Zygote

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FGF18 modulates *CTGF* mRNA expression in cumulus–oocyte complexes and early bovine embryos: preliminary data

Elisabeth Schmidt da Silva[1], Carolina Amaral[1], Marcos Barreta[2], Alfredo Antoniazzi[1], Leonardo Guedes de Andrade[4], Rogério Ferreira[3], Fernando Mesquita[4], Valério Marques Portela[1] and Paulo Bayard Gonçalves[1,2,3,4]

[1]Biotechnology and Animal Reproduction Laboratory, Universidade Federal de Santa Maria, Santa Maria, RS, 97105-900, Brazil; [2]Laboratory of Animal Reproduction Physiology, Universidade Federal de Santa Catarina, SC, 89520-000, Brazil; [3]Department of Animal Science, Universidade do Estado de Santa Catarina, SC, 88035-901, Brazil and [4]Molecular and Integrative Physiology of Reproduction Laboratory, Universidade Federal do Pampa, Uruguaiana, RS, 97501-970, Brazil

Research Article

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Author for correspondence:
Paulo Bayard Gonçalves. Biotechnology and Animal Reproduction Laboratory, Universidade Federal de Santa Maria, Santa Maria, RS, 97105–900, Brazil.
E-mail: pbayardg@gmail.com

Summary

The Hippo pathway is involved in the proliferation of intrafollicular cells and in early embryonic development, mainly because effectors of this pathway are key transcription regulators of genes such as *CTGF* and *CYR61*, which are involved in cell proliferation. Recent studies by our group found that fibroblast growth factor 18 (FGF18) is present in the fallopian tube during early embryonic development, leading to the hypothesis that FGF18 may have a role during embryonic development. Therefore, the aim of the following study was to determine whether FGF18 modulates the expression of Hippo pathway target genes, *CTGF* and *CYR61*, during oocyte maturation and early embryonic development. Three experiments were carried out, with *in vitro* maturation (IVM) of cumulus–oocyte complexes (COCs) and embryo culture. In experiment one, FGF18 (100 ng/ml) induced an increase ($P < 0.05$) in *CTGF* gene expression at 12 h post-exposure. In experiment two, FGF18 (100 ng/ml) induced a reduction ($P < 0.05$) in *CTGF* expression at 3 h post-exposure. In the third experiment, day 7 embryos exposed to FGF18 during oocyte IVM expressed greater *CTGF* mRNA abundance, whereas FGF18 exposure during embryo *in vitro* embryo culture did not alter *CTGF* expression in comparison with untreated controls. The preliminary data presented here show that FGF18 modulates *CTGF* expression in critical periods of oocyte nuclear maturation, cumulus expansion and early embryonic development in cattle.

Introduction

The fibroblast growth factor (FGF) family is composed of 22 proteins, which are proteins that span the plasma membrane of a cell and contain an extracellular domain that binds to its ligands, and an intracellular domain important for signalling relay. FGFs have been associated with several biological activities including angiogenesis, embryonic development, endocrine signalling pathway, cell proliferation and differentiation (Böttcher and Niehrs, 2005; Ocón-Grove *et al.*, 2008).

As a member of the FGF family, FGF18 has been detected in theca and granulosa cells (Jiang *et al.*, 2013). FGF18 is secreted by theca cells and acts on granulosa cells during atresia. Intrafollicular injection experiments in cows revealed that exogenous FGF18 supplementation caused the interruption of follicular growth and steroidogenesis (Portela *et al.*, 2010; da Silva *et al.*, 2019).

The granulosa cells have a close connection with the oocyte and the cumulus cells. In this regard, whereas mouse oocytes express FGF18 (da Silva *et al.*, 2019), the growth factor has not been detected in bovine oocytes (Portela *et al.*, 2010). The oocyte interacts intimately with cumulus cells forming the cumulus–oocyte complex (COC), which is essential for the formation and development of the female gamete. Such an intimate interaction helps in the nuclear and cytoplasmic maturation of the oocyte, leading to oocyte competence in supporting early embryonic development (Sánchez and Smitz, 2012). Preliminary studies by this group showed that the oviduct secretes FGF18 during embryonic development, therefore indicating that this protein may have an important role in cell proliferation during the initial embryonic development in cattle.

