EGFR
Relative mRNA abundance
BMP15 Dose (ng/ml)
0
10
50
100

Figure 4

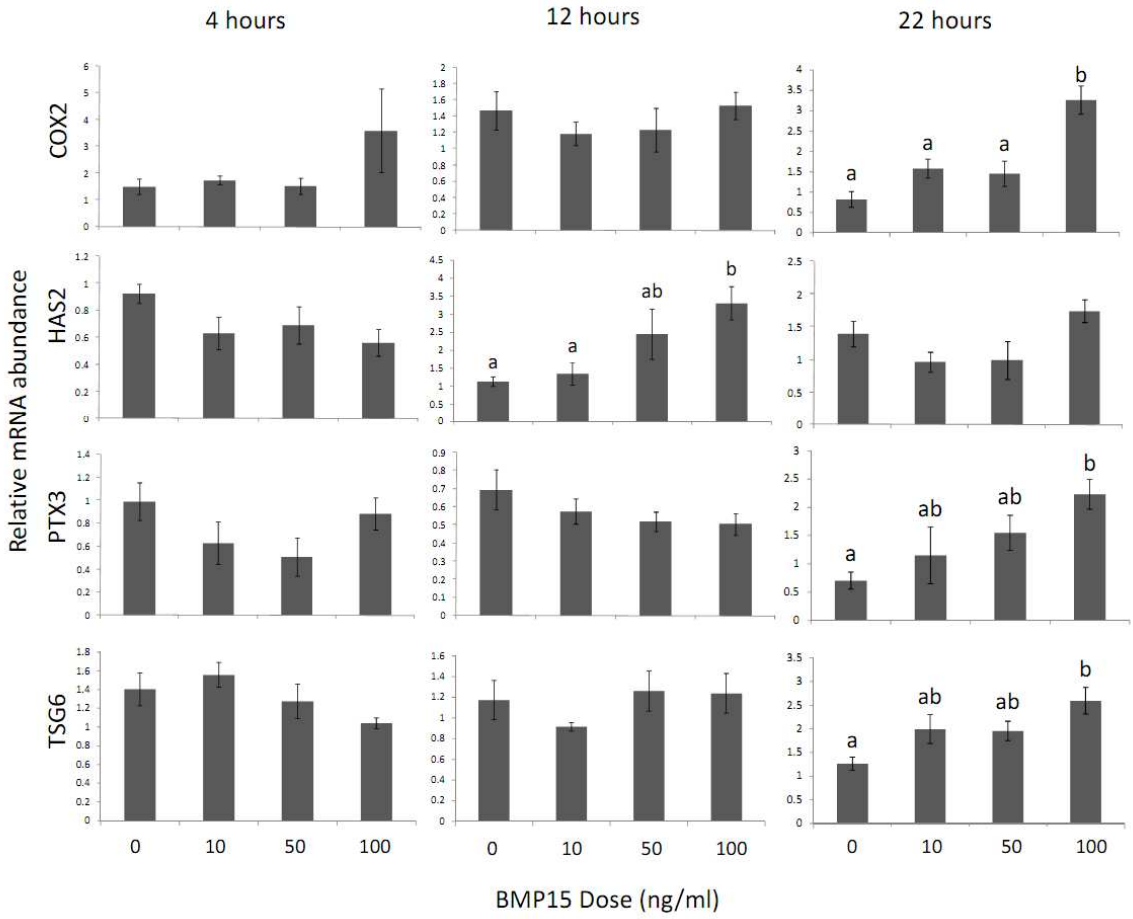

4 hours
12 hours
22 hours
COX2
HAS2
PTX3
TSG6
Relative mRNA abundance
a
a
a
b
a
a
ab
b
a
ab
ab
b
a
ab
ab
b
0
10
50
100
BMP15 Dose (ng/ml)

Figure 5

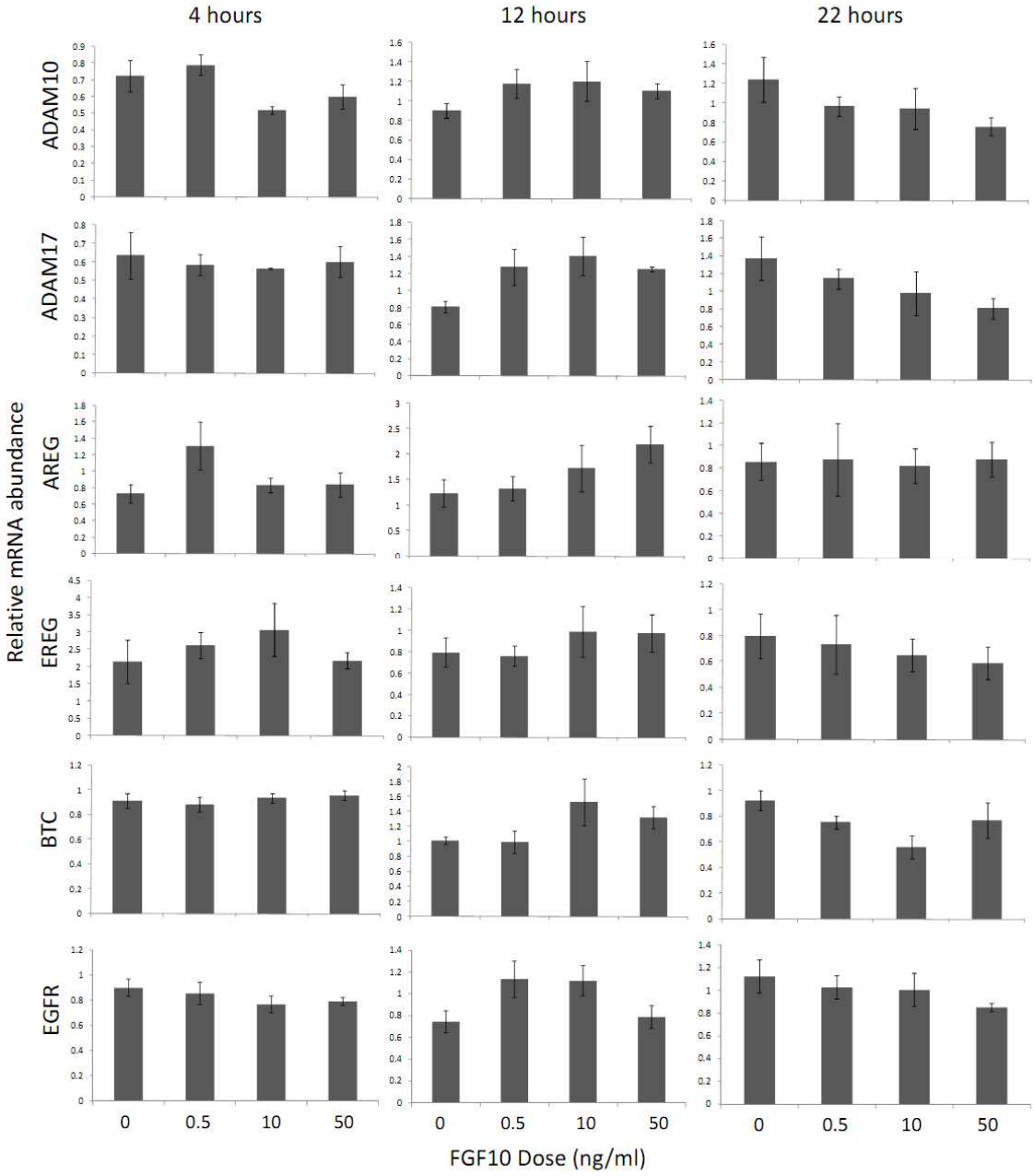

4 hours
12 hours
22 hours
ADAM10
ADAM17
AREG
EREG
BTC
EGFR
Relative mRNA abundance
0
0.5
10
50
FGF10 Dose (ng/ml)

