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Regulation of fibrillins and modulators of TGFβ in fetal bovine and human ovaries.

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Abstract
Fibrillins 1-3 are stromal extracellular matrix proteins that play important roles in regulating TGFβ activity, which stimulates fibroblasts to proliferate and synthesise collagen. In the developing ovary the action of stroma is initially necessary for formation of the ovigerous cords and subsequently for the formation of follicles and the surface epithelium of the ovary. \(FBN3\) is highly expressed only in early ovarian development and then it declines. In contrast, \(FBN1\) and \(2\) are up regulated in later ovarian development. We examined the expression of \(FBN1-3\) in bovine and human fetal ovaries. We used cell dispersion and monolayer culture, cell passaging and tissue culture. Cells were treated with growth factors, hormones or inhibitors to assess the regulation of expression of \(FBN1-3\). When bovine fetal ovarian tissue was cultured, \(FBN3\) expression declined significantly. Treatment with TGFβ-1 increased \(FBN1\) and \(FBN2\) expression in bovine fibroblasts, but did not affect \(FBN3\) expression. Additionally, in cultures of human fetal ovarian fibroblasts (9-17 weeks gestational age) the expression of \(FBN1\) and \(FBN2\) increased with passage whereas \(FBN3\) dramatically decreased. Treatment with activin A and a TGFβ family signalling inhibitor, SB431542, differentially regulated expression of a range of modulators of TGFβ signalling and of other growth factors in cultured human fetal ovarian fibroblasts suggesting that TGFβ signalling is differentially involved in regulation of ovarian fibroblasts. Additionally since the changes in \(FBN1-3\) expression that occur in vitro are those that occur with increasing gestational age in vivo, we suggest that the fetal ovarian fibroblasts mature in vitro.

Keywords: Stroma, bovine, human, fetal ovary, fibrillin, TGFβ-1, activin A, SB431542.
Introduction

Fibroblasts or stromal cells are of mesenchymal origin (Wong et al. 2007) and are a major cell type present in the stroma of many organs (Birchmeier & Birchmeier 1993). They play an important role in the production and deposition of collagen in tissues (Varga et al. 1987; Christner & Ayitey 2006) and ensure proper organ development and function (Saxen & Sariola 1987; Birchmeier & Birchmeier 1993). Perturbations within the stroma can result in defects in the functions of organs such as pulmonary fibrosis (Rock et al. 2011), cardiac fibrosis (Chen et al. 2000), renal fibrosis (Ito et al. 1998) and polycystic ovary syndrome (PCOS) (Hughesdon 1982).

Fibroblasts are activated by a pro-fibrotic cytokine, TGFβ (Roberts et al. 1986; Chen et al. 2000; Raja-Khan et al. 2014). There are three TGFβs that are secreted as inactive latent homodimeric complexes of precursor molecules each consisting of a signal peptide, a latency-associated peptide (LAP) and a mature peptide (Saharinen et al. 1999). These LAPs form covalent disulphide bonds with other chaperone proteins called latent TGFβ binding proteins (LTBPs) (Saharinen et al. 1999), specifically LTBP1, LTBP3 and LTBP4 (Isogai et al. 2003). LTBPs also play a role in regulating the secretion of latent TGFβ from cells and targeting latent TGFβ to the extracellular matrix (ECM) (Taipale et al. 1994; Isogai et al. 2003), where glycoproteins called fibrillins are present.

Fibrillins are major structural components in stromal ECM (Ramirez & Pereira 1999). In addition to their structural function, they regulate growth factor/cytokine activity by binding LTBPs 1 to 4 (Isogai et al. 2003; Zilberberg et al. 2012) and thus sequestering latent TGFβ in the ECM (Chaudhry et al. 2007). This is important for the regulation of TGFβ activation and bioavailability in the ECM (Saharinen et al. 1999; Chaudhry et al. 2007) and hence for stromal fibroblast function. TGFβ is only released from the ECM and activated via proteolytic cleavage (Saharinen et al. 1999). Activated TGFβ can then activate fibroblasts to proliferate and synthesise ECM proteins such as collagens and fibronectin (Fine & Goldstein 1987; Varga et al. 1987; Leask & Abraham 2004). In 2004 another member of the fibrillin family, fibrillin 3 was discovered (Corson et al. 2004). The expression of the FBN3 gene was shown to be significantly higher in fetal compared to adult tissues of the same organs (Corson et al. 2004; Sabatier et al. 2010; Hatzirodos et al. 2011). Furthermore, in addition to humans, FBN3 is expressed in cows, sheep and chickens, but not in rodents (Corson et al. 2004).