The Hippo pathway has a crucial role in the regulation of embryonic development as, in its inactive state, it leads to delayed embryonic development or embryonic death (Lorthongpanich and Issaragrisil, 2015). When the Hippo pathway is inactive, transcription activators YAP and

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TAZ are maintained unphosphorylated, being allowed to translocate into the nucleus and interact mainly with transcription factor TEA domain (TEAD1/2/3/4) family members. In the nucleus, the complex YAP/TAZ–TEAD acts as transcription factors for target genes such as *CTGF* and *CYR61* (Serrano *et al.*, 2013), which are known regulators of cell proliferation and tissue growth.

Oocyte maturation can be understood as the set of biological modifications by which the oocyte acquires competence to support fertilization and the beginning of embryonic development. Several molecules and pathways that regulate cell proliferation are closely related to the regulation of oocyte maturation and early embryonic development. This paper aims to determine whether the set of target molecules regulated by FGF18 include the Hippo-related regulators of cell proliferation, CTGF and CYR61, during oocyte maturation and early embryonic development.

Material and methods

Cow ovaries were obtained from a local abattoir and transported to the laboratory in saline solution (0.9% NaCl; 30°C) containing 100 UI/ml of penicillin and 50 µg/ml of streptomycin sulfate. COCs from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 20 ml of water/min). COCs were recovered and selected under a stereomicroscope. Grades 1 and 2 COCs were randomly distributed into 500 µl of maturation medium in four-well plates (Nunc, Roskilde, Denmark) and cultured in incubators at 38.5°C (101.3°F) in a 5% CO_2 and 95% air saturation humidity atmosphere for 22–24 h. The maturation medium consisted of TCM199, containing Earle's salts and L-glutamine (Gibco Laboratories, Grand Island, NY, USA), supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/ml sodium bicarbonate, 5.0 µg/ml luteinizing hormone (LH) (Lutropin-V®), 0.5 µg/ml follicle-stimulating hormone (FSH) (Follitropin-V®), and 10% fetal bovine serum (FBS; Gibco Laboratories, Grand Island, NY, USA), 100 UI/ml penicillin and 50 µg/ml streptomycin sulfate.

In vitro *fertilization (IVF)*

After *in vitro* maturation (IVM), bovine oocytes were fertilized *in vitro* with tested semen after thawing and separation through a discontinuous Percoll (GE Healthcare, São Paulo, SP, Brazil) gradient. Sperm cells were diluted and added to the COC plate with the final concentration adjusted to 2×10^6 sperm/ml in Fert-TALP medium containing 10 µg/ml heparin, 30 µg/ml penicillamine, 15 mM hypotaurine and 1 mM epinephrine. Fertilization was carried out by co-culture of sperm and oocytes for 18–20 h in four-well plates under the same atmospheric conditions used for maturation. The day of IVF was considered as day 0 of embryo development.

In vitro *embryo culture (IVC)*

After IVF, presumptive bovine zygotes were denuded by vortexing, and then cultured in a culture chamber at 38.5°C and a 5% CO_2, 5% O_2 and 90% N_2 saturated humidity atmosphere in 500 µl of synthetic oviductal fluid (SOF) medium in four-well plates (Nunc, Roskilde, Denmark). Cleavage rates were evaluated 48 h after fertilization and blastocyst rates were assessed on day 7 of embryo development. Blastocysts assessed on day 7 were rinsed three times in phosphate-buffered saline (PBS) and collected in TRIzol® reagent at 80°C for subsequent RNA extraction.

Table 1. Primers used for expression analysis of candidate genes

Gene	Accession number	Sequence
RPS18	NM_001033614.2	F: CTTCCGCGAGGATCCATTG R: GCTCCCAAGATCCAACTAC
GAPDH	NM_001034034.2	F: GATTGTCAGCAATGCCTCCT R: GTCATAAGTCCCTCCACGA
CTGF	AF000137.1	F: AGCTGGAGCGACTTGTGT ACC R: TCCGAAAATGTAGGGGGGCAC
CYR61	NM_001034340.2	F: GGCTCCCCGTTTGGAATG R: TCATTGGTAACGCGTGTGGA

