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LEONARDO GUEDES DE ANDRADE

REGULAÇÃO DAS CÉLULAS DA GRANULOSA BOVINA POR EFETOR DA VIA DE SINALIZAÇÃO HIPPO

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Dissertação apresentada ao Programa de Pós-Graduação Stricto Sensu em Ciência Animal da Universidade Federal do Pampa, como requisito parcial para obtenção do título de Mestre em Ciência Animal.

Orientador: Prof. Dr. Paulo Bayard Dias Gonçalves

Coorientador: Prof. Dr. Valério Marques Portela

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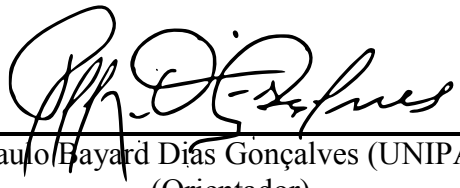

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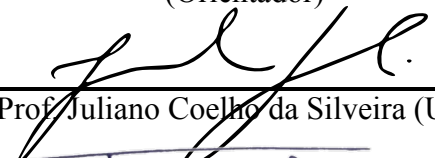

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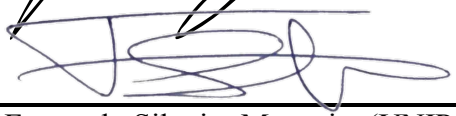

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"Não sou nada.

Nunca serei nada.

À parte isso, tenho em mim todos os sonhos do mundo"

Fernando Pessoa

RESUMO

O crescimento folicular em mamíferos é um evento complexo e dinâmico que requer uma interação de diferentes moléculas e vias de sinalização celular. Em bovinos, principalmente as células da granulosa sofrem diferenciações morfofisiológicas fundamentais para a síntese do 17β-estradiol (E2), através da expressão do citocromo P450 da família 19 do membro 1 da subfamília A (CYP19A1). A via de sinalização Hippo regula a homeostase tecidual, controlando o equilíbrio entre a proliferação e a apoptose das células. O efetor YAP1 controla a transcrição de genes proliferativos e anti-apoptóticos ao interagir com fatores de transcrição membros da família de domínios TEA (TEAD), e entre os genes alvo de YAP1 destaca-se o connective tissue growth factor (CTGF). Porém, a participação da via Hippo na dinâmica do crescimento folicular em bovinos ainda não foi totalmente compreendida. No presente trabalho, buscamos entender como ocorre a regulação de YAP1, principalmente sob o estímulo de FSH, durante o processo de divergência folicular. Para isso, utilizamos um cultivo de células da granulosa que mimetiza aspectos fisiológicos (in vitro), bem como células da granulosa de animais ovariectomizados (in vivo). Nossos resultados mostram que os tratamentos com FSH parece aumentama fosforilação de YAP1 para que ocorra a diferenciação celular. Além disso, quando adicionados inibidores da ligação YAP1-TEADo cultivo de células da granulosa, houve aumento da expressão de CYP19A1. Portanto, sugerimos que os níveis de YAP1 fosforilado aumentem à medida que ocorra o estabelecimento da dominância folicular.

Palavras-chave: YAP1; Hippo pathway; CYP19A1; FSH

ABSTRACT

Follicular growth in mammals is a complex and dynamic event that requires an interaction of different molecules and cell signaling pathways. In cattle, mainly granulosa cells undergo fundamental morphophysiological differentiations for the synthesis of 17β-estradiol (E2), through the expression of cytochrome P450 family 19 subfamily A member 1 (CYP19A1). The Hippo signaling pathway regulates tecidual homeostasis controlling the balance between proliferation and apoptosis. The effector YAP1 controls the transcription of proliferative and anti-apoptotic genes by interacting with transcription factors of the TEA domain family member (TEAD); among the target genes of YAP1, we are particularly interested in the connective tissue growth factor (CTGF). In the present work, we seek to understand how YAP1 regulation occurs, mainly under FSH stimulation, during the follicular divergence process. To accomplish this aim we use a culture of granulosa cells that mimics physiological aspects (in vitro) and also granulosa cells of ovariectomized animals (in vivo). Our results show that FSH appears to increase YAP1 phosphorylation, allowing cell differentiation to occur. In addition, when YAP1 inhibitors were added to the granulosa cell culture, there was an increase in CYP19A1 expression. Therefore, we suggest that the levels of phosphorylated YAP1 will increase as follicular dominance is established.

Keywords: YAP1; Hippo pathway; CYP19A1; FSH

SUMÁRIO

1 INTRODUÇÃO

Durante o ciclo estral na fêmea bovina, podem ocorrer duas ou três ondas de crescimento folicular (MIURA, 2019). Durante cada onda, ocorre um período de emergência de vários folículos seguido de atresia ou ovulação de um gameta originário do folículo dominante (SIROIS; FORTUNE, 1988). As funções do folículo são reguladas pela interação entre hormônios do hipotálamo, hipófise anterior, ovários e útero, sendo as gonadotrofinas FSH (hormônio folículo estimulante) e LH (hormônio estimulante) importantes reguladores da foliculogênese e esteroidogênese. Já está bem estabelecido que o FSH estimula o crescimento folicular sincronizado, caracterizando uma onda folicular (GINTHER; KASTELIC; KNOPF, 1989).

Depois de estabelecido o folículo dominante, o mesmo passa a secretar inibina e estradiol, comprometendo o desenvolvimento dos demais folículos subordinados, momento correspondente a divergência folicular (GINTHER et al., 1996). Mas, além das gonadotrofinas citadas, diversos fatores podem influenciar neste estádio, interferindo na eficiência reprodutiva tanto em animais quanto em mulheres. Desta forma, o conhecimento da fisiologia do sistema reprodutivo é essencial para entender o mecanismo de ação de cada um dos fatores, visando geração e/ou aprimoramento de biotecnologias bem como a possibilidade de novas abordagens terapêuticas.

Durante o crescimento, seleção folicular e atresia ou ovulação, as células da granulosa do folículo respondem a estímulos, através dos quais podem sofrer diferenciações morfofuncionais, principalmente relacionadas com a secreção de hormônios e com a aquisição de receptores de FSH (DUGGAVATHI; MURPHY, 2009). Considerando que todos os folículos recrutados estão sob o mesmo ambiente endócrino, é evidente que a seleção do dominante é determinada por fatores endócrinos, mas também por fatores parácrinos que atuam localmente (RIVERA; FORTUNE, 2001). Embora já sejam conhecidos alguns mecanismos controladores da divergência folicular, a completa elucidação deste processo ainda não foi atingida.

Ao longo dos anos, a foliculogênese tem sido alvo de pesquisas envolvendo a fisiologia da reprodução, pois é um pré-requisito essencial para o melhor aproveitamento do potencial reprodutivo de espécies de interesse econômico ou ameaçadas de extinção. Nas últimas décadas, inúmeros mecanismos reguladores e rotas de sinalização celular foram identificados nesse

processo, o qual é altamente complexo e dinâmico. Atualmente, a via de sinalização celular Hippo vem sendo estudada no contexto da fisiologia ovariana (HSUEH, A. J. W. et al., 2015).

Considerada uma via altamente conservada com funções bem definidas no controle do crescimento de tecidos, diferenciação, proliferação e apoptose celular, a via de sinalização Hippo foi descoberta em 1995, utilizando *Drosophila melanogaster* como organismo modelo para estudo de genes de supressão tumoral (JUSTICE; WOODS; BRYANT, 1995). Os efetores principais da sinalização Hippo são: Yes-associated protein 1 (YAP1) e transcriptional co-activator with PDZ-binding motif (TAZ), essas proteínas possuem semelhanças estruturais (ZHAO, B. et al., 2010), o que implica em desempenharem basicamente a mesma função, atuam regulando a transcrição de genes alvo, principalmente *connective tissue growth factor* (CTGF) e cysteine-rich angiogenic inducer 61 (CYR61), membros da família de proteínas CCN (SHOME et al., 2020). O controle da transcrição gênica ocorre através da acumulação dos efetores no núcleo da célula, onde permanecem ligados, sob a forma de complexos, a fatores de transcrição, especialmente aos fatores da família transcriptional enhanced associate domain (TEAD1-4) (HUH et al., 2019).

