RESEARCH

Discovery of a possible role of asprosin in ovarian follicular function

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Abstract

Asprosin is a novel fasting-induced protein encoded by fibrillin-1 (*FBN1*) gene, produced when *FBN1* is cleaved by the enzyme furin, and is associated with insulin resistance and polycystic ovarian syndrome in humans. To characterize mRNA abundance of *FBN1*, *FURIN*, and the presumed asprosin receptor, olfactory receptor family 4 subfamily M member 1 (*OR4M1*) in granulosa (GC) and theca cells (TC), and identify hormones regulating *FBN1* mRNA expression, GC and TC from small (1–5 mm; SM) and large (>8 mm; LG) follicles were collected from ovaries of heifers obtained at an abattoir and used for real-time PCR gene expression analysis or *in vitro* evaluation of hormone regulation and asprosin effects. SMTC had 151-fold greater ($P < 0.05$) *FBN1* mRNA abundance than SMGC, and LGTC had 50-fold greater *FBN1* mRNA than LGGC. In contrast, *OR4M1* mRNA was 81-fold greater in SMGC than LGGC and did not differ from SMTC, but LGTC had 9-fold greater *OR4M1* mRNA than LGGC. *FURIN* mRNA was 2.6-fold greater in SMTC than SMGC, but did not differ among follicular sizes. In cultured TC, leptin, insulin, LH, IGF1 and steroids did not affect *FBN1* mRNA, but TGFB1 increased ($P < 0.05$) *FBN1* mRNA by 2.2-fold; EGF and FGFs increased *FBN1* mRNA by 1.3- to 1.5-fold. Asprosin enhanced LH-induced TC androstenedione production, reduced IGF1-induced TC proliferation, and had no effect on progesterone production. Developmental regulation of *FBN1*, *FURIN* and *OR4M1* along with direct effects of asprosin on TC suggests that asprosin may be a novel regulator of ovarian follicular function.

Key Words

- ▶ asprosin
- ▶ fibrillin-1
- ▶ theca cells

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Introduction

Asprosin is a recently discovered protein, released by white adipose tissue that targets the liver activating the G-protein-cAMP-PKA pathway to release glucose in the blood stream (Duerschmid *et al.* 2017). Both asprosin and fibrillin are encoded by *FBN1* gene and synthesized together as a pro-protein, and subsequently separated by the enzyme furin at the C-terminal end of *FBN1* (Romere *et al.* 2016). Asprosin increases in the blood with fasting and decreases after feeding linking asprosin to glucose

metabolism, obesity, insulin resistance and metabolic disorders like type II diabetes (Romere *et al.* 2016, Duerschmid *et al.* 2017, Li *et al.* 2019, Wang *et al.* 2019) and polycystic ovarian syndrome (PCOS) (Li *et al.* 2018, Alan *et al.* 2019). Interestingly, oocyte-specific deletion of furin leads to female infertility by causing early secondary follicle arrest in mice (Meng *et al.* 2017) but whether asprosin has direct effects on ovarian function is unknown.

