



## Regulation of MMP2 and MMP9 metalloproteinases by FSH and growth factors in bovine granulosa cells

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### Abstract

Matrix metalloproteinases (MMP) are key enzymes involved in tissue remodeling. Within the ovary, they are believed to play a major role in ovulation, and have been linked to follicle atresia. To gain insight into the regulation of MMPs, we measured the effect of hormones and growth factors on *MMP2* and *MMP9* mRNA levels in non-luteinizing granulosa cells in serum-free culture. FSH and IGF1 both stimulated estradiol secretion and inhibited *MMP2* and *MMP9* mRNA abundance. In contrast, EGF and FGF2 both inhibited estradiol secretion but had no effect on *MMP* expression. At physiological doses, none of these hormones altered the proportion of dead cells. Although we cannot link MMP expression with apoptosis, the specific down regulation by the gonadotropic hormones FSH and IGF1 *in vitro* suggests that excess *MMP2* and *MMP9* expression is neither required nor desired for follicle development.

*Key words:* metalloproteinase, gonadotropin, growth factor, granulosa.

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### Introduction

Bovine antral follicles are composed of several cell types including granulosa and theca cells, fibroblasts and endothelial cells. Extracellular matrix (ECM) influences basic cellular processes such as proliferation, differentiation, migration and adhesion, and is therefore fundamental to normal development (Hulboy *et al.*, 1997). Although MMPs can be activated by vasoactive peptides in some tissues (Tsuruda *et al.*, 2004), most MMP activation occurs through the action of plasmin (Rundhaug, 2005). Plasmin is synthesized from plasminogen through plasminogen activators, and is an active protease that promotes degradation of components of the ECM in normal and malignant tissue as well as activation of matrix metalloproteinases (MMP) (Castellino and Ploplis, 2005; Rundhaug, 2005). Significant tissue remodeling occurs in the ovary because of constant follicular recruitment and atresia, as well as ovulation (Curry and Osteen, 2001; Hagglund *et al.*, 2001; Irving-Rodgers *et al.*, 2006). Changes associated with atresia appear initially in the granulosa cell (GC) layer. The death of GCs leads to a virtually complete destruction of the GC layer lining the inner follicular wall (Carson *et al.*, 1981). The enzymes that degrade the protein material present in the ECM during its remodeling include the MMP and tissue

plasminogen activator families. MMPs are a family of zinc-dependent endopeptidases possessing proteolytic activities against several components of the ECM (Erickson, 1986; Richards *et al.*, 1987; Hulboy *et al.*, 1997). In spite of current knowledge of the factors involved in follicular growth, little is known regarding the mechanisms by which those factors act on ECM remodeling in the bovine ovary. MMP gelatinase activity has been detected in cultured bovine theca and granulosa cells (Smith *et al.*, 2005), although the factors that control the expression of these proteins are unknown. Thus, the objective of the present study was to determine the effects of FSH and growth factors on granulosa cell MMP expression in bovine granulosa cells, and to determine if changes in ECM remodeling activity are associated with cell survival.

### Materials and Methods

#### Cell culture

Granulosa cell culture was based on the culture system described elsewhere (Gutiérrez *et al.*, 1997) with slight modifications. This is a serum-free culture that maintains an estrogenic phenotype with a minimum of luteinization (Sahmi *et al.*, 2004). The reagents were obtained from Invitrogen except where otherwise stated. Briefly, bovine ovaries were collected from adult cows at an abattoir, and were transported to the laboratory in PBS at 35 °C, containing penicillin (100 IU/mL) and streptomycin (100 µg/mL).

Follicles between 2 and 5 mm diameter were bisected and cells were collected by rinsing the follicle walls with DMEM/F12 (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12). A viable cell count was performed in the presence of 0.4% Trypan Blue, and  $110^6$  viable cells/mL were cultured in 24-well plates in DMEM/F12 with added sodium bicarbonate (10 mM), sodium selenite (4 ng/mL), BSA (0.1%; Sigma-Aldrich), penicillin (100 U/mL), streptomycin (100 µg/mL), transferrin (2.5 µg/mL), non-essential amino acid mix (1.1 mM), androstenedione ( $10^{-7}$  M at start of culture, and  $10^{-6}$  M at each medium change) and bovine insulin (10 ng/mL). Cultures were maintained at 37 °C in 5% CO<sub>2</sub> in air for 6 days, with 700 µL medium being replaced every two days.