A via Hippo vem sendo estudada em diversos tecidos celulares, já foi comprovado que YAP1 é expresso em células da granulosa de camundongos e que a via Hippo-YAP1 contribui significativamente para a fertilidade feminina, cooperando com múltiplas vias de sinalização (LV et al., 2019). Recentemente, foi comprovada a importância de YAP1 para a proliferação e síntese de estradiol em células da granulosa bovina (PLEWES et al., 2019). Embora já existam estudos enfatizando a importância da via Hippo no sistema reprodutivo da fêmea, ainda não foi demonstrado se há participação de YAP1 para o estabelecimento da dominância folicular. Baseado nisso, a hipótese do presente estudo é que a via Hippo é diferentemente sinalizada no folículo dominante quando comparado aos subordinados.

A função da via de sinalização Hippo na regulação da foliculogênese através das células da granulosa bovina foi o foco deste trabalho. Foram analisados diferentes genes alvo da interação de YAP1-TEAD com objetivo de demonstrar, através de abordagens *in vitro* e *in vivo*, a importância da via Hippo no processo de divergência folicular.

2 REVISÃO DE LITERATURA

2.1 Foliculogênese

Considerados como unidades morfofuncionais do ovário, os folículos são caracterizados por um conjunto de células circundando um oócito, onde a cada onda de crescimento folicular, os folículos saem do estado de quiescência e passam a se desenvolver de forma gradual e continua (HURK, VAN DEN; BEVERS; BECKERS, 1997). Os folículos primordiais, primários e secundários são classificados como pré-antrais. O crescimento dos folículos pré-antrais é controlado basicamente por fatores intraovarianos (MAGALHÃES et al., 2011). Após a formação do antro, os folículos antrais passam a ser dependentes das gonadotrofinas, de hormônios esteroides e também de fatores ovarianos de ação parácrina e endócrina (FORTUNE, 1994).

A citodiferenciação das células da granulosa é regulada endocrinamente pela ação de gonadotrofinas já mencionadas e também por fatores de crescimento, entre os quais destacam-se: os fatores de crescimento epidérmico (EGFs), fatores de crescimento semelhantes à insulina (IGF-1) (PALMA et al., 2012) e fatores de crescimento fibroblásticos (FGFs) (FAUSTINO; FIGUEIREDO, J. R., 2011). O grupo dos FGFs é composto por mais de 20 subtipos, dentre os quais, os FGF-17 e 18 são conhecidos por induzirem a proliferação e diferenciação celular em diferentes tecidos em processos fisiopatológicos (BROCKI; WEARDEN, 2006). Entretanto, o FGF-18 já foi relacionado à atresia folicular, esse fator induziu a morte celular e alterou a expressão gênica da regulação do ciclo celular, no mesmo estudo também foi constatada uma maior quantidade de mRNA de FGF-18 no início do processo de atresia do folículo subordinado (PORTELA et al., 2010).

A superfamília TGF-β também participa da foliculogênese. Essa superfamília é formada por um grupo de proteínas que atuam como ligantes em vários processos celulares, como a proliferação e diferenciação (WOTTON; MASSAGUE, 2006). A subfamília TGF-β prototípica

compreende os subtipos TGF-β1, TGF-β2, TGF-β3, também faz parte desse grupo uma subfamília de proteína morfogenética óssea extensa (BMP), a subfamília do fator de crescimento e diferenciação (GDF), entre outros (KNIGHT; GLISETER, 2006). Membros da família TGF-β estão envolvidos no desenvolvimento folicular, os quais podem regular a diferenciação das células foliculares.

Em relação aos fatores foliculares locais, IGF-1 livre é essencial para proliferação normal de células da granulosa, modulando o desenvolvimento e a esteroidogênese folicular. Camundongos knockout para IGF-1 apresentam crescimento folicular retardado (KADAKIA et al., 2001). A ação do IGF-1 é mediada por suas proteínas de ligação (IGFBPs) e sua expressão estimula a expressão de receptores de FSH e, consequentemente, aumenta a resposta das células da granulosa ao hormônio (ZHOU, Jian et al., 1997). O sinergismo entre IGF-1 e FSH também é responsável por maior atividade da aromatase (CYP19A1) e consequente síntese de estradiol (GENC et al., 2011). Portanto, a disponibilidade de IGF-1 livre também é um determinante importante para que o folículo se torne dominante (GADELLA et al., 2003). Recentemente, foi demonstrado que o IGF-1 também pode promover a sobrevivência do folículo através do aumento a resistência à apoptose (QUIRK et al., 2018).

O FSH ao se ligar em seu receptor, inicia a cascata de sinalização primária via AMP cíclica / proteína quinase A (cAMP), que atua como um segundo mensageiro levando a ativação *downstream* de proteínas quinases, como a proteína quinase A (PKA), as quais são responsáveis por ativar diferentes fatores de transcrição (NAVALAKHE et al., 2013). Nos últimos anos, a via das proteínas quinases ativadas por mitógeno P38 (MAPK) vem sendo estudada em células foliculares. Em células esteroidogênicas, essas quinases atuam como sinalizadores de morte, envolvendo p53 e outras caspases necessárias para regular a atresia de folículos subordinados (CASARINI; CRÉPIEUX, 2019). Em células da granulosa tratadas com PD-98059 (inibidor de MAPK) houve aumento na expressão da aromatase (SILVA et al., 2006). Por outro lado, a via PI3K/Akt está relacionada a dominância folicular (RYAN et al., 2007). Um trabalho in vitro, mostrou que as vias PI3K/AKT e Hippo estão envolvidas na ativação folicular (DEVOS; GROSBOIS; DEMEESTERE, 2020). Juntos, esses resultados demonstram o quão complexa e dinâmica é a rede de sinalização e transdução de sinais em células foliculares.

2.2 Via de sinalização Hippo

A via de sinalização Hippo foi descrita na década de 90, utilizando *Drosophila melanogaster* como modelo para estudo de genes de supressão tumoral (JUSTICE; WOODS; BRYANT, 1995). Possui funções bem definidas na homeostase tecidual, pois atua controlando o equilíbrio entre a proliferação e a apoptose celular. Por ser uma via que controla a proliferação celular, desde sua descoberta, a via Hippo é amplamente estudada em algumas patologias, como o câncer (ZHAO, B. et al., 2010). Em *D. melanogaster*, a mutação de qualquer um dos componentes da Hippo pode resultar em tecidos e órgãos aumentados, essa constatação inspirou o nome da via (MAUVIEL; VARELAS, 2011).

A via Hippo vem sendo estudada em modelos roedores e humanos e estudos mostraram que o mau funcionamento da via está intimamente ligado a insuficiência ovariana primária e a síndrome do ovário policístico em camundongos e mulheres, respectivamente (CHENG et al., 2015; LI, T. et al., 2012). Em mamíferos, a via Hippo é caracterizada inicialmente pela ação das quinases MST1/2 que fosforilam LATS1/2, os quais fosforilam e inativam YAP1 e TAZ (MAUVIEL; VARELAS, 2011), caracterizando o estado ativo da via. YAP1 e TAZ atuam como reguladores transcricionais de genes alvo da via Hippo, tornando-se responsáveis diretos pelas funções da via (ZHAO, B. et al., 2010). Apesar de ser pouco conhecida a regulação, YAP1 e TAZ podem ser rapidamente fosforilados e desfosforilados por uma ampla variedade de sinais (MA, S. et al., 2019) e estímulos extracelulares podem regular YAP1/TAZ por meio dos mecanismos independentes ou dependentes da via de sinalização do Hippo (YAMAGUCHI; TAOUK, 2020). YAP1/TAZ atuam na mecanotransdução celular, processo molecular que converte sinais mecânicos extracelulares em respostas intracelulares (PANCIERA et al., 2017). A via Hippo, no seu estado inativo, caracteriza-se por um acúmulo de YAP1 e TAZ no núcleo, onde permanecem ligados em forma de complexos, associados a diferentes fatores de transcrição, entre eles aos fatores TEAD (TEAD 1-4) (HUH et al., 2019). Entre os genes alvo conhecidos do complexo YAP1/TAZ-TEAD, estão CTGF, CYR61 e *ankyrin repeat domain* 1 (ANKRD1) (WANG, K. et al., 2016). A figura 1 representa a cascata de sinalização Hippo.