The family of fibrillin proteins include the TGF β binding proteins as part of the extracellular matrix that are localized and expressed in different parts of the ovary and follicle (Rodgers *et al.* 2003, Prodoehl *et al.* 2009). Specifically, Prodoehl *et al.* (2009) found that FBN1 protein was localized in the outer regions of tunica albuginea and in the stroma, appearing as fibrillar structures which forms microfibrils in connective tissues for structure and mechanical stability. In larger antral follicles, FBN1 protein is localized closest to the epithelial granulosa layers and highly expressed in most of the theca interna layer (Prodoehl *et al.* 2009). The theca interna plays an essential role in forming the structure of an ovarian follicle, delivering nutrients to the granulosa cell (GC) layer, cumulus cells and oocyte and by releasing endocrine and paracrine factors (Tajima *et al.* 2007, Young & McNeilly 2010, Richards *et al.* 2018). In addition to FBN1, the theca layer of growing follicles is composed of extracellular matrix factors including collagen, laminin, fibronectin, and proteoglycans (Rodgers *et al.* 2003, Richards *et al.* 2018). GC and theca cell (TC) communication is important in regulating cell proliferation and differentiation, steroidogenesis and overall ovarian function (Yada *et al.* 1999, Tajima *et al.* 2007, Hsueh *et al.* 2015). Theca factors modulate the hormone producing capacity of GC biphasically during follicular maturation, while GC factors promote both differentiation and growth of TC during follicular development (Yada *et al.* 1999, Liu *et al.* 2019). Although, both cell types are working together, TC play a more crucial role in diseases like PCOS due to excessive production of androgens (Rosenfield & Ehrmann 2016). Recently, it was reported that elevated plasma asprosin levels in women are associated with increased risk for developing PCOS and correlated positively with free androgen index (Alan *et al.* 2019) and total testosterone in obese PCOS women (Li *et al.* 2018).

In mice, the asprosin receptor, olfactory receptor (*Olfr734*) has been identified recently in different tissues including liver, kidney and testis, but has not been studied in ovaries. In a knockout mouse model, it was observed that hepatic *Olfr734* protein binds to asprosin to promote hepatic gluconeogenesis (Li *et al.* 2019). Moreover, mice that are *Olfr734*-deficient were observed to have attenuated cAMP levels and hepatic glucose production and increased insulin sensitivity, and when fasted and given a high-fat diet, mice with knockout *Olfr734* produced less glucose (Li *et al.* 2019). *OR4M1*, a homolog of *Olfr734*, was also highly expressed in the testis and olfactory bulb but moderately expressed in liver tissue, both of these asprosin receptors were recently identified to promote

hepatic gluconeogenesis and negatively regulate insulin sensitivity (Li *et al.* 2019, Hoffmann *et al.* 2020). Whether *OR4M1* mRNA is expressed in ovarian cells such as GC or TC is unknown.

With asprosin playing a significant role in insulin resistance and perhaps PCOS, and that *FBN1* is highly expressed in TC, there is a possibility for a gene-protein interaction in ovarian cells. Thus, it was decided to study *FBN1* and asprosin relationship in ovarian function. Specifically, the present study aimed to characterize *FBN1*, *FURIN* and *OR4M1* mRNA abundance in TC and GC in small and large follicles, and evaluate the possible hormone regulators for expression of *FBN1* mRNA in TC. The effects of asprosin on steroid production and proliferation of TC were also evaluated.

Materials and methods

Reagents and hormones

Reagents used for cell culture were: Dulbecco's modified Eagle medium (DMEM), Ham's (F12), insulin, enzymes, antibiotics, TRI reagent, estradiol (E2), dihydrotestosterone (DHT), progesterone (P4), leptin, and androstenedione (A4) were obtained from Sigma-Aldrich Co.; purified ovine FSH (FSH activity, 15 $\times$ NIH-FSH-S1 U/mg) and ovine LH (LH activity, 2.3 $\times$ NIH-LH-S1 U/mg) from the National Hormone and Peptide Program (Torrance, CA); carrier-free recombinant human IGF1, fibroblast growth factor (FGF2 and FGF9), EGF, transforming growth factor-beta 1 (TGF β 1), and wntless-type MMTV integration site family member 3A (WNT3A) from R&D Systems (Minneapolis, MN); testosterone from Steraloids (Wilton, NH); recombinant human asprosin from Biologics Inc. (Cary, NC); and fetal calf serum (FCS) from Atlanta Biologicals (Flowery Branch, GA).

The reagents used for RIA were [125 I] iodo-P4 (MP Biomedicals, Irvine, CA) and anti-P4 rabbit antiserum (X-16) were provided by Dr P. Natashima Rao (Southwestern Foundation for Research Education, San Antonio, TX), normal rabbit serum (Equitech, Kerville, TX) and Androstenedione Double Antibody – 125 I RIA Kit (MP Biomedicals).