RNA extraction, reverse transcription and real time PCR

Total RNA was extracted from 25 COCs and five day-7 blastocysts in accordance with TRIzol instructions. Briefly, the extraction used 1000 µl TRIzol reagent (Thermo Fischer, Waltham, MA, USA) and 200 µl chloroform, followed by purification of the aqueous phase with GlycoBlue (Thermo Fisher, Walthan, MA, USA) and 700 µl isopropyl alcohol. Quantification and assessment of RNA purity was performed using a NanoDrop spectrophotometer (Thermo Fisher, Walthan, MA, USA; 260/280 nm absorbance ratio). Complementary DNA was synthesized from 200 ng of RNA, which was treated with 0.1 U DNase Amplification Grade (Thermo Fisher, Walthan, MA, USA) for 15 min at 27°C to neutralize any DNA molecules. DNase was inactivated with 1 µl ethylenediaminetetraacetic acid (EDTA) for 10 min at 65°C. Reverse transcription was performed adding 1 U iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) for 5 min at 25°C followed by 30 min at 42°C and 5 min at 85°C. Quantitative polymerase chain reaction (qPCR) was conducted in a thermocycler (Bio-Rad, Hercules, CA, USA) using 2.5 ng of cDNA in 2 µl and 8 µl of Mix containing forward and reverse bovine specific primers (Table 1), nuclease-free water and GoTaq® Master Mix (Promega Corporation, Madison, USA). Amplification was performed with initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. To optimize the qPCR assays, serial dilutions of cDNA templates were used to generate a relative standard curve. Samples were run in duplicate and the results of all genes were analyzed as relative fold difference, using RPS18 and GAPDH as reference genes, according to Pfaffl (2001).

Experimental design

The study assessing the effect of FGF18 exposure during IVM on COC expression of Hippo-related molecules, CTGF and CYR61, used an FSH-induced cumulus expansion model, and it was divided into two experiments. Experiment 1 assessed the effects of different concentrations of recombinant human FGF18 (0, 10 and 100 ng/ml) in FSH-induced (100 ng/ml) cumulus expansion, at different time points, while each sampling time had three treatment groups. The COCs were distributed randomly in 400 µl of maturation medium in a Nunc four-well plate, where they were matured and were collected at 6, 12 and 24 h of *in vitro* maturation (IVM). After sampling, the COCs were stored in TRIzol at −80°C for later RNA extraction. The experiment was replicated five times and, in total, 25 COCs per replicate were used for each treatment condition. This experiment was conducted especially to assess the effect of different FGF18 concentrations on Hippo pathway-related target molecules (i.e. CTGF and CYR61) during IVM.

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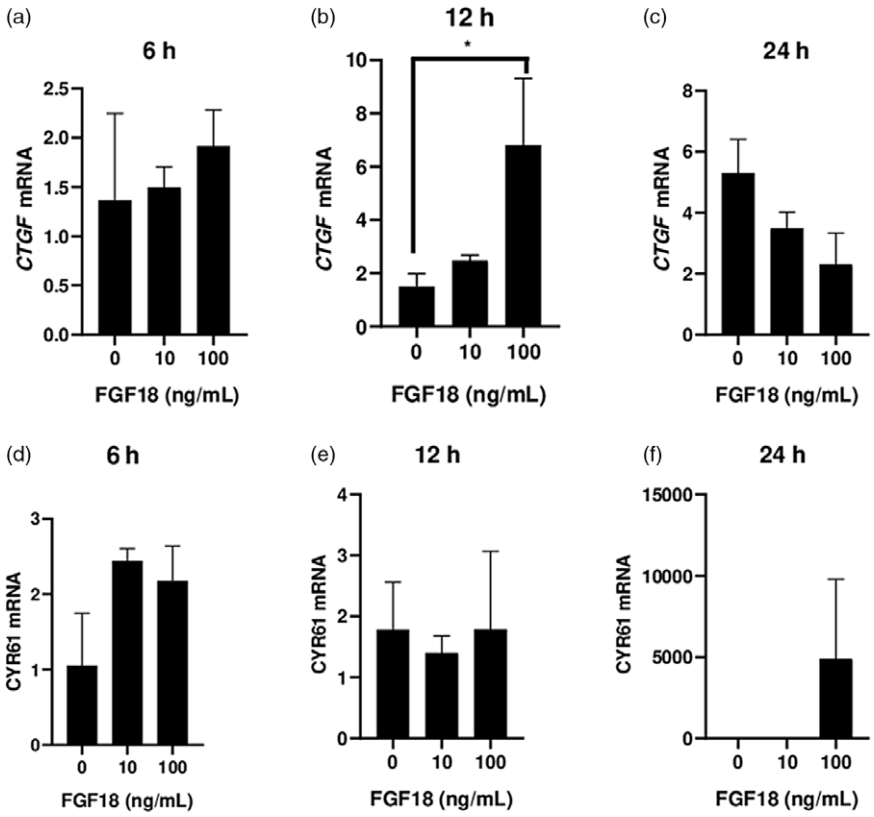