Figure 6

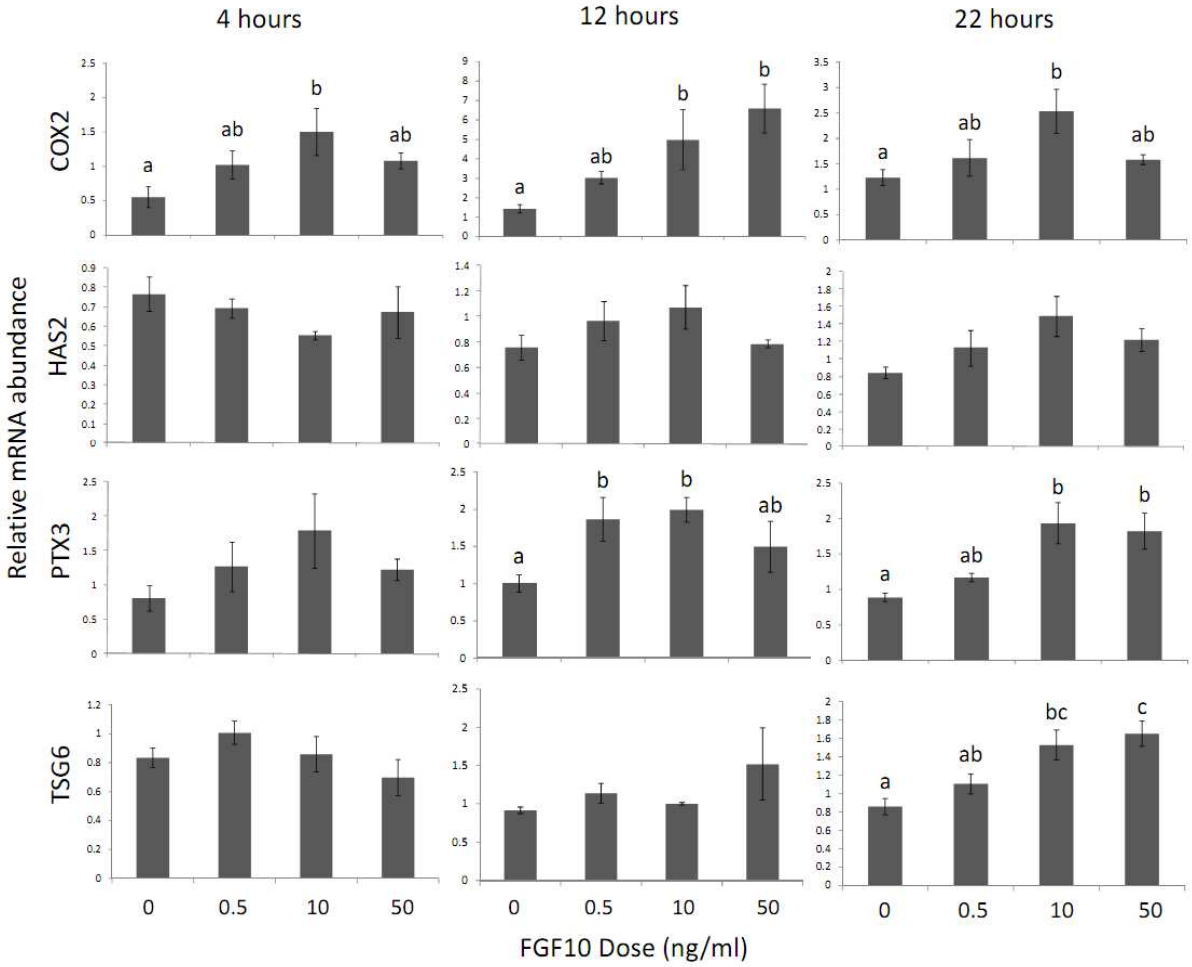
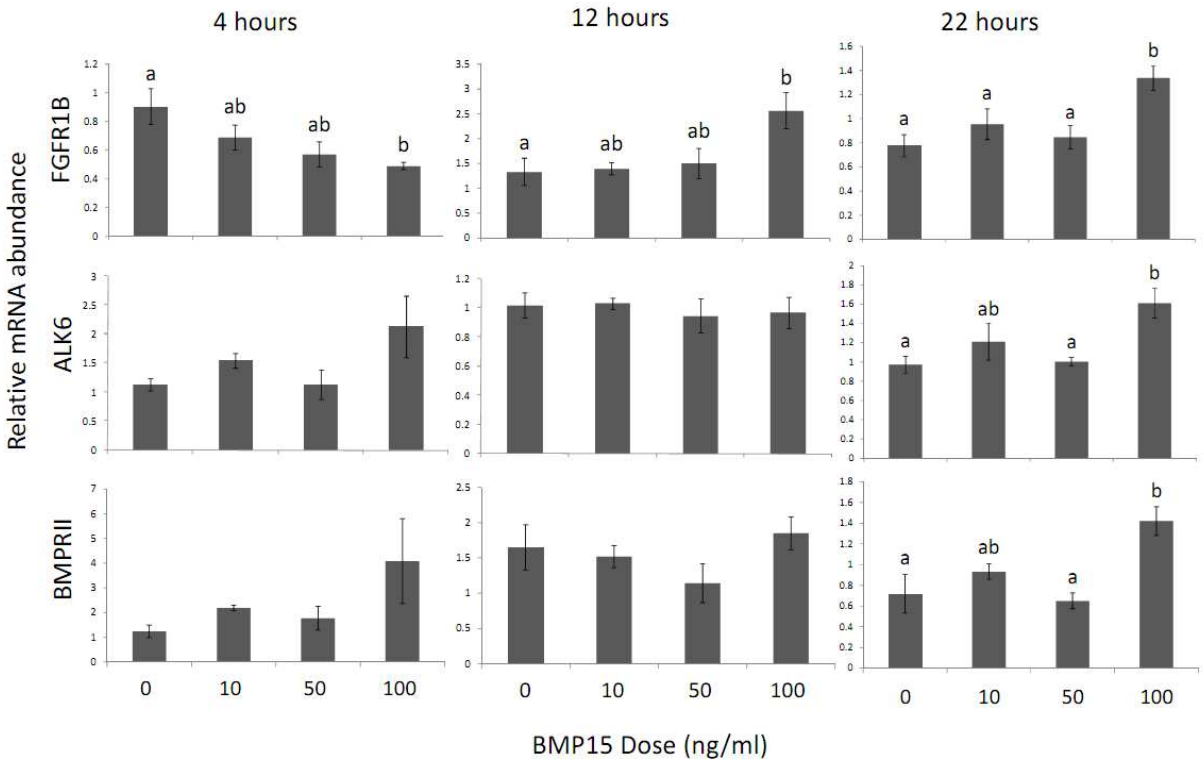

4 hours
12 hours
22 hours
COX2
HAS2
PTX3
TSG6
Relative mRNA abundance
FGF10 Dose (ng/ml)
0
0.5
10
50
a
ab
b
ab
a
ab
b
b
a
ab
b
ab
a
b
b
ab
a
ab
b
b
a
ab
bc
c

Figure 7


4 hours
12 hours
22 hours
Relative mRNA abundance
FGFR1B
ALK6
BMPRII
a
ab
ab
b
a
ab
ab
b
a
a
a
b
a
ab
a
b
a
ab
a
b
a
ab
a
b
0
10
50
100
BMP15 Dose (ng/ml)

CAPÍTULO 3

FSH REGULATES THE EXPRESSION OF RECEPTORS FOR OOCYTE SECRETED FACTORS (OSFs) AND MEMBERS OF THE EGF-LIKE FAMILY DURING *IN VITRO* MATURATION IN CATTLE

O presente artigo foi submetido para publicação no periódico *Reproduction Fertility and Development* e encontra-se de acordo com as normas de submissão exigidas pelo periódico.

FSH regulates the expression of receptors for oocyte secreted factors (OSFs) and members of the EGF-like family during *in vitro* maturation in cattle.

Short Title: FSH regulates expression of OSF receptors and EGF-like factors

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ABSTRACT

FSH induces expansion of bovine cumulus-oocyte complexes (COCs) in cattle, which can be enhanced by oocyte secreted factors (OSFs). In this study we hypothesized that FSH stimulates COC expansion in part from direct stimulation of the EGF-like ligands amphiregulin (*AREG*), epiregulin (*EREG*) and betacellulin (*BTC*), but also in part through regulation of OSFs or their receptors in cumulus cells. Bovine COCs were cultured in defined medium with graded doses of FSH. In the absence of FSH, COCs did not expand. FSH caused cumulus expansion, and increased the abundance of mRNA *AREG* and *EREG* in time and dose dependent manners, but decreased *BTC* mRNA levels. FSH had modest stimulatory effects on levels of mRNA encoding the bone morphogenetic protein 15 (BMP15) receptor, *ALK6* in cumulus cells, but not those of the growth and differentiation factor 9 (GDF9) receptor, *ALK5*. More interestingly, FSH dramatically stimulated levels of mRNA encoding two fibroblast growth factor (FGF) receptors, *FGFR2C* and *FGFR3C* in cumulus cells. This study demonstrates that FSH stimulates the expression of EGF-like factors in bovine cumulus cells, and more strikingly, provides novel evidence that FSH enhances the expression of receptors for OSFs in cumulus cells.

Key words: FSH / Cumulus expansion / EGF-like ligands / oocyte secreted factors

INTRODUCTION

The *in vitro* maturation (IVM) of oocytes is rising in importance as a key component of assisted reproduction in numerous species including cattle (Thibier, 2006), but developmental competence of *in vitro* matured oocytes is notably lower than that of oocytes matured *in vivo* in cattle, sheep, mice and women (Thompson *et al*. 1995; Child *et al*. 2002; Rizos *et al*. 2002; Vanhoutte *et al*. 2009). There is thus a medical and economic incentive to improve the efficiency of IVM.

The preovulatory LH surge triggers the resumption of meiosis of the oocyte, cumulus expansion and ovulation of the cumulus-oocyte complex (COC; Richards *et al*. 2002). The effects of LH on the COC are mediated by epidermal growth factor (EGF)-like family members, amphiregulin (AREG), epiregulin (EREG) and betacellulin (BTC), that are secreted by mural granulosa cells in response to LH and which activate the EGF receptor (EGFR) on cumulus cells (Park *et al*. 2004; Ashkenazi *et al*. 2005; Conti *et al*. 2006).

Oocyte secreted factors (OSFs) also regulate cumulus cell function and developmental competence of the oocyte (reviewed by Gilchrist *et al*. 2008). Bone morphogenetic protein 15 (BMP15) and growth and differentiation factor 9 (GDF9) are the best-known OSFs and enhance cumulus expansion in mice and cattle (Elvin *et al*. 1999; Leyens *et al*. 2004; Dragovic *et al*. 2005; Yoshino *et al*. 2006) and blastocyst production in cattle (Hussein *et al*. 2006, 2011). Furthermore, the addition of GDF9 to IVM medium increased the number of surviving fetuses after blastocyst transfer in mice (Yeo *et al*. 2008). BMP15 and GDF9 signaling involves two receptors; both bind to the BMP receptor II (BMPRII) but they employ distinct co-receptors for pathway activation. The downstream actions of BMP15 are mediated by the BMP receptor 1B (also named ALK6) and the Smad1/5/8 proteins (Moore *et al*. 2003), whereas actions of GDF9 are mediated by the TGFB type-I receptor (also named ALK5) and the Smad 2/3 proteins (Mazerbourg *et al*. 2004). All of these receptors are present within the follicle (Fatehi *et al*. 2005).

The fibroblast growth factor (FGF) family also contains potential OSFs, including FGF8 in the mouse (Valve *et al*. 1997) and FGF8, FGF10 and FGF17 in cattle (Buratini *et al*. 2005, 2007, Machado *et al*. 2009). Addition of FGF10 enhanced cumulus expansion and blastocyst formation in cattle *in vitro* (Zhang *et al*. 2010). FGF signaling is complex as there are four receptor (FGFR) genes, three of which give rise to splice variants (‘b’ and ‘c’ forms) with specific ligand binding properties. FGF10 activates predominantly the ‘b’ isoform of FGFR1 and 2 (Itoh and Ornitz 2004), while FGF8 and FGF17 efficiently activate the ‘c’

isoform of FGFR2 and 3 (Ornitz *et al*. 1996). Expression of these receptors has been reported in cumulus cells (Cho *et al*. 2008; Zhang *et al*. 2010).

FSH can also directly stimulate expansion of COCs in cattle (Choi *et al*. 2001; Sutton-McDowall *et al*. 2004). This is likely to be through increased expression of EGF-like growth factors, as FSH increased *AREG* mRNA abundance in isolated mouse and pig COCs *in vitro* (Downs and Chen, 2008; Procházka *et al*. 2011), although this has not been demonstrated in the cow. FSH may also act through OSF signaling, as it decreased the expression of *BMPRII* in serum-free cultures of bovine and ovine mural granulosa cells (Jayawardana *et al*. 2006; Chen *et al*. 2009) and increased *FGFR* expression in bovine granulosa cells (Buratini *et al*. 2005, 2007). The effect of FSH on cumulus cells is less clear; *BMPRII* mRNA levels in COCs increased during IVM in the presence of serum and gonadotropins, although the specific effects of FSH were not evaluated (Kyasari *et al*. 2011). Thus, the objectives of the present study were to determine the effect of FSH on genes involved with cumulus expansion and OSF signaling in bovine IVM under defined conditions.

MATERIALS AND METHODS

Unless specified, all chemicals and reagents were purchased from Sigma (St. Louis, MO, USA).