Granulosa and theca cell collection

The first experiment was designed to determine if the abundance of *FBN1*, *FURIN*, and *OR4M1* mRNA in GC and TC changes during follicle growth. Ovaries from non-pregnant beef heifers were collected at an abattoir where

humane slaughter practices were followed, according to USDA guidelines and transported to the lab on ice in antibiotic saline (0.9% saline solution with 1% penicillin–streptomycin) as previously described (Langhout *et al.* 1991, Schreiber *et al.* 2012). For large-follicle (LG; 8–20 mm) GC and TC, each of 6 or 7 samples was collected from one LG healthy follicle from one ovary as previously described (Zhang *et al.* 2017). For small follicle (SM; 1–5 mm) GC and TC, each sample had pooled cells from 3 to 5 small follicles from one ovary. Each cell type had 6 or 7 samples collected from at least three animals. These fresh cells were lysed in TRI reagent and RNA was extracted as described subsequently.

Ovaries from beef heifers were collected at an abattoir as described earlier, and follicular fluid was aspirated from SM follicles (1–5 mm) and LG follicles (8–20 mm) to collect the GC. Then follicles were dissected to isolate TC via enzymatic digestion (i.e. collagenase, DNase, protease, and hyaluronidase) and filtration as previously described (Stewart *et al.* 1995, Schreiber *et al.* 2012, Schütz *et al.* 2016). GC and TCs were suspended in a serum-free medium (1:1 DMEM/F12 supplemented with 2.0 mM glutamine, 0.12 mM gentamycin, 38.5 mM sodium bicarbonate, 1.25 mg/mL collagenase and 0.5 mg/mL deoxyribonuclease). Prior to cell culture, viability of cells was measured by trypan blue exclusion method, and approximately 2.0×10^5 viable cells were plated on 24-well culture plates with each well containing 1 mL of culture medium with 10% of FCS. This culture system has been shown to support the growth of TC that are responsive to LH and IGF1 (Stewart *et al.* 1995, Schreiber & Spicer 2012). Culture plates were incubated in a humidified incubator with 5% CO₂ and 95% air at 38.5°C for 48 h with a medium change every 24 h. Cells were then treated for another 24 h or 48 h, with medium change every 24 h using serum-free medium. Treatments were applied for 24 h or 48 h depending on experimental design, and cells were harvested for either RNA isolation or cell counting. Medium was collected for steroid RIA.

RNA extraction and quantitative PCR

Cell culture medium was aspirated, replaced with 250 µL of TRI reagent, and wells were scraped to collect the cells. Samples were stored at –80°C until RNA was extracted as described previously (Spicer *et al.* 2006, Zhang *et al.* 2017). Briefly, RNA from cell lysates were isolated using TRI reagent protocol and Phase Lock Gel Heavy tubes (5 Prime Inc., Germantown, MD). Each RNA sample was solubilized in DEPC water and measured quantitatively

using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE) at 260 nm and stored at –80°C for future use.