Granulosa cells were cultured with graded doses of FSH (AFP-5332B; NIDDK) (0, 0.1, 0.5, 1 ng/mL), IGF1 (Sigma, CA) (0, 5, 10, 100 ng/mL) or with epidermal growth factor EGF (10 ng/mL; R&D Systems, Minneapolis, MN) and FGF2 (10 ng/mL; PeproTech, Rocky Hill NJ) starting on day two of culture.

To measure steroid secretion, the medium was removed for steroid assay on day six and stored at -20 °C. The cells were lysed with 200 µL of 1 N NaOH for 2 h followed by neutralization with 200 µL of 1 N HCl for total cell protein measurement with Bradford protein assay (Bio-Rad, Mississauga, Ontario, Canada). To measure mRNA abundance, cells were collected into Trizol and stored at -80 °C until RNA extraction. All series of cultures were performed on at least three different pools of cells collected on different occasions.

### Real-time RT-PCR

Gene expression was assessed by relative real-time RT-PCR. Total RNA (1 µg) was first treated with 1 U DNase (Invitrogen) to digest any contaminating DNA. The RNA was reverse transcribed in the presence of 1 mM oligo (dT) primer and 4 U Omniscript RTase (Omniscript RT Kit; Qiagen, Mississauga, Ontario Canada), according to the manufacturer's instructions.

Real-time PCR was conducted in an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) and bovine-specific primers for amplifying histone H2AFZ (sense: 5'-GAGGAGCTGAACAAGCTGTTG-3', anti-sense: 5'-TTGTGGTGGCTCTCAGTCTTC-3'), MMP2 (sense: 5'-ACGAAGACCCACAGGAGGAG-3', antisense: 5'-TAGCCAGTCGATTTGATGC-3'), MMP9 (sense: 5'-TCCAGGAGAACCACGAACCAA-3', antisense: 5'-GCGGCAGGTCTCCGAGTAA-3'). Common thermal cycling parameters (3 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C) were used to amplify each transcript. Melting curve analyses were performed to verify product identity. Samples were run in du-

plicate, and were expressed relative to histone H2AFZ as housekeeping gene. Data were normalized to a calibrator sample using the  $\Delta\Delta C_t$  method with correction for amplification efficiency (Pfaffl, 2001).

### Steroid assay

Estradiol was measured in follicle fluid and in conditioned medium in duplicate as described (Bélanger *et al.*, 1990), without solvent extraction. Intra- and inter-assay coefficients of variation were 6% and 9%, respectively. The sensitivity of estradiol assay was 10 pg per tube, equivalent to 0.3 ng/µg protein, respectively. Estradiol concentrations in culture medium were corrected for cell number by expressing per unit mass of total cell protein.

### Flow cytometry

The effect of FSH, IGF1, EGF and FGF2 on cell survival was measured by flow cytometry essentially as described previously (Blondin *et al.*, 1996). Granulosa cells from 2-5 mm follicles were cultured in serum-free medium for 6 days and treated with insulin (10 ng/mL), FSH (1 ng/mL), IGF1 (10 ng/mL), EGF (10 ng/mL) and FGF2 (10 ng/mL) for the last 4 days of culture. Cells were recovered on day 6 of culture by scraping the plate with a rubber spatula. The cells were washed three times in ice-cold PBS then fixed overnight in 70% ethanol before staining with propidium iodide (50 µg/mL in PBS with 0.1% Triton X and 20 mg/mL RNase A). A minimum of 25,000 cells/sample were sorted on a FACSVantage SE instrument (BD Biosciences, Oakville, ON, Canada) and analyzed with Cell Quest Pro software (BD Biosciences). The number of cells in the "sub-G1" peak was quantified and represented the number of apoptotic (dead) cells (Krysko *et al.*, 2008).