Figure 1. Relative abundance of target gene mRNA of the Hippo pathway (*CTGF* and *CYR61*), during IVM of bovine COCs produced *in vitro*. COCs were matured in the presence of different concentrations of recombinant human FGF18 (0, 10 or 100 ng/ml) in FSH-induced (100 ng/ml) cumulus expansion for 6 h (a, d), 12 h (b, e) or 24 h (c, f). Cultures were replicated five times with, in total, 25 COCs. *Indicates statistical difference ($P < 0.05$).

In the second experiment, the IVM treatment groups were 0 and 100 ng/ml of FGF18 in FSH-induced (100 ng/ml) cumulus expansion. In this experiment, the COCs were also randomly distributed into 400 µl of maturation medium and then collected after 0, 3, 6 or 9 h of IVM.

In the third experiment, early embryonic development was evaluated. In this experiment, three groups were placed: control group (untreated), IVM group (treated with 100 ng/ml of FSH and FGF18 only during IVM), and IVC group (100 ng/ml of FSH and FGF18 added to the presumptive embryo only during the embryo culture period). As there was no difference between the concentrations tested in experiment 1, FGF18 concentration was 100 ng/ml. All groups were collected at embryonic day 7 and stored in TRIzol until RNA extraction. The experiment was replicated five times; 150 COCs were used for each treatment group.

Statistical analysis

The data were tested for normal distribution using the Shapiro–Wilk test and normalized when necessary. All data were analyzed through analysis of variance with the treatment as main effect and replicates as a random variable. Differences between the means were tested using the Tukey multiple comparison test and JMP software [Tukey–Kramer honest significant difference (HSD) test] using the JMP software (SAS Institute Inc., Cary, NC, USA). The results are presented as mean ± standard error of the mean. A P-value < 0.05 was considered significant.

Results

Concentration–effect relationship of FGF18 and mRNA expression for *CTGF* and *CYR61* in COCs during 6-h intervals of oocyte maturation (Experiment 1)

The effect of different concentrations of FGF18 (0, 10 and 100 ng/ml) on Hippo-dependent gene expression, in FSH-induced cumulus expansion, was assessed at 6, 12 and 24 h of IVM. *CTGF* mRNA abundance revealed that FGF18 affected the Hippo pathway during bovine oocyte maturation. There was increased *CTGF* mRNA expression in COCs induced by 100 ng/ml of FGF18 at 12 h of *in vitro* maturation ($P < 0.05$). However, there was no effect of FGF treatment on *CYR61* mRNA levels at all the examined time intervals and on *CTGF* at 6 and 24 h of IVM (Fig. 1).

Effect of FGF18 on the expression of Hippo pathway-related genes (*CTGF* and *CYR61*) in bovine COCs during 3-h intervals up to 9 h of oocyte maturation (Experiment 2)

In this experiment, COCs were cultured in the presence of 100 ng/ml of FGF18 and the abundance of *CTGF* and *CYR61* mRNA was assessed at 0, 3, 6 and 9 h of oocyte maturation. *CTGF* mRNA abundance was reduced ($P < 0.05$) at 3 h compared with the untreated control group. Hippo pathway target gene expression did not change at 0, 6 and 9 h of IVM. The mRNA level for CYR61 was not affected by FGF18 treatment in any of the evaluated times (Fig. 2).