The primers and probes for *FBN1*, asprosin receptor (*OR4M1/Olfr734*) and *FURIN* were designed using IDT technologies (Coralville, IA). The *FBN1* forward primer: 5'-GTACGAACACAGTCAGCAGTTA-3'; reverse primer: 5'-TATCCTGGGCGGACATCTAT-3'; probe: 5'-/56-FAM/ACACCTCTC/ZEN/CAGACGGTACCAGAT/3IAbkFQ/-3'. While, for the *OR4M1* primers and probe, a multiple sequence alignment was first done with the *Olfr734* sequence as identified in mice and the *OR4M1* as the homolog for the bovine species. After getting the sequences aligned, primers were designed and the forward primer: 5'-TGATGGCCTTTGACCGATATG-3'; reverse primer: 5'-CAGGAGAAAGCCACCAGAATAC-3'; probe: 5'-/56-FAM/ACCATCATG/ZEN/AATCGACGTCTC TGCT/3IAbkFQ-3'. The forward primer for *FURIN* was 5'-CGTGGCATTGTGGTCTCTATC-3'; reverse primer: 5'-CAGGGTCCTGATCATTGACATC-3' and probe: 5'-/56-FAM/AGAAGAACC/ZEN/ACCCAGACTTGGCAG/3IAbkFQ/-3'. Primer sequences were submitted to BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to check for highly similar sequences, to ensure primer and probe specificity, and to determine if there were non-homologous regions coding for other genes. The target gene expression was normalized using 18S rRNA (Taqman rRNA Control Reagent, Applied Biosystems Inc.). The housekeeping gene, 18S rRNA was selected because previous studies reported it to be a stable gene over a variety of treatments (Schmittgen & Zakrajsek 2000, Sekar *et al.* 2000, Tsuji *et al.* 2002, Voge *et al.* 2004, Zhang *et al.* 2017). The quantitative PCR used a one-step method and a CFX96 Touch Real-Time PCR Detection System (BioRad) as follows: 10 min at 50°C for RT, 5 min at 95°C for denaturation, 50 cycles of 10 s at 95°C for annealing and 30 s at 60.4°C for extension. Relative quantities of the genes were expressed as $2^{-\Delta\Delta Ct}$ based on the relative comparative threshold values (Ct) as previously described (Dentis *et al.* 2017, Morrell *et al.* 2020). Briefly, the ΔCt was calculated by subtracting the Ct of the 18S rRNA to the Ct of the target gene, followed by subtracting the highest ΔCt from all other ΔCt values to obtain the $\Delta\Delta Ct$. The fold change of mRNA abundance for all the target genes were calculated and expressed as fold of control values.

Radioimmunoassay (RIA)

Medium samples from the culture plates were collected and stored at –20°C for future RIA. For P4, a double

antibody RIA was conducted as previously described (Langhout *et al.* 1991, Spicer *et al.* 2006). For the A4 RIA, an Androstenedione Double Antibody – ¹²⁵I RIA Kit was used following the manufacturer's protocol and as previously described (Stewart *et al.* 1995, Pizzo *et al.* 2015). Intra- and inter-assay coefficients of variation were 7.7 and 6.6%, respectively, for the P4 RIA, and 9.4 and 10.2%, respectively, for the A4 RIA.

Cell counting

Numbers of TC were determined using a Coulter counter (Model Z2; Beckman Coulter Inc., Hialeah, FL) as previously described (Spicer *et al.* 2006, Pizzo *et al.* 2016). Initially, wells were exposed to 0.5 mL of trypsin (0.25% (w/v) in 0.5 M NaCl) for 20 min at 37°C, then cells were scraped from each well, and were disrupted via repetitive pipetting back and forth through a 500 µL pipette tip, and diluted in 1:10 at 0.5 M NaCl. Cell suspensions were then counted and values were expressed as cells per well. Intra-assay coefficient of variation averaged 8.4%.

Experimental design

Experiment 1 was an *in vivo* study conducted to determine if *FBN1*, *FURIN*, and *OR4M1* mRNA expression differs in GC and TC of small and large follicles. Cells were isolated from various sized follicles and RNA was extracted as described earlier.

Theca cells were selected for Experiments 2–5 because *FBN1* mRNA levels were found to be several fold greater in TC vs GC of Experiment 1. Experiment 2 was conducted to test the effect of steroid hormones on *FBN1* mRNA abundance in LG-follicle TC collected *in vitro*. Cells were prepared as described earlier and cultured for 48 h in 10% FCS supplemented medium, washed, and then treated for 24 h in serum-free medium containing P4 (300 ng/mL), E2 (300 ng/mL), A4 (300 ng/mL) and DHT (300 ng/mL). Doses of steroids were based on previous studies (Spicer 2005, Zhang *et al.* 2017).