In vitro maturation

Ovaries of adult cows (predominantly Nellore, *Bos indicus*) were obtained in an abattoir local to the Sao Paulo State University campus in Botucatu and transported to the laboratory in saline solution (0.9% NaCl) containing antibiotics (penicillin G; 100 IU/ml and streptomycin; 100 ug/ml) at 35-37°C. COCs were aspirated from 3 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 ml conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogenous cytoplasm and compact multilayer of cumulus cells were used (Grade 1 and 2). COCs were washed and transferred in groups of 20 to a 100 µl drop of maturation medium (TCM199 containing Earle's salts supplemented with 22 ug/ml sodium pyruvate and 75 ug/ml amicacin). Drops were covered with mineral oil and incubated at 38.5°C in 5% of CO_2 in humidified air.

To assess the effects of time and FSH on gene expression during IVM a time course experiment was designed. COCs were cultured under conditions described above for 4, 8, 12, 16 and 20 hours with (10 ng/ml; porcine FSH, Folltropin-V® Bioniche, ON, CA) or without FSH and cumulus cells were recovered for gene expression analysis from pools of 20 COCs

(n=4). Immature COCs were used to represent 0 hours. Subsequently, to test the effects of FSH dose on gene expression, COCs were cultured for 12 hours in maturation medium supplemented with grading doses of FSH (0, 0.1, 1, 10 and 100 ng/ml; n=4), after which cumulus cells were separated for total RNA extraction.

To assess the effect of IVM on mRNA expression of OSFs, oocytes were separated for total RNA extraction from pools of 20 COCs before (immature) and after culture for 20 hours in maturation medium supplemented with FSH (10 ng/ml; n=4).

Gene expression analysis

Cumulus cells and oocytes (n=20/group) were mechanically separated by repeated pipetting in PBS from immature COCs and cultured COCs. Denuded oocytes were recovered and washed three times in PBS. Cumulus cells were transferred to a 1.5 ml tube, centrifuged twice for 5 minutes at 700g, the supernatant was discarded and 350 µl of the RNA extraction lysis buffer was added to the cell pellets. The cell suspension and oocytes were stored at -80°C until RNA extraction.

Total RNA was extracted from pools of 20 oocytes and cumulus cells from groups of 20 COCs using the RNeasy® kit (Qiagen, Mississauga, ON, CA) as recommended by the manufacturer. After purification, RNA samples were eluted in 30 µl of RNAse free water. Total RNA concentration in cumulus cells samples were measured by spectrophotometer using a NanoDrop ND® 1000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (100 ng/reaction for cumulus samples and entire RNA sample for oocyte samples) was incubated with DNAse I (1 U/µg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using Oligo-dT primers and according with the protocol provided by the Omniscript or Sensiscript kits (Qiagen, Mississauga, ON, CA) for cumulus and oocyte samples, respectively. The reagents were incubated at 37ºC for 60 min and then at 93º for 3 min for enzyme inactivation.

Real time RT-PCR analysis was performed with an ABI 7500 thermocycler using Power SYBR Green PCR Master Mix (Applied Biosystems) for all target and housekeeping genes, except for FGF17. The detection system TaqMan Assay by Design (Applied Biosystems, Sao Paulo, Brazil) was used for FGF17 as it provided a higher amplification efficiency in comparison with SYBR Green. The final volume of the PCR mix was 25 µl and PCR cycling conditions were: 95°C for 10 min (1 cycle), denaturing at 95°C for 10 sec followed by annealing for 1 min (40 cycles). The primers sequences, amplicons sizes and annealing temperatures for each target gene are shown in Table 1. Reactions were optimized to provide maximum amplification efficiency for each gene. Each sample was run in

duplicates, and the specificity of the PCR products was assessed by the melting curve analysis and amplicon size determined by electrophoresis in agarose 2%. Negative controls (water replacing cDNA) were run in every plate.

To select the most stable housekeeping gene Cyclophilin-A (*CYC-A*), Glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) and Histone H2AFZ (*H2AFZ*) amplification profiles were compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/genorm; Vandesompele et al., 2002); the most stable housekeeping gene was Cyclophilin-A (*CYC-A*) for oocytes and cumulus cells.

The relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as calibrator (Pfaffl, 2001). Mean efficiency values for each gene were calculated from the amplification profile of individual samples with LinRegPCR software (Ramakers et al., 2003).

Statistical Analysis

The effects of time of maturation and FSH treatment on gene expression of oocytes and cumulus cells were tested by analysis of variance (ANOVA), and means were compared with the Tukey-Kramer HSD test. Gene expression data were transformed to logarithms if not normally distributed; when the log-transformed data departed signifiantly from normality, a non-parametric test (Kruskal-Wallis) was used. The analyses were performed with JMP software (SAS Institute, Cary, NC, USA) and the results are presented as mean± standard error of the mean (SEM). Differences were considered significant when $P<0.05$.

RESULTS

Effects of time and FSH on expression of expansion-related genes in cumulus cells

To determine the impact of FSH on cumulus cells, we first assessed the effect of FSH on cumulus expansion during COC culture in defined medium. In the absence of FSH, negligible expansion was observed, whereas in the presence of 10 ng/ml FSH, 100% of COCs expanded. During culture without FSH, abundance of mRNA encoding *EREG* and *BTC* did not change over 22 hours, whereas there was a significant increase in *AREG* mRNA levels (Fig 1). Addition of FSH significantly increased *AREG* and *EREG* mRNA levels above controls (without FSH) from 4 to 12 hours of culture, and significantly decreased *BTC* mRNA levels from 8 to 20 hours culture (Fig 1). Abundance of mRNA encoding *EGFR* increased at 4 hours relative to immature COC, and was only affected (inhibited) by FSH at 20 hours of IVM.

The effect of FSH was confirmed by a dose response study performed at 12 hours of culture; FSH significantly increased *AREG* and *EREG* mRNA levels, decreased *BTC* mRNA levels and was without effect on *EGFR* mRNA levels (Fig 2).

Effects of time and FSH on OSF receptor gene expression in cumulus cells

Cumulus cells consistently expressed mRNA encoding *FGFR1B*, *FGFR2B*, *FGFR2C* and *FGFR3C*, with average Ct values of 26, 30, 32 and 35, respectively. In the absence of FSH, abundance of mRNA encoding *FGFR2C* but not the other *FGFRs* increased with time in culture. FSH significantly increased *FGFR1B*, *FGFR2C* and *FGFR3C* mRNA levels, but had no effect on *FGFR2B* mRNA abundance (Fig 3). Receptors for GDF9 and BMP15 were also expressed in cumulus cells, with average Ct values of 28, 30 and 28 for *BMPRII*, *ALK5* and *ALK6*, respectively. Abundance of mRNA encoding *BMPRII* and *ALK5* did not change during culture with or without FSH, whereas *ALK6* mRNA levels were stimulated by FSH at 12 and 16 hours of culture (Fig 3).

The stimulatory effect of FSH on *FGFR1B*, *FGFR2C* and *FGFR3C* was confirmed by the dose-response study at 12 hours of culture (Fig 4). FSH had no effect of *FGFR2B*, *BMPRII* or *ALK5* mRNA levels, and increased *ALK6* mRNA only at a dose higher than that used in the time course study.

Effects of maturation on OSF gene expression in oocytes

Abundance of mRNA encoding three oocyte-derived FGFs and, for comparison, of *BMP15* and *GDF9*, were measured before and after maturation with FSH. Relative levels of *FGF8* and *FGF10* mRNA were low in immature oocytes (Ct values of 36-37) compared to *BMP15* and *GDF9* (Ct values of 24). Abundance of mRNA encoding *BMP15*, *GDF9* and *FGF10* were not altered by IVM, however, *FGF8* mRNA was undetectable in 3 of 4 samples at 20 hours (Fig 5). *FGF17* was weakly expressed in immature oocytes (Ct values of 38), and was undetectable at 20 hours culture.

DISCUSSION

FSH can induce cumulus expansion in bovine COC *in vitro*, and this effect can be enhanced by addition of BMP15 or FGF10 (Choi *et al*. 2001; Yoshino *et al*. 2006; Zhang *et al*. 2010). It is therefore likely that FSH regulates expansion-related genes and expression of BMP/FGF receptors in cumulus cells. In the present study we demonstrate that FSH directly stimulates *AREG* and *EREG* expression in bovine cumulus cells, as previously shown for

mice and pigs (Downs and Chen, 2008; Procházka *et al*. 2011), and show for the first time upregulation of *BMP* and *FGF* receptor mRNAs by FSH in the cumulus.

The most striking result of the present study is the rapid and significant increase in levels of mRNA encoding the 'c' splice variants of *FGFR2* and *FGFR3* in cumulus cells by FSH. Relative abundance increased by approximately 500-fold within 4 h of culture compared to culture in the absence of FSH. A similar but less marked effect was noted for *FGFR1B*, whereas FSH had no effect on *FGFR2B*. These data show clear regulation of specific receptors in cumulus cells, and point to potential roles for ligands that bind to these receptors.

The 'c' splice variants of FGFR2 and FGFR3 are activated by a broad range of FGFs, including FGF1, FGF2, FGF8, FGF9 and FGF17 (Zhang *et al*. 2006), all of which have been detected in the oocyte of several species (van Wezel *et al*. 1995; Valve *et al*. 1997; Ben-Haroush *et al*. 2005; Buratini *et al*. 2005; Zhong *et al*. 2006; Machado *et al*. 2009), although other FGFs that activate these receptors may also be present within the follicle.

FSH also increased *ALK6* mRNA levels in cumulus cells, which is consistent with the increase of these messages with time during IVM in sheep (Kyasari *et al*. 2011), although different from studies in rat granulosa cells in which FSH did not alter *ALK6* mRNA levels (Nakamura *et al*. 2010; Miyoshi *et al*. 2007). On the other hand, *BMPRII* mRNA abundance in cumulus was not affected by FSH treatment, in agreement with data from rat granulosa cells (Nakamura *et al*. 2010; Miyoshi *et al*. 2007), but in contrast to the inhibition in sheep and cattle granulosa cells (Jayawardana *et al*. 2006; Chen *et al*. 2009), suggesting different responses in granulosa *vs.* cumulus cells, or the different nature of the culture systems involved. The increase in *ALK6* observed here is consistent with the effect of BMP15 on cumulus expansion previously reported (Yoshino *et al*. 2006).

FSH induces cumulus expansion in basal media (Sutton-McDowall *et al*. 2004), and expansion is dependent on expression of EGF-like ligands and COX2 (Park *et al*. 2004, Conti *et al*. 2006). Consistent with this, FSH increased *AREG* and *EREG* mRNA abundance in the present study in time- and dose-dependent manners. In the absence of FSH, and therefore of expansion, basal *EREG* mRNA levels did not increase, however, there was a significant increase in *AREG* mRNA levels. Clearly this increase was not able to induce expansion, most likely because FSH is necessary for sheddase activity and release of bioactive AREG. Interestingly, these data demonstrate differential regulation of *AREG* and *EREG* mRNA abundance. This is supported by data from bovine granulosa cells, in which LH increases *EREG* mRNA levels several hours before *AREG* mRNA levels increased (Portela *et al*. 2011).