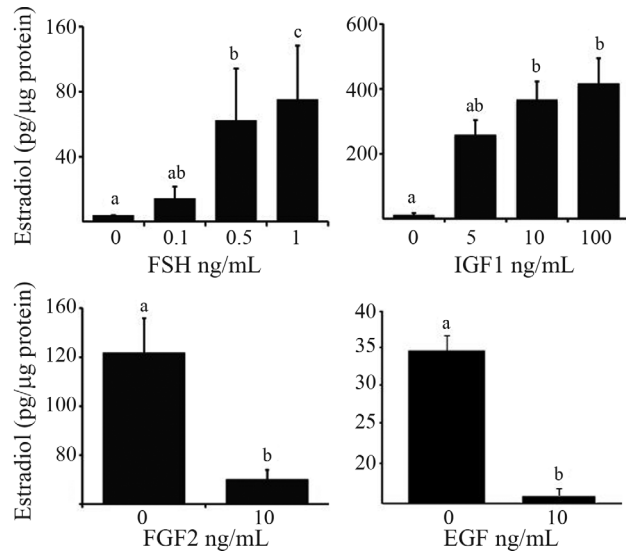
### Statistics

The data that did not follow a normal distribution (Shapiro-Wilk test) were transformed to logarithms. Homogeneity of variance was tested with O'Brien and Brown-Forsythe tests. Analysis was performed with JMP software (SAS Institute) with treatment as main effect and culture replicate as a random variable in the F-test. Differences between means were tested with the Tukey-Kramer HSD test. Data are presented as means  $\pm$  SEM.

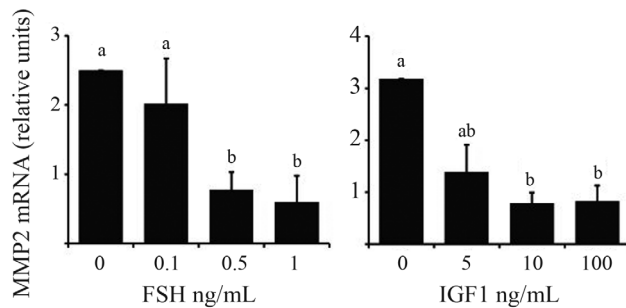
### Results

Estradiol secretion was significantly up-regulated by FSH and IGF1 in dose dependent manners, and inhibited by EGF and FGF2 treatment ( $p < 0.05$ ; Figure 1).

Real-time PCR revealed the presence of *MMP2* and *MMP9* mRNA in granulosa cells. In granulosa cell culture, FSH and IGF1 decreased *MMP2* ( $p < 0.05$ ; Figure 2) and *MMP9* ( $p < 0.05$ ; Figure 3) mRNA abundance in a dose de-



**Figure 1** - Effect of FSH, IGF1, EGF and FGF2 on secretion of estradiol and progesterone from bovine granulosa. Granulosa cells from 2-5 mm follicles were cultured in serum-free medium for 6 days and treated with the stated doses of FSH (0, 0.1, 0.5, 1 ng/mL), IGF1 (0, 5, 10, 100 ng/mL), EGF (0, 10 ng/mL), FGF2 (0, 10 ng/mL) for the last 4 days of culture. Data are means  $\pm$  SEM of three independent cultures and the differences between bars indicated by different letters are significant ( $p < 0.05$ ).



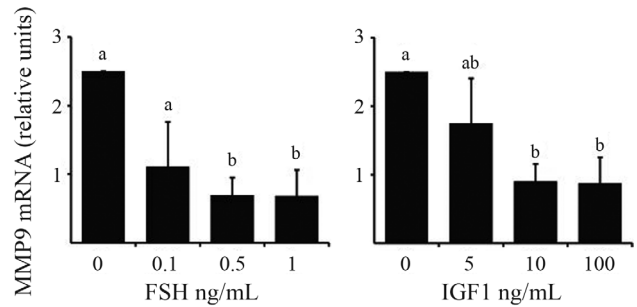
**Figure 2** - Regulation of *MMP2* mRNA abundance in granulosa cells. Granulosa cells from 2-5 mm follicles were cultured in serum-free medium for six days and treated with FSH (0, 0.1, 0.5, and 1 ng/mL) and IGF1 (0, 5, 10, and 100 ng/mL). Steady-state mRNA levels were measured by real-time PCR and expressed relative to a calibrator sample with the  $\Delta\Delta Ct$  method. Data are means  $\pm$  SEM of three independent cultures and the differences between bars indicated by different letters are significant ( $p < 0.05$ ).

pendent manner. However EGF and FGF2 had no effect on *MMP2* and *MMP9* mRNA abundance.

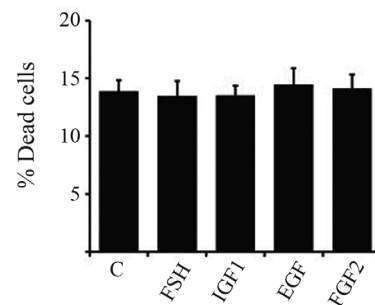
Flow cytometry revealed that these treatments did not alter the proportion of dead cells (Figure 4;  $p > 0.05$ ).