Experiments 3–5 were conducted to evaluate the effect of various growth factors and metabolic hormones on *FBN1* mRNA abundance in LG-follicle TC *in vitro*. Cells were cultured as described in Experiment 2 for 48 h in 10% FCS, washed, and treated with 300 ng/mL of P4, E2, A4 and DHT for 24 h in a serum-free medium. For Experiment 3, treatments used were leptin (Lep; 100 ng/mL), WNT3A (300 ng/mL), insulin (Ins; 100 ng/mL), TGFB1 (30 ng/mL) and leptin-insulin combination (Lep-Ins). For Experiment 4, cells were treated with 30 ng/mL of EGF, FGF2 and FGF9.

For Experiment 5, treatments were LH (30 ng/mL) and IGF1 (30 ng/mL). Doses of hormones were based on the results from previous studies (Spicer & Stewart 1996, Spicer 2005, Schreiber *et al.* 2012).

Experiment 6 was conducted to evaluate the effect of asprosin (200 ng/mL) treatment on cell proliferation and steroid production of TC treated with LH (30 ng/mL), IGF1 (30 ng/mL) and LH+ IGF1 *in vitro*. Cells were cultured for 48 h in 10% FCS, washed, and treatments applied for another 48 h in serum-free medium. Medium was changed every 24 h. Doses of LH and IGF1 were based on previous studies (Stewart *et al.* 1995, Spicer 2005). Dose of asprosin was selected to represent the higher end of concentrations reported in systemic blood of women (Romere *et al.* 2016, Wang *et al.* 2018) and mice (Romere *et al.* 2016).

Statistics

In Experiment 1, data for mRNA abundance of *FBN1*, *FURIN* and *OR4M1* gene in different cell types (GC and TC) from SM and LG follicles were analyzed with general linear models procedure of SAS using 2 × 2 factorial ANOVA (version 9.4, SAS Institute Inc., Cary, NC). In Experiments 2–5, *FBN1* mRNA abundance data were analyzed using one-way ANOVA. In Experiment 6, data for the effects of asprosin on cell proliferation and steroidogenesis were analyzed using 2 × 2 × 2 factorial ANOVA. If necessary, values were transformed to natural log (x+1) to ensure homogeneity of variance. Data were presented as either individual data points or means ± S.E.M. Mean differences were assessed using Fisher's protected least significant differences test, if significant treatment effects in ANOVA were detected. Differences among groups or treatments were considered significant at $P < 0.05$ and a trend at $0.05 < P < 0.10$. For the *in vitro* experiments (Experiments 2–6), three different pools were used as experimental (i.e. biological) replicates; replicate was included in the statistical model.

Results

Experiment 1: Determination of *FBN1*, *FURIN* and *OR4M1* mRNA abundance in GC and TC of small and large follicles

All data have been included in the analyses. A significant follicle size × cell type interaction influenced *FBN1* mRNA abundance such that in both SM and LG follicles, *FBN1* mRNA abundance was greater ($P < 0.05$) in TC than GC (Fig. 1A). SMTC had 152-fold greater ($P < 0.05$) abundance of *FBN1* mRNA than SMGC, while LGTC had 50-fold greater

Figure 1

Changes in *FBN1*, *OR4M1* and *FURIN* mRNA during follicular development (Experiment 1). Panel A: *FBN1* mRNA abundance in granulosa (GC) and theca (TC) cells from small (SM) and large (LG) follicles. Means $\pm$ S.E.M. without a common letter differ ($P < 0.05$); note log scale. Panel B: *OR4M1* mRNA abundance in GC and TC from SM and LG follicles. Means $\pm$ S.E.M. without a common letter differ ($P < 0.06$); note log scale. Panel C: *FURIN* mRNA abundance in GC and TC from SM and LG follicles. Means $\pm$ S.E.M. without a common letter differ ($P < 0.10$). For all panels, means represent $n = 6-7$. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0218>.