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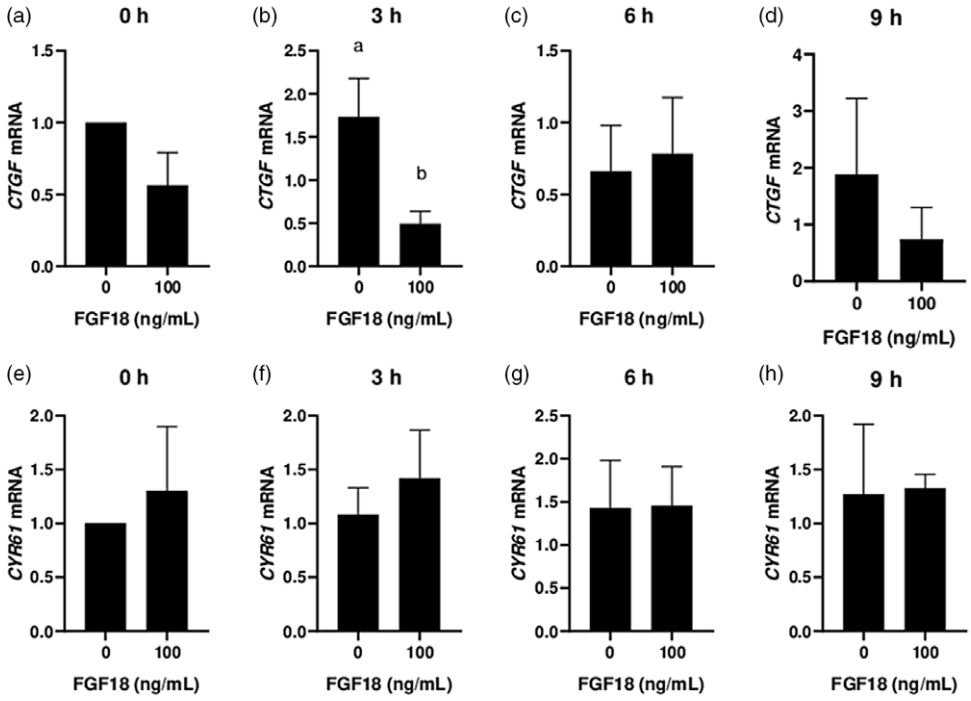

Figure 2. Relative abundance of target gene mRNAs of the Hippo pathway (*CTGF* and *CYR61*), during IVM of bovine COCs produced *in vitro*. COCs were matured in the presence of recombinant human FGF18 (0 or 100 ng/ml) in FSH-induced (100 ng/ml) cumulus expansion for 0 h (Control; a, e), 3 h (b, f), 6 h (c, g) or 9 h (d, h). Cultures were replicated five times with, in total, 25 COCs. [a,b]Indicates statistical difference ($P < 0.05$).

Regulation of Hippo pathway-related gene expression (*CTGF* and *CYR61*) at day 7 embryo after FGF18 exposure during IVM or IVC of bovine embryos (Experiment 3)

The presence of 100 ng/ml FGF18 during embryo development impaired blastocyst rate (*c.* 20%) at day 7 post-insemination (unpublished data). *CTGF* and *CYR61* mRNA abundance was assessed in a study of FGF18 exposure, either during oocyte maturation or during embryo culture, to analyze whether FGF18 would affect the Hippo pathway in different moments of *in vitro* embryo production. Treatment with FGF18, during oocyte maturation or during embryonic culture, did not affect mRNA levels for *CYR61* in the blastocyst. However, embryos cultured in the presence of FGF18 had higher levels of CTGF mRNA ($P < 0.05$) compared with embryos cultured without FGF18, or those embryos derived from oocytes matured in the presence of FGF18 (Fig. 3).