BTC is an EGF-like ligand that is upregulated by LH in rodents (Park *et al*. 2004), but is downregulated in granulosa cells by gonadotropins in monkeys *in vivo* (Fru *et al*. 2007), and decreases in cumulus cells during IVM in pigs (Procházka *et al*. 2011). In the present study, *BTC* mRNA levels did not change in the absence of FSH, but were inhibited by FSH in a dose-dependent manner. Together, these data suggest specific downregulation of BTC in cattle, pigs and primates, but not in mice.

Gene expression of the major OSFs, *BMP15* and *GDF9* in oocytes did not differ between immature (germinal vesicle stage) and mature oocytes. This is generally consistent with data from cattle (Lonergan *et al*. 2003; Fair *et al*. 2007) and sheep (Kyasari *et al*. 2011) although other studies have reported significant decreases in *GDF9* mRNA in metaphase II oocytes of cattle (Lequarre *et al*. 2004), pigs (Li *et al*. 2008; Zhu *et al*. 2008; Zhang *et al*. 2009) and mice (Su *et al*. 2007; Sanchez *et al*. 2009). Abundance of mRNA encoding *FGF8* and *FGF17* decreased in mature oocytes, in agreement with data for *FGF8* in mice (Zhong *et al*. 2006; Su *et al*. 2007), although *FGF10* mRNA levels were not altered. Thus degradation of OSF mRNA in matured bovine oocytes is restricted to specific OSFs, at least under the present conditions.

In conclusion, the present data demonstrate that FSH stimulates expression of EGF-like ligands and specific OSF receptors in bovine cumulus cells during IVM. The marked stimulation of *FGFR2C* and *FGFR3C* mRNA levels in cumulus by FSH suggests important roles for FGFR signaling in the process of cumulus expansion in cattle.

ACKNOWLEDGEMENTS

We thank Drs R Bueno da Silva, A C S Castilho and F M Dalanezi for their technical assistance.

FUNDING

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

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FIGURE LEGENDS

Figure 1. – Effects of time and FSH on *AREG*, *EREG*, *BTC* and *EGFR* mRNA abundance in cumulus cells during IVM. COCs were cultured for 4, 8, 12, 16 and 20 hours with (10 ng/ml) or without FSH. Groups of immature COCs were used to represent 0 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample and were calculated by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05). Bars with asterisks represent differences in relation to time 0 hours (P<0.05). Data were derived from four independent replicates.

Figure 2. - Effects of grading doses of FSH on *AREG, EREG*, *BTC* and *EGFR* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of FSH for 12 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample are were calculated by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05). Data were derived from four independent replicates.

Figure 3. - Effects of time and FSH on *FGFR1B, FGFR2B, FGFR2C, FGFR3C, BMPRII, ALK5* and *ALK6* mRNA abundance in cumulus cells during IVM. COCs were cultured for 4, 8, 12, 16 and 20 hours with (10 ng/ml) or without FSH. Groups of immature COCs were used to represent 0 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample and were calculated by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05). Bars with asterisks represent differences in relation to time 0 hours (P<0.05). Data were derived from four independent replicates.

Figure 4. - Effects of grading doses of FSH on *FGFR1B, FGFR2B, FGFR2C, FGFR3C, BMPRII, ALK5* and *ALK6* mRNA abundance in cumulus cells. COCs were cultured with increasing doses of FSH for 12 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample are were calculated by the ΔΔCt method with efficiency correction. Bars with different letters are significantly different (P<0.05). Data were derived from four independent replicates.

Figure 5. – Effects of *in vitro* maturation on *BMP15, GDF9, FGF10, FGF8* and *FGF17* mRNA abundance in oocytes. Oocytes were separated from COCs before (immature) and after culture for 20 hours. Messenger RNA abundance was measured by real-time PCR. Data are presented as mean values (±S.E.M.) relative to a calibrator sample were calculated by the ΔΔCt method with efficiency correction. Asterisks indicate differences between 0 and 20 hours of culture ($P<0.05$). Data were derived from four independent replicates.

Table I. Information of specific primers used for amplification in real time PCR.

Genes	Primer Sequence	Fragment size (bp)	Annealing Temperature (°C)
CYC-A	F 5'-GCCATGGAGCGCTTTGG-3' R 5'-CCACAGTCAGCAATGGTGATCT-3'	65	60
GAPDH	F 5'-GGCGTGAACCACGAGAAGTATAA-3' R 5'-CCCTCCACGATGCCAAAGT-3'	119	62
H2AFZ	F 5'-GAGGAGCTGAACAAGCTGTTG-3' R 5'-TTGTGGTGGCTCTCAGTCTTC-3'	74	60
FGF8	F 5'-TGAGACAGGCCTCTACATCTGCAT-3' R 5'- ATTGTTCTCCAGCACGATCTCCGT-3'	106	60
FGF10	F 5'-AAGGAGATGTCCGCTGGAGAAAGCT-3' R 5'-ACTGTACGGGCAGTTCTCCTTCTT-3'	104	60
FGF17	F 5'-CCGGGTGCGCATCAAG-3' R 5'-GCTTGCCCCTCTTATTCATACAGA-3' Probe FAM 5'-CTGAGAGTGAGAAATAC-3'	62	60
BMP15	F 5'-GTCAGCAGCCAAGAGGTAGTG–3' R 5'-CCCGAGGACATACTCCCTTAC–3'	360	59
GDF9	F 5'-TGGTCCTTGCTGAAGCATCTAGA–3' R 5'-ACAGTGTTGTAGAGGTGGCTTCT–3'	202	59
FGFR1b	F 5'-ACGTCCTGGTGACGGAGG-3' R 5'-CCGGTGCCATCCATTTGA-3'	126	60
FGFR2b	F 5'-TGTGGTTGGAGGTGATGT-3' R 5'-CGAGTGCTTCAGAACCTTG-3'	141	58
FGFR2c	F 5'-CACCACGGACAAAGAAAT-3' R 5'-ATGCAGAGTGAAAGGATATCC-3'	84	60
FGFR3c	F 5'-ACTGGTACAACACCTATGCCTCCA-3' R 5'-TCTGGACATGGTGGGCAACTTAGA-3'	84	59
ALK6	F 5'-CAAACCAGCAATTGCCCATCGAGA–3' R 5'-AAGCCCAGGTCAGCTATACAGCAA–3'	87	60
ALK5	F 5'-ATCTGGCCTTGGTCCTGTTGAACT-3' R 5'-ACGAGGGATCCTCTTCATTTGGCA-3'	143	59
BMPRII	F 5'-CCCACTCTTCGGCACCCTGG-3' R 5'-CCCCGCAGTTATTTCCCCCG-3'	100	59
AREG	F 5'-CTTTCGTCTCTGCCATGACCTT-3' R 5'-CGTTCTTCAGCGACACCTTCA-3'	100	60
EREG	F 5'-ACTGCACAGCATTAGTTCAAACTGA-3' R 5'-TGTCCATGCAAACAGTAGCCATT-3'	100	60
BTC	F 5'-GCCCCAAGCAGTACAAGCAT–3' R 5'-GCCCCAGCATAGCCTTCATC–3'	100	59
EGFR	F 5'-AAAGTTTGCCAAGGGACAAG-3' R 5'-AAAGCACATTTCCTCGGATG-3'	253	53

F= forward primer; R= reverse primer

Figure 1

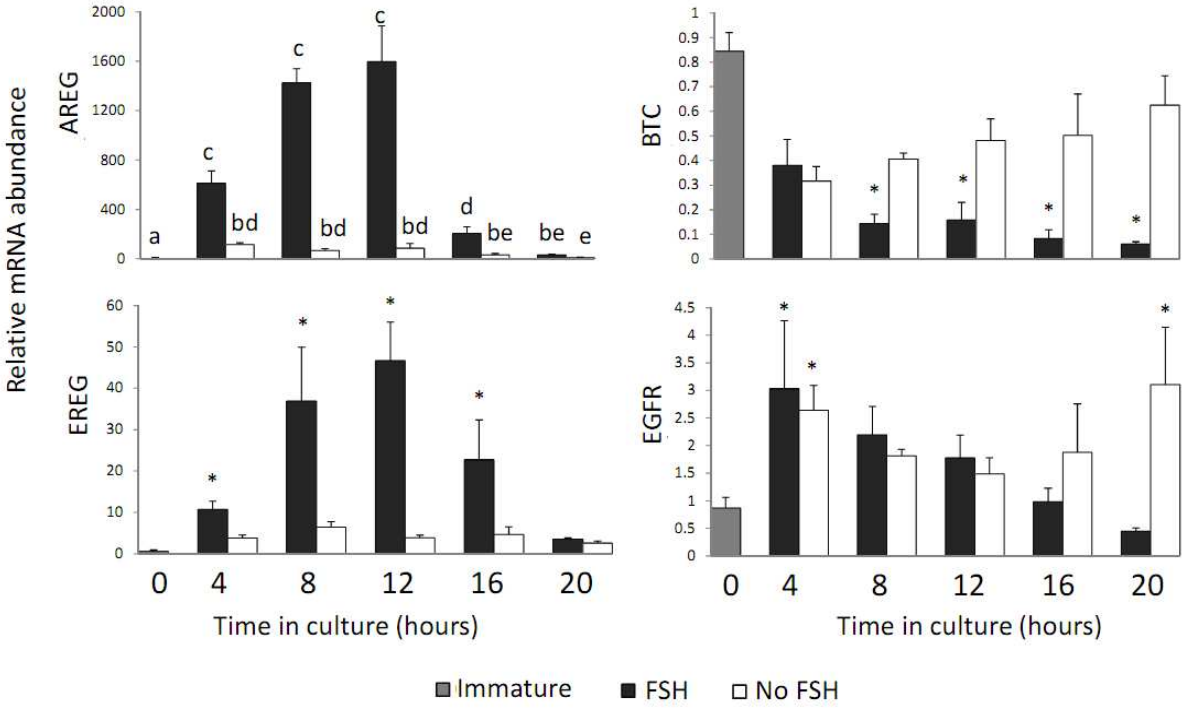

Relative mRNA abundance
AREG
BTC
EREG
EGFR
a
c
bd
d
be
e
Time in culture (hours)
Time in culture (hours)
Immature
FSH
No FSH