($P < 0.05$) *FBN1* mRNA than LGGC. SMTC had 6-fold greater ($P < 0.05$) abundance of *FBN1* mRNA than LGTC.

A significant follicle size $\times$ cell type interaction influenced *OR4M1* mRNA such that *OR4M1* mRNA abundance was 81-fold greater ($P < 0.05$) in SMGC than LGGC, and tended to be greater than in SMTC ($P < 0.06$; Fig. 1B). LGTC had 9-fold greater ($P < 0.05$) *OR4M1* mRNA abundance than LGGC.

Follicle size $\times$ cell type interaction tended to influence ($P < 0.10$) *FURIN* mRNA abundance whereas cell type ($P < 0.01$) but not size ($P > 0.10$) influenced *FURIN* mRNA abundance such that TC had 1.8-fold greater ($P < 0.05$) *FURIN* mRNA abundance than GC. Specifically, *FURIN* mRNA abundance was greatest in SM and LG follicle TC and least in SMGC ($P < 0.05$; Fig. 1C).

Experiments 2-5: effect of hormones on *FBN1* mRNA abundance in TC *in vitro*

In Experiment 2, treatment of E2, A4, DHT and P4 did not alter *FBN1* mRNA abundance in TC (Fig. 2; $P > 0.05$). In Experiment 3, WNT3A, insulin, leptin and leptin-insulin combination did not affect ($P > 0.05$) *FBN1* mRNA abundance in TC compared with controls (Fig. 3), whereas TGF β 1 stimulated ($P < 0.05$) *FBN1* mRNA abundance by 2.23-fold. In Experiment 4, FGF2, FGF9 and EGF caused 1.33- to 1.52-fold increases (Fig. 4; $P < 0.05$) in TC *FBN1* mRNA abundance. In Experiment 5, LH or IGF1 treatment

Figure 2

FBN1 mRNA abundance in steroid treated large theca cells (Experiment 2). E2, estradiol; A4, androstenedione; DHT, dihydrotestosterone; P4, progesterone. Means $\pm$ S.E.M. without a common letter differ ($P < 0.05$), and represent $n = 6$ for each mean; no significant treatment effect was observed. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0218>.

Figure 3

TGFβ1 induced *FBN1* mRNA abundance in theca cells (Experiment 3). TGFβ1, transforming growth factor beta 1; WNT3A, wingless type 3A; Lep + Ins, leptin + insulin. Means $\pm$ S.E.M. without a common letter differ ($P < 0.05$), and represent $n = 6$ for each mean. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0218>.

had no significant effect (controls = 1.0 ± 0.3 , LH = 1.5 ± 0.2 , IGF1 = 1.0 ± 0.3 , $n = 6$) on *FBN1* mRNA abundance.

Experiment 6: The role of asprosin in cell proliferation and steroidogenesis in TC

Main effects on cell numbers included asprosin ($P < 0.05$), LH $\times$ IGF1 interaction ($P < 0.001$) and asprosin $\times$ IGF1

Figure 4

Stimulatory effects of EGF, FGF2 and FGF9 on *FBN1* mRNA abundance in theca cells (Experiment 4). EGF, epidermal growth factor; FGF, fibroblast growth factor. Means $\pm$ S.E.M. without a common letter differ ($P < 0.05$), and represent $n = 6$ for each mean. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0218>.

interaction ($P < 0.11$). Alone, IGF1 ($P < 0.05$) increased TC numbers by 30%, whereas LH alone tended ($P < 0.08$) to increase TC numbers (Fig. 5). Asprosin treatment alone had no effect on TC numbers but inhibited ($P < 0.05$) the IGF1-induced TC numbers by 11% in the absence of LH, and tended ($P < 0.07$) to decrease TC numbers in the presence of IGF1 plus LH (Fig. 5).

