UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

Júlia Koch

INTERAÇÃO YAP1-TEAD REGULA A CASCATA DE SINALIZAÇÃO DO EGF EM CÉLULAS DO CUMULUS EM BOVINOS

Santa Maria, RS
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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Sanidade e Reprodução Animal da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Medicina Veterinária**.

Orientador: Prof. Paulo Bayard Dias Gonçalves

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Koch, Júlia
INTERAÇÃO YAP1-TEAD REGULA A CASCATA DE SINALIZAÇÃO DO EGF EM CÉLULAS DO CUMULUS EM BOVINOS / Júlia Koch.- 2020.
70 p.; 30 cm

Orientador: Paulo Bayard Gonçalves
Coorientador: Valério Portela
Dissertação (mestrado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Medicina Veterinária, RS, 2020

1. Reprodução Animal 2. Ovulação 3. Expansão do cumulus 4. Via Hippo 5. Via de sinalização do EGF I. Gonçalves, Paulo Bayard II. Portela, Valério III. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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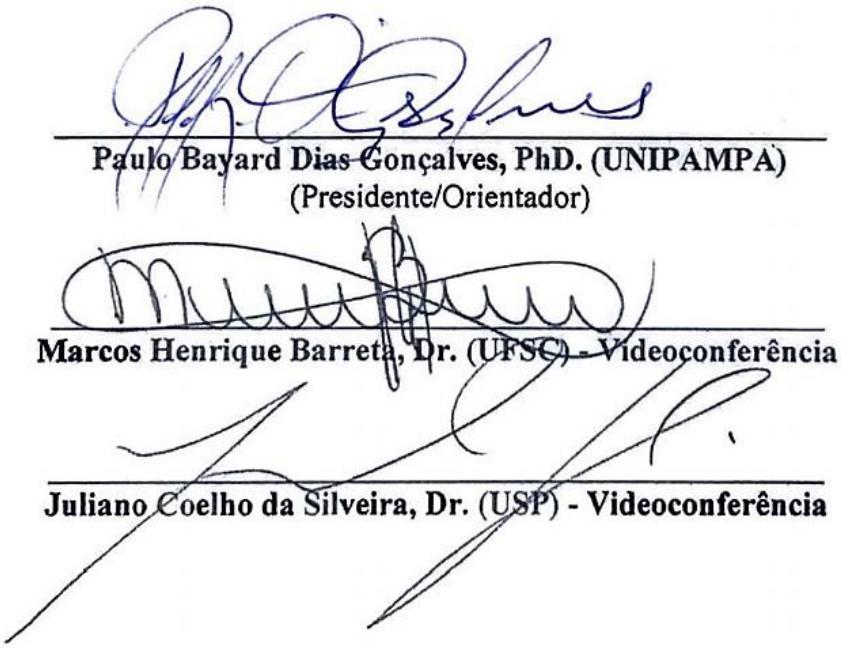
Aprovado em 07 de fevereiro de 2020:



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Santa Maria, RS
2020

Dedico esta dissertação

Aos meus pais e minha irmã que sempre me incentivam a lutar pelos meus sonhos.

Ao meu noivo por todo o seu amor, apoio, compreensão e incentivo. Por ser luz na minha vida.

Ao meu avô Eugênio (*in memoriam*), o qual deve estar orgulhoso de eu estar seguindo os seus passos.

AGRADECIMENTOS

A Deus pelo dom da minha vida. Por toda a proteção, saúde e bênçãos derramadas nesse tempo de mestrado permitindo superar todas as dificuldades que surgiram. E por ter colocado pessoas incríveis no meu caminho.

À Universidade Federal de Santa Maria e ao Programa de Pós-Graduação em Medicina Veterinária pela oportunidade de realizar esse sonho em um programa de excelência.

Aos órgãos de fomento CNPq, CAPES, FAPERGS e FINEP por proporcionar as condições necessárias para a realização dessa pesquisa. Agradeço especialmente ao CNPq pela bolsa de estudos que me permitiu concretizar o mestrado.

Ao meu orientador, professor Paulo Bayard Dias Gonçalves, pela oportunidade de realizar o mestrado. Pela sua orientação profissional e espiritual.

Ao meu coorientador, professor Valério Valdetar Marques Portela Jr., por toda a sua disponibilidade, dedicação e orientação para que esse trabalho fosse concretizado.

Ao professor Gustavo Zamberlam pela colaboração intelectual e ao incentivo financeiro, juntamente com a Université de Montréal (UdeM), para a realização dessa pesquisa.

À minha amiga e companheira de laboratório, Daniele Missio, por todas as experiências compartilhadas e desafios superados. Por toda a sua ajuda, zelo e dedicação para que esse trabalho fosse efetivado.

Aos meus amigos e colegas de laboratório Zigomar, Leonardo, Camila Cupper e Camila Azzolin que colaboraram intelectualmente e braçalmente nesse trabalho.

Ao Dirvano Donadel pela sua disponibilidade e auxílio nos experimentos.

À secretária do PPGMV, Maria da Rosa, pela paciência e dedicação.

Às colaboradoras, Maria e Denise, que com zelo proporcionaram um ambiente de trabalho agradável e limpo para a realização desta pesquisa. E a todos os colaboradores que mantem a funcionalidade da Universidade.

A todos os colegas, estagiários e professores do BioRep pela convivência e experiências compartilhadas.

À Dra. Susane Lopes e ao Laboratório Central de Microscopia Eletrônica (LCME) da Universidade Federal de Santa Catarina (UFSC) pela colaboração e dedicação com as imagens de fluorescência.

Ao Frigorífico Silva pela paciência, compreensão e doação dos ovários para que os experimentos fossem realizados.

Os que desprezam os pequenos acontecimentos nunca
farão grandes descobertas.
Pequenos momentos mudam grandes rotas.

- Augusto Cury -

RESUMO

INTERAÇÃO YAP1-TEAD REGULA A CASCATA DE SINALIZAÇÃO DO EGF EM CÉLULAS DO CUMULUS EM BOVINOS

AUTOR: Júlia Koch
ORIENTADOR: Paulo Bayard Dias Gonçalves

O mecanismo de expansão das células do cumulus (CC) e ovulação *in vivo* é desencadeado pelo pico de LH nas células da granulosa e CC, culminando com uma série de comunicações parácrinas e autócrinas entre estas células ovarianas. Está estabelecido que fatores secretados pela granulosa atuam sobre as CC estimulando a transcrição de genes importantes para a expansão, maturação oocitária e ovulação. Embora já sejam conhecidas diversas vias de sinalização envolvidas com esse processo, ainda não se tem o completo entendimento. Recentemente, a via de sinalização Hippo e seus efetores (YAP1 e TAZ) vêm sendo relacionados com diferentes contextos da fisiologia ovariana, inclusive com o processo ovulatório, em camundongos e humanos. Porém, ainda são escassos os estudos relacionando essa via com espécies de produção. O objetivo deste estudo foi demonstrar a função da ligação YAP1 com fatores de transcrição TEAD na modulação de genes-chave para a expansão e mecanismo ovulatório nas células do cumulus de bovinos e a sua regulação. Para isso, complexos cumulus-oócito (CCO) foram cultivados *in vitro* durante 0 e 12h e submetidos a imunofluorescência para demonstrar a localização intracelular de YAP1 e pYAP1. Os resultados sugerem que YAP1 transloca do citoplasma para o núcleo das CC durante o processo de maturação *in vitro* (MIV). Para elucidar a regulação de *YAP1*, receptores de EGF (EGFR; com AG 1478) foram inibidos e então, os CCOs foram estimulados com EGF ou FSH. Com isso, foi demonstrado que a inibição de EGFR resulta na redução da expressão de *YAP1* e *CTGF* (um dos principais genes-alvo de YAP1-TEAD) nas CC. Então, os CCOs foram tratados com diferentes concentrações de verteporfina, um inibidor da interação YAP1-TEAD e obteve-se a redução de maneira concentração-dependente de genes-chave para a expansão (*HAS2* e *PTX3*) e ovulação (*ADAM17*, *EREG* e *PTGS2*) nas CC. A interação YAP1-TEAD está envolvida na expressão de genes-chave para a expansão e mecanismo ovulatório em células do cumulus de bovinos e a regulação de *YAP1* é mediada através da estimulação de EGFR, por ação direta de EGF, ou indireta de FSH.

Palavras-chave: Complexo cumulus-oócito. Expansão do cumulus. Ovário. Ovulação.

ABSTRACT

YAP1-TEAD INTERACTION REGULATES EGF SIGNALING CASCADE IN CUMULUS CELLS IN BOVINE

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The mechanism of cumulus cell (CC) expansion and ovulation *in vivo* is triggered by LH surge in granulosa cells and CC, which culminating in a series of paracrine and autocrine communications between these ovarian cells. It is well established that granulosa-secreted factors act on CC, stimulating important genes involved in cumulus expansion, oocyte maturation and ovulation. Although several signaling pathways involved in this process are already known, there is no complete understanding in this physiological process. Recently, the Hippo signaling pathway and its effectors (YAP1 and TAZ) have been related to different contexts of ovarian physiology, including the ovulatory process, in mice and humans. However, few studies relating this pathway are available in economic relevant species. The aim of this study was to demonstrate the function of YAP1-TEAD interaction in the modulation of key genes for expansion and ovulatory mechanism, as well as, their regulation in bovine cumulus cells. For this, cumulus-oocyte complexes (COCs) were *in vitro* cultured during 0 or 12h and subjected to immunofluorescence to demonstrate YAP1 and pYAP1 intracellular localization. Our results suggest that YAP1 translocates from the cytoplasm to the nucleus during *in vitro* maturation (IVM) time. To elucidate YAP1 regulation, we inhibited EGF receptor (EGFR, with AG 1478) and stimulated COCs with EGF or FSH. Thus, EGFR inhibition results in reduction of YAP1 and CTGF (one of the major YAP1-TEAD target genes) expression. Then, COCs were subjected to different concentrations of verteporfin, an YAP1-TEAD inhibitor, and it was obtained dose-dependent reduction of cumulus expansion (*HAS2* and *PTX3*) and ovulation (*ADAM17, EREG* and *PTGS2*) related genes. Based on that, it is suggested that YAP1-TEAD interaction is involved in the expression of key genes for cumulus expansion and ovulation in bovine cumulus cells. Also, YAP1 regulation is mediated through EGFR stimulation, by EGF direct action, or FSH indirect action.

Keywords: Cumulus-oocyte complex. Cumulus expansion. Ovarian. Ovulation.