Figure 2

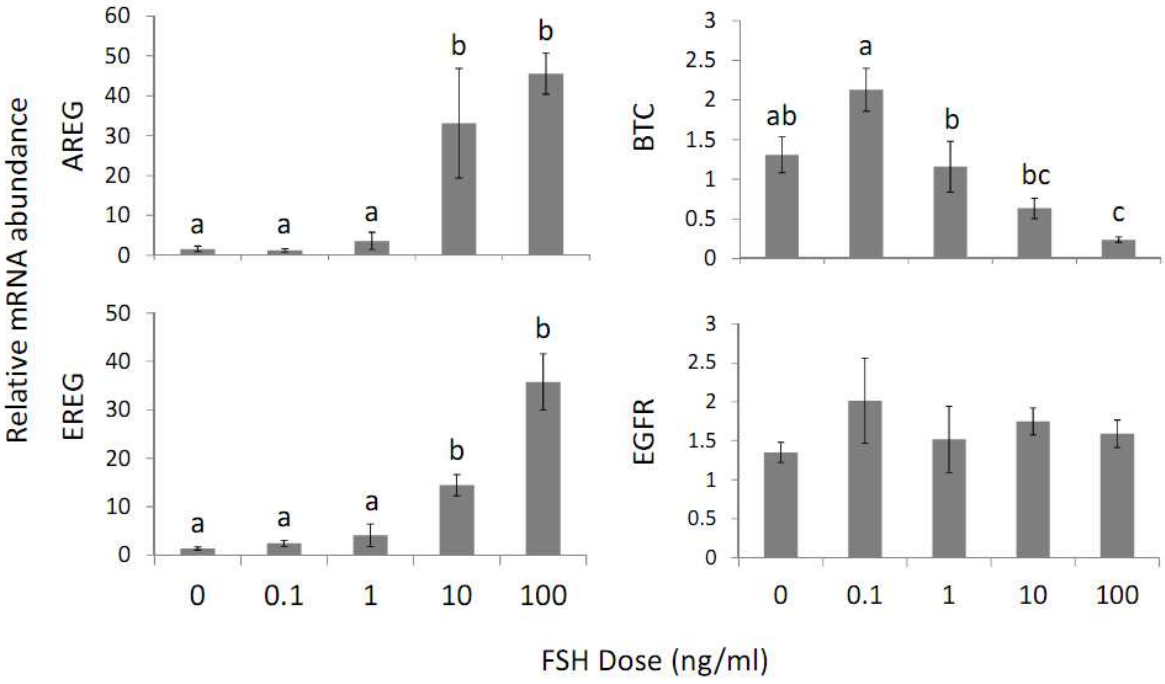

Relative mRNA abundance
AREG
EREG
BTC
EGFR
a
a
a
b
b
a
a
a
b
b
ab
a
b
bc
c
0
0.1
1
10
100
FSH Dose (ng/ml)

Figure 3

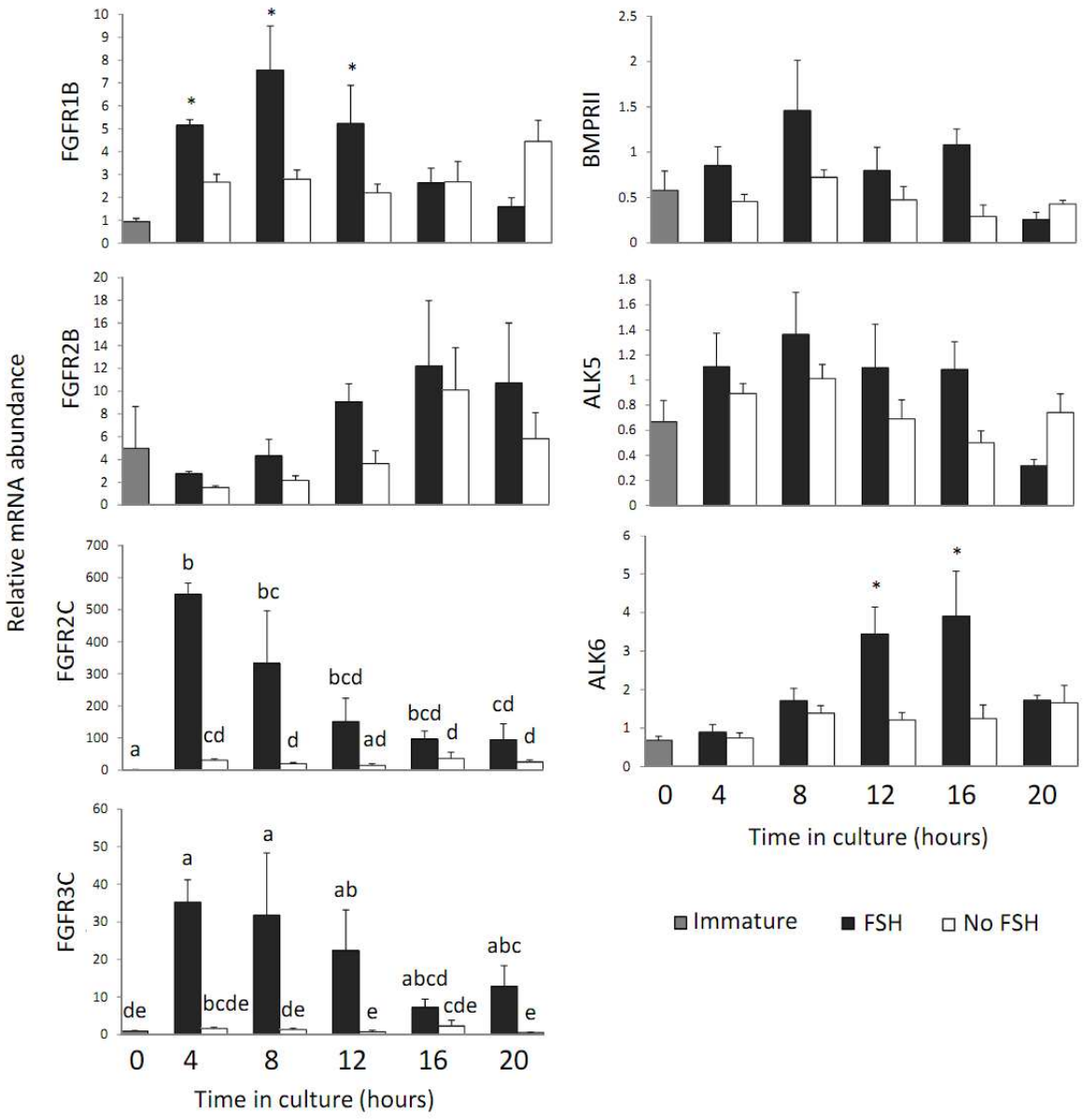

FGFR1B
BMPRII
FGFR2B
ALK5
FGFR2C
ALK6
FGFR3C
Relative mRNA abundance
Time in culture (hours)
Immature
FSH
No FSH

Figure 4

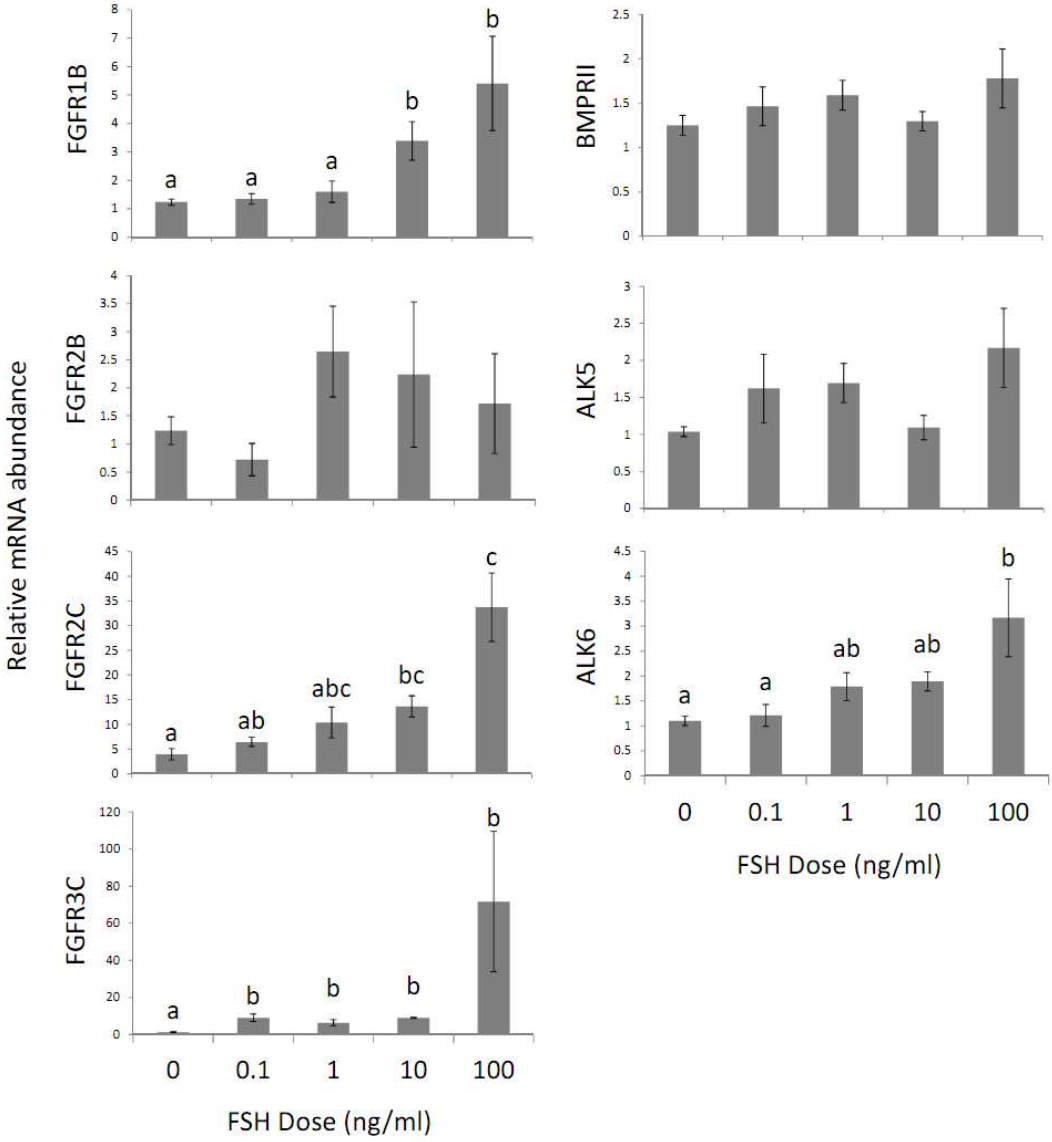
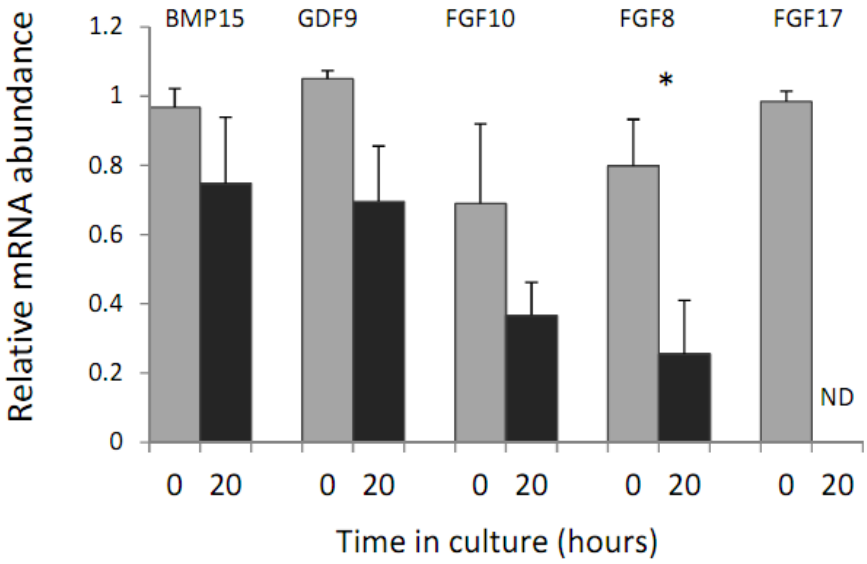