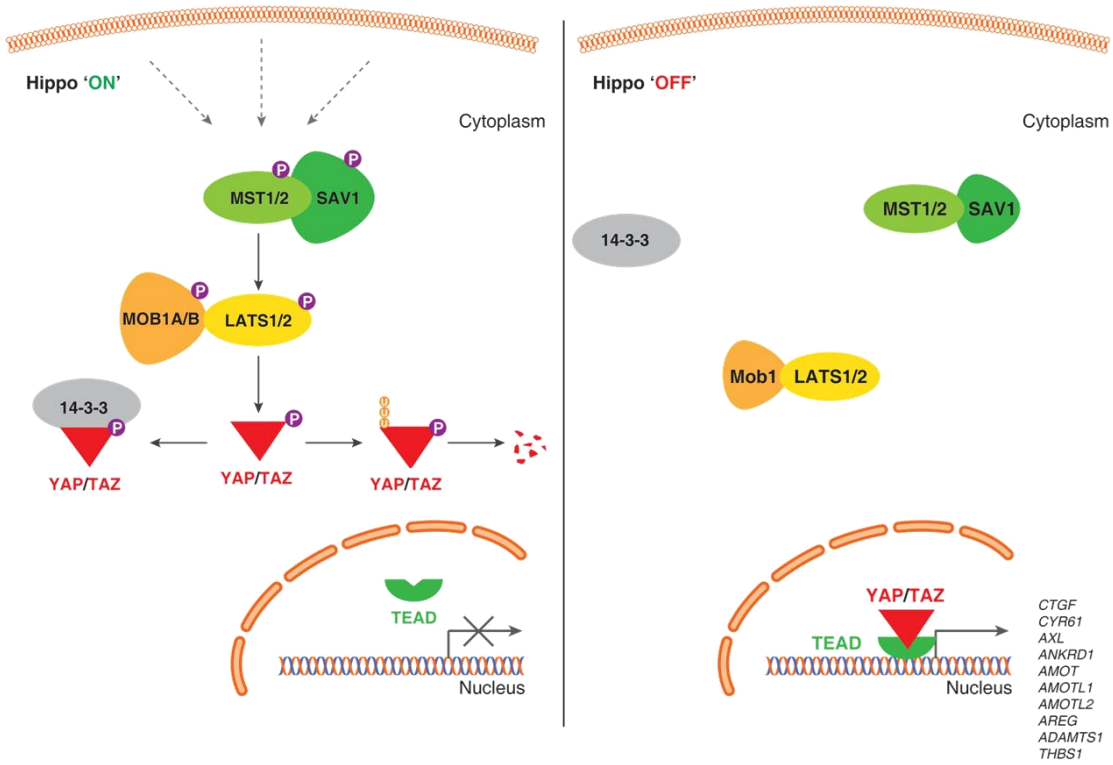

FIGURA 1- Figura representativa da via de sinalização Hippo. Quando a via de sinalização Hippo está ativa, através de sinais upstream, regulam a fosforilação das quinases MST1/2, LATS1/2 e YAP1/TAZ. A fosforilação de YAP1 recruta proteínas 14-3-3 que estimulam a retenção citoplasmática, ou são alvos da degradação proteolítica. Quando a via Hippo está inativa, YAP1 não é fosforilado, formando um complexo com o fator de transcrição TEAD, regulando genes alvo.
Fonte: Adaptado de (BOOPATHY; HONG, 2019).

2.3 Via Hippo na fisiologia ovariana

A literatura atual relaciona a Via Hippo à fisiologia ovariana em roedores (LV et al., 2019). Talvez pela íntima relação desta via com a formação tumoral, os estudos concentram-se em patologias. Em camundongos, Cheng e colaboradores sugerem que YAP1/TAZ regulam a expressão de genes estimulados durante a cascata pré-ovulatória de LH (CHENG et al., 2015). Além disso, a superexpressão de YAP1 compromete a ação do LH em células da granulosa (LI, T. et al., 2012). YAP1 também mostrou-se necessário para a proliferação das células da granulosa em camundongos, na qual, sob efeito de LH, ocorreu a inativação de YAP1 através da via ERK1/2, fundamental para que ocorra ovulação (JI et al., 2017).

Em 2019, foi comprovado que YAP1 é espaço-temporariamente expresso nas células da granulosa em camundongos, sendo nuclear em células proliferativas, YAP1 também interagiu com o receptor do fator de crescimento epidérmico (EGFR) e com a via de sinalização de TGF-β para regular a proliferação, diferenciação e sobrevivência das células da granulosa (LV et al., 2019). A deleção condicional em células foliculares de CTGF afeta o desenvolvimento folicular, ovulação e luteólise em camundongos (NAGASHIMA et al., 2011).

Os componentes da via Hippo estão presentes no endométrio de primatas, e resultados indicam que a via está envolvida na fisiologia uterina durante o ciclo menstrual (STRAKOVA et al., 2010). Em mulheres, YAP1 é altamente expresso em células da granulosa tumorais da linhagem KGN, estimulando significativamente a proliferação celular e levando a hiperplasia. No mesmo artigo, é sugerido que a via Hippo é um dos principais reguladores da progressão tumoral nessas células (FU et al., 2014). Também foi comprovado que a interrupção da sinalização Hippo, induzida por fragmentação ovariana, pode promover o crescimento do folículo (KAWAMURA, K. et al., 2013).

Em bovinos, uma análise do perfil de miRNAs em vesículas extracelulares de folículos ovarianos demonstrou diversos genes envolvidos com a via Hippo, sendo mais expressos em folículos submetidos a menores concentrações de progesterona do que em altas concentrações (ÁVILA, DE et al., 2020). Além disso, um estudo examinou a expressão e localização dos componentes de sinalização da via Hippo no ovário de bovinos e as possíveis funções de YAP1 e TAZ em células da granulosa, os resultados indicam que esses co-ativadores da transcrição desempenham papéis importantes na proliferação da granulosa e na síntese de estradiol, uma vez que o knockdown de YAP1 em cultivo de células da granulosa comprometeu a síntese de estradiol sob estímulo de FSH (PLEWES et al., 2019).

Durante a foliculogênese, ocorre um processo de diferenciação progressiva bem ajustado em todos os constituintes do folículo (GOUGEON, 1996), onde apenas um se torna dominante em relação aos demais. Pesquisas sugerem que a sinalização de Hippo também pode estar envolvida nas comunicações interfoliculares (BAKER; SPEARS, 1999). Além disso, estudos genômicos fornecem suporte para os papéis essenciais da sinalização do Hippo na polimerização da actina para regulação do desenvolvimento do folículo (HSUEH, A. J. W. et al., 2015). De uma forma geral, as pesquisas relacionando a via Hippo à fisiologia ovariana são bastante amplas, consequentemente, o papel da sinalização Hippo via YAP1 no ovário bovino ainda é

desconhecido. Desta forma, estudar a influência do FSH na sinalização da via Hippo em células da granulosa bovina pode levar a um melhor entendimento dos mecanismos moleculares durante a seleção folicular.

3 ARTIGO CIENTÍFICO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico. As seções Material e Métodos, Resultados, Discussão e Referências estão descritas no próprio manuscrito, o qual será submetido para publicação no periódico **Biology of Reproduction.**

Artigo a ser enviado para publicação:

FSH regulation of the Hippo signaling main effector (YAP1) during follicular divergence in cattle: an in vivo and in vitro approach

Running title: Role of YAP1 in follicular divergence in granulosa cells.

Summary sentence: FSH increases phosphorylation of the Hippo signaling pathway main effector YAP1, which seems to be necessary for granulosa cell differentiation.

Keywords: granulosa cells, Yes Associated protein 1, Hippo pathway, estradiol.