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LISTA DE ABREVIAÇÕES E SIGLAS

ADAM17	*ADAM metallopeptidase domain 17*
AMPc	Adenosina 3',5'-monofosfato cíclico
AREG	Ampiregulina
Adamts1	*Metallopeptidase with thrombospondin type 1 motif 1*
BMP15	*Bone morphogenetic protein 15*
BTC	Betacelulina
CCO	Complexo cumulus-oócito
CTGF	*Connective tissue growth factor*
Cyr61	*Cysteine-rich angiogenic inducer 61*
EGF	Fator de crescimento epidérmico
EGF-like	Fatores semelhantes ao EGF
EGFR	Receptor de EGF
EREG	Epiregulina
ERK1/2	Quinases reguladas por sinal extracelular 1 e 2
FSH	Hormônio folículo estimulante
GDF9	*Growth differentiation factor-9*
GnRH	Hormônio liberador de gonadotrofina
HAS2	*Hyaluronan synthase 2*
LH	Hormônio Luteinizante
LHR	Receptores de LH
MAPK	Proteína quinase ativada por mitógeno
NOV	*Nephroblastoma overexpressed*
PGE2	Prostaglandina E2
PKA	Serina proteína quinase A
PTGS2	Prostaglandina sintetase 2
PTX3	*Pentraxin 3*
p38MAPK	Proteínas cinases ativadas por mitogênio P38
RNAm	Ácido ribonucleico (RNA) mensageiro
TAZ	*Transcriptional co-ativator with PDZ-binding motif*
TEAD	*TEA domain family*
TNFAIP6	*Tumor necrosis factor-alpha-induced protein 6*
YAP	*Yes-associated protein*
YAP1	*Yes-associated protein 1*

SUMÁRIO

1 INTRODUÇÃO

O sucesso reprodutivo da fêmea é dependente de uma série de eventos fisiológicos, dentre os quais, a ovulação de um gameta apto a ser fecundado é crucial para a perpetuação das espécies. É de amplo conhecimento que diversos fatores podem influenciar nesse processo, afetando a eficiência reprodutiva tanto em animais quanto em mulheres. Desta forma, o completo entendimento da fisiologia e das patologias que acometem o sistema reprodutivo se tornam fundamentais para a elucidação desses mecanismos bem como para o desenvolvimento e aplicação de biotecnologias, permitindo que ocorra o sucesso na ovulação.

A ovulação é consequência de uma dinâmica regulação hipotalâmica-hipofisária-gonadal que induz mudanças funcionais de caráter endócrino, bioquímico e molecular no folículo pré-ovulatório, culminando com a ruptura do estigma ovulatório e liberação do gameta feminino (Shimada *et al.*, 2006). O fator desencadeador do processo ovulatório é decorrente do pico do hormônio luteinizante (LH) liberado pela adenohipófise. O LH se liga em seus receptores nas células da granulosa, ativando uma série de eventos intracelular (Richards, 1994). Como consequência, ocorre a transcrição de genes relacionados com o mecanismo ovulatório, com a esteroidogênese, expansão do cumulus e maturação nuclear (Park *et al.*, 2004).

Além disso, o pico pré-ovulatório de LH induz a mudanças estruturais e funcionais no complexo cumulus-oócito (CCO), resultando na produção de ácido hialurônico e expansão do cumulus (Eppig, 1979), processos fundamentais para que ocorra a posterior ovulação e fecundação. A ação do LH sob o cumulus ocorre de maneira indireta por ação parácrina de prostaglandina E2 (PGE2) e fatores semelhantes ao fator de crescimento epidérmico (EGF-*like*) oriundos das células murais (Sirois e Richards, 1992; Park *et al.*, 2004; Shimada *et al.*, 2006).

Embora já sejam conhecidos alguns mecanismos reguladores do processo ovulatório, a completa elucidação deste processo ainda não foi atingida. Na última década, um novo mecanismo de ação, a via de sinalização Hippo, vem sendo relacionada com o sistema reprodutivo, principalmente com a embriogênese, cistos foliculares e tumores ovarianos (Hall *et al.*, 2010; Li *et al.*, 2012; Yu e Guan, 2013; Meng *et al.*, 2016).

A Hippo é uma via de sinalização considerada altamente conservada com funções bem definidas no controle do crescimento de órgãos, na diferenciação celular, proliferação e apoptose (Halder e Johnson, 2011; Meng *et al.*, 2016). Essa via representa uma cascata de proteína quinases que regula a atividade de dois efetores principais, YAP1 (*yes-associated*

protein) e TAZ (*transcriptional co-activator with PDZ-binding motif*). YAP1 e TAZ se ligam a fatores de transcrição da família TEAD (*TEA domain family member*; TEAD1, TEAD2, TEAD3 e TEAD4) e regulam a expressão de diversos genes (Zhao, Li, Lei, *et al.*, 2010; Mauviel *et al.*, 2012), sendo os mais conhecidos o CTGF (*connective tissue growth factor*), Cyr61 (cysteine-rich angiogenic inducer 61) e NOV (*nephroblastoma overexpressed*; Heath *et al.*, 2008; Lai *et al.*, 2011; Malik *et al.*, 2015).

A via Hippo vem sendo estudada em diversos tecidos celulares (Yu e Guan, 2013; Meng *et al.*, 2016), inclusive na embriogênese (Manzanares e Rodriguez, 2013; Frum *et al.*, 2018) e na fisiopatologia da reprodução na vida adulta. Estudos têm demonstrado que a desregulação de YAP1 está relacionada com a insuficiência ovariana primária (Cheng *et al.*, 2015) e a síndrome do ovário policístico (Li *et al.*, 2012). Enquanto a ativação de YAP1 é essencial para o desenvolvimento folicular em camundongos (Lv *et al.*, 2019) e bovinos (Plewes *et al.*, 2019). Além disso, camundongos nocaute para CTGF apresentaram desenvolvimento folicular interrompido e redução nas taxas de ovulação, através de uma significante redução de *Adamts1*, uma metaloproteínase fundamental para o remodelamento da matriz extracelular de folículos pré-ovulatórios (Nagashima *et al.*, 2011).

Similar a camundongos, o processo ovulatório em bovinos também é dependente de uma metaloproteínase, a ADAM17 (*ADAM metallopeptidase domain 17*; Qinglei *et al.*, 2009). ADAM17 é uma enzima proteolítica ativada por LH nas células da granulosa, que estimula a liberação de EREG (epiregulina) pré-sintetizado e armazenado na forma de pró-EREG na membrana celular, a qual ativa o receptor do fator de crescimento epidérmico (EGFR) estimulando a expressão de EGF-*like*: EREG, AREG (ampiregulina) e BTC (betacelulina; Portela *et al.*, 2011). Em contrapartida, as células do cumulus e os oócitos não expressam receptores para LH, não respondendo ao estímulo direto desse hormônio (Park *et al.*, 2004). Assim, o estímulo para a ovulação nas células do cumulus ocorre por ação parácrina de EGF-*like* sobre o seu receptor (EGFR; Espey e Richards, 2002; Sekiguchi *et al.*, 2004; Panigone *et al.*, 2008), associada ao estímulo de FSH (Richards, 1980; Regan, 2003). A expressão de AREG e EREG nas células do cumulus é essencial para a ativação de vias que culminam com a expansão do cumulus (Shimada *et al.*, 2006) e a maturação do oócito (Park *et al.*, 2004). A expansão do cumulus, associada com a expressão de genes ovulatórios-chave, são fundamentais para a ovulação de um CCO apto a ser fecundado (Kawashima e Kawamura, 2018).

Embora já existam estudos demonstrando a importância da via Hippo na reprodução da fêmea, ainda não foi demonstrado a importância dos efetores YAP1 e TAZ no mecanismo ovulatório e na expansão do cumulus das espécies de produção. Dados não publicados do nosso

grupo de pesquisa demonstraram que a ligação de YAP1-TEAD é fundamental para desencadear a cascata pré-ovulatória de EGF-like nas células murais da granulosa. Com isso, surge a hipótese de que a ligação de YAP1 com as TEAD é fundamental para a transcrição de genes relacionados com o mecanismo ovulatório. O objetivo deste estudo foi demonstrar a função da ligação YAP1-TEAD na modulação de genes-chave para a expansão e mecanismo ovulatório nas células do cumulus de bovinos, bem como, elucidar a sua regulação.

2 REVISÃO DA LITERATURA

2.1 OVULAÇÃO

A ovulação é marcada por uma dinâmica interação de fatores ainda não completamente elucidados que culminam com a ruptura do estigma ovulatório e liberação de um gameta apto a ser fecundado. Para que esse processo ocorra, uma dinâmica comunicação hormonal entre o eixo hipotalâmico-hipofisário-gonadal é necessária, bem como, a comunicação parácrina entre células somáticas e o oócito é crucial para o sucesso da ovulação (Gilula et al., 1978; Su et al., 2010).

Após a determinação da dominância, o folículo dominante aumenta exponencialmente as concentrações de estradiol intrafolicular, que associada aos baixos níveis de progesterona circulantes (Baird et al., 1976; Baird et al., 1981), induzem a um aumento súbito e de grande amplitude de GnRH no hipotálamo, que consequentemente estimula a liberação do pico pré-ovulatório de LH pela adenohipófise (Espey, 1980; Russell e Robker, 2007). O pico de LH induz mudanças funcionais de caráter endócrino, bioquímico e molecular no folículo pré-ovulatório, permitindo que ocorra a ovulação (Shimada et al., 2006).

Porém, a ovulação só é desencadeada quando as células da granulosa se tornam responsivas ao LH (Sartori et *al.*, 2001), ou seja, quando as mesmas adquirem receptores de LH (LHR; Robert et *al.*, 2003), que em *Bos taurus* ocorre em folículos com diâmetro superior a 11 mm (Evans e Fortune, 1997). Anterior a isso, somente as células da teca possuem LHR funcionais em suas membranas (Xu et *al.*, 1995). E embora ocorra a expressão de RNAm para LHR em células da granulosa de folículos antrais pequenos (<4 mm), esse RNAm sofre *splicing* alternativo, não dando origem à proteína funcional (Robert et *al.*, 2003). Já as células do cumulus de bovinos não expressam LHR mesmo no período pré-ovulatório (Robert et *al.*, 2003). Desta forma, o estímulo desencadeador do processo ovulatório e para a maturação oocitária nessas células ocorre através de mediadores oriundos das células da granulosa (Hsieh e Conti, 2005; Conti et al., 2006).