Main effects on P4 production included LH, IGF1 and LH $\times$ IGF1 interaction ($P < 0.01$). Asprosin or its interaction with LH or IGF1 had no significant effect on P4 production. Treatments of either IGF1 or LH alone increased P4 production by 2- to 4-fold above controls, whereas combined treatment of LH and IGF1 further increased ($P < 0.05$) P4 production by 3- to 5-fold above either treatment alone (Fig. 6A). However, asprosin had no effect on these responses ($P > 0.10$).

Main effects on A4 production included LH ($P < 0.01$), IGF1 ($P < 0.01$), LH $\times$ IGF1 interaction ($P < 0.01$), and asprosin $\times$ LH interaction ($P < 0.08$). Alone, LH and IGF1 increased A4 production by 5.1- and 1.54-fold, respectively ($P < 0.05$); combined, LH and IGF1 increased A4 production by 5.5- to 18-fold above either treatment alone (Fig. 6B). Asprosin increased ($P < 0.05$) A4 production by 26% in LH treated cells (Fig. 6B), but asprosin had no effect ($P > 0.10$) on IGF1 and LH+IGF1 induced A4 production.

Discussion

This research is one of the first to elucidate the regulation of *FBN1* gene expression and the possible role of its protein

Figure 5

Effect of asprosin (200 ng/mL) on theca cell (TC) proliferation in the presence of LH, IGF1 or a combined treatment of LH and IGF1 (Experiment 6). Treatments were applied for 48 h. Means $\pm$ S.E.M. without a common letter differ ($P < 0.05$), and represent $n = 9$ for each mean.

Figure 6

Effect of asprosin on LH- and IGF1-induced steroidogenesis in theca cells (TC) (Experiment 6). Panel A: Progesterone production in asprosin-treated, large-follicle TC concomitantly treated with LH, IGF1 or both. Means $\pm$ s.e.m. without a common letter differ ($P < 0.05$). Panel B: Androstenedione production in asprosin-treated, large-follicle TC concomitantly treated with LH, IGF1 or both. Means $\pm$ s.e.m. without a common letter differ ($P < 0.05$), and represent $n = 9$ for each mean.

counterpart, asprosin in ovarian follicular function in mammals. The present studies revealed that (1) *FBN1* mRNA abundance was greater in TC than GC, whereas, *OR4M1* mRNA was greatest in SM-follicle GC; (2) *FURIN* mRNA abundance was greater in TC than GC but did not differ among follicle sizes; (3) TGFB1, EGF, FGF2 and FGF9 stimulated *FBN1* mRNA abundance in TC whereas steroids (E2, A4, DHT and P4), leptin and insulin did not affect *FBN1* mRNA abundance; and (4) asprosin enhanced LH-induced A4 production and inhibited IGF1-induced cell proliferation but did not affect P4 production.

Most hormones that are involved in the development of follicles from pre-antral to ovulatory size act via endocrine signaling (Jones & Shikanov 2019), while

growth factors (i.e. keratinocyte growth factor, hepatocyte growth factor, wntless signaling type factor, etc.) stimulate DNA synthesis in one cell type and promote a response in another (Monget & Monniaux 1995, Robker & Richards 1998, Palma *et al.* 2012). Within the follicle, these subsequent responses could generally stimulate or suppress cell proliferation, hormone release and/or oocyte maturation. Intrafollicular growth factors can be produced in TC and affect GC function, and vice versa (Monget & Monniaux 1995, Nilsson *et al.* 2003). In the present study, abundance of *FBN1*, *FURIN* and *OR4M1* mRNA differed between large and small follicles with *FBN1* and *FURIN* mRNA being expressed mostly in TC, while *OR4M1* mRNA was expressed more abundantly in SM-follicle GC. This may suggest a paracrine-autocrine signaling role for asprosin in ovarian follicles. It would be expected that asprosin would have greater effects in GC where *OR4M1* mRNA is more abundantly expressed than in TC, but further studies will be required to verify this suggestion.