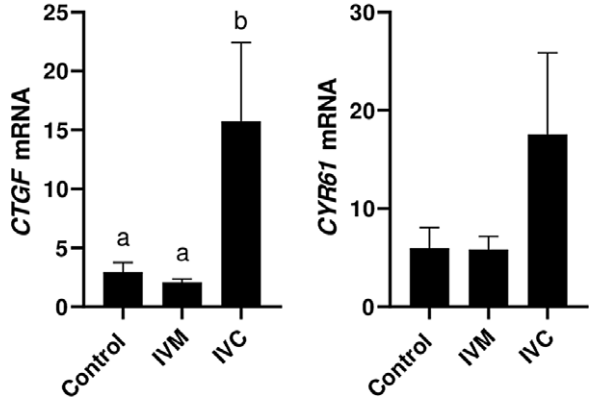

Figure 3. Relative abundance of mRNA of Hippo pathway genes (*CTGF* and *CYR61*) in day 7 blastocyst. COCs were matured in the presence of 100 ng/ml of FSH or without [Control and *in vitro* embryo culture (IVC)] or with FGF18 (100 ng/ml, IVM). In the IVC group, the presumptive embryos were cultured with FGF18 (100 ng/ml). The experiment was replicated five times and, in total, 150 COCs were used for each treatment group. Different letters indicate statistical significance ($P < 0.05$) among the groups.

Discussion

This exploratory study on FGF18 sheds light on its effect on Hippo pathway-regulated genes during early embryonic development (preimplantation). This study is the first to assess the effect of FGF18 on *CTGF* and *CYR61* mRNA expression during early embryonic development. It was possible to demonstrate that, whereas FGF18 alters *CTGF* mRNA expression, no effect on *CYR61* mRNA abundance was observed. These findings strongly suggested that FGF18 regulates YAP/TAZ. The increase in *CTGF* mRNA levels occurs mainly when YAP/TAZ were dephosphorylated and transported from the cytoplasm to the nucleus, binding to TEADs for the transcription of target genes (Boopathy and Hong, 2019).

In the first experiment, the FGF18 concentration was determined and, in the same experiment, its action at different critical moments (6, 12 and 24 h) of oocyte maturation was evaluated. It is important to note that differential abundance in mRNA must be caused by FGF18 stimulation in cumulus cells, because oocytes removed from the follicle resume meiosis and cease transcription (Pincus and Enzmann, 1935; Hyttel *et al.*, 2001). Supplementation of FGF18 to culture medium in maturation did not change mRNA levels for *CYR61* in any experiment. However, the abundance of *CTGF* mRNA increased at 12 h when COCs were treated with FGF18. *CTGF* acts on the activation of MAP kinase (MAPK) and Smad-dependent pathways, which are important for oocyte maturation (Ohashi *et al.*, 2003; Jiang *et al.*, 2013; Higaki *et al.*, 2017). These findings are important because, in cattle, MAPK is

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activated during GVBD, reaches the maximum activity in MI, and remains elevated until the formation of pronuclei, not decreasing in MII (Tian *et al.*, 2002). Therefore, there is evidence that the Hippo pathway, through MAPK, is important for maintaining the oocyte in MII and entering interphase. In this study, CTGF was regulated by FGF18, which may activate MAPK, however we did not investigate those pathways.

After determining the concentration at 6-h intervals, the effect of FGF18 was evaluated from the beginning of oocyte maturation until the end of the GVBD at 3-h intervals (0, 3, 6 and 9 h). Interestingly, FGF18 caused downregulation of CTGF in COCs at 3 h of maturation. At these time points, bovine oocytes undergo GVBD and cumulus granulosa cells go through expansion and mucification. These results agreed with previous studies by Liu and colleagues that observed an increase in CTGF expression at the end of cumulus expansion (Liu *et al.*, 2018).

The level of *CTGF* mRNA was increased when zygotes were cultured *in vitro* up to the blastocyst stage in the presence of FGF18. This finding is interesting because CTGF plays an important role in embryonic development (Krupska *et al.*, 2015). During embryonic development, the abundance of *CTGF* (but not *CYR61*) mRNA was higher in day 7 blastocysts compared with the control when the embryos were treated with FGF18. *CTGF* and *CYR61* genes seem to be expressed by the trophectoderm in the blastocyst (Sanz-Ezquerro *et al.*, 2017). The Hippo pathway may participate in the regulation of cell proliferation and differentiation during embryo development, as observed in other cell types (Yu and Guan, 2013). Although we have not directly investigated this mechanism, there is evidence that FGF18 regulates, through the Hippo pathway, cell proliferation and, probably, differentiation processes during embryonic development.

The preliminary data presented here show that FGF18 modulates *CTGF* mRNA expression at critical periods of oocyte nuclear maturation, cumulus expansion and early embryonic development in cattle.

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Ethical approval. Not applicable

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

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