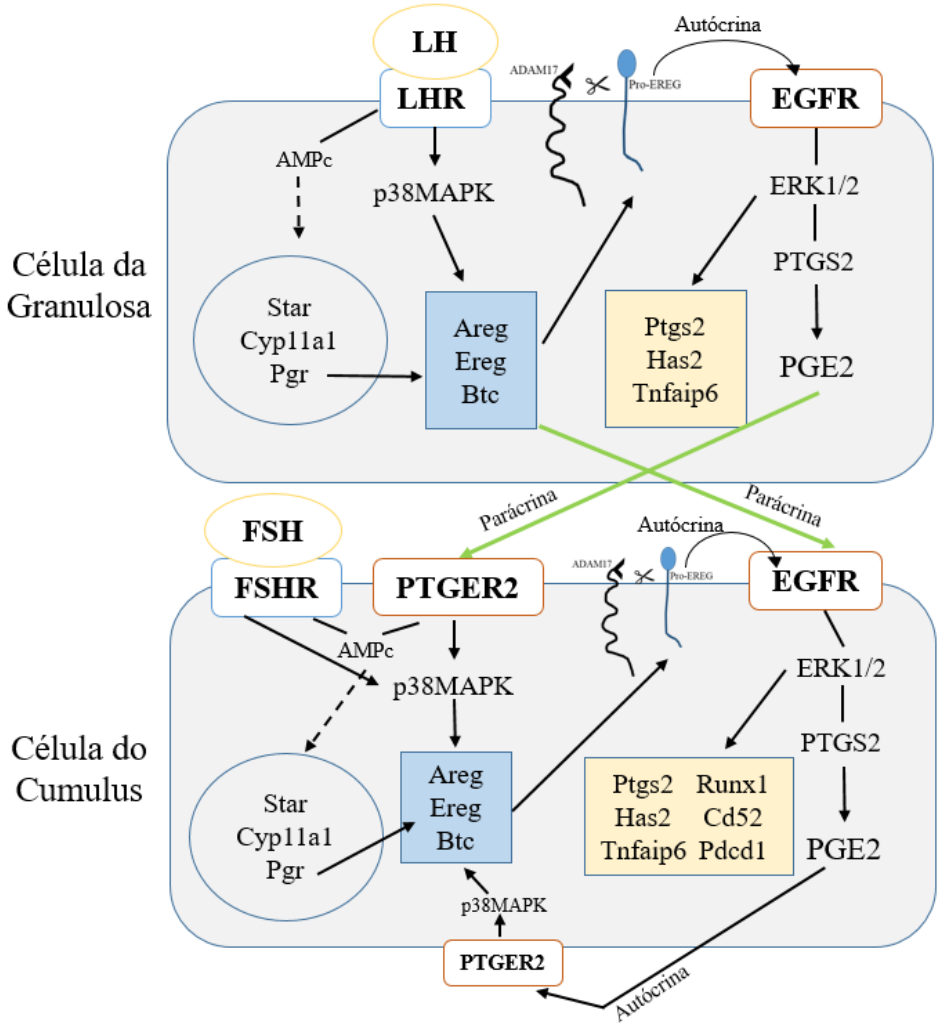
Nas células da granulosa, o LHR é acoplado à proteína G, portanto, ao se ligar ao seu receptor, o LH ativa adenilato ciclase, resultando no aumento intracelular de AMPc seguido da ativação de PKA dependente de AMPc (Marsh, 1976; Richards, 1994; Puri et *al.*, 2016). Por sua vez, a PKA ativa diferentes vias que culminam com a transcrição de genes relacionados com o mecanismo ovulatório (Arias et al., 1994; Mukherjee et al., 1996; Russell et al., 2003; Zhang et al., 2014; Wang et al., 2016; Egbert et al., 2018), com a esteroidogênese, expansão do

cumulus e maturação nuclear (Park et al., 2004), os quais atuam de maneira autócrina nas próprias células da granulosa ou de maneira parácrina, nas células do cumulus (Shimada et *al.*, 2006).

Da mesma forma que o LH nas células da granulosa, nas células do cumulus, o FSH se liga ao seu receptor ativando AMPc, bem como a fosforilação e ativação de p38MAPK (Richards, 1980; Regan, 2003), que por sua vez é induzida também de forma parácrina pela ação da PGE2 oriunda da granulosa sobre o seu receptor nas células do cumulus (Shimada et al., 2006). A ativação de p38MAPK é responsável pela indução da expressão de AREG, EREG e BTC (Shimada *et* al., 2006), fatores de crescimento semelhantes ao EGF (EGF-like; Park et al., 2004).

A transativação do EGFR ocorre nas células da granulosa por ação da enzima proteolítica ADAM17, a qual libera o domínio de AREG e EREG para se ligar em EGFR e regula a fosforilação de MAPK (Yamashita et al., 2007; Panigone et al., 2008; Yamashita et al., 2009; Yamashita e Shimada, 2012). O EREG se liga ao seu receptor de membrana, EGFR, ativando a via ERK1/2 que induzem a produção de prostaglandina sintetase 2 (PTGS2) e prostaglandina E2 (PGE2), de genes envolvidos com a esteroidogênese (Shimada et al., 2006), expansão do cumulus (Conti et al., 2006) e com a função imune celular (Hernandez-Gonzalez et al., 2006) (Figura 1). A PGE2 possui efeitos similares ao LH, participando do processo de ovulação por induzir a síntese de EGF-like via AMPc/PKA e MAPK (Shimada et al., 2006).

Figura 1 – Ilustração esquemática da regulação da expressão gênica e da comunicação entre células da granulosa e do cumulus de folículos pré-ovulatórios.



Fonte: Adaptado de Shimada et al., 2006.

2.2 EXPANSÃO DO CUMULUS E OVULAÇÃO

O folículo é composto por diferentes tipos celulares, cada qual desempenhando suas determinadas funções para permitir que ocorra o desenvolvimento e posterior ovulação de um gameta apto a ser fecundado. No folículo pré-ovulatório, o oócito é circundado pelas células do cumulus, dando origem ao CCO. As células do cumulus são conectadas umas com as outras e com o oócito através de junções aderentes e comunicantes (*gap junctions*; Eppig, 1991; Kidder e Mhawi, 2002), produzindo fatores específicos que atuam de forma parácrina controlando a parada meiótica e as funções do cumulus (Larsen et al., 1987; Zhang et al., 2010).

O pico pré-ovulatório de LH induz mudanças estruturais e funcionais no CCO, resultando na produção de matriz rica em ácido hialurônico, processo denominado de mucificação e expansão do cumulus (Eppig, 1979), fundamental para que ocorra a ovulação e posterior fecundação (Chen et al., 1993). Durante esse processo o LH estimula as células da granulosa a produzir os *EGF-like*, os quais se ligam em EGFR expressos nas células do cumulus e da granulosa (Park et a*l.*, 2004).

O estímulo de LH também induz a expressão de PTGS2, resultando no aumento da produção de PGE2, a qual estimula a expressão de EGF-*like* nas células da granulosa e do cumulus (Sirois e Richards, 1992; Shimada et al., 2006). Sinergicamente EGF-like e PGE2 estimulam as células do cumulus a produzir ácido hialurônico via HAS2 e fatores estabilizadores hialurônicos, tais como, TNFAIP6 e PTX3 (Richards, 2005), culminando com a expansão do cumulus. A expansão do cumulus associada com a expressão de genes ovulatórios-chave são essenciais para a liberação do CCO de dentro do folículo (Kawashima e Kawamura, 2018).

Porém, para que o LH tenha ação efetiva, torna-se necessária a expressão de LHR funcionais. É estabelecido que as células da teca e da granulosa apresentam LHR (Camp et al., 1991; Park et al., 2004), mas esses receptores são ausentes em oócitos e células do cumulus (Van Tol et al., 1996; Robert et a*l.*, 2003). A não expressão de LHR pelas células do cumulus ocorre através da ação inibitória de GDF9 (*Growth differentiation factor-9*) e BMP15 (*Bone morphogenetic protein 15*) secretadas pelo oócito (Eppig et al., 1997).

No contexto *in vitro*, onde o CCO é cultivado de maneira isolada, tem sido demonstrado que o FSH melhora a maturação oocitária, a expansão do cumulus, a fecundação *in vitro* e o desenvolvimento embrionário inicial em bovinos (Van Tol et al., 1996; Ali e Sirard, 2002; Nivet et al., 2012). Assidi et al. (2013) realizaram uma revisão comparativa entre as respostas de FSH *in vitro* versus de LH *in vivo* nas células do cumulus e propuseram que os dois hormônios apresentam similaridades no que diz respeito ao estímulo gênico. Aparentemente, o FSH *in vitro* estimula vias de expressão gênica comuns ao LH, reproduzindo suas funções *in vivo* e substituindo pelo menos parcialmente a atividade *in vivo* de LH (revisado por Assidi et al., 2013).

2.3 VIA HIPPO

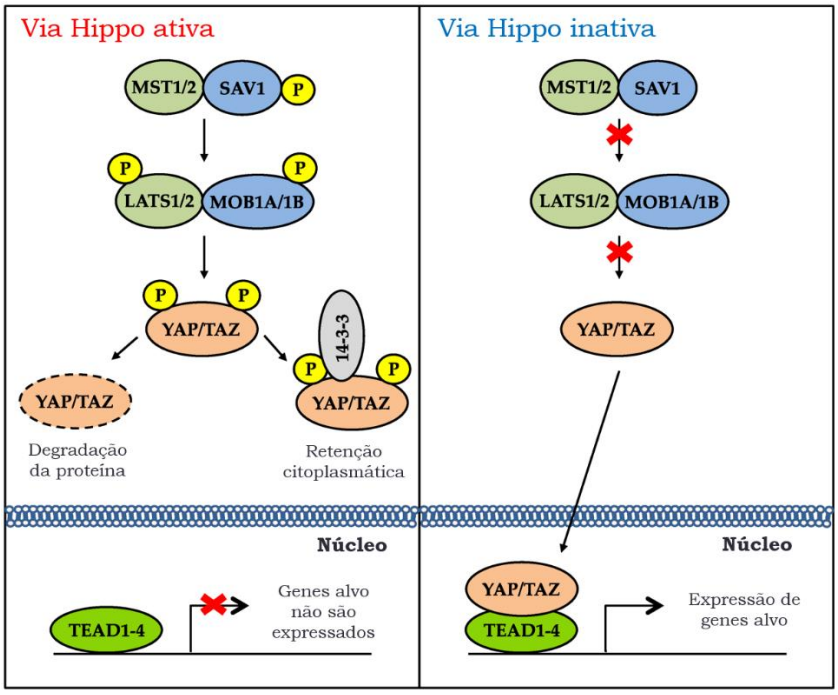
A via Hippo é uma via de sinalização evolutivamente conservada e com funções bem estabelecidas em uma variedade de tecidos. Ela foi inicialmente descoberta em 1995 a partir de

estudos de genes de supressão tumoral utilizando *Drosophila melanogaster* como modelo experimental (Justice et al., 1995). Desde então, vem sendo estudada em diferentes contextos celulares, sendo considerada como reguladora chave do destino celular para proliferar, permanecer inativa ou levar à morte celular (Halder e Johnson, 2011).

A via Hippo consiste em uma cascata de proteínas quinases que regulam negativamente a atividade de dois efetores principais, YAP1 e TAZ. Quando a via Hippo está na sua forma ativa, as quinases MST1 e MST2 fosforilam LATS1 e LATS2 (Callus et al., 2006; Praskova et al., 2008), as quais fosforilam YAP1 e TAZ. YAP1 e TAZ fosforiladas translocam para o citoplasma onde ficam retidas ou são degradadas (Figura 2; Zhao et al., 2007; Hao et al., 2008; Lei et al., 2008; Liu et al., 2010; Zhao, Li, Tumaneng, et al., 2010).

Por outro lado, quando inativa, YAP1 e TAZ acumulam no núcleo, formando complexos com fatores de transcrição, principalmente da família TEAD (*transcriptional enhancer factor TEF-1*, também conhecido como *TEA domain family member*; TEAD1, TEAD2, TEAD3 e TEAD4), resultando na modulação da atividade transcricional de genes alvo (Figura 2) envolvidos com a sobrevivência e proliferação celular (Mauviel et al., 2012). Além de TEAD1-4, foi demonstrada a capacidade de YAP1/TAZ em formar complexos com os fatores de transcrição SMAD, RUNX1/2, p63/p73 e OCT4 (Yu et al., 2015), atuando na regulação do desenvolvimento e crescimento celular.