Recent studies have proposed a new model of mammalian ovarian development which emphasises the importance of stromal penetration and expansion as a crucial processes.
in the developing fetal bovine and human ovaries (Hummitzsch et al. 2013; Heeren et al. 2015; Hummitzsch et al. 2015). The stromal tissue first penetrates the developing ovary from the mesonephros and whilst doing so it subdivides and thus partitions the oogonia and the precursor gonadal-ridge epithelial (GREL) cells of the ovary into ovigerous cords. It then segregates the ovigerous cords into smaller groups of cells thus contributing to the formation of follicles. Finally it penetrates to just below the surface of the ovary before spreading laterally, thus isolating some GREL cells at the surface of the ovary. These GREL cells then begin to form an epithelium on the surface. Throughout ovary development the stroma expresses fibrillin genes but different ones at different times. In the bovine and human, \textit{FBN1} is expressed during fetal ovarian development and increases significantly in the adult ovary (Hatzirodos et al. 2011). \textit{FBN2} is also expressed in fetal ovaries but declines in the later stages of bovine fetal ovarian development and increases in the adult bovine ovary. In humans, \textit{FBN2} is expressed during fetal ovarian development but is very low in the adult ovary (Hatzirodos et al. 2011). \textit{FBN3} is highly expressed in the early bovine and human fetal ovary, however its expression declines and is undetectable in adult bovine and human ovaries (Hatzirodos et al. 2011). Histochemical localisation of fibrillin 3 revealed an extensive network of fibrillin 3 fibres in the stromal compartment as it penetrates between ovigerous cords in fetal bovine and human ovaries (Hatzirodos et al. 2011; Hummitzsch et al. 2013). These findings suggest that fibrillin 3 plays a crucial role in the fetal ovary during early ovary development, when stromal tissue is expanding from the mesonephros into the developing ovary (Hatzirodos et al. 2011; Hummitzsch et al. 2013) but as development progresses fibrillin 1 in particular becomes more important.

Although the stromal matrix protein fibrillin-3 appears to have an important role early in fetal development, in contrast to all other fibrillins, LTBPs and TGFβ molecules, there is very little known about its regulation or function. A recent study by Davis et al. (2014) identified the promoters of the human fibrillin genes and the transcription factors that bind to these promoters (Davis et al. 2014). This study was heavily biased to adult tissues present in the FANTOM database at that time, thus limiting the information about the promoter of \textit{FBN3} and identification of potential transcription factor binding motifs present within this promoter. Interestingly it was found that the transcription factor binding motifs in the \textit{FBN3} promoter do not overlap with those of other two fibrillin genes (Davis et al. 2014). This would be consistent with the differential expression of the fibrillin genes seen in ovaries (Hatzirodos et al. 2011).
The FBN3 gene may be associated with the occurrence of PCOS (Urbanek et al. 2007). The PCOS ovary phenotype has the hallmarks of increased TGFβ activity with expanded stroma and collagen deposition (Hughesdon 1982). Additionally the behaviour of the specialised stromal thecal cells is different, with increased steroidogenic activity (Nelson et al. 1999; Polla et al. 2003). Thus aberrant FBN3 activity during fetal development could be related to the altered stroma phenotype in the PCOS ovary (Hatzirodos et al. 2011; Raja-Khan et al. 2014). In this study our goals were to determine the factors that affect FBN1-3 expression in human and bovine fetal ovaries.