Previous research has shown that *FBN1* is a structural protein that is present in the tunica albuginea, ovarian stroma and theca layers with major roles in the structural formation of microfibrils and as an environmental niche for growth factor signaling (Prodoehl *et al.* 2009, Sengle & Sakai 2015). Fibrillin-1 protein has been localized in fetal and adult bovine ovaries, specifically an intense stain in the tunica albuginea and theca interna but lesser in the stroma (Rodgers *et al.* 2003, Hatzirodos *et al.* 2011). Similarly, *FBN1* mRNA abundance was greatest in TC of the present study. The GC and TC communication-interaction are important for the maintenance of follicular structure and function, like growth and differentiation of these cells (Skinner & Coffey 1988, Nilsson *et al.* 2003, Tajima *et al.* 2007). Interestingly, *FBN1* mRNA levels in TC were increased the greatest by TGFB1, and increased to a lesser extent by EGF and FGFs in the present study, whereas steroids, leptin, insulin and IGF1 had no effect on *FBN1* expression, and IGF1 had little effect on asprosin action. Similarly, Bastian *et al.* (2016) reported that TGFB1 increased *FBN1* mRNA in bovine fetal fibroblasts, whereas steroids and IGF1 had no effect, supporting findings of the present study. Also, in a tight-skin mouse model, TGFB1 stimulated *FBN1* assembly (Lemaire *et al.* 2010), and in human dermal fibroblasts, TGFB1 induced *FBN1* protein incorporation (Kissin *et al.* 2002). As a signaling protein, *FBN1* regulates growth factor/cytokine activity by binding to latent TGFB1 binding proteins (LTBP) 1 to 4 and controls TGFB1 availability and function (Zilberberg *et al.*

2012), is greater in stroma of adult than fetal bovine ovaries (Hatzirodos *et al.* 2011), and increases in fetal bovine ovarian stroma during gestation (Hatzirodos *et al.* 2019). Thus, ovarian *FBN1* gene expression may function as a matrix protein as well as to provide an intraovarian source of asprosin. As mentioned earlier, elevated plasma asprosin levels in women are associated with increased risk for developing PCOS and correlated positively with free androgen index (Alan *et al.* 2019) and total testosterone in obese PCOS women (Li *et al.* 2018), the latter of which is consistent with results of the present study where asprosin increased LH-induced A4 production by TC. However, *FBN1* mRNA levels in ovarian stroma did not differ between PCOS and non-PCOS women (Prodoehl *et al.* 2009), suggesting further research is needed to clarify the relative contribution of systemic vs locally produced asprosin as a source of intraovarian asprosin. It is important to emphasize that attempts to use Western blotting to detect asprosin protein in GC and TC lysates in the present study were unsuccessful, most likely because of the small quantities of protein produced. Thus, additional developmental work will be required to ascertain if changes in cellular or secreted asprosin protein levels parallel *FBN1* mRNA levels during follicular growth and atresia.

In the present study, asprosin increased LH-induced A4 production by TC providing the first evidence that asprosin has direct effects on ovarian follicular function. Interestingly, IGF1-induced TC proliferation was weakly inhibited by asprosin in the present study. Moreover, asprosin did not affect P4 production stimulated by IGF1 and LH treatments. In support of the present study, Li *et al.* (2018) found that plasma asprosin levels correlated positively with total testosterone levels in obese PCOS women but not normal-weight PCOS women, and asprosin levels did not significantly correlate with plasma progesterone or 17-OH-progesterone levels. The weak effects of asprosin on TC steroidogenesis and cell proliferation are reflected by low asprosin receptor expression (i.e. *OR4M1* mRNA) observed in LG-follicle TC of Experiment 1. Nonetheless, the stimulatory effect of asprosin on A4 production and inhibitory effect on IGF1-induced cell proliferation suggests that asprosin may directly contribute to TC differentiation and thus asprosin may serve as a paracrine and/or autocrine regulator of ovarian function, but further research is required to confirm this. Future research should also evaluate if asprosin has direct effects on granulosa cell function.

Declarations of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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