Figura 2 – Ilustração esquemática da via Hippo.



Fonte: adaptado de Juan e Hong, 2016.
Quando na forma ativa, MST1/2 (em mamíferos) fosforila LATS1/2, que por sua vez, fosforila YAP1/TAZ, resultando na degradação ou na retenção citoplasmática da proteína. Quando na forma inativa, não ocorre a fosforilação de YAP1/TAZ, as quais translocam para o núcleo formando complexos com os fatores de transcrição da família TEAD e estimulando a transcrição de genes alvo.

Os principais genes alvo da relação YAP1/TAZ-TEADs são o CTGF (connective tissue growth fator), Cyr61 (cysteine-rich angiogenic inducer 61) e NOV (nephroblastoma overexpressed), genes relacionados com a sobrevivência e proliferação celular (Heath et al., 2008; Lai et al., 2011; Malik et al., 2015). Além desses, YAP1 estimula a transcrição de PTGS2 e AREG em células tumorais de ratos e humanos (Guerrant et al., 2016).

Diferentemente do que vinha sendo demonstrado quanto a regulação e retenção de YAP1 no citoplasma, recentemente tem sido relatada que a regulação da via Hippo é dinâmica. Estudos em fibroblastos de mamíferos apresentaram uma rápida translocação de YAP1 entre o citoplasma e o núcleo (Ege et al., 2018). Além disso, foi demonstrado que YAP1/TAZ são rapidamente fosforilados e defosforilados apesar de ser pouco conhecido a regulação das fosfatases responsáveis por esses eventos (Ma et al., 2019).

2.4 VIA HIPPO NO CONTEXTO REPRODUTIVO

Como descrito, a via Hippo é considerada uma via de sinalização celular altamente conservada, com funções bem definidas na determinação do tamanho de órgãos, participando dos mecanismos de diferenciação, proliferação e apoptose de células de diversos tecidos, sobretudo durante a embriogênese (Yu e Guan, 2013; Meng et al., 2016). No entanto, no contexto da fisiopatologia da reprodução, já na vida adulta, existe um aumento crescente no número de evidências, ligando a via Hippo tanto à fisiologia ovariana (Nagashima et al., 2011; Ji et al., 2017; Plewes et al., 2019) quanto a processos patológicos, ocasionados por sua desregulação, como a formação de cisto folicular ovariano ou mesmo câncer ovariano (Hall et al., 2010; Li et al., 2012).

A inativação de Lats1/2, reguladores da atividade de YAP1/TAZ, nas células da granulosa de roedores, tanto *in vivo* quanto *in vitro*, culminou na alteração da morfologia, na função e na expressão gênica da granulosa. A não expressão de Lats1/2 resultou no aumento dos níveis de YAP1 e TAZ total, bem como de seus genes alvo, Cyr61, CTGF e NOV, provavelmente sendo responsável pela transdiferenciação das células da granulosa em outras linhagens celulares (Tsoi et al., 2019).

Em camundongos, YAP1 shRNA levou a inibição da ativação dos folículos primordiais. O mesmo ocorreu quando realizada a inibição de AKT, porém revertida quando associada com o aumento da expressão de YAP1. Concluindo, que YAP1 regula a ativação de folículos primordiais mediada pela via de sinalização AKT (Hu et al., 2019). Além disso, a YAP1 é necessária para a proliferação das células da granulosa de camundongos, onde sob efeito de LH ocorre a inativação de YAP1 através da via ERK1/2, fundamental para que ocorra a luteinização e ovulação (Ji et al., 2017).

Recentemente, foi demonstrado que YAP1 é espaço-temporariamente expresso nas células da granulosa. Quando na sua forma ativa (nuclear), foi predominantemente expresso em células da granulosa proliferativas, enquanto que sua forma inativa (citoplasmática), foi principalmente detectado em células luteais (Lv et al., 2019). Nesse mesmo estudo, evidenciaram que YAP1 interage com as vias de sinalização de EGFR e TGF-β para controlar a proliferação, diferenciação e sobrevivência das células da granulosa (Lv et al., 2019).

Também em camundongos, foi relacionada a regulação da via Hippo no CCO com a ovulação, onde na ausência do estímulo ovulatório o oócito secreta fatores que suprimem a atividade da via, ativando YAP1. Em contrapartida, quando estimulada a ovulação, ocorre a degradação e fosforilação de YAP1, permitindo a diferenciação das células do cumulus (Sun e Diaz, 2019). A deleção condicional em células foliculares de CTGF, talvez o mais importante gene alvo de YAP1/TAZ-TEAD, afeta o desenvolvimento folicular, ovulação e luteólise em camundongos, através de uma significante redução de *Adamts1* (Nagashima et al., 2011).

Em relação a fisiologia ovariana na espécie bovina, apenas foi relatada a importância da função de YAP1/TAZ na proliferação das células da granulosa e na esteroidogênese em bovinos (Plewes et al., 2019). Plewes et al. (2019) demonstraram que a desregulação de YAP1 inibiu a proliferação das células da granulosa de bovinos, bem como, reduziu a síntese de estrógeno estimulada por FSH *in vitro*. Além disso, uma análise do perfil de microRNA em vesículas extracelulares de folículos ovarianos bovinos, demonstrou 115 genes envolvidos com a via Hippo sendo mais expressos em folículos submetidos a menores concentrações de progesterona (4,87 ng/mL), do que em altas concentrações (9,33 ng/mL; De Avila et al., 2019). Porém, a regulação e interação da via Hippo durante o processo ovulatório tanto *in vivo* quanto *in vitro* ainda não foi elucidada nessa espécie.

3 ARTIGO

ARTIGO SUBMETIDO PARA PUBLICAÇÃO:

IS THE HIPPO EFFECTOR YAP1 EXPRESSED IN CUMULUS CELLS A POTENTIAL TARGET TO IMPROVE THE EFFICIENCY OF IN VITRO MATURATION (IVM) SYSTEMS?

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Human Reproduction, 2020

IS THE HIPPO EFFECTOR YAP1 EXPRESSED IN CUMULUS CELLS A POTENTIAL TARGET TO IMPROVE THE EFFICIENCY OF IN VITRO MATURATION (IVM) SYSTEMS?

Running title: YAP1 regulates EGF-like signaling in cumulus cells

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Abstract

Study question: Is the regulation and function of the Hippo effector YAP1 critical for the cumulus expansion-related events induced by EGF signaling in COCs subjected to IVM?

Summary answer: YAP1 regulation in cumulus cells during IVM is mediated by EGFR downstream signaling and its interaction with TEADs is critical for the expression of important cumulus expansion and oocyte maturation-related genes.

What is known already: Analysis of the human EGFR proximal promoter region indicates that YAP1 positively regulates EGFR expression transcription through an intact TEAD binding site. In addition, it has also been demonstrated that YAP1 interacts with EGFR to regulate mural granulosa cell function during follicle development and ovulation in rodents.

Study design, size, duration: This study consisted of a series of experiments (each with multiple replicates) using cumulus oocyte complexes (COCs) subjected to an IVM protocol for up 24 hours in the presence or not of growth factors or gonadotropins with or without pharmacological inhibitors.

Participants/materials, setting, methods: Immature COCs were collected from bovine ovaries obtained from a local abattoir. The experimental treatments along the IVM varied according to the aim of each study. To confirm the presence of phospho and total YAP1 protein levels in cumulus cells by immunofluorescence, we collected COCs from control groups at two distinct timepoints: at 0 and 12h of IVM. To evaluate the effects of EGFR activity on YAP1 mRNA abundance and its correlation with cumulus expansion, COCs were cultured with EGF recombinant protein or FSH in the presence or not of AG 1478 (a selective

inhibitor of EGF receptor protein) and then submitted to cumulus expansion evaluation up to 24 h or collected at 6, 12, 18 and 24 h for RT-qPCR analyses. Finally, to determine if important genes for cumulus expansion are transcriptional targets of YAP1-TEAD interaction in cumulus cells, COCs were subjected to IVM in the presence of FSH with or without distinct concentrations of Verteporfin (VP; a small molecule inhibitor that interferes with YAP1 binding to TEAD). COCs were then collected at 6, 12, 18 and 24 h for total RNA extraction and RT-qPCR.

Main results and the role of chance: EGF and FSH treatments significantly increased YAP1 mRNA abundance in cumulus cells in a time-dependent manner ($P<0.05$). A similar pattern was observed in mRNA levels of the connective tissue growth factor (CTGF), a classic YAP1-TEAD transcriptional target gene. When COCs were subjected to IVM in the presence of FSH without or with pre-treatment of distinct concentrations of VP, CTGF levels were reduced in a dose-dependent manner ($P<0.05$), while YAP1 and FSHR did not change significantly in comparison to respective controls at each timepoint evaluated ($P>0.05$). Most importantly, this experiment indicated that VP inhibits in a time- and concentration-dependent manner ($P<0.05$) distinct cumulus expansion and oocyte maturation-related genes, including the critical gene for EGF signaling in cumulus cells, EGFR and its downstream targets as ADAM17, EREG and PTGS2 as well HAS2, PTX3 and PLAT.

Limitations, reasons for caution: Although we have reported basal post-translational regulation of YAP1 in cumulus cells up to 12 h, the results presented herein suggest the existence of a regulated YAP1 post-translational mechanism in cumulus cells along the IVM that should better elucidated to potential manipulation. In addition, to complement the herein

reported findings, future studies should also assess the direct effect of VP on cumulus expansion, oocyte maturation and early embryo development.

Wider implications of the findings: The present study present considerable insight into the regulation and functional relevance of a completely novel signaling pathway underlying cumulus expansion and oocyte maturation in mono-ovulatory species. YAP1 and/or CTGF may represent potential targets to improve the efficiency of IVM systems, not only for mono-ovulatory species of agricultural importance as the cow, but for human embryo production.

Study funding/competing interest(s): This work was supported by grants and fellowships from the National Council for Scientific and Technological Development (CNPq; Brazil), Coordination for the Improvement of Higher Education Personnel (CAPES; Brazil), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS – Edital PRONEX 12/2014 -FAPERGS/CNPq, 16/2551-0000494-3; Brazil); and from the Natural Sciences and Engineering Research Council of Canada (NSERC). We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Keywords: ovary /oocyte in vitro maturation / cumulus / YAP1 / CTGF / EGF-signaling

Introduction

In vitro maturation (IVM) systems employed in domestic species such as cows has been a successful tool in applied reproductive biotechnology (Dieci, et al., 2016, Hirao, et al., 2013, Hirao, et al., 2014, Van den Hurk, et al., 2000). On the other hand, IVM of human oocytes has been applied, however it presents variable success rates (Chian, et al., 2013, Nogueira, et al., 2012). Current IVM systems do not mimic the environment where the cumulus-oocyte complex (COC) differentiates in vivo during the preovulatory period, which may result in compromised cumulus cells metabolism, asynchronous oocyte nuclear and cytoplasmic maturation and reduced developmental competence (Coticchio, et al., 2012, Sirard, et al., 2006). For this reason, better understanding the cumulus cells functions and its interaction with the oocyte during IVM is extremely useful to improve the efficiency of in vitro maturation and fertilization protocols employed for the in vitro embryo production, a major economic activity in the applied reproductive biotechnology industry for both human and animals worldwide.