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ABSTRACT

Follicular growth in mammals is a complex and dynamic event that requires an interaction of different molecules and cell signaling pathways. In cattle, mainly the granulosa cells undergo fundamental morphophysiological differentiation for the synthesis of 17β-estradiol (E2), through the expression of cytochrome P450 family 19 subfamily A member 1 (CYP19A1). The Hippo pathway regulates the number of cells, controlling the balance between proliferation and apoptosis. The effector YAP1 controls the transcription of proliferative and anti-apoptotic genes, for this, the effectors interact with transcription factors of the TEA domain family member (TEAD), among the target genes downstream of YAP1, highlighting the tissue growth factor (CTGF). In the present work, we seek to understand how YAP1 regulation occurs, mainly on FSH stimulation, during the follicular divergence process. To accomplish this aim we use a culture of granulosa cells that mimics physiological aspects (in vitro) and granulosa cells of ovariectomized animals (in vivo). Our results indicate that FSH regulates YAP1 in granulosa cells. FSH increased phosphorylation of YAP1, which is likely to be necessary for cell differentiation. In addition, the abundance of *CYP19A1* mRNA increased when YAP1 was inhibited by pharmacological inhibitors (VP or P17). Therefore, there are strong evidence that phosphorylated YAP1 is required to establish the follicular dominance in cattle.

INTRODUCTION

Follicular growth in mammals is a complex and dynamic event that requires the interaction of different molecules and cell signaling pathways [1]. In cattle, especially granulosa cells undergo fundamental morphophysiological differentiation for the synthesis of 17β-estradiol (E2), through the expression of Cytochrome P450 family 19 subfamily A member 1 (CYP19A1) induced by Follicle-Stimulating Hormone (FSH) [2].

The interaction of FSH with its FSH-specific G protein-coupled receptor (FSHR) in bovine granulosa cells of antral follicles is intense until follicular divergence, a period when a single follicle becomes dominant [3]. After follicular divergence, the circulating FSH decreases and the intrafollicular bioavailability of insulin-like growth factor (IGF) increases, which is crucial to the success of the dominant follicle [4]. Although the follicles are under the same environment during the estrous cycle, it is likely that there are many particularities in the dominant follicle in addition to those previously described [5]. Despite years of research, the molecular mechanisms that control follicular divergence have not yet been completely unveiled.

It is known that follicle growth depends on cell proliferation and different pathways such as MAPK, TGF-beta, and Hippo are involved in the processes of cell proliferation, differentiation, and apoptosis [6]. The Hippo pathway is formed by different kinases, and its main effectors are Yes-associated Protein 1 (YAP1) and transcriptional coactivator with PDZ-binding motif (TAZ); these proteins have similar structural characteristics and act as transcriptional coactivators [7].

The Hippo pathway regulates the tecidual hoemostasis controlling the balance between proliferation and apoptosis. The effectors YAP1 and TAZ mainly control the transcription of proliferative and anti-apoptotic genes [8]. The YAP and TAZ proteins are translocated to the cell nucleus for specifics gene transcription pathways. In the nucleo the effectors interact with transcription factors of the member of the TEA domain family (TEAD) [9]. Among the target genes downstream of YAP1, tissue growth factor (CTGF) and cysteine-rich angiogenic protein 61 (CYR61), members of CCN family proteins [10], and the regulator ankyrin repeat domain 1 (ANKRD1) [11] are the targets of this study.

Studies in cattle show that the Hippo pathway kinases are expressed in follicular cells and YAP1 is important for the proliferation of granulosa cells and estradiol synthesis [12]. In mice, it

was demonstrated that YAP1 activity is essential for the proliferation, differentiation, and survival of granulosa cells during folliculogenesis [13].

In mammals, YAP1 is a protein expressed in different tissues, which changes according to the cellular context [14,15]. In women, nuclear YAP1 is predominantly highly expressed in granulosa cells tumor (GCT), being considered a critical regulator of proliferation, migration, and steroidogenesis in the human GCT cell line KGN [16], suggesting the regulation of YAP1 by FSH. Therefore, our hypothesis is that FSH activates the Hippo signaling pathway during the establishment of follicular dominance.

Although the expression of the Hippo pathway components in cattle ovary has been explored [16], the regulation of effectors downstream of YAP1in follicular divergence is unknown. In the present study, experimental models in vivo and in vitro were used to evaluate the transcriptional profile of downstream YAP1 genes in granulosa cells. In vivo, we collect granulosa cells before, during, or after the follicular divergence. The phosphorylation of YAP1 was also evaluated under FSH stimulation.

MATERIAL AND METHODS

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee (ACUC no. 23081.009594/2007-41). All chemicals used in the present study were purchased from Sigma Chemicals Company, unless otherwise indicated in the text.

Experimental design I: Transcriptional profile of Hippo pathway target genes in granulosa cells cultured with or without FSH.

To test the hypothesis that the Hippo pathway is activated in the dominant follicle, bovine granulosa cells were cultured in vitro for six days using a culture model that mimics cellular cytodifferentiation that occurs after follicular divergence [17]. In the initial 48 hours, cells were cultured with insulin (10 ng/ml) and without FSH. After this initial culture period, different concentrations of FSH were added in four groups: 1) Control 0 ng/ml; 2) 0.1 ng/ml; 3) 1.0 ng/ml and 4) 10 ng/ml. Concomitantly, other cultures were performed to quantify the YAP1 protein (total and phosphorylated; pYAP1) under two concentrations of FSH: 1) 1 ng/ml and 2) 10 ng/ml, as described below. The level of CYP19A1 mRNA was also evaluated after treating the cells with YAP1-TEAD binding inhibitors. All series of cultures were performed on at least three different pools of cells collected on different occasions.

Experimental design II: Transcriptional profile of Hippo pathway target genes before, during and after follicular divergence in vivo.

Thirty-six weaned beef cows (predominantly Hereford and Aberdeen Angus) were injected with two doses of PGF2α analogue (Cloprostenol, 125 µg; Schering-Plough Animal Health, Brazil) intramuscularly (i.m.), 12 h apart. They were observed in estrus within 3–5 days after PGF2α. Therefore, the experiment was performed in the first follicular growth wave of the estrous cycle. Ovaries were then examined once a day by transrectal ultrasonography, using an 8-MHz linear-array transducer (Aquila Vet scanner, Pie Medical, Netherlands), and all follicles larger than 5 mm were drafted using three to five virtual slices of the ovary allowing a three-dimensional localization of follicles and monitoring individual follicles during follicular wave [18]. The day of the follicular emergence was designated as day 0 of the wave and was retrospectively identified as the last day on which the dominant follicle was 4 or 5 mm in diameter [20]. The cows were randomly assigned to be ovariectomized by colpotomy at days 2, 3

or 4 of the follicular wave (four cows for each day) to recover the largest (dominant follicle - F1) and the second largest (subordinate follicle - F2) follicles from each cow. After ovariectomy, the granulosa cells were recovered from F1 and F2 and stored at -80ºC for further RNA extraction. Levels of abundance of CYP19A1 mRNA were also analyzed, as described by Ferreira, et al. [20]. This approach allowed the analysis of gene expression of target genes of the Hippo pathway before (D2), during (D3) and after (D4) follicular divergence.

Immunohistochemistry

Bovine follicles were isolated and fixed in formaldehyde 10% solution for 24 h, rinsed, and dehydrated in alcohol. Whole follicles were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Immunohistochemistry (IHC) was performed on 7 μm tissue sections using VectaStain Elite avidin–biotin complex method kits (Vector Laboratories). Sections were probed with a primary antibody against YAP (1:300, #14074, Cell Signaling). Staining was done using the 3,3′-diaminobenzidine peroxidase substrate kit (Vector Laboratories). Negative controls consisted of slides for which the primary antibody was omitted. Photomicrographs were taken using a Carl Zeiss Axio Imager M1 microscope (Carl Zeiss Canada Ltd., Toronto, Canada) at ×1,000 magnification and using the Zen 2012 blue edition software (Carl Zeiss, Oberkochen, Germany).

Cell culture

The granulosa cells (GC) were obtained from 2 to 5 mm ovarian follicles of adult cows, regardless of estrous cycle stage, collected in a local abattoir. The follicles were dissected from the ovarian stroma and sectioned in DMEM F12 medium. Granulosa cells were filtered through a Cell Dissociation Sieve - Tissue Grinder Kit/ 150 Mesh (Sigma). The basal medium for all

cultures was DMEM/F12 with added sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), bovine serum albumin (1 mg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), human transferrin (5 ng/ml), nonessential amino acid mix (10 mM), androstenedione (10 −7 M), insulin (10 ng/ml supplemented at the beginning of culture) and FSH (0.1; 1 or 10 ng/ml). A viable cell count was performed in the presence of 0.4% trypan blue, and 1×106 viable cells/ml were cultured in 24-well plates at 37ºC in 5% CO2 in air for six days, with 70% (700 μl) medium being replaced every two days. To measure steroid secretion, the medium was removed for steroid assay on day 6 and stored at -20ºC. To measure mRNA abundance, cells were collected into Trizol and stored at -80ºC until RNA isolation.