Relative mRNA abundance
FGFR1B
a a a b b
BMPRII
FGFR2B
ALK5
FGFR2C
a ab abc bc c
ALK6
a a ab ab b
FGFR3C
a b b b b
0 0.1 1 10 100
FSH Dose (ng/ml)
0 0.1 1 10 100
FSH Dose (ng/ml)

Figure 5


BMP15
GDF9
FGF10
FGF8
FGF17
Relative mRNA abundance
1.2
1
0.8
0.6
0.4
0.2
0
*
ND
0 20
0 20
0 20
0 20
0 20
Time in culture (hours)

RESULTADOS ADICIONAIS

EFEITOS DA PROTEÍNA MORFOGÊNICA ÓSSEA (BMP15) E DO FATOR DE CRESCIMENTO FIBROBLÁSTICO 10 (FGF10) SOBRE A VIA METABÓLICA DE EXPANSÃO DO CUMULUS

1 INTRODUÇÃO

Durante o desenvolvimento deste trabalho tivemos a oportunidade de discutir os resultados apresentados nos capítulo anteriores com os pesquisadores Robert Gilchrist e Jeremy Thompson do *"Research Centre for Reproductive Health, Robinson Institute"* da Universidade de Adelaide – Austrália, um grupo de pesquisa referência em estudos relacionados à interação oócito-células do cumulus e à investigação dos processos metabólicos envolvidos na maturação do complexo cumulus-oócito (COC). De posse da informação de que o fator de crescimento fibroblástico 10 (FGF10) e a proteína morfogênica óssea 15 (BMP15) são capazes de melhorar a expansão do cumulus pelo controle transcricional de genes reguladores da expansão, outros genes reguladores do metabolismo da expansão do cumulus se tornaram alvo interessante de avaliação. Em adição, consideramos pertinente investigar dados funcionais relacionados ao consumo de glicose, um substrato essencial para a síntese de ácido hialurônico.

A glicose é captada para dentro das células do cumulus através da ação dos facilitadores do transporte da glicose (GLUT; Morita *et al*., 1992; Watson e Pessin, 2001). Após ser captada, a glicose influencia vários aspectos da maturação oocitária e o seu metabolismo está relacionado com a capacidade de desenvolvimento do oócito (Sutton-McDowall *et al*., 2006). A glicose consumida pelo COC pode ser utilizada para a produção de energia, homeostase celular, maturação nuclear e substrato para a produção de matriz extracelular. Até o presente momento 4 vias metabólicas da glicose foram identificadas: a glicólise (produção de energia), a via pentose fosfato, a via biossíntese da hexosamina e a via poliol (Figura 1; Sutton-McDowall *et al*., 2010).

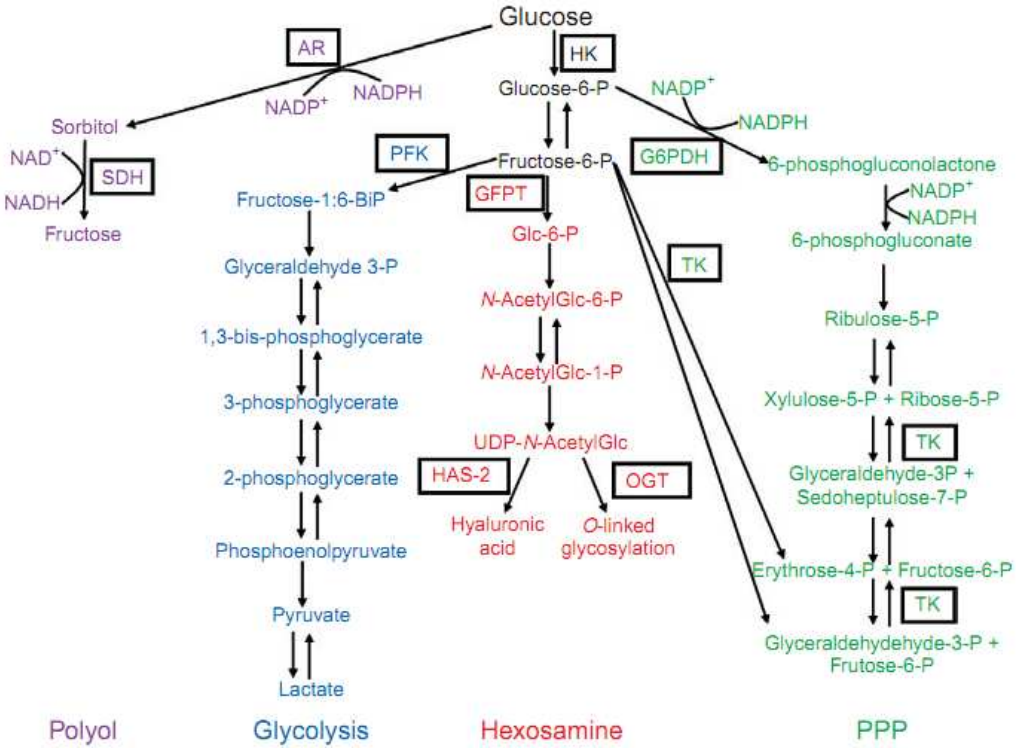


Figura 1 – Vias metabólicas através das quais a glicose pode ser utilizada no complexo cumulus-oócito (COC). AR, aldose redutase; ECM, matriz extracelular; G6PDH, glicose-6-fosfato desidrogenase; GFPT, glicosamina:frutose-6-fosfato acetil transferase; HAS-2, hialurona sintetase 2; HK, hexoquinase; OGT, glicosilação O-*linked*; PFK, fosfofrutoquinase; SDH, sorbitol desidrogenase (Sutton-McDowall *et al*., 2010).

Durante a maturação *in vitro* (MIV), o ácido láctico é o primeiro produto do metabolismo da glicose pelo COC bovino, indicando que a glicólise é o destino predominante da glicose (Sutton-McDowall *et al*., 2006). No entanto, foi demonstrado que ao longo das 24 horas da MIV, o consumo de glicose aumenta enquanto a produção de L-lactato permanece constante, sugerindo um destino alternativo para uma parte da glicose consumida (Sutton-McDowall *et al*., 2004). Em adição, foi observado que uma considerável proporção de glicose é cada vez mais utilizada pelo compartimento somático para a produção da matriz extracelular e mucificação das células do cumulus, estimuladas pelo FSH (Sutton-McDowall *et al*., 2004).

A via da biossíntese da hexosamina permite que a glicose seja utilizada para a síntese de glicosaminoglicanos, como o ácido hialurônico, necessário para a expansão do cumulus (Sutton-McDowall *et al*., 2010). Enquanto nas células somáticas não reprodutivas esta via é responsável por somente 1 a 3% do metabolismo da glicose (Sutton-McDowall *et al*., 2006),

nas células do cumulus estima-se que 23% da glicose consumida seja dirigida para a via da hexosamina (Gutnisky *et al*., 2007). Isto sugere que o aumento na taxa do consumo de glicose pelos COCs no final da MIV está relacionado com a produção da matriz extracelular e expansão do cumulus através da via hexosamina (Sutton-McDowall *et al*., 2010).

A principal enzima reguladora da via da hexosamina é a L-glicosamina:D-frutose-6-fosfato acetil transferase (GFPT). Tal enzima é responsável pela conversão da frutose-6-fosfato em glicosamina-6-fosfato, sendo a UDP-N-acetil glicosamina o produto final da via. Nas células do cumulus, a maioria da UDP-N-acetil glicosamina é convertida pela enzima HAS2 em ácido hialurônico, o principal componente da matriz extracelular das células do cumulus (Sutton-McDowall *et al*., 2006; Sutton-McDowall *et al*., 2010). A Figura 2 ilustra a via metabólica de utilização da glicose para a síntese de ácido hialurônico.

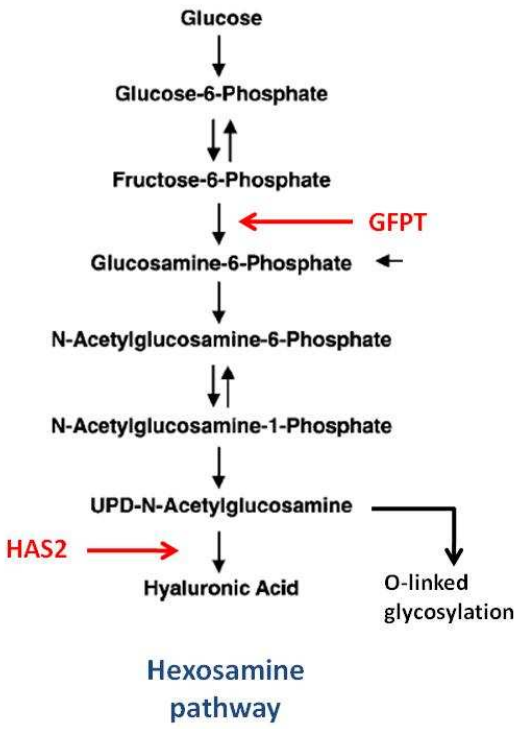


Figura 2 – Utilização da glicose pela via da hexosamina para a síntese de ácido hialurônico (Adaptado de Sutton-McDowall *et al*., 2004).

Levando em consideração as informações acima, foi sugerida pelos pesquisadores Jeremy Thompson e Robert Gilchrist, a investigação dos efeitos da BMP15, do FGF10 e da interação de ambos sobre as taxas de consumo de glicose e produção de lactato ao final da MIV, assim como, sobre a expressão dos RNAm das duas isoformas da enzima envolvida na

via metabólica de expansão do cumulus, a GFPT1 e a GFPT2. Foram avaliados também os efeitos da BMP15 e do FGF10 sobre a expressão de dois membros da família dos facilitadores do transporte da glicose (GLUT), o facilitador do transporte da glicose sensível à insulina (GLUT4) e o GLUT1. Para tanto, foram conduzidos dois experimentos com o objetivo de investigar se os fatores secretados pelos oócitos (FSOs) podem impactar a atividade da via biossíntese da hexosamina.