Materials and Methods

Bovine fetal tissues

Bovine fetal ovaries from a range of gestational ages were collected from fetuses of Bos taurus cows from a local abattoir (Thomas Foods International, Murray Bridge, SA, Australia). The crown-rump-length of the fetuses was measured to determine the approximate ages of fetuses (Russe 1983) and the fetal ovaries were transported on ice in Hank’s Balanced-Salt Solution containing Mg²⁺ and Ca²⁺ (HBSS⁺⁺; Sigma-Aldrich Pty Ltd, Castle Hill, NSW, Australia) to the laboratory. The connective tissue surrounding the fetal ovaries was removed and a small portion of the ovaries was excised and frozen at -80°C for subsequent RNA extraction. The ovaries were rinsed once in 70% ethanol and twice in HBSS⁺⁺ and then dissected into small pieces and minced with a scalpel. The fetal ovaries were digested in 3-5 ml of 1 mg/ml collagenase type I (GIBCO/Life Technologies Australia Pty Ltd, Mulgrave, VIC, Australia) in HBSS⁺⁺ at 37°C shaking at 150 rpm. The durations of digestion for the fetal ovaries depended on the crown-rump lengths of the fetuses. After the first digestion, the samples were centrifuged at 1500 rpm for 5 min and the supernatant was removed. The samples were then digested in 2 ml of 0.025% trypsin/EDTA (GIBCO/Life Technologies) in Hank’s Balanced-Salt Solution without Mg²⁺ and Ca²⁺ (HBSS⁻⁻; Sigma-Aldrich) for 5 min at 37°C at 150 rpm. After centrifugation at 1500 rpm for 5 min, the cell pellets were resuspended in DMEM/F12 medium containing 5% FCS, 1% penicillin and streptomycin sulphate, and 0.1% fungizone (all GIBCO/Life Technologies) and the cells were dispersed further by pipetting up and down. The fetal fibroblasts were cultured in 6-well
plates or 10 cm petri dishes at 38.5°C and 5% CO₂ until confluent. Once the fetal fibroblast cultures were confluent, the cells were detached by treatment with 0.25% trypsin/EDTA, the total number of viable cells was estimated with the trypan blue method using a haemocytometer and the cells subsequently stored in liquid nitrogen for later use.

**Human fetal tissues**

Morphologically normal human fetal ovaries (9-17 weeks gestation) were obtained following medical termination of pregnancy. Maternal consent was obtained and the study was approved by the Lothian Research Ethics Committee (ref 08/S1101/1). Gestational age of the fetuses was determined by ultrasound scan and by direct measurement of the fetal foot length. Extraneous tissue was removed from ovaries in HBSS (GIBCO/Life Technologies). Ovaries were manually dispersed under a dissection microscope using 19 gauge needles in a total of 500 µl of 10 mg/ml Collagenase IV (Sigma, Dorset, UK) in HBSS. The tissue/collagenase suspension was incubated in a thermomixer at 37°C, shaking at 1000 rpm for 10 min and pipetted up and down to ensure complete disaggregation of the tissue. Fifty µl of DNase I (7 mg/ml HBSS; Sigma) was added to the suspension and incubated for a further 5 min, shaking at 37°C. The single cell suspension was then centrifuged at 600 g for 5 min and the cell pellet was washed twice with 1 ml HBSS; centrifuging between each wash. The cell pellet was then resuspended in 1 ml of DMEM (without phenol red) (GIBCO/Life Technologies) supplemented with 10% FCS, 2mM L-glutamine, 1X MEM Non-Essential Amino Acids (NEAA; all GIBCO/Life Technologies) and 1X penicillin/streptomycin/amphotericin (GIBCO/Life Technologies). The cell suspension was filtered through a 70 µm filter and the resulting filtrate centrifuged. The cell pellet was resuspended in 1.2 ml of culture medium. 200 µl of the initial cell suspension was transferred to a separate fresh 1.5 ml tube and centrifuged. The cell pellet was washed in 1X phosphate-buffered saline (PBS), resuspended in 350 µl of buffer RLT (Qiagen) with 2-mercaptoethanol (Sigma Aldrich) and stored at -80°C for RNA isolation (T₀). The remaining cells were cultured in 2 wells of a 12-well plate at 37°C and 5% CO₂ overnight. After 13-17 h, the cells were washed twice with culture medium and these washes were collected and centrifuged. The pellet was washed with PBS and resuspended in 350 µl of buffer RLT plus 2-mercaptoethanol and stored at -80°C for RNA isolation (S₀). Fresh culture medium was added to each well and the cells were cultured further until confluent (P₀ culture). Once the cell cultures were confluent, the cells were detached through trypsinization. An aliquot of the cells was collected for RNA extraction. The
remaining cells were passaged into either a 6-well plate or 25 cm² tissue culture flask. Passaging of cells and freezing down aliquots of cells in Bambanker™ (Anachem, Luton, Beds, UK) freezing medium were continued for several passages.