In vivo, cumulus cells expansion in the periovulatory period is an important marker for oocyte maturation, and is critical for its fertilization, subsequent cleavage and blastocyst development. Defective cumulus expansion results in reduced ovulation rates and, consequently, affects fertility (Bromer, et al., 2008, Fortune, 1994). The proper functioning of cumulus cells to support for the acquisition of oocyte competence in the periovulatory period is, however, highly dependent on mural granulosa cells (Davis, et al., 1999). The main trigger of the preovulatory cascade is LH, which activates a cascade of signaling events, which are propagated throughout the ovarian preovulatory follicle to promote ovulation of a mature egg (Espey, 1980, Shimada, et al., 2006). Although LH directly stimulates mural granulosa cells, its effects on cumulus cells and oocytes are probably indirect, as both cell types express few or no LH receptors and fail to respond when directly stimulated by LH (Russell, et al., 2007).

The ovulatory process in mammals is, therefore, initiated by LH surge that acts upon its receptors on mural granulosa cells. This leads to activation of proteolytic sheddase enzymes (ADAMs) that cause the release of the membrane-bound proteins epiregulin (EREG) and amphiregulin (AREG), members of the epidermal growth factor (EGF) family. These proteins then activate the EGF receptor (EGFR) on mural granulosa cells, thereby stimulating the expression of prostaglandin endoperoxide synthase 2 (PTGS2) as well as increasing the transcriptional activity of the AREG and EREG genes, creating a positive feedback loop (autocrine action). The EGF signaling network in mural granulosa cells is then transmitted to the cumulus cells (paracrine action) (Portela, et al., 2011, Shimada, et al., 2006), modulating the expression of important genes related to gap junctions closure and production of an extensive extracellular matrix by cumulus cells and downregulation of the meiotic inhibitory signaling network that ultimately will lead to cumulus expansion and oocyte meiotic maturation (Park, et al., 2004, Shimada, et al., 2006). It is actually demonstrated that sustained activity of the EGFR is essential for LH-induced oocyte maturation and cumulus expansion (Reizel, et al., 2010).

There is a growing awareness of the involvement of the Hippo pathway in the regulation of ovarian function, including its potential contribution to key preovulatory events (Ji, et al., 2017, Nagashima, et al., 2011, Sun, et al., 2019). The core Hippo pathway consists of a kinase cascade that ultimately regulates the activity of the transcriptional activators yes-associated protein 1 (YAP1) and transcriptional co-activator with PDZ-binding motif (TAZ). When these Hippo effectors are accumulated in the nucleus they form complexes with numerous transcription factors, notably those of the TEAD (TEA domain family member; TEAD1, TEAD2, TEAD3 and TEAD4) family of transcription factors, resulting in the modulation of the transcriptional activity of target genes in a cell type- and context-specific manner (Heath, et al., 2008, Lai, et al., 2011, Malik, et al., 2015, Mauviel, et al., 2012). A

recent study in mice showed that YAP1 is involved in cumulus cell function during the ovulatory cascade and oocyte maturation (Sun, et al., 2019). In addition, it has also been demonstrated in another study in rodents that YAP1 interacts with EGFR to regulate mural granulosa cell function during follicle development. Briefly, it was shown that knockdown of YAP1 dramatically suppressed expression of EGFR (Lv, et al., 2019). Interestingly, analysis of the human EGFR proximal promoter region indicates that YAP1 positively regulates EGFR expression transcription through an intact TEAD binding site (Song, et al., 2015). Moreover, a study evaluating chromatin accessibility demonstrated that TEAD1 is a regulator of migration in human glioblastoma through a mechanism that involves EGFR regulation (Tome-Garcia, et al., 2018). Taken together, all these findings brought us to hypothesize that YAP1 also exerts an important role in the signaling that culminates with cumulus expansion and oocyte maturation, at least in part, regulating EGFR expression in cumulus cells.

As the physiological importance of YAP1-TEAD interaction for cumulus expansion-related events in mono-ovulatory species (as the women) has not been established, in the present study, we aimed to determine whether YAP1 is regulated following EGFR activation or inhibition in cumulus cells in vitro; Moreover, we investigated whether critical cumulus expansion-related genes, as EGFR, are transcriptional targets of YAP1-TEAD interaction in cumulus cells during an established IVM protocol (Campbell, et al., 2003). Because of ethical and practical reasons, we decided to employ herein a bovine model. Besides being predominantly mono-ovulatory, both species share important similarities at the ovarian physiology level. Sizes of follicles at different stages of development and the dynamics of follicle wave emergence are similar in both species (Adams, et al., 1992, Baerwald, et al., 2003, Baerwald, et al., 2003, Campbell, et al., 2003, Sirois, et al., 1988). In addition, reproductive ageing in cattle and women share many features (Malhi, et al., 2005).

Materials and methods

All chemicals used in the present study were purchased from Sigma Chemicals Company (San Luis, MO, EUA), unless otherwise indicated in the text.

Bovine oocyte collection and in vitro maturation (IVM) protocol

Bovine ovaries were obtained from a local abattoir and transported to the laboratory in saline solution (0.9% NaCl; 30 °C) containing 100 IU/mL penicillin and 50 μg/mL streptomycin sulphate. Cumulus oocyte complexes (COCs) from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 15 mL of water/minute). COCs were recovered and selected as previously described (Leibfried, et al., 1979) under a stereomicroscope. After selection, only grade 1 COCs were randomly distributed to four-well culture dishes (Nunc®, Roskilde, Zealand, DNK), containing 200 μL of maturation medium with the appropriate treatment and cultured in an incubator at 39 °C in a saturated humidity atmosphere containing 5% CO2 and 95% air, for 6, 12, 18 or 24 hours. The basic maturation medium used was Medium 199 (1X) containing Earle's salts, L-glutamine, 2.2 mg/mL sodium bicarbonate and 25 mM Hepes (Gibco Labs, Grand Island, NY, USA), supplemented with 0.2 mM pyruvic acid, 0.4% (v/v) bovine serum albumin (BSA), 100 IU/mL penicilin and 50 μg/mL streptomycin sulphate. The experimental treatments varied according to the aim of each study as described below:

Study 1; YAP1 phosphorylation pattern and intracellular localization in cumulus cells of bovine COCs subjected to IVM

To confirm the presence of phospho and total YAP1 protein levels in cumulus cells of bovine COCs subjected to IVM, we collected COCs from control groups at two distinct

timepoints: at 0 and 12h. We decided to employ immunofluorescence (IF) analysis in attempt to better understand the basal intracellular localization pattern of YAP1 during the first 12h of IVM, a critical window for the signaling events that lead to cumulus expansion and oocyte maturation.

Study 2; effects of EGFR activity on YAP1 mRNA abundance in cumulus cells during IVM

To determine the effects of EGFR activation or inhibition on YAP1 transcriptional regulation in cumulus cells, we performed two series of experiments in vitro. In both series, COCs were randomly divided into groups (n= 5 COCs/group) and placed in 200 μL of maturation medium. COCs were then pretreated for 1h with 6 μM of AG 1478 (a selective inhibitor of epidermal growth factor receptor protein) or equivalent amount of vehicle (control; Dimethyl Sulfoxide:Methanol (DMSO:MeOH)). One hour later it was then added to the IVM culture medium 10 ng/mL of recombinant human EGF (R&D Systems, Oakville, ON, CAN; first series of experiments) or 500 ng/mL of bovine FSH (Bioniche, Belleville, ON, CAN; second series of experiments). For both series, two experiments were performed: in one, COCs were cultured for 6, 12, 18 and 24h post-EGF or FSH to be then collected at the each timepoint for total RNA extraction and qRT-PCR analyses; and in a second experiment COCs were kept in the plates for cumulus expansion assessment up to 24h of IVM.

Study 3; YAP1-TEAD interaction inhibition affects the expression of critical cumulus expansion-related genes

To determine if important genes for cumulus expansion are transcriptional targets of YAP1-TEAD interaction in cumulus cells in vitro, COCs were subjected to the above-mentioned IVM protocol in which maturation is stimulated with FSH. This protocol has been

published several times (Barreta, et al., 2008, De Cesaro, et al., 2015, Stefanello, et al., 2006) and it is closer (if not similar) to IVM protocols employed commercially. Briefly, COCs were randomly divided into groups (n= 5 COCs/group) and placed in 200 μL of maturation medium. COCs were then pretreated for 1h with distinct concentrations of Verteporfin (VP; a small molecule inhibitor that interferes with YAP1 binding to TEAD family transcription factors): 0.1, 0.3, 0.5 μM of VP or equivalent amount of vehicle (control; DMSO). One hour later it was then added to the IVM culture medium of all groups (including control) 500 ng/ml of FSH. COCs were then collected at 6, 12, 18 and 24h post-FSH for total RNA extraction and qRT-PCR analyses.

Evaluation of cumulus cells expansion

COCs were photographed using an inverted microscope (Leica DMI 4000B; Leica Microsystems, Wetzlar, HE, GER). Images of same COCs were captured through Leica Application Suite (LAS, Version 3.8) software at 0 and 24h of culture. Total surface area expressed in pixels of each COC appearing on the 2-dimensional image was measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence (IF)

COCs were fixed in 4% paraformaldehyde for 15 minutes and maintained in a maintenance solution of the nuclear membranes containing Phosphate Buffered Saline (PBS), 0.2 % Triton X-100 and 1% BSA until evaluation. For assessment of YAP1 or pYAP1 signal, COCs were permeabilized in PBS with 0.3% BSA and 0.2% TritonX-100 for 1 hour at 37 °C and then incubated with rabbit monoclonal antibodies (Cell Signaling Technology, Danvers, MA, EUA) for total YAP1 (1:300, No. 14074) and pYAP1 (Ser127; 1:300, No. 13008) overnight at 4 ºC. As a negative control, some COCs were incubated with blocking solution

(PBS, 3% BSA and 0.2% Tween20) only. After washing in blocking solution three times during 20 minutes, then COCs were incubated with Alexa Fluor 488-conjugated antirabbit IgG antibody (1:500; Molecular Probes, Carlsbad, CA, EUA) during 1 hour and 10 μM of bisBenzimide (Hoechst 33342) for 15 minutes. COCs were washed two times with blocking solution for 10 minutes each time and placed on blade and coverslip. COCs stained for YAP1 and pYAP1 were observed with a confocal microscope (Leica DMI6000 B).

RNA isolation, Reverse Transcription and Quantitative Real-Time PCR (qRT-PCR)

After collecting COCs, cumulus cells were removed, recovered and immediately stored at -80 ºC. Total RNA was extracted using PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, EUA) according to the manufacturer's instructions and was quantified at 260 nm wavelength using a spectrophotometer (NanoDrop1000, Thermo Scientific, Wilmington, DE, USA). 50 ng of total RNA was reverse transcribed (RT) using the iScript™ cDNA Synthesis Kit (Bio-Rad, Des Plaines, IL, USA) at 25 °C for 5 minutes and 46 °C for 30 minutes. The reaction was ended by incubation at 95 °C for 5 minutes.