2 EFEITOS DA BMP15 E DO FGF10 SOBRE O CONSUMO DE GLICOSE E PRODUÇÃO DE LACTATO

O experimento para a avaliação dos efeitos da BMP15, do FGF10 e da combinação de ambos sobre o consumo de glucose e produção de lactato no final da MIV foi realizado pelo grupo do pesquisador Jeremy Thompson na Universidade de Adelaide. Foi utilizada a mesma composição do meio de maturação (meio base, concentração e fonte dos suplementos), assim como as condições de incubação adotadas nos experimentos realizados em nosso laboratório (descritas no artigo que compõe o capítulo 2).

Grupos de 10 COCs bovinos foram cultivados em 200 µl de meio de maturação por 22 horas, a 38,5°C, 5% CO_2, em atmosfera umidificada. O meio de maturação consistia de TCM199 com sais de Earl's suplementado com pFSH (1 µg/ml), pLH (10 UI/ml), piruvato de sódio (22 µl/ml), amicacina (75 µg/ml) e BSA (4 mg/ml). Para testar os efeitos dos FSOs foi adicionado ao meio de maturação BMP15 recombinante humana (100 ng/ml; n=3), FGF10 recombinante humano (10 ng/ml; n=3) e BMP15 (100 ng/ml) em combinação com o FGF10 (10 ng/ml; n=3). Ao final da maturação por 22 horas, o meio gasto foi coletado e estocado à -80°C.

Para a determinação quantitativa do consumo de glicose e produção de lactato, 100 µl do meio gasto foram analisados pelo sistema automatizado Roche Hitachi 912, utilizando os kits para ensaio de glicose (Gluco-quant Glicose HK®, Roche Diagnostics, NSW, Australia) e lactato (Lactato LACT®, Roche Diagnostics, NSW, Australia). Os dados estão expressos como pmol/COC/h. Os resultados foram analisados utilizando um modelo linear geral e o teste Bonferroni post-hoc.

A presença de ambas as proteínas, BMP15 e FGF10, aumentou o consumo de glicose em comparação com o grupo controle (ausência das proteínas; $P<0,05$). Entretanto, não houve efeito aditivo da BMP15 e FGF10 em combinação sobre o consumo de glicose em relação aos grupos tratados com cada fator individualmente (Figura 3A). Em adição, não foram notadas

diferenças na produção de lactato entre os tratamentos (Figura 3B), indicando que apesar do aumento no consumo de glicose na presença de BMP15 e FGF10, a proporção de glicose metabolizada via glicólise para a produção de lactato não foi alterada.

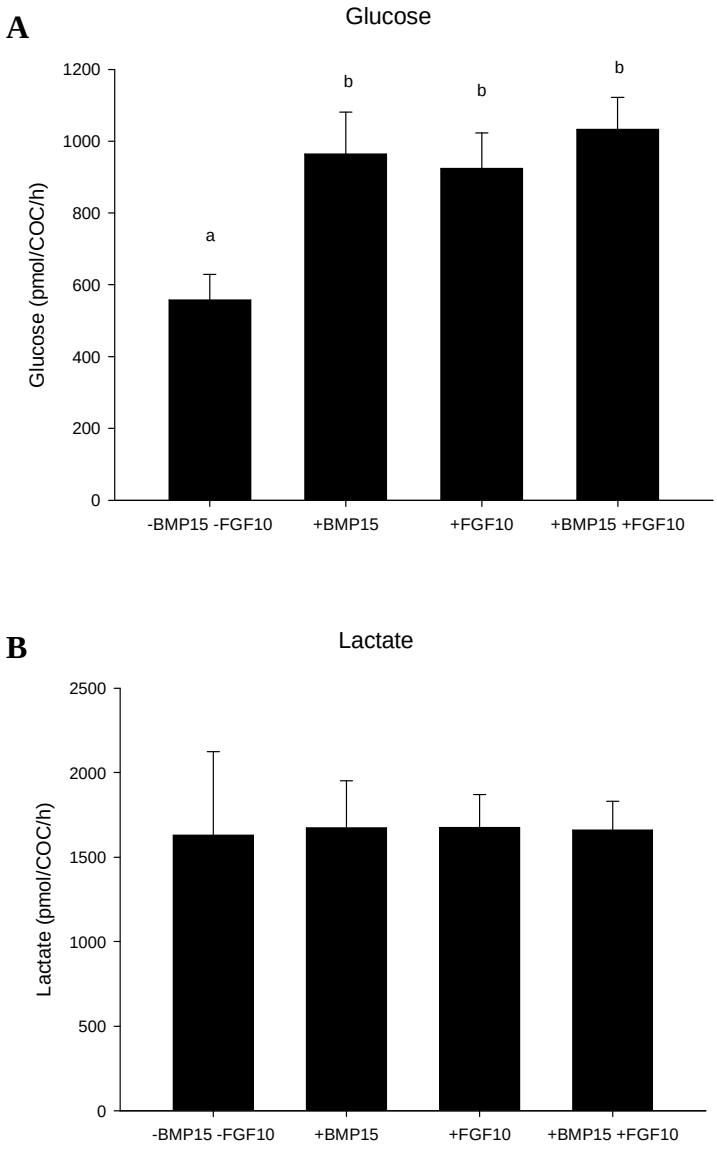


Figura 3 – Consumo de glicose (A) e produção de lactato (B) pelos complexos cumulus-oócito (COCs) após 22 horas de maturação em meio controle (-BMP15 -FGF10), +BMP15 (100ng/ml), +FGF10 (10ng/ml) ou +BMP15 +FGF10. Os dados estão apresentados como média ±EPM. As barras com diferentes letras indicam valores significativamente diferentes (P<0,05). Os dados foram obtidos a partir de 3 réplicas para cada tratamento.

3 EFEITOS DA BMP15 E DO FGF10 SOBRE A EXPRESSÃO GÊNICA DAS ENZIMAS GFPT1 E GFPT2 E DOS FACILITADORES DO TRANSPORTE DA GLICOSE (GLUT1 E GLUT4)

A expressão do RNAm da GFPT1, GFPT2, GLUT1 e GLUT4 foi avaliada nas células do cumulus dos experimentos de dose resposta de FGF10 e BMP15, assim como no experimento de interação da BMP15 com o FGF10, nos momentos 4, 12 e 22 horas de maturação. Toda a metodologia adotada em tais experimentos encontra-se descrita no artigo que compõe o capítulo 2.

A BMP15 na dose de 100ng/ml estimulou a expressão do RNAm da GFPT2 a partir das 12 horas de maturação e da GFPT1 e do GLUT1 às 22 horas de maturação ($P<0,05$; Figura 4). A expressão do GLUT4 não foi afetada pelo tratamento com a BMP15. Já o FGF10 estimulou (50ng/ml) a abundância do RNAm GFPT1 às 12 horas de maturação ($P<0,05$), porém não alterou a expressão da GFPT2, GLUT1 e GLUT4 (Figura 5). A combinação da BMP15 com o FGF10 não modificou a expressão do RNAm da GFPT1, GFPT2, GLUT1 e GLUT4 diferentemente da BMP15 ou do FGF10 adicionados individualmente (dados não mostrados).

Em resumo, os resultados de expressão gênica indicam que a BMP15 pode regular a captação de glicose através da modulação do facilitador do transporte da glicose, GLUT1. Em adição, a regulação do FGF10 e, em especial, da BMP15 sobre a expressão gênica da enzima GFPT, em conjunto com os dados referentes ao aumento do consumo de glicose e manutenção na produção de lactato, sustentam a hipótese de que estes FSOs podem modular o processo metabólico de expansão do cumulus através de um aumento na atividade da via da biossíntese da hexosamina. Para confirmar a hipótese de que a maior proporção de glicose consumida após o tratamento com BMP15 e FGF10 é direcionada para a produção da matriz extracelular e mucificação das células do cumulus é necessário primeiramente avaliar a incorporação de glicose na matriz extracelular. Um experimento com este objetivo está em andamento no “Research Centre for Reproductive Health, Robinson Institute” Universidade de Adelaide - Austrália, sob a supervisão dos pesquisadores Jeremy Thompson e Robert Gilchrist.

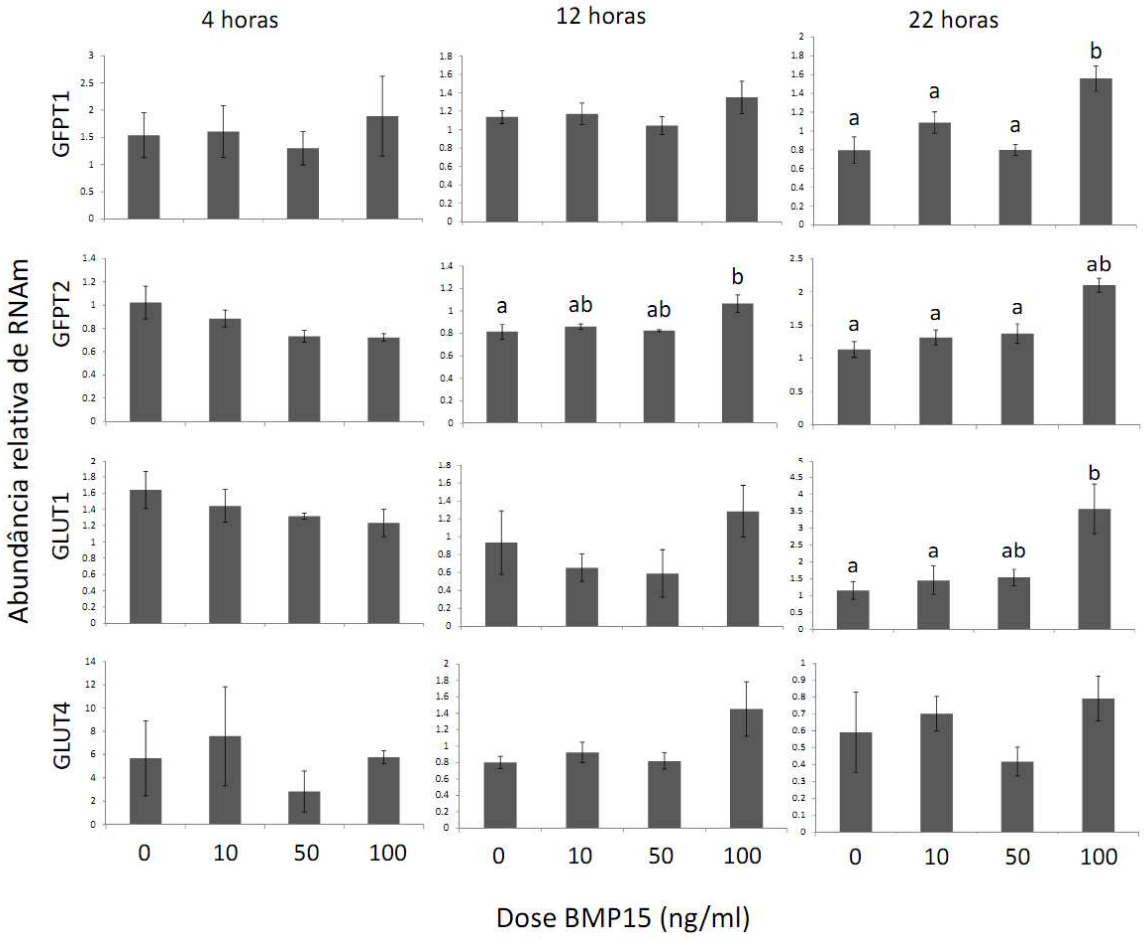


Figura 4 – Efeito das doses crescentes de BMP15 sobre a abundância de RNAm da GFPT1, GFPT2, GLUT1 e GLUT4 nas células do cumulus. Os COCs foram cultivados com doses crescentes de BMP15 por 4, 12 e 22 horas. A abundância do RNAm foi mensurada por PCR em tempo real. Os dados estão apresentados como média (±EPM) em relação a uma amostra calibradora pelo método de ΔΔCt com correção da eficiência. As barras com diferentes letras indicam valores significativamente diferentes (P<0,05). Os dados foram obtidos a partir de 4 réplicas para cada tratamento.