**Screening for possible regulators of FBN3 expression in cultured bovine fetal fibroblasts**

Bovine fetal fibroblasts (n = 5 from weeks 13, 14, 17, 19 and 33 of gestation) previously stored in liquid nitrogen were thawed and 30,000 cells/well seeded in 24-well plates in DMEM/F12 medium containing 5% FCS, 1% penicillin and streptomycin sulphate and 0.1% fungizone, and incubated for 24 h at 38.5°C and 5% CO₂ until 60-70% confluent. The wells were washed with 1X PBS, and subsequently the different chemical treatments added. All treatments were prepared in DMEM/F12 medium containing 1% FCS, 1% penicillin and streptomycin sulphate, and 0.1% fungizone. After 18 h, the cells were harvested for RNA extraction by lysis in 500 µl Trizol® (Ambion/Life Technologies) each and stored at -80°C. To limit the number of samples for the qRT-PCR, the treatments were used at concentrations previously reported in the literature instead of dose-response experiments for each of the 31 agents (Table 1).

**Treatment of bovine fibroblast cultures with TGFβ1 and TGFβ-inhibitor SB431542**

Bovine fetal fibroblasts (n=5 for weeks 9-15 in the first trimester, n=6 for weeks 19-26 in the second trimester) were seeded at 30,000 cells/well in 24-well plates in DMEM/F12 medium containing 5% FCS, 1% penicillin and streptomycin sulphate and 0.1% fungizone, and incubated for 24 h at 38.5°C and 5% CO₂ until 60-70% confluent. After 24 h, the wells were washed with 1X PBS and immediately treated with 5 ng/µl or 20 ng/µl TGFβ-1 with or without the TGFβ inhibitor SB431542 (10 µM, dissolved in DMSO, Sigma-Aldrich), in DMEM/F12 medium containing 1% FCS, 1% penicillin and streptomycin sulphate and 0.1% fungizone. DMSO (0.78%), the vehicle for SB431542, was added to the control wells and the wells treated with TGFβ-1 alone. After 18 h, the cells were harvested for RNA.

**Culture of bovine ovarian tissue slices**
Bovine fetal ovaries (n=4 12 to 18 weeks) were excised into two portions, one portion was stored at -80°C (0 h tissue) for subsequent RNA extraction and the second portion was cultured in DMEM/F12 medium containing 5% FCS, 1% penicillin and streptomycin sulphate and 0.1% fungizone, and incubated for 24 h at 38.5°C and 5% CO₂. After 24 h, the ovarian tissue slices were collected and frozen at -80°C for RNA extraction.

**Treatment of human fetal ovarian fibroblasts with TGFβ-1, activin-A and SB431542**

Human fetal ovarian fibroblasts (n=3, 15-17 weeks gestation) were cultured in 75 cm² tissue culture flasks until sub-confluent. Cells were then seeded in culture medium in 6- or 12-well plates at 37°C for 7 hours. Once 70% confluent, the cells were washed and serum-starved overnight in culture medium containing only 1% FCS. The next day, the medium was replaced with fresh culture medium containing 1% FCS and the relevant treatments or vehicle; 5 ng/ml TGFβ-1, 100 ng/ml activin A, and 10 µM SB431542. The cells were incubated at 37°C for 24 h and then harvested for RNA extraction.