Real-time qPCR was performed using CFX384™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) using the GoTaq® DNA Polymerase (Promega, Madison, WI, USA) and specific primers (Table 1). After an initial denaturation step at 95 °C for 3 minutes, 40 cycles at 95 °C for 10 seconds were carried out, followed by 1 minute at 60 °C to amplify each transcript. The reaction was performed in duplicate, and the melting-curve was analyzed to determine the product's identity. Two housekeeping gene (H2AFZ and RPS18) were tested, however, the target mRNA concentration was just normalized to the amplification of the constitutional gene H2AFZ, which was the one that best behaved with the samples. Relative mRNA levels calculation was performed as described by Pfaffl (Pfaffl,

2001). All primers used were designed based on sequences from GenBank, using Primer-BLAST platform.

Statistical Analysis

All experiments were performed on three or four independent replicates, with each replicate using ovaries collected at different times. For the in vitro data, doses of hormones and VP were used as the main effects and culture replicate was included in the model as a random effect in the F-test. Data were transformed to logarithms when not normally distributed (Shapiro–Wilk test). Differences between means were tested with the Tukey–Kramer HSD test or t-tests. All analyses were performed with JMP software (SAS Institute, Cary, NC). Data are presented as means ± SEM.

Results

YAP1 phosphorylation pattern and intracellular localization in cumulus cells of bovine COCs subjected to IVM

Before proceeding to our regulatory and functional experiments in COCs, more precisely to cumulus cells expansion-related events, we performed a first experiment to confirm the presence of YAP1 protein levels in bovine cumulus cells during the in vitro maturation. For this, we employed immunofluorescence (IF) instead of the immunoblotting, once the first one not only allow us to determine the amount of our target protein as its intracellular localization. Interestingly, YAP1 roles are dependent on its phosphorylation status and intracellular localization (Mauviel, et al., 2012). In its nonphosphorylated form, YAP1 is able to accumulate in the nucleus and form complexes with numerous transcription factors, notably with TEADs. The results shown in the **Figure 1** suggest that basal phospho-YAP1 (Ser127) levels decrease in a time-dependent manner. On the other hand, total YAP1

protein levels seem to remain stable up to 12h, but more importantly, there was an increase in nuclear total YAP1 levels with time (Control 0h vs Control 12h). Taking together, these results suggest that YAP1 may gradually translocate from the cytoplasm into the nucleus of cumulus cells during in vitro maturation of COCs.

YAP1 regulation in cumulus cells during IVM is mediated by EGFR downstream signaling

For the second study, we decided, first, to determine whether YAP1 mRNA abundance is regulated in cumulus cells of COCs subjected to IVM in the presence or not of EGF recombinant protein. In such model, COCs clearly respond to EGF, as this growth factor significantly increases mRNA levels for EREG ($P<0.05$, **Figure 2B**) and induces cumulus expansion ($P<0.05$, **Figure 2A**), which were both blunted by the pretreatment with Tyrphostin AG 1478, a specific EGFR inhibitor. Most interestingly, our results indicated that EGF significantly increased YAP1 mRNA abundance only at 18 h post-treatment, which was also inhibited by the pretreatment with AG 1478 ($P<0.05$, **Figure 2C**). In addition, a similar pattern was observed in mRNA levels of the connective tissue growth factor (CTGF), a classic YAP1-TEAD transcriptional target gene (Lai, et al., 2011). In all timepoints which EGF significantly increased CTGF levels and such effect was also blunted by the pretreatment with AG 1478 ($P<0.05$, **Figure 2D**).

Taking into account that most of IVM commercial protocols use FSH as stimulator of cumulus expansion and oocyte maturation, we then decided to test the effects of FSH on YAP1 mRNA abundance regulation in cumulus cells of COCs during IVM. As expected, FSH

increased mRNA levels for EREG ($P<0.05$, **Figure 3B**) and induced cumulus expansion ($P<0.05$, **Figure 3A**) in a EGF-dependent manner. However, unlike EGF, which significantly

induced YAP1 mRNA levels only at 18 h post-treatment, FSH increased significantly YAP1 mRNA abundance only at 6 h post-treatment in comparison to controls. Such increase was, however, also abrogated by AG 1478 pretreatment ($P<0.05$, **Figure 3C**). In addition, FSH-induced CTGF mRNA levels increase at 12 h was also inhibited by the pretreatment with AG 1478 ($P<0.05$, **Figure 3D**).

YAP1-TEAD interaction inhibition affects the expression of critical cumulus expansion-related genes

To start elucidating the physiological relevance of YAP1 to cumulus cells expansion in vitro, COCs were cultured in maturation medium containing FSH without or with pre-treatment of distinct concentrations of VP (a small molecule inhibitor that interferes with YAP1 binding to TEAD family transcription factors) for 6, 12, 18 and 24h. The results confirmed the pharmacological specificity of VP, as CTGF levels were reduced in a dose-dependent manner ($P<0.05$, **Figure 4A**), while YAP1 and FSHR did not change significantly in comparison to respective controls at each timepoint evaluated ($P>0.05$, **Figure 4B and C)**. Most importantly, this experiment clearly indicated that VP inhibits in a time- and concentration-dependent manner distinct cumulus expansion-related genes, including the critical gene for EGF signaling in cumulus cells, EGFR and its downstream targets as ADAM17, EREG and PTGS2 ($P<0.05$, **Figure 4D, E and F**). Besides that, YAP1-TEAD inhibition reduced other important markers for cumulus expansion, matrix remodeling and oocyte maturation, including the hyaluronan synthase 2 (HAS2), pentraxin-related protein 3 (PTX3) and the plasminogen activator tissue-type A (PLAT) mRNA levels ($P<0.05$, **Figure 4 H, I and J**).

Discussion

Studies demonstrating the roles of Hippo effectors in ovarian follicle cells, particularly in the periovulatory events, are scarce. To our knowledge, this is the first report to indicate that YAP1-TEAD interaction may be critical for the EGF-induced signaling cascade that requires cumulus cells to promote cumulus expansion, oocyte meiotic resumption and ovulation in mono-ovulatory species. Briefly, we have reported herein that YAP1-TEAD interaction inhibition affects the expression of critical cumulus expansion-related genes, at least in part, regulating EGFR transcription. Although we have used bovine COCs in the present study, the recent findings indicating that the human EGFR promoter region is positively regulated by YAP1 through an intact TEAD binding site (Song, et al., 2015), allow us to strongly suggest that the YAP-TEAD interaction may be also critical for cumulus expansion and oocyte maturation-related events in women.

A recent study in bovine granulosa cells evaluated the expression of both Hippo effectors (YAP1 and TAZ) in mural granulosa cells (but not cumulus) from follicles of increasing size (2–5, 5–10, >10 mm). While YAP1 protein levels were expressed in granulosa cells from follicles of all stages of development, TAZ expression decreased in granulosa cells with increasing of follicle size, being almost undetectable by western blotting in granulosa cells from follicles larger than 5 mm (Plewes, et al., 2019). Indeed, we have assessed by immunoblotting, YAP1 and TAZ in bovine mural preovulatory granulosa cells in vitro, and the results indicate that total YAP1 protein levels are consistently expressed along the culture, but TAZ protein levels are barely detected in both untreated or treated cells (data not shown herein). Taking into account these parallel findings, for the herein presented experiments in cumulus cells, we decided to focus exclusively on YAP1 expression transcriptional regulation. To better elucidate its interaction with TEADs in this context, we have also

described results for CTGF, a classic YAP1-TEAD transcriptional target gene (Lai, et al., 2011).

The present study clearly shows that YAP1 and CTGF are regulated in cumulus cells at the transcriptional level following direct activation of EGFR with recombinant EGF or, indirectly, through FSH stimulation. In the latter scenario, the results from the groups pretreated with EGFR inhibitor indicated that both YAP1 and CTGF mRNA levels increase following FSH are dependent on EGFR activity. Interestingly, in these FSH-treated cumulus cells, YAP mRNA levels increase (at 6 h) preceded CTGF mRNA levels augmentation (at 12 h). Instead, the results presented in the **Figure 2** indicate an EGF-induced CTGF mRNA levels increase (at 6 and 12 h) prior to YAP mRNA augmentation, which was verified only at 18 h. Taking into account that CTGF is a classic target gene of the interaction of YAP1 with transcription factors of the TEAD family, these latter results clearly suggest the existence of a transitory accumulation of YAP1 in the nucleus before 6 h. Although we did not assess YAP1 phosphorylation pattern and intracellular localization in cumulus cells following EGF treatment (particularly in the first 6 h post-treatment), our first study (Figure 1) indicated, even at basal levels, a gradual accumulation of YAP1 in the nucleus of cumulus cells along the IVM. In spite of the fact that it must be better elucidated, these results strongly suggest the existence of a YAP1 post-translational regulation mechanism in cumulus cells along the IVM.

One obvious question raised by all our regulatory studies was the physiological relevance of both basal and EGF- or FSH-induced YAP and CTGF mRNA levels increase in cumulus cells subjected to our IVM protocol. Hippo is an evolutionarily conserved signaling pathway with established roles in cell differentiation, proliferation and apoptosis in a variety of tissues, particularly during embryogenesis (Halder, et al., 2011, Meng, et al., 2016). However, recent studies (mainly using mice models), however, have reported some of the physiological roles of Hippo effectors in the ovarian physiology in the adult life (Hu, et al.,

2019, Lv, et al., 2019, Nagashima, et al., 2011, Plewes, et al., 2019). Nevertheless, studies showing the importance of Hippo effectors to cumulus functions and to cumulus expansion-related events are, to our knowledge, extremely scarce. A recent study in mice also tested the effects of the YAP1-TEAD inhibitor VP on cumulus expansion events in COCs subjected to IVM (Sun, et al., 2019). Intriguingly, COCs pretreated with VP presented not only increased expression for cumulus expansion-related genes in a dose-dependent manner (PTGS2, HAS2 and PTX3) as well increased cumulus expansion in comparison to controls (Sun, et al., 2019). These data are the opposite of what we are demonstrating in the present study, in which we clearly show that VP inhibits in a dose-dependent manner critical genes for cumulus expansion and oocyte maturation. Although such discrepancies may be explained by eventual species-specific differences, other functional studies in mice suggest that the interaction of YAP1 with TEADs may be important to allow and/or even modulate the preovulatory cascade that leads to cumulus expansion and ovulation. It has been demonstrated that knockdown of YAP1 dramatically suppressed expression of EGFR in mouse granulosa cells (Lv, et al., 2019). Moreover, there is also evidence in tumor cells that Hippo signaling effectors, YAP1 and TAZ, can modulate the two main EGFR-downstream signaling pathways, ERK and AKT, at least in part, regulating the EGFR expression and activity (Andrade, et al., 2017, Yang, et al., 2016). Based on these reports and other reports, we then hypothesized that the YAP1-TEAD interaction is also critical for the EGFR signaling that regulates cumulus cells function. Indeed, the results presented herein (**Figure 4**) confirm that VP decreases significantly in a dose-dependent manner EGFR mRNA levels in cumulus cells from COCs cultured at 12 and 24 h. Such decline in EGFR can explain, at least in part, the decrease of mRNA levels for ADAM17 (at 12, 18 and 24 h), EREG (at 18 and 24 h) and PTGS2 (at 24 h), as for HAS2 and PTX3 mRNA levels from 12 h on, once all these genes are classic targets of EGFR downstream signaling (Ochsner, et al., 2003, Park, et al., 2004, Portela, et al., 2011, Salustri,

et al., 2004, Su, et al., 2003). On the other hand, we did not find a consistent VP-induced decrease of EGFR mRNA levels at 6 h, even so, ADAM17 EREG and PLAT levels were decreased by VP treatment at the same timepoint suggesting an event independent to EGFR mRNA levels downregulation. One possible explanation is that EGFR expression pattern could be transiently affected by the VP treatment between 0 and 6 h (not evaluated herein), what would explain such results. Nevertheless, we cannot exclude the fact that VP may decrease the expression of some of these genes in a mechanism independently of the EGFR reduction.