Immunoblotting

Immunoblotting was performed as previously described [21] using primary antibodies (anti-YAP, #14074 and anti-phosphoYAP (Ser127), #13008; both from Cell Signaling Technology) or β-Actin Antibody (#sc-47778, 1:10000, Santa Cruz Biotechnology, Dallas, TX, USA) diluted in 5% bovine serum albumin. After washing three times with TTBS, membranes were incubated for 1 h at room temperature with anti-rabbit HRP-conjugated IgG (GE Healthcare Life Sciences, Baie d'Urfé, QC, Canada) diluted 1:10000 in 5% non-fat dry milk in TTBS. Protein bands were visualized by chemiluminescence (Immobilon; Millipore, Billerica, MA, USA) and quantified using a ChemiDoc MP detection system (Bio-Rad) and Image Lab™ software.

RNA extraction, Reverse Transcription and Quantitative PCR (qPCR)

Total RNA was isolated from cell culture (experiment I) using Trizol protocol according to the manufacturer's instructions. Total RNA of the experiment II was extracted using silica

column-based protocol (granulosa cells; Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. The quantification of the extracted RNA was determined by wavelength using a spectrophotometer (NanoDrop1000, Thermo Scientific, Waltham, MA, USA; absorbance at 260/280nm). The RNA was treated with 0.1 U DNAse Amplification Grade (Thermo Fisher, Waltham, MA, USA) for 15 minutes at 27ºC to digest any contaminating DNA molecule. DNAse was inactivated with 1µl EDTA for 10 minutes at 65ºC. The RNA was reverse-transcribed using the enzyme 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 terminated by incubation at 95 ° C for 5 minutes. The quantitative polymerase chain reaction (qPCR) was performed using the CFX384™ Real Time System (Bio-Rad Laboratories, Hercules, CA, USA), using 2 µl of cDNA and 8 µl of MIX containing specific primers (Table 1), nucleases, water and GoTaq® DNA Polymerase (Promega, Madison, WI, USA). Amplification was performed with initial denaturation at 95 ° C for 5 minutes, followed by 40 cycles of denaturation at 95 ° C for 10 seconds, followed by 1 minute at 60 ° C to amplify each transcript. The reaction was performed in duplicate and serial dilutions of cDNA were used to generate a relative standard curve. The target mRNA concentration of experiment I was normalized to the amplification of the housekeeping gene H2AFZ, whereas the samples from experiment II were analyzed relative to GAPDH. Housekeeping genes were determined and used in subsequent experiments because they were stable between the treated and control groups. Relative mRNA levels calculation was performed as described by Pfaffl et al. [22].

Steroid Assay

Estradiol concentration were measured from the culture media. The concentration was determined by the chemiluminescence kit (ADVIA Centaur, Siemens) in a specialized clinical analysis laboratory following the manufacturer's recommendations.

Statistical Analysis

Continuous data were tested for normal distribution using Shapiro-Wilk test and normalized when necessary. All data were analyzed by ANOVA followed by multiple pairwise comparisons (Tukey-Kramer HSD test) using the JMP Software (14; SAS Institute Inc., Cary, NC). Results are presented as mean ± standard error of the mean (SEM) and $P<0.05$ was considered significant.

RESULTS

Effect of FSH on the expression of Hippo pathway genes in granulosa cells

To determine whether FSH regulates the expression of classic YAP1-TEAD transcriptional target genes in bovine granulosa cells, we used a non-luteinized granulosa cell culture model. The cells were cultured in the presence of increasing doses of FSH for the last 4 days of culture. This is a completely serum-free long-term granulosa cell culture system that allows the induction/maintenance of CYP19A1 expression and estradiol (E2) secretion in response to physiological doses of FSH, as it can be confirmed by the results presented in **Fig 1**. FSH upregulated *CYP19A1* mRNA and consequently stimulates E2 secretion (particularly at the concentration of 10 ng/ml; $P<0.05$, **Fig 1A** and **B**, respectively). In contrast, FSH downregulated *CTGF*, *ANKRD1* and *CYR61* mRNA in a concentration-dependent manner (**Fig 2**). The mRNA levels for these well-known YAP1-TEAD target genes were decreased by high concentrations of FSH ($P <0.05$, **Fig 2A, 2B, and 2C**).

Effect of FSH on phosphorylation of Yes-associated protein 1 (YAP1) in granulosa cells

When Hippo signaling is inactive, YAP1 accumulates in the nucleus and form complexes with numerous transcription factors, notably those of the TEAD family, resulting in the modulation of the transcriptional activity of target genes as *CTGF*, *ANKRD1* and *CYR61* [14]. Conversely, when Hippo signaling is activated, YAP1 is phosphorylated and translocated from nucleus to cytoplasm [23]. To better elucidate the mechanism by which FSH downregulated YAP1-TEAD target genes in bovine GC, the cells were cultured in the presence of 0,1 or 10 ng/ml of FSH and the abundance of phosphorylated YAP1 protein was determined by Western blot after 4-day culture system. The results indicated that FSH treatments did not alter total YAP1 protein levels ($P > 0.05$, **Fig 3A**) but significantly promoted YAP1 phosphorylation on serine 127 (Ser127; $P < 0.05$, **Fig 3B**). The phosphorylation of this site generates a binding site for 14-3-3 protein leading to their sequestration in the cytoplasm [8].

To determine whether FSH-induced YAP1 phosphorylation pattern can be observed in GC from follicles of different sizes, cells from ovarian follicles of sizes compatible with those found in emergence to dominance were analyzed by IHC. Interestingly, the results suggested that total YAP1 expression pattern does not differ in GC collected from small (<5 mm) and medium (5–10 mm) follicles (**Fig 4**). On the contrary, while the positive signal for phospho-YAP1 (Ser127) was barely detected in GC from small follicles, strong signal was detected in the cytoplasm of medium follicles (**Fig 4**). This finding suggests that as the follicle develops a significant amount of YAP1 is translocated to or remains in the cytoplasm, which prevents the YAP1 to bind to the nuclear TEAD family of transcription factors.

The mRNA abundance of classic YAP1-TEAD transcriptional target genes is higher in subordinate follicles after follicular divergence

In the in vitro studies, we observed that YAP1 phosphorylation increased in the presence of FSH, which decreased the expression of classic YAP1-TEAD target genes in follicles with size compatible with the establishment of dominance. To confirm that, an *in vivo* experiment was performed to obtain samples from ovaries collected at days 2 (D2), 3 (D3), and 4 (D4) of the first follicular wave (those days correspond to the day before, during and after follicular deviation, respectively). The results confirmed our hypothesis and clearly indicated that mRNA levels for *CTGF*, *CYR61* and *ANKRD* are slightly increased in granulosa cells of subordinate follicles at D3 ($P > 0.05$, **Fig 5**) but are significantly higher is GC from subordinate follicles at D4 ($P < 0.05$, **Fig 5).** Day 4 corresponds to the first day after divergence when the dominant follicle has established its dominance, as well the *CYP19A1* mRNA and E2 levels are higher in GC and in follicular fluid, respectively, than those from D3 [20].

Pharmacological inhibition of YAP1-TEADs interaction in vitro increases basal levels for mRNA encoding CYP19A1.

The sequence of experiments (in vitro and in vivo) previously performed in this study indicated an inverse relationship between the expression levels of CYP19A1 and YAP1-TEAD target genes. To elucidate the nature of such relationship (cause vs consequence), we decided to perform a series of in vitro experiments using pharmacological inhibitors that interfere with YAP1 binding to TEAD family transcription factors. In the first series of cultures, GC were cultured in the absence or presence of different concentrations of verteporfin (VP), a well-known and commonly used YAP1-TEAD inhibitor molecule [24]. The results indicated that VP increased *CYP19A1* basal mRNA levels in a concentration-dependent manner ($P < 0.05$, Fig 6A).