**RNA extraction and cDNA synthesis**

For our bovine study, the ovarian tissue samples previously frozen were homogenised in 1 ml of Trizol® with 0.5 g of ceramic beads in homogenisation tubes using the Mo Bio Powerlyser 24 (Mo Bio Laboratories Inc., Carlsbad, CA, USA). The cells previously harvested for RNA and the homogenised tissue samples underwent further treatment for RNA extraction as per manufacturer’s instructions (Ambion/Life Technologies). Using a Nanodrop spectrophotometer (NanoDrop 1000 3.7.1, Thermo Fisher Scientific, Inc., USA), the RNA concentrations were determined based on the 260 λ (wavelength) absorbance. All samples had a 260/280 λ absorbance ratio > 1.8 indicating sufficient RNA purity for analysis. 200 ng of each DNAse-treated RNA underwent cDNA synthesis as described in a previous study (Matti et al. 2010).

For the human samples, RNA was extracted from cells using the RNeasy Micro Kit (Qiagen, Crawley, UK) with on-column DNase I digestion as per manufacturer’s instructions. After quantification on a Nanodrop spectrophotometer, reverse transcription was carried out using 200ng RNA/reaction with the Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc, USA).
Quantitative real-time PCR

Quantitative real-time PCR of the bovine samples for the target genes FBN1, FBN2 and FBN3 and the housekeeping gene 18S was performed using a Rotor-Gene 6000 series 1.7 thermal cycler (Corbett Life Science, Concord, NSW, Australia). cDNA dilutions were amplified in 10 µl reactions containing 5 µl of Power SYBR™ Green PCR Master Mix (Applied Biosystems/Life Technologies), 0.1 µl each of reverse and forward primers (Geneworks; Table 4) respectively for the genes of interest, 1 µl of the 1:100 cDNA dilution (for the housekeeping gene 18S) or 1:10 cDNA dilution (for FBN1, FBN2, FBN3) and 3.6 µl of DEPC-treated water. PCR amplification of the cDNA samples was carried out in duplicates at 95°C for 15 sec, followed by 60°C for 60 sec for a total of 40 cycles. The Rotor-Gene 6000 software (Q Series, Qiagen) was used to determine the cycle threshold (Ct) values at a threshold of 0.05 normalized fluorescence units. Gene expression was determined by the mean of 2⁻ⁿΔCt, where ΔCt represents the target gene Ct – 18S Ct. The standard error of the mean (+/-SEM) for the power calculation was determined accordingly: 2⁻ⁿ(ΔCt + SEMΔCt) - 2⁻ⁿΔCt/2⁻ⁿ(ΔCt - SEMΔCt) - 2⁻ⁿΔCt.

Gene expression in human fetal ovaries and ovarian cell cultures was analysed by qRT-PCR using the ABI7900 Fast system with SDS2.4 software (Life Technologies, Paisley, UK), and Brilliant III SYBR Green Master Mix (Agilent Technologies, Wokingham, UK), with melt curve analysis as described previously (Bayne et al. 2015). Primers used for the qRT-PCR are shown in Table 2.

Statistical analyses

All statistical calculations were performed using Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) and GraphPad Prism version 6.00 (GraphPad Software Inc., La Jolla, CA, USA). For the treatment experiments on bovine and human samples, statistical comparisons of the ΔCt data between the untreated control and the treatments for each fetal fibroblast sample were conducted using log transformed data where appropriate by ANOVA with Dunnett’s post-hoc test and a value of P<0.05 was considered significant. For the bovine ovarian tissue culture experiment, statistical comparisons of the ΔCt data between the 0 h tissue and the 24 h cultured tissue for each fetal ovary sample were conducted by unpaired T-tests and a value of P<0.05 was considered significant.
Results

Screening for possible regulators of FBN3

To identify possible regulators of FBN3, we treated bovine fetal ovarian fibroblasts (n = 5 ovaries, each from 13, 14, 17, 19 and 33 weeks of gestation) with 31 different reagents (Table 1) for 18 h and observed their effects on FBN3 expression (Fig. 1). The range of treatments used included cAMP regulators, growth factors, steroid hormones, peptide hormones, prostaglandins and cytokines, previously shown to play roles in adult ovarian function, such as cell proliferation and extracellular matrix production. No substantial effects were seen in any individual culture and the data were therefore combined across the gestational ages for statistical analyses which showed that there were no significant differences in FBN3 expression between the control and any of the treated cultures. Furthermore, we also observed that expression FBN3 in these cultures was very low.