## Discussion

Granulosa cells cultured under serum-free non-luteinizing conditions present the best model available to study granulosa cell differentiation. As estrogenic granulosa cells secrete *MMP2* and *MMP9* (Smith *et al.*, 2005) and other ECM remodeling proteins (Cao *et al.*, 2004), we used this



**Figure 3** - Regulation of *MMP9* mRNA abundance in granulosa cells. Granulosa cells from 2-5 mm follicles were cultured in serum-free medium for 6 days and treated with FSH (0, 0.1, 0.5, and 1 ng/mL) and IGF1 (0, 5, 10, and 100 ng/mL). Steady-state mRNA levels were measured by real-time PCR and expressed relative to a calibrator sample with the  $\Delta\Delta Ct$  method. Data are means  $\pm$  SEM of three independent cultures, and the differences between bars indicated by different letters are significant ( $p < 0.05$ ).



**Figure 4** - Effect of control (insulin), FSH, IGF1, EGF and FGF2 on cell survival. Granulosa cells from 2-5 mm follicles were cultured in serum-free medium for six days and treated with insulin (10 ng/mL), FSH (1 ng/mL), IGF1 (10 ng/mL), EGF (10 ng/mL) and FGF2 (10 ng/mL) for the last 4 days of culture. Data generated by flow cytometry are shown as means  $\pm$  SEM of three replicate cultures. No significant difference between groups was detected ( $p > 0.05$ ).

system to determine the factors that control *MMP2* and *MMP9* expression. The present study demonstrates that the gonadotrophic hormones FSH and IGF1 decrease *MMP2* and *MMP9* expression, whereas growth factors known to inhibit granulosa cell differentiation, EGF and FGF2, had no effect. These data point to specific regulation of MMPs in differentiating granulosa cells. Therefore the relationship between hormone action, cell death and MMP expression is complex and requires further study.

In this culture system, FSH and IGF1 stimulate estradiol secretion (Cao *et al.*, 2006; Portela *et al.*, 2008) and the expression of genes encoding steroidogenic enzymes (Sahmi *et al.*, 2004). In human granulosa cells, FSH also stimulated the expression of genes involved in signal transduction (Sasson *et al.*, 2003). Both FSH and IGF1 inhibited *MMP* expression, suggesting that *MMP* activity is not required or desired for follicle development. This is in apparent contrast to a study with granulosa cells from eCG-treated immature rats, in which *MMP9* activity was stimu-

lated by FSH (Ke *et al.*, 2004). In non-luteinizing bovine granulosa cells, LH was reported to have no effect on MMP2 activity and inconsistent effects on MMP9 activity (Smith *et al.*, 2005).

FSH and IGF1 stimulate follicle development and are anti-apoptotic factors, therefore it is reasonable to assume that they downregulate genes involved in apoptosis. This is supported from microarray studies in ovarian surface epithelium (Ji *et al.*, 2004). A study on bovine follicles demonstrated that MMP2 and MMP9 enzyme activities and mRNA levels were significantly higher in atretic compared to healthy follicles (Yahia Khandoker *et al.*, 2001). Our data are therefore consistent with lower MMP expression in healthy granulosa cells under the influence of FSH or IGF1. In the present study, we did not see an effect of FSH on cell viability, but this may be an artifact of the cell culture system. Medium was changed during the treatment and dead cells may have been aspirated at this time, leading to an underestimation of the proportion of dead cells.

Several growth factors inhibit estradiol secretion from granulosa cells, and this is taken as an inhibition of differentiation. This includes EGF and FGF2 (Cao *et al.*, 2006; present study). However, despite the decrease in estradiol secretion induced by these factors, they did not alter MMP expression, in agreement with a study on luteinized human granulosa cells (Ben-Shlomo *et al.*, 2003). It has been reported that FGF2 inhibits estradiol without inducing apoptosis (Tilly *et al.*, 1992), therefore the lack of an effect of FGF2 on MMPs in the present study may be because apoptosis was not induced.

In conclusion, *MMP2* and *MMP9* mRNA are regulated by FSH and IGF1 but not by EGF and FGF2 in bovine granulosa cells *in vitro*. Although no apparent relationship was found between MMP expression and apoptosis in granulosa cells, anti-apoptotic factors (FSH, IGF1) may act to inhibit excess MMP synthesis during follicle development.

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