To further investigate whether VP action was specific or not, we then decided to use an alternative inhibitor, peptide 17 (P17), an engineered peptide that also disrupts YAP-TEAD interaction but with affinity to TEAD1 [25]. Similarly, CYP19A1 basal mRNA levels also increased in a concentration-dependent manner ($P < 0.05$, Fig 6B), suggesting that CTGF and/or other YAP1-TEAD target may exert a direct or indirect inhibitory regulation of CYP19A1 transcriptional regulation in bovine granulosa cells.

DISCUSSION

Most of the studies that relate the Hippo signaling pathway to ovarian physiology were developed in vitro using mice as an experimental model. As far as we know, this is the first work carried out with an in vivo approach using cattle, a monovulatory species with similar physiological characteristics to women. In the present study, we explored the mRNA abundance of three genes regulated by the interaction of YAP1 / TAZ-TEAD in granulosa cells. Collectively, our data suggest that YAP1 phosphorylation, in other words, activation of the Hippo pathway may be a direct or indirect response to the increase in FSH. In assessing CTGF mRNA levels in granulosa cells from rats, Harlow and colleagues also observed inhibition of the CTGF by FSH, the authors suggest that FSH, by increasing cAMP levels, inhibits CTGF expression, acting as an antagonist to stimulators of this factor, as is the case with members of the TGFβ Family [26].

A recent study in bovine granulosa cells evaluated the expression of both Hippo effectors (YAP1 and TAZ) in granulosa cells of different follicle size (2–5, 5–10, >10 mm), in this study, YAP1 protein levels were expressed in granulosa cells of follicles of all stages of development whereas TAZ protein expression decreased in granulosa cells with increasing follicle size [12]. Based on this, we decided to investigate only the effector most expressed in this cell type.

Considering that CTFG is a downstream target for YAP1, we decided to analyze two other target genes and observed similar patterns in the abundance of mRNA, which reinforced our conclusion. The ANKRD1 belongs to the family of preserved muscle ankyrin repeat proteins (MARP) and among its functions it is known that it can act as a positive transcriptional co-activator of p53 [27]. In mice, ANKRD1 participate in the apoptosis process, inducing Bax expression via p53 [28]. In the ovarian context, ANKRD1 is expressed by granulosa cells from follicles containing a less competent oocyte, leading to apoptosis and, eventually, atresia [29]. This association justifies the fact that we found high levels in the subordinate (atresic) follicle, but not in the dominant follicle (fig 5).

CYR61 has functions in cell migration, proliferation, and adhesion, interfering with biological activities that vary according to the cellular context [30]. The balance between proliferation and apoptosis is regulated at different tissue levels, as well as in angiogenesis, a process of blood vessel formation, necessary for nutrition and oxygenation of all tissues in the body, with which CYR61 is closely related [10]. Also, CYR61 is involved in the processes of angiogenesis of the corpus luteum. In cattle, it was demonstrated, using an in vivo approach, that CYR61 is expressed in the developing corpus luteum, stimulated by PGF2α to promote the survival of endothelial cells, which maintain the blood vessels during the onset of luteolysis [31]. In the present study, the mRNA for this target of Hippo pathway was found at lower levels in the dominant when compared to the subordinate follicle after follicular divergence in vivo.

Considering that CTGF, CYR61, and ANKRD1 are classic targets of the interaction of YAP1 with transcription factors of the TEAD family [11], our results clearly indicate an activation of the Hippo pathway in the dominant follicle. There are likely other regulatory pathways for the genes studied here; however, our hypothesis is reinforced when we analyze the levels of phosphorylated YAP1 under FSH stimulation in vitro (Fig 3).

It is worth mentioning that during the wave of follicular growth, estradiol levels vary [32], first due to the serum levels of FSH and later by the greater availability of free IGF-1, both induce increased expression of CYP19A1 [33]. Our in vitro experiment showed estradiol levels directly proportional to FSH treatments. In addition, the inactivation of YAP1 by the use of pharmacological blockers (VP and P17) also induced greater expression of CYP19A1 (fig 6), together these findings demonstrate the need for YAP1 to be phosphorylated for the synthesis of estradiol.

Curiously in Sertoli cells, the Hippo pathways components are differentially expressed in neonates and puberty mice (with cells functionally mature), and FSH appears to regulate YAP1 expression and phosphorylation [34]. In these cells, YAP1 regulates the cAMP-PKA pathway as well as the expression of physiologically important genes for spermatogenesis [35]

As expected, our in vivo experiment showed higher levels of estradiol [20] and less CTGF mRNA in dominant than in subordinate follicles. In bovine granulosa cells cultured in vitro, overexpression of CCN2 (CTGF) decreased estradiol secretion [36] indicating feedback between these substances. Interesting assumption if we take into account the inversely proportional relationship between CTGF and estradiol in the subordinate follicles of our in vivo experiment.

In one a previous study, the expression of Serine/threonine protein kinase 3/4 (STK3 / 4) varies in the murine endometrium during the estrous cycle, being regulated by estrogen, because the pretreatment with estrogen receptor antagonist reduced the expression and phosphorylation of STK 3/4, in the cascade of Hippo signaling STK 3/4 is responsible for phosphorylating large tumor suppressor kinases 1/2 (LATS 1/2) [37]. LATS 1/2 activated phosphorylates directly YAP1 and TAZ keeping them in the cell cytoplasm [38]. This reinforces the role of the Hippo pathway also in uterine dynamics and increases the complexity of the possible interactions of the upstream components to YAP1.

In summary, the data presented here, using bovine granulosa cells, indicate the participation of the Hippo signaling pathway in the establishment of follicular dominance. So far, we can conclude that the activation of the Hippo pathway, by increasing phosphorylated YAP1, is necessary to momentarily for cell differentiation to occur. It is tempting to state that the activation of the hippo pathway in these cells can also be mediated by estradiol; however, new experiments are being developed to confirm this.

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FIGURE LEGENDS

Fig 1. Effect of FSH on CYP19A1 mRNA abundance and estradiol secretion in vitro. Granulosa cells were cultured for 6 days under non-luteinizing conditions and treated with graded doses of FSH. A) Abundance of CYP19 mRNA was measured by real-time PCR and B) estradiol secretion in culture medium by chemiluminescence kit. Data are means ± SEM of three independent cultures. Bars with different letters are significantly different ($P < 0.05$).

Fig 2. FSH regulation of mRNA levels for CTGF and other classic YAP1-TEAD transcriptional target genes in vitro. Granulosa cells were cultured for 6 days under non-luteinizing conditions and treated with graded doses of FSH. Abundance of CTGF, ANKRD1 and CYR61 mRNA levels was measured by real-time PCR. Data are means ± SEM of three independent cultures. Bars with different letters are significantly different ($P < 0.05$).

Fig 3. YAP1 phosphorylation is regulated by FSH in vitro. Granulosa cells were cultured for 6 days under non-luteinizing conditions and treated with two distinct doses of FSH. Total and phospho-YAP1 (Ser127) protein levels were measured by Western blot. A) Representative blots (n=2 replicates). B) Data are means ± SEM of four independent cultures. Bars with different letters are significantly different ($P < 0.05$).

Fig 4. Localization and expression pattern of total YAP1 and phospho-YAP1(Ser127) in follicles of increasing size. Representative immunohistochemistry (IHC) micrographs showing expression of total and phospho-YAP1(Ser127) in granulosa cells (GC) and theca cells (TC) from follicles of small (<5 mm) and medium (5–10 mm) sizes. Negative controls consisted of slides for which the primary antibody was omitted.

Fig 5. Classic YAP1-TEAD transcriptional target genes are regulated during establishment of the dominant follicle in vivo. Granulosa cells were recovered from the largest (F1: black bar) and the second largest (F2: white bar) follicle (mean ± SEM) collected at days 2 (n = 4), 3 (n = 4), and 4 (n = 4) of the first follicular wave of a cycle estrous. D2 corresponds to the day before the divergence; D3 corresponds to the day of follicular divergence; and D4 corresponds to the first day after divergence. Asterisk indicates statistical difference between the largest and the second largest follicle.