Treatment of bovine fetal fibroblasts with TGFβ-1 and TGFβ-inhibitor SB431542

A partial dose response experiment was carried out using 5 or 20 ng/ml TGFβ-1 with or without the TGFβ-signalling inhibitor SB431542, which selectively inhibits the TGFβ superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7 (Inman et al. 2002a), using fetal ovarian fibroblast cultures (n = 5 ovaries from weeks 9-15 in first trimester, n = 6 ovaries from weeks 19-26 in the second trimester). Gene expression analyses showed that the expression of FBN1 in fetal ovarian fibroblasts from 9-15 weeks of gestation was not significantly affected when these cells were treated with TGFβ-1 (Fig. 2). However, it was observed that compared to the untreated control, there was a significant increase in FBN1 expression in the TGFβ-1-treated 19-26 week fibroblasts, with the higher TGFβ-1 concentration causing a more significant increase in FBN1 expression. This effect of TGFβ-1 was prevented by the antagonist SB431542, which had no effect alone. TGFβ-1 did not cause a significant effect on FBN2 expression in the 9-15 week gestation cells (Fig. 2) but SB431542 caused a significant reduction in FBN2 expression, with or without TGFβ-1 (Fig. 2). In later gestation TGFβ-1 stimulated FBN2 expression and SB431542 inhibited this stimulation, similarly to the effect on FBN1 expression. None of the treatments significantly affected FBN3 expression in fibroblast cultures of either gestational age (Fig. 2).
Expression of FBN1-3 in bovine fetal ovarian tissue

We then measured expression levels of fibrillin genes in fetal ovarian tissue slices before (0 h) and after culturing for 24 h (n = 1 ovary from 12 weeks of gestation and n = 3 ovaries from weeks 16-18). There were no significant differences (P > 0.05) in FBN1 and FBN2 expression respectively between the 0 h ovarian tissue and the ovarian tissue cultured for 24 h (Fig. 3). However, we observed a significant decline in FBN3 expression by 24 h.

Expression of FBN1-3 in human fetal ovarian cells

The expression levels of FBN1-3 were analysed in disaggregated human fetal ovarian tissue (n = 4 ovaries from 9-17 weeks of gestational age) before culture, in adherent ovarian fibroblasts before the first passage and up to the eighth cell passage. FBN1 and FBN2 expression increased in all cultures across passages (Fig. 4). FBN3 was expressed higher in the disaggregated cells of 9 week old fetal ovary compared to later gestation fetal ovaries (Fig. 4). However the ovarian cells of all fetal ovaries showed a dramatic decline in FBN3 expression in culture and a loss of FBN3 expression after the first passage.

TGFβ-1, activin A and SB431542 treatment of human fetal ovarian cells

Human fetal ovarian fibroblasts (n = 3 ovaries from 15-17 weeks gestation) were treated with 5 ng/ml TGFβ-1, 100 ng/ml activin A and 10 µM SB431542 separately. TGFβ1-3, LTBP1-4, FBN1-3, AR, INHBA, HTRA1 and BDNF expression levels were subsequently measured. SB431542 treatment significantly lowered TGFBI, LTBP1, TGFBI, INHBA and BDNF expression, whereas TGFβ-1 treatment significantly reduced AR expression (Fig. 5). None of the treatments significantly affected TGF2-3, LTBP1, LTBP3-4, FBN1-2, and HTRA1 expression (Fig. 5). FBN3 expression was not detectable in these cultures.

Discussion
In this study we investigated the regulation of fibrillins and related TGFβs and latent TGFβ binding proteins in vitro using bovine and human fetal ovaries under different culture conditions: monolayer culture, passaging of these monolayer cultures and culture of pieces of tissue. We initially carried out a screen for possible regulators of FBN3. The effects of TGFβ and its pathway inhibitor SB431542 were examined in more detail and the effects on all fibrillins were examined. In humans the effects of these and activin were also examined in detail. A consistent observation was that during culture FBN3 was down regulated and FBN1 was up regulated, as occurs in vivo with increasing gestation (Hatzirodos et al. 2011).