For many authors, the data with VP should be interpreted with some caution, since YAP1-TEAD independent effects of the drug are reported in cancer cells (Zhao, et al., 2018). In our model, however, the pharmacological effects of VP seem to be specific, once CTGF was downregulated in cumulus cells in a dose-dependent manner following VP treatment, while some genes as FSHR and YAP1 itself were not affect by VP at any dose or timepoints tested. In terms of mechanism of action, we strongly believe that most of the results observed herein following VP treatment are consequence of its effect on EGFR. As mentioned previously, it has been shown in distinct human cell types that YAP1 positively regulates EGFR transcription through the interaction with an intact TEAD binding site at the EGFR promoter (Song, et al., 2015) and that TEAD1 is a critical regulator of EGFR transcription in vitro (Tome-Garcia, et al., 2018). On the other hand, CTGF levels decrease post-VP may contribute to the altered expression profiled observed in cumulus cells herein. CTGF knockout mice showed disrupted follicle development and decreased ovulation rates, through of significant downregulation of the disintegrin and metalloproteinase Adamts1, which is critical for remodeling of extracellular matrix surrounding granulosa cells of preovulatory follicles (Nagashima, et al., 2011). These latter findings may explain the herein observed decrease in ADAM17 and PLAT mRNA levels already 6 h post-VP treatment. In addition, it

453 has been demonstrated that supplementation of culture media with CTGF recombinant protein
454 benefits ovine oocyte IVM and in vitro embryo production (Wang, et al., 2018). Briefly,
455 CTGF and/or its combination with other factors significantly promoted cumulus cell
456 expansion, inhibited oocyte/cumulus apoptosis, induced oocyte nuclear maturation and
457 improved early embryo developmental competence.

458 In summary, the data presented herein in cattle provide, for the first time, considerable
459 insight into the regulation and functional relevance of a completely novel signaling pathway
460 underlying cumulus expansion and oocyte maturation in mono-ovulatory species, as the
461 women. The present study allowed us to define YAP1 and/or CTGF as potential targets to
462 improve the efficiency of in vitro maturation (IVM) systems. Future studies involving the
463 manipulation of the post-translational regulation of YAP1 in COCs undergoing IVM can
464 represent a potential tool to increase rates of fertilization, subsequent cleavage and blastocyst
465 development in existent IVM protocols used by the industry of reproductive biotechnology in
466 both humans and animals.

467

468 **Authors' roles**

469 J.K., V.M.P., P.B.D.G and G.Z were involved in the study conception and design; J.K,
470 V.M.P., D.M., L.G.A., Z.S and A.Q.A performed experiments and were involved in the
471 acquisition and analyzes of data; G.Z., P.B.D. G. and B.G.G contributed with required
472 reagents acquisition; J.K. wrote the main manuscript text and prepared the tables and figures;
473 P.B.D.G and G.Z. edited the reviewed the manuscript to be published.

474

475 **Acknowledgements**

476 The authors are thankful to Susane Lopes and Laboratório Central de Microscopia
477 Eletrônica (LCME) from the Universidade Federal de Santa Catarina (UFSC) for her help

with photomicrographs; to Camila Azzolin de Souza and Camila Cupper for their support in this study; to Frigorífico Silva for ovaries donation.

Funding

This work was supported by grants and fellowships from the National Council for Scientific and Technological Development (CNPq; Brazil), Coordination for the Improvement of Higher Education Personnel (CAPES; Brazil) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS – Edital PRONEX 12/2014 -FAPERGS/CNPq, 16/2551-0000494-3; Brazil) and Natural Sciences and Engineering Research Council of Canada (NSERC).

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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697 **Figure Legends**

698

699 **Figure 1.** COCs subjected to IVM were collected from control groups at 0 and 12h of IVM.
700 Immunofluorescence signals obtained from antibodies against total YAP or phospho-YAP
701 (Ser127) are shown (**A, D, G, J**). The nuclei are labeled with Hoechst (**B, E, H, K**). The merge
702 of YAP or phospho-YAP (Ser127) and Hoechst are shown on **C**, **F** and **I**, **L,** respectively.
703 Arrows (**F**) indicate cumulus cells with clear total YAP protein accumulation in the nucleus.
704 Photomicrographs were taken at 1000x magification and zoomed 2x; bars = 20 µm.

705

706 **Figure 2.** COCs were randomly divided into groups (n= 5 COCs/group), subjected to IVM and
707 then pretreated for 1 h with 0 or 6 µM of AG 1478 (a selective inhibitor of EGFR) before adding
708 0 or 10 ng/mL of EGF. **(A)** Cumulus area (µm²) was measured in each COCs at 0 and 24 h post-
709 EGF to assess cumulus expansion —●— EGF 0 ng/mL + AG 1478 0 uM, —■— EGF 10 ng/mL +
710 AG 1478 0 uM, —▲— EGF 10 ng/mL + AG 1478 6 uM. **(B, C, D)** Messenger RNA abundance
711 was measured by real-time PCR. Data represent the mean ± SEM of three-four replicate cultures
712 and different letters or asterisk (*) represent statistical difference (P<0.05).

713

714 **Figure 3**. COCs were randomly divided into groups (n= 5 COCs/group), subjected to IVM and
715 then pretreated for 1 h with 0 or 6 µM of AG 1478 (a selective inhibitor of EGFR) before adding
716 500 ng/mL of FSH. **(A)** Cumulus área (um²) was measured in each COCs at 0 and 24 h post-
717 EGF to assess cumulus expansion, —●— FSH 0 ng/mL+AG 1478 0 uM, —■— FSH 500 ng/mL+AG
718 1478 0 uM, —▲— FSH 500 ng/mL+AG 1478 6 uM. **(B, C, D)** Messenger RNA abundance was
719 measured by real-time PCR. Data represent the mean ± SEM of three-four replicate cultures
720 and different letters or asterisk (*) represent statistical difference (P<0.05).

721

Figure 4. COCs were randomly divided into groups (n= 5 COCs/group), subjected to IVM and then pretreated with distinct concentrations of Verteporfin (VP; a small molecule inhibitor that interferes with YAP binding to TEAD family transcription factors) 0.1, 0.3, 0.5 μM of VP before adding 500 ng/ml of FSH. Messenger RNA abundance was measured by real-time PCR. Data represent the mean ± SEM of three-four replicate cultures and different letters represent statistical

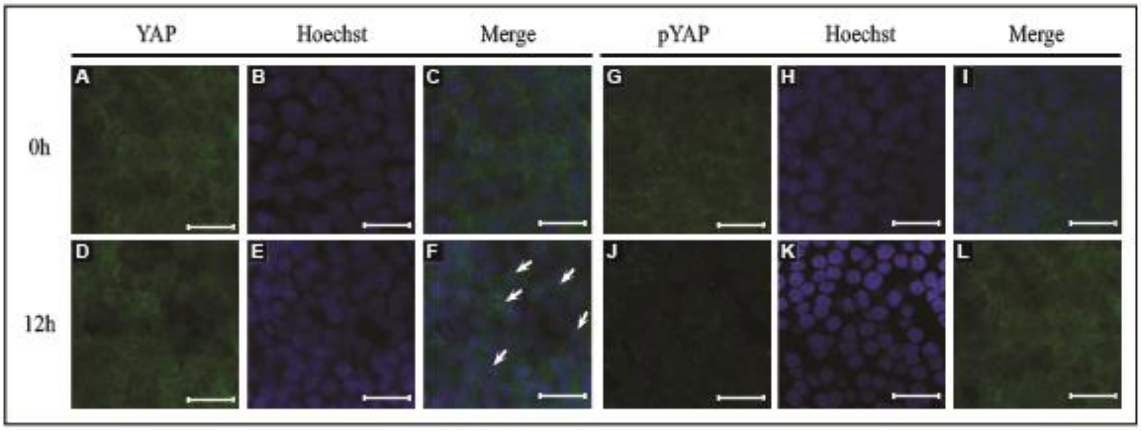
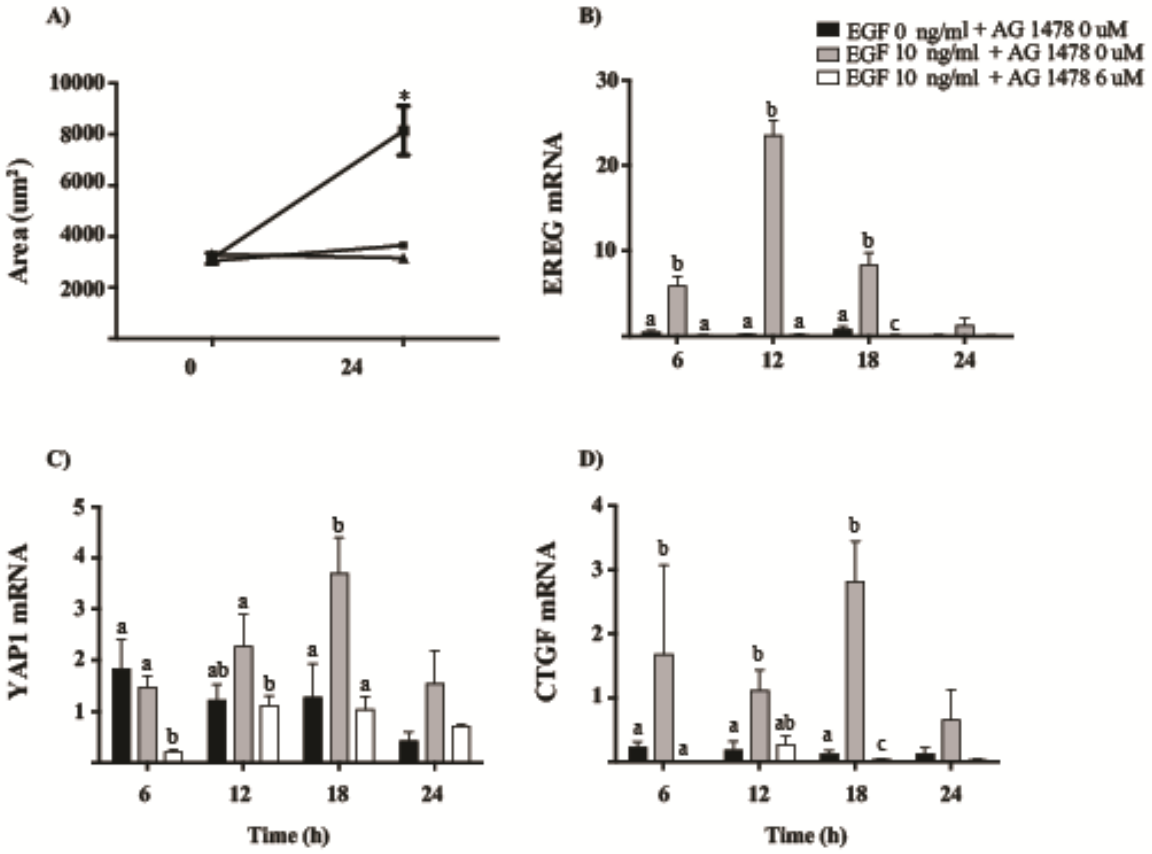


Figure 1.