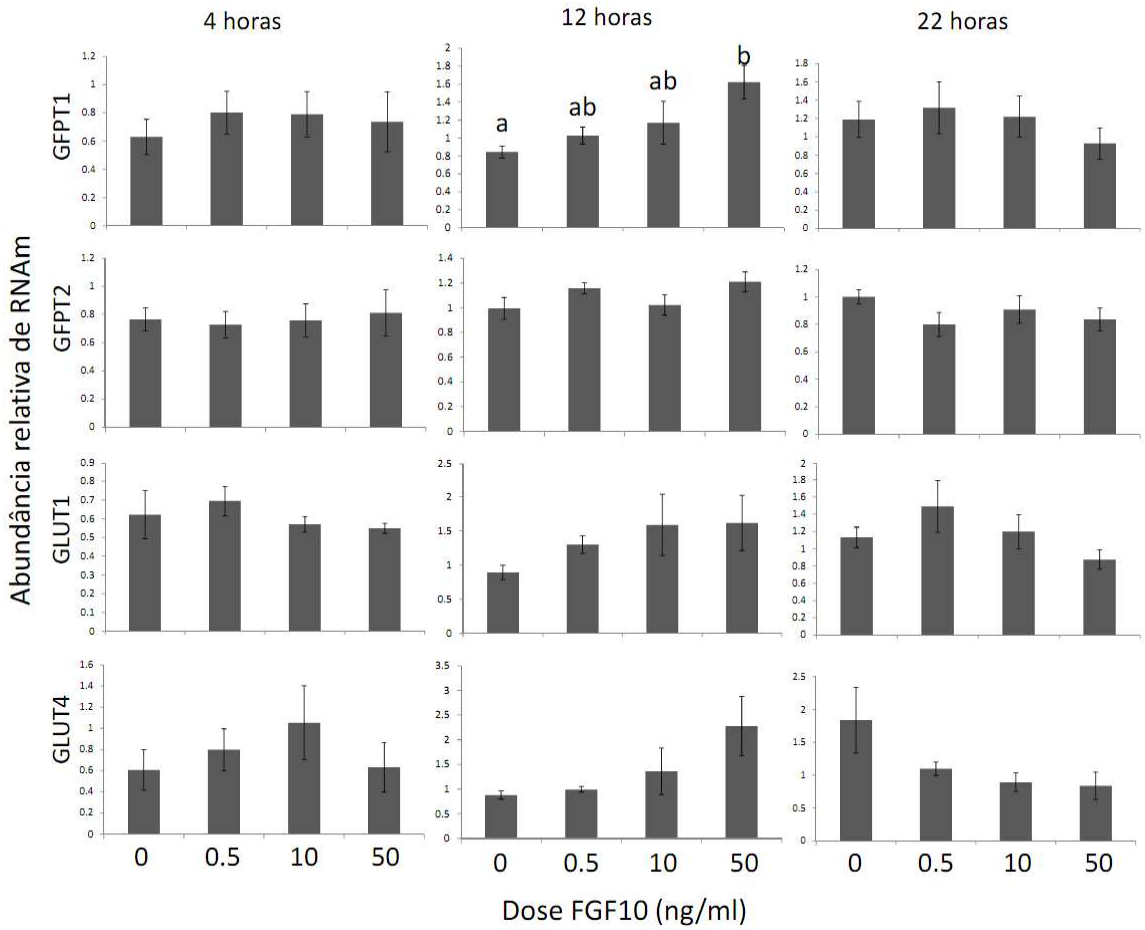


Figura 5 – Efeito das doses crescentes de FGF10 sobre a abundância de RNAm da GFPT1, GFPT2, GLUT1 e GLUT4 nas células do cumulus. Os COCs foram cultivados com doses crescentes de FGF10 por 4, 12 e 22 horas. A abundância do RNAm foi mensurada por PCR em tempo real. Os dados estão apresentados como média (±EPM) em relação a uma amostra calibradora pelo método de ΔΔCt com correção da eficiência. As barras com diferentes letras indicam valores significativamente diferentes (P<0,05). Os dados foram obtidos a partir de 4 réplicas para cada tratamento.

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CONSIDERAÇÕES FINAIS

CONSIDERAÇÕES FINAIS

Diante de evidências crescentes acerca do papel modulador do oócito sobre a diferenciação das células do cumulus, bem como da importância desta diferenciação para a competência do oócito e para o desenvolvimento embrionário, este trabalho estudou algumas funções e a regulação de importantes fatores secretados pelo oócito (FSOs) durante a maturação *in vitro* em bovinos. Em resumo, foi demonstrado um efeito positivo da BMP15 e do FGF10 sobre a expansão do cumulus, associado à regulação da expressão gênica, por ambos os fatores, em diferentes estágios da cascata molecular que induz a expansão, conforme modelo sugerido na Figura1. Estes resultados são consistentes com dados da literatura que mostram ações benéficas da BMP15 e do FGF10 nas taxas de desenvolvimento embrionário, uma vez que a expansão do cumulus foi previamente associada a melhores taxas de fecundação e desenvolvimento embrionário *in vitro* (Slotte *et al*., 1993; Gutnisky *et al*., 2007). Adicionalmente, foi demonstrado que o FSH regula a expressão de genes relacionados com a expansão do cumulus, assim como a expressão de receptores para FGFs e BMP15 nas células do cumulus durante a maturação *in vitro*. Estes dados reforçam a hipótese de que a sinalização por meio dos FGFRs e de receptores da BMP15 é importante no processo de expansão do cumulus em bovinos e, possivelmente, para outros aspectos da diferenciação do complexo cumulus-oócito. Vale ainda ressaltar que resultados preliminares sugerem que a BMP15 e o FGF10 modulam o processo metabólico de expansão do cumulus através da intensificação da via da biossíntese da hexosamina, responsável pela produção do ácido hialurônico.

Vale destacar que a pesquisa básica, tal como o presente trabalho apresenta, tem permitido um substancial avanço na compreensão do eixo de comunicação entre o oócito e as células somáticas foliculares e dos eventos moleculares que desencadeiam a retomada da meiose e a expansão do cumulus durante a maturação oocitária. Este conhecimento é fundamental para o aperfeiçoamento da técnica de maturação oocitária *in vitro*, uma etapa chave na reprodução assistida como uma importante plataforma tecnológica para a fecundação e produção embrionária *in vitro*, tecnologia de células tronco embrionária, clonagem e trangenia.

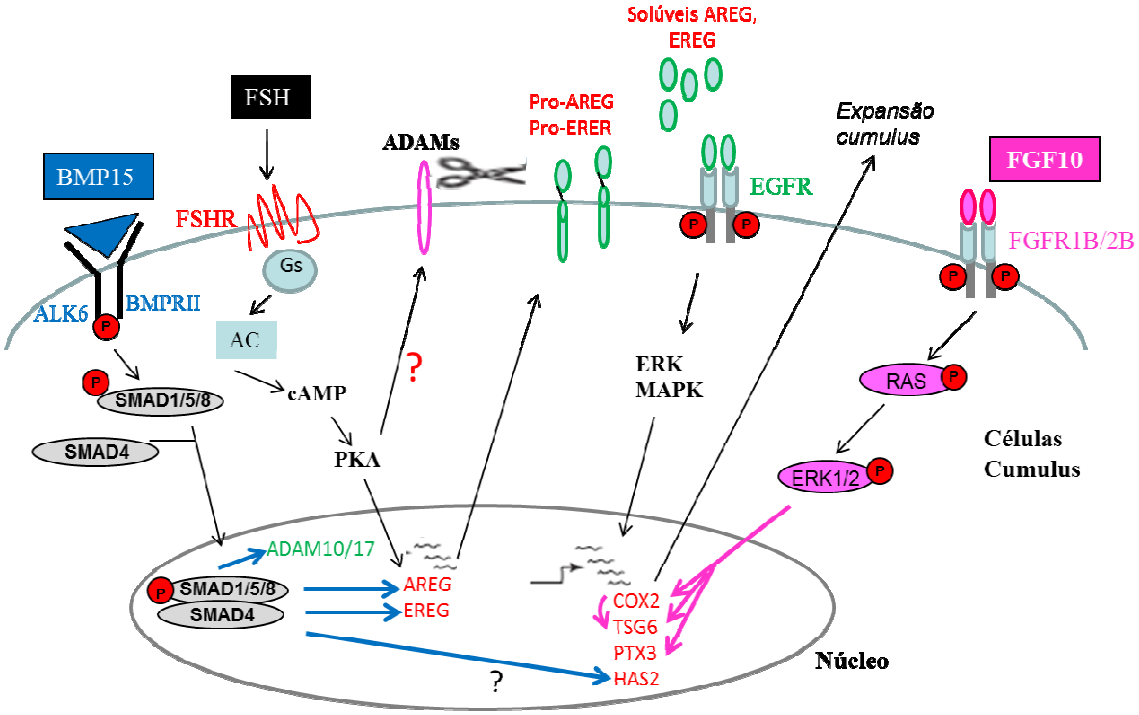

Figura 1 – Modelo sugerido para a regulação da expressão de genes indutores da expansão do cumulus pela BMP15 e FGF10. Enquanto a BMP15, via SMADs1/5/8, parece estimular a expressão de genes do início da cascata que leva à expansão (ADAM10, ADAM17, AREG e EREG), e por intermádio deles, a expressão de genes que diretamente estimulam a expansão do cumulus (COX2, TSG6, PTX3 e HAS2), o FGF10 parece agir especificamente sobre esses últimos, particularmente a COX2.