Fig 6. Effects of pharmacological inhibition of YAP1-TEADs interaction *in vitro* on mRNA encoding CYP19A1. Granulosa cells were cultured without or in the presence of different concentrations of **A)** Verteporfin (VP) or **B)** Peptide 17 (P17), both known as molecules that interfere with YAP1 binding to TEAD family transcription factors. FSH treatment (1ng/ml) was included as a positive control for CYP19A1 stimulation. Abundance of CYP19 mRNA was measured by real-time PCR. Data are means ± SEM of three independent cultures. Bars with different letters are significantly different ($P < 0.05$).

Table 1. Primers designed for quantitative real-time PCR analysis.

Target	Accession number	Primer sequence
GAPDH	NM_001034034.2	F: TGACCCCTTCATTGACCTTC
		R: CGTTCTCTGCCTTGACTGTG
CTGF	NM_174030.2	F: AGCTGAGCGAGTTGTGTACC
		R: TCCGAAAATGTAGGGGGCAC
ANKRD1	NM_001034378.2	F: ATCAGTGCGCGGGATAAGTT
		R: GGGAGTATCTCCTTCCCGGT
CYR61	NM_001034340.2	F: GGCTCCCCGTTTTGGAATG
		R: TCATTGGTAACGCGTGTGGA
H2AZ	[39]	F: GAGGAGCTGAACAAGCTGTTG
		R: TTGTGGTGGCTCTCAGTCTTC
CYP19A1	NM_174305.1	F: GTGTCCGAAGTTGTGCCTATT
		R: GGAACCTGCAGTGGGAAATGA

Abbreviations: F: Forward; R: Reverse.

FIGURES

Figure 1.

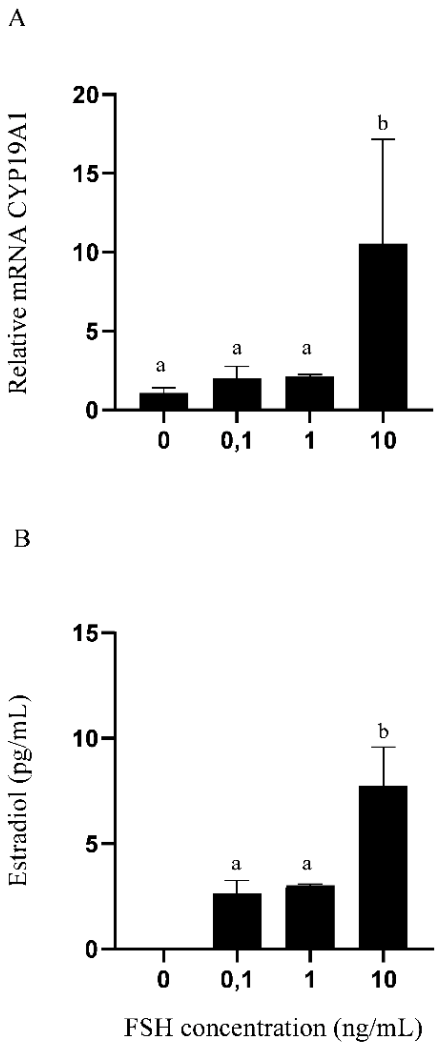


Figure 2.

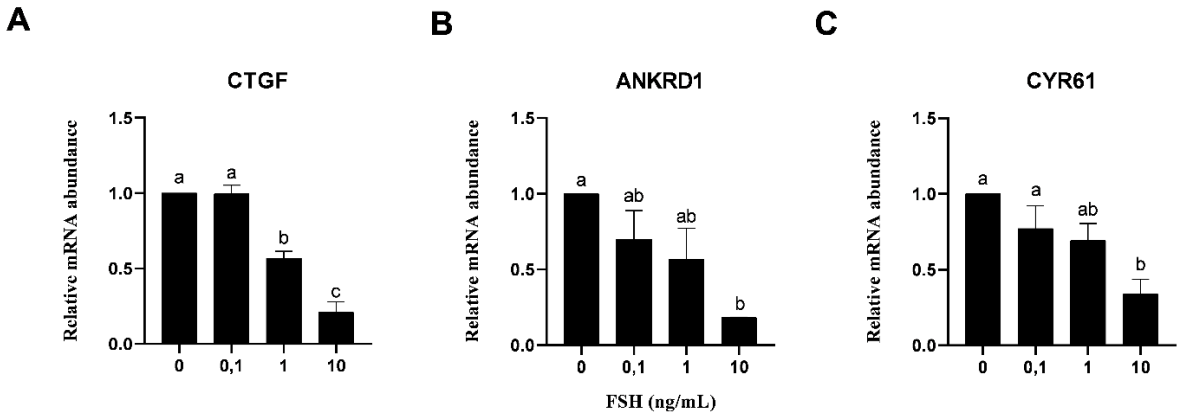

A
CTGF
Relative mRNA abundance
1.5
1.0
0.5
0.0
a
a
b
c
0
0,1
1
10
B
ANKRD1
Relative mRNA abundance
a
ab
ab
b
FSH (ng/mL)
C
CYR61
Relative mRNA abundance
a
a
ab
b

Figure 3.

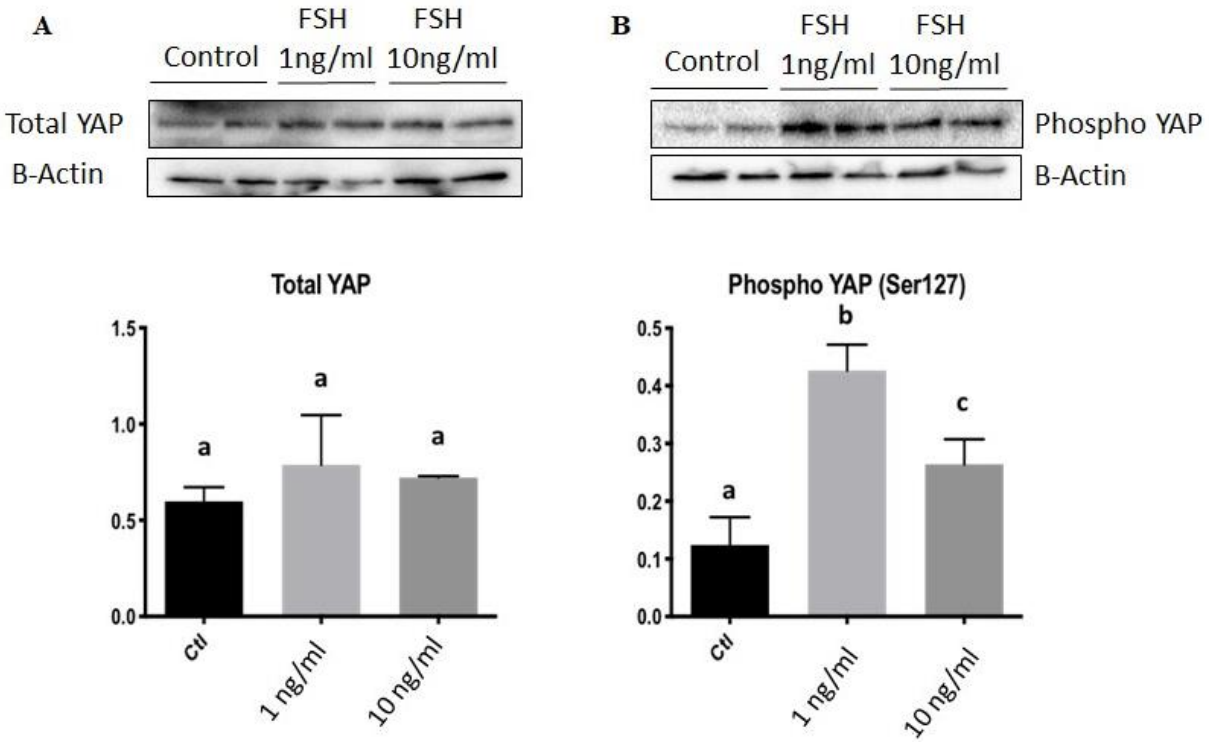

A
Control
FSH 1ng/ml
FSH 10ng/ml
Total YAP
B-Actin
B
Control
FSH 1ng/ml
FSH 10ng/ml
Phospho YAP
B-Actin
Total YAP
1.5
1.0
0.5
0.0
a
a
a
ctl
1 ng/ml
10 ng/ml
Phospho YAP (Ser127)
0.5
0.4
0.3
0.2
0.1
0.0
a
b
c
ctl
1 ng/ml
10 ng/ml

Figure 4.