To date, there has only been one study that has investigated the effects of TGFβ signalling on the expression of fibrillin genes in fetal tissues. This study found that TGFβ-1 increased FBN1 and FBN2 expression in murine fetal skin (Samuel et al. 2003). Since a murine model was used for that study, an examination of the expression of FBN3 was not possible as this gene is inactivated in mice (Corson et al. 2004). In adult fibroblasts TGFβ-1 was shown to increase FBN1 and FBN2 expression (Samuel et al. 2003) and the involvement of the TGFβ superfamily is well characterised in ovarian follicle development (Knight & Glister 2006). We therefore examined the effects of TGFβ-1 on expression of the fibrillin genes. In the bovine study, we found that treatment with TGFβ-1 caused a significant increase in FBN1 and FBN2 expression in the second trimester fibroblasts. These results suggest that in the bovine, TGFβ-1 regulation of the expression of FBN1 and FBN2 only becomes active as development progresses. However, TGFβ-1 treatment did not affect FBN1 and FBN2 expression in cultured human fetal ovarian fibroblasts at 15-17 weeks gestation. FBN2 has different expression profiles in the bovine and human both during gestation and in the adult ovaries (Hatzirodos et al. 2011), suggesting differential regulation in these species. Furthermore, it appears that TGFβ-1 regulates FBN2 expression differently in the bovine and human later in gestation.

We also examined human fetal ovarian fibroblasts from later gestation when steroidogenic enzymes are expressed (Fowler et al. 2011). We observed that TGFβ-1 treatment caused a significant decrease in the expression of the androgen receptor gene (AR). There are no previous studies that have investigated the effects of TGFβ directly on AR expression. However, it is known that interaction of Smad3, a mediator of intracellular TGFβ signalling, with the androgen receptor represses AR-mediated transcription, but the exact mechanisms of this repression are not well understood (Kang et al. 2001; Chipuk et al. 2002). Treatment of monkey kidney cells and human prostate cells with TGFβ-1 caused a reduction
The findings of our study suggest that TGFβ signalling may repress AR-dependent transcription by inhibiting expression of the androgen receptor itself. On the other hand, we also observed significant reductions in the expression of the TGFBI, LTBP2, TGFBI, and INHBA genes when the human fetal ovarian fibroblasts were treated with the TGFβ antagonist SB431542. SB431542 selectively inhibits TGFβ type I receptors, activin-like receptor kinase 4, 5 and 7 (ALKs 4, 5 and 7) (Inman et al. 2002a). Therefore, ALK5 remains inactive (Callahan et al. 2002) and unable to phosphorylate Smads 2 and 3 (Callahan et al. 2002; Inman et al. 2002a) and the classical TGFβ/smard pathway is disrupted (Heldin et al. 1997; Inman et al. 2002b; Derynck & Zhang 2003). TGFβ-1 treatment causes a small and non-significant increase in the expression of these genes thus the effect of SB431542 is likely to reflect antagonism of stimulation by endogenous TGFβ signalling. Likewise the reduction in BDNF expression with SB431542 treatment suggests that endogenous TGFβ signalling is capable of stimulating BDNF expression in these cultured fibroblasts.

In culture of bovine fetal fibroblasts from the first trimester, SB431542 either alone or in combination with TGFβ-1 caused a significant decrease in FBN2 expression compared to the control even though TGFβ-1 had no effect on these fibroblasts. Therefore, as with the human cell experiments, we speculate that SB431542 is inhibiting endogenous TGFβ signalling via the TGFβ type I and II receptors which is stimulatory of FBN2 expression. The TGFβ superfamily ligands that bind to these receptors include TGFβs, activins, nodal and GDF8 (myostatin) (Heldin et al. 1997; Reissmann et al. 2001; Derynck & Zhang 2003; Rebbapragada et al. 2003). Currently, we have not identified which of these endogenous ligands are involved. On the other hand, in the second trimester bovine fibroblasts, the expression levels of FBN1 and FBN2 were similar to the control when SB431542 in combination with TGFβ-1 was present in these cultures. Since we observed that TGFβ-1 increased the expression of FBN1 and FBN2 in these fibroblasts, these observations suggest that SB431542 is alleviating the effects of exogenous TGFβ-1, with no evidence of stimulation of the expression of these genes by endogenous TGFβ. Overall, our observations show that FBN1 and FBN2 are differentially regulated in both the bovine and human ovary.