A)
Area (um²)
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*
B)
EGF 0 ng/ml + AG 1478 0 uM
EGF 10 ng/ml + AG 1478 0 uM
EGF 10 ng/ml + AG 1478 6 uM
EREG mRNA
30
20
10
6
12
18
24
C)
YAP1 mRNA
Time (h)
D)
CTGF mRNA
Time (h)

Figure 2.

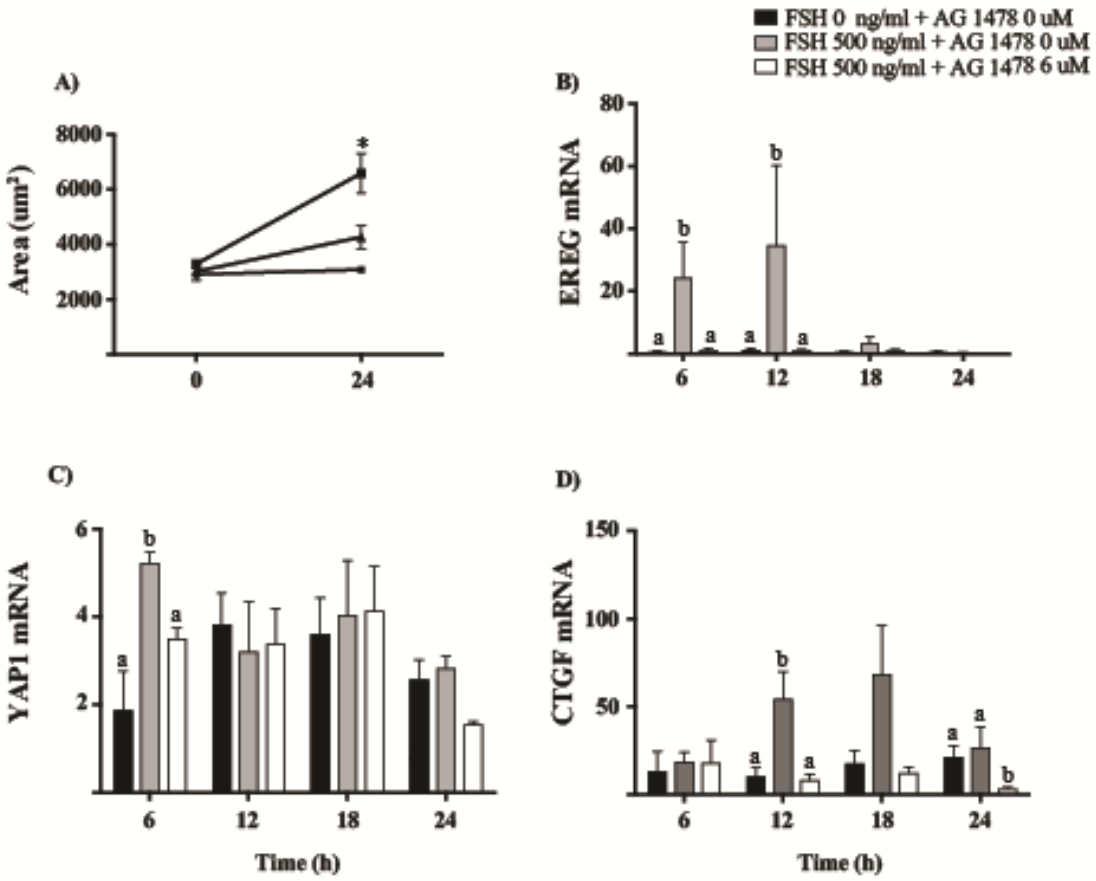
FSH 0 ng/ml + AG 1478 0 uM
FSH 500 ng/ml + AG 1478 0 uM
FSH 500 ng/ml + AG 1478 6 uM
A)
Area (um²)
8000
6000
4000
2000
0
24
B)
EREG mRNA
80
60
40
20
6
12
18
24
C)
YAP1 mRNA
6
4
2
Time (h)
D)
CTGF mRNA
150
100
50
Time (h)

Figure 3.

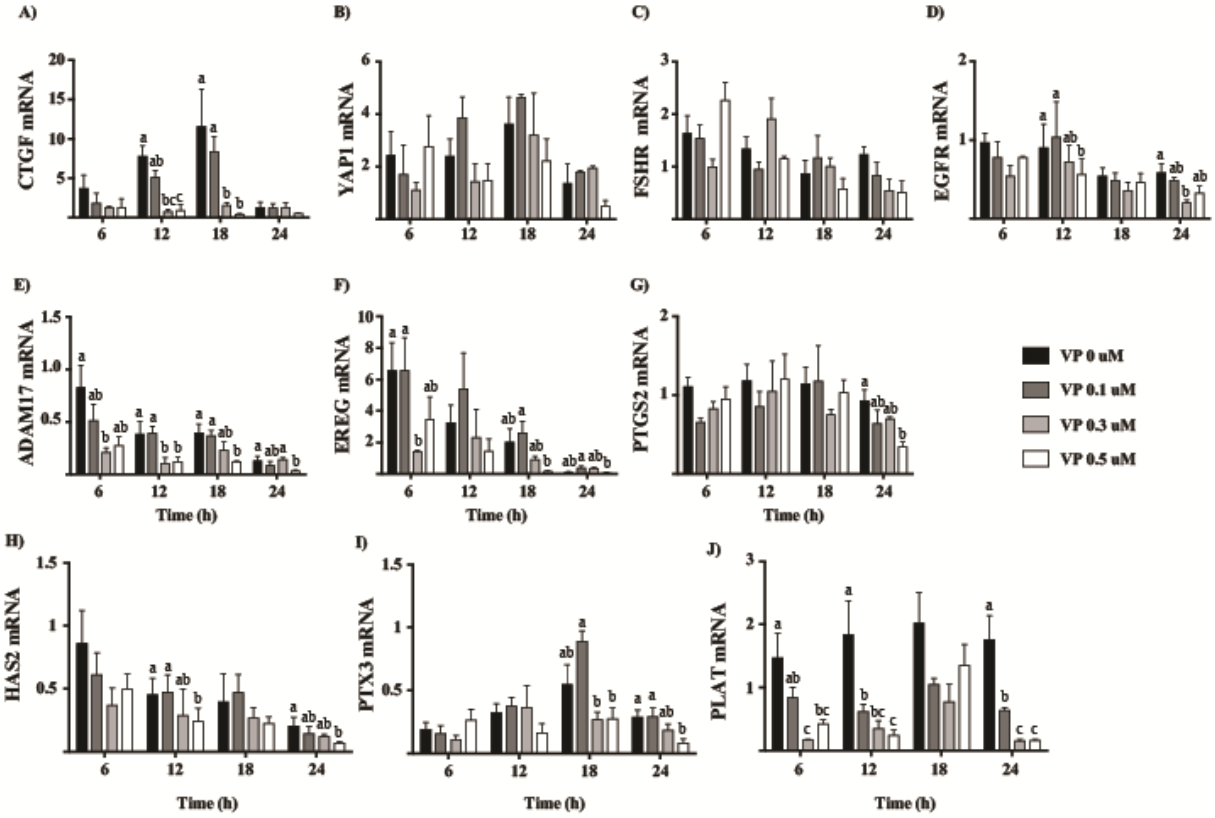
A)
CTGF mRNA
B)
YAP1 mRNA
C)
FSHR mRNA
D)
EGFR mRNA
E)
ADAM17 mRNA
F)
EREG mRNA
G)
PTGS2 mRNA
VP 0 uM
VP 0.1 uM
VP 0.3 uM
VP 0.5 uM
H)
HAS2 mRNA
I)
PTX3 mRNA
J)
PLAT mRNA
Time (h)

Figure 4.

Table

Table 1 – Primers used for Quantitative Real-Time PCR in cumulus cells.

Gene	Sense (5'-3')	Antisense (5'-3')	Accession number
ADAM17	TTCATGGGACAATGCAGGTTT	GAAGTGCCTTTCACCAGGTTTT	XM_002691486.2
CTGF	AGCTGAGCGAGTTGTGTACC	TCCGAAAATGTAGGGGGCAC	NM_174030.2
EGFR	ACCACCCATCCTGCCTGTATCAA	TGCCCAAACGGACAACATTCTTCC	NC_037349.1
EREG	ACTGCACAGCATTAGTTCAAACTGA	TGTCCATGCAAACAGTAGCCATT	XM_010806226.3
FSHr	AGCCCCTTGTCACAACTCTATGTC	GTTCCTCACCGTGAGGTAGATGT	NM_174061.1
HAS2	GCATGTCACCCAGTTGGTCT	TGGGTCAAGCATGGTGTCTG	NM_174079.3
H2A	GAGGAGCTGAACAAGCTGTTG	TTGTGGTGGCTCTCAGTCTTC	(37)
PLAT	GGGGAAGCACAACCACTG	AGCTGATCAGGATCCCCC	NM_174146.3
PTGS2	TTTGACCCAGAGCTGCTTTT	GAAAGACGTCAGGCAGAAGG	(8)
PTX3	CCTCAGCTATCGGTCCATAA	ATTGAAGCCTGTGAGGTCTGC	NM_001076259.2
RPS18	CCTTCCGCGAGGATCCATTG	CGCTCCCAAGATCCAACTAC	NM_001033614.2
YAP1	TCCTTTGAGATCCCTGACGATG	TGACGTTCATCTGGGAGAGC	XM_024975715.1

F: Forward primer; R: Reverse primer.

4 CONCLUSÃO

Este estudo inédito demonstra a regulação de YAP1 e a importância da interação YAP1-TEAD na regulação das células do cumulus em bovinos. Com isso, tornou-se possível aprimorar o conhecimento acerca da fisiologia ovariana através do perfil de expressão de genes-chave para os mecanismos de expansão do cumulus e ovulação, mediadas pelas células do cumulus. Além disso, a compreensão de que a regulação de YAP1 é mediada através da estimulação de EGFR por ação direta de EGF ou, indireta de FSH, permite o futuro desenvolvimento de protocolos capazes de melhorar os resultados de maturação de oócitos *in vitro*.

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