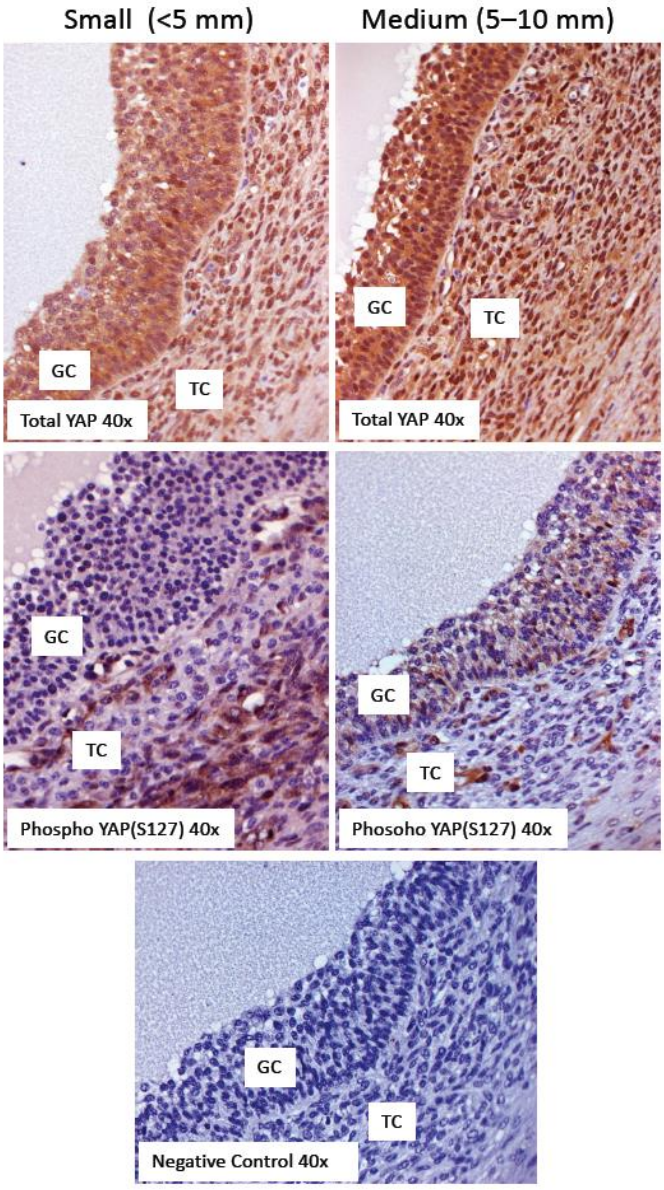

Small (<5 mm)
Medium (5–10 mm)
GC
TC
Total YAP 40x
GC
TC
Total YAP 40x
GC
TC
Phospho YAP(S127) 40x
GC
TC
Phosoho YAP(S127) 40x
GC
TC
Negative Control 40x

Figure 5.

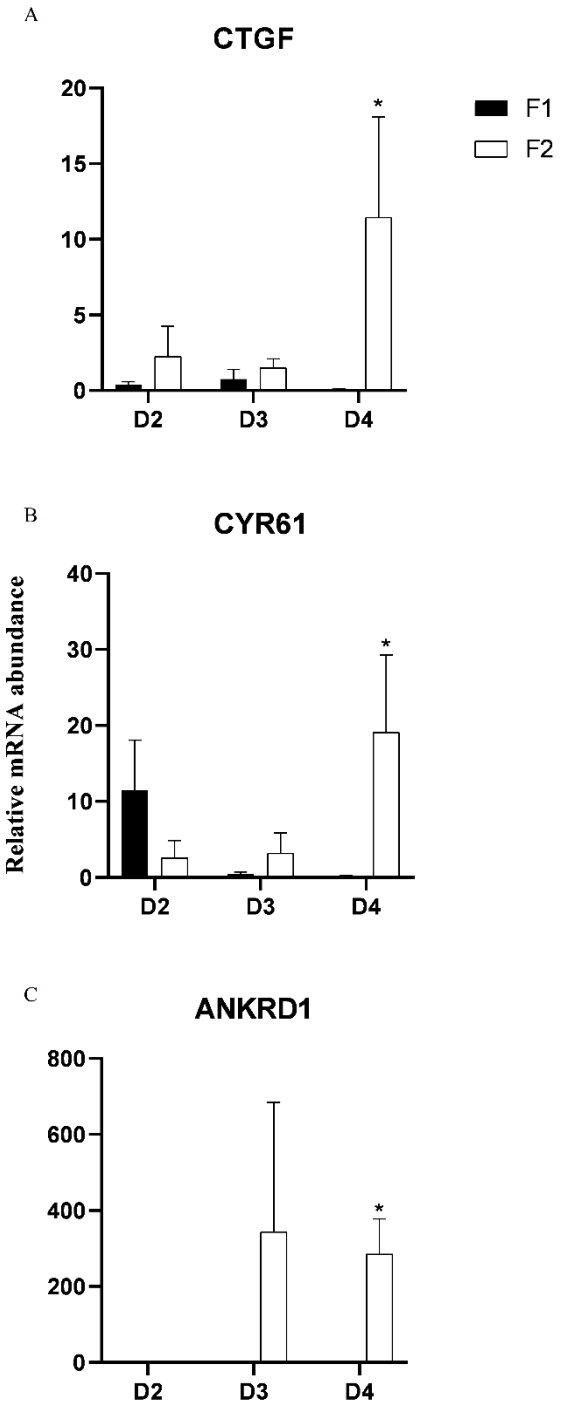
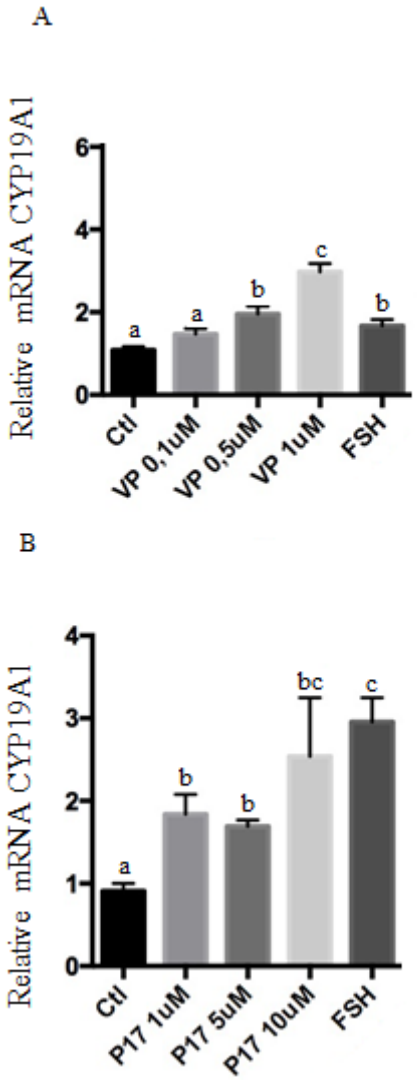

A
CTGF
F1
F2
20
15
10
5
0
*
D2
D3
D4
B
CYR61
Relative mRNA abundance
40
30
20
10
0
*
D2
D3
D4
C
ANKRD1
800
600
400
200
0
*
D2
D3
D4

Figure 6.


A
Relative mRNA CYP19A1
6
4
2
0
a
a
b
c
b
Ctl
VP 0,1uM
VP 0,5uM
VP 1uM
FSH
B
Relative mRNA CYP19A1
4
3
2
1
0
a
b
b
bc
c
Ctl
P17 1uM
P17 5uM
P17 10uM
FSH

4 CONCLUSÃO

Os resultados apresentados aqui, utilizando células da granulosa bovina, indicam a participação da via de sinalização Hippo no estabelecimento da dominância folicular, através da fosforilação de YAP1 por estímulo do FSH. Através dos experimentos in vitro e in vivo, sugerimos que a ativação da via do Hippo é necessária momentaneamente para que ocorra a diferenciação celular no folículo dominante.

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