To date, there have not been any studies that have investigated the effect of activin A on the gene expression of fibrillins. However, previous studies have shown that activin A is capable of causing an increase in the proliferation of human lung fibroblasts (Heeren et al. 2015) as well as promoting proliferation of cultured rat renal interstitial fibroblasts and
increasing the expression of type I collagen (Yamashita et al. 2004). We have observed that activin A did not affect the expression of FBN1 or FBN2 or the other TGFβ-associated genes examined in our human samples. The levels of FBN3 expression in the treated human fetal ovarian fibroblasts cultures were undetectable.

The fetal ovary consists of a mixture of cell types and interactions between fibroblasts, GREL cells, pregranulosa/ granulosa cells and germ cells might be required to maintain FBN3 expression. The other cell types in the ovary may also be producing factors required by fibroblasts to stimulate FBN3 expression. Thus we carried out tissue culture experiments, but even a short-term culture (24 h) of bovine fetal ovarian tissue sections, which represent a cross-section of the three-dimensional ovarian structure containing all cell types and its extracellular matrix, showed a decline in the expression of FBN3. Similarly, we observed a drastic decline in FBN3 expression but an increase in FBN1 and FBN2 expression in the cultured human fetal ovarian fibroblasts, consistent with their developing a more mature phenotype in culture. This indicates very stringent and possibly complex regulation of FBN3 expression in vivo, which is not maintained in vitro, limiting our ability to study the expression of this gene.

A previous study showed that ovarian FBN2 and FBN3 are fetal fibrillins (Hatzirodos et al. 2011). FBN3 is initially expressed highly and then begins to decline at the end of the first trimester and FBN2 declines sometime between the fetal stages and adulthood at least in human (Hatzirodos et al. 2011). FBN1 persists through fetal ovary development and is increased in the adult ovary (Hatzirodos et al. 2011) - it is an adult fibrillin. These data therefore imply that the three fibrillin genes have independent regulatory mechanisms to account for their different expression profiles in the bovine and human. This is also supported by the study of Davis et al. (2014) which found that there was little overlap in the transcription factor motifs present on the human FBN3 promoter and those of FBN1 and 2 promoters, suggesting that these genes are differentially regulated and differentially expressed (Davis et al. 2014).

In summary, FBN3 expression is rapidly reduced in both cell and tissue culture, and was not maintained or stimulated by a range of growth factors. This study has also shown that in the bovine, there is differential regulation of FBN1 and FBN2 between the early and later stages of gestation which is partially mediated through the signalling pathways involving either ALK 4, 5 or 7. TGFβ regulates its own signalling both directly through TGFβ expression, and through regulation of expression of other binding proteins such as LTBP2.
These data therefore demonstrate that the regulation of TGFβ signalling appears to change during fetal ovarian development.

Declaration of Interest
None declared.

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Authors’ contribution statement
N.A.B., K.H., N.H., W.M.B., M.D.H., H.F.I-R, R.J.R. were responsible for planning the experiments on bovine samples, conducting cell culture and treatment experiments, RT-PCR, data analysis and interpretation as well as revising the manuscript. R.A.B. and R.A.A. conducted cell culture and treatment experiments on human samples, RT-PCR, data analysis and reviewed the manuscript.

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References


Christner PJ & Ayitey S 2006 Extracellular matrix containing mutated fibrillin-1 (Fbn1) down regulates Col1a1, Col1a2, Col3a1, Col5a1, and Col5a2 mRNA levels in Tsk/+ and Tsk/Tsk embryonic fibroblasts. *Amino Acids* **30** 445-51.


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### Table 1 Treatments for cultured bovine fetal ovarian fibroblasts.

<table>
<thead>
<tr>
<th>Category</th>
<th>Treatments</th>
<th>Concentration</th>
<th>Distributor</th>
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</thead>
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<tr>
<td>Hormones</td>
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<td>Sigma-Aldrich</td>
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<tr>
<td></td>
<td>Estradiol</td>
<td>100 ng/ml</td>
<td>Sigma-Aldrich</td>
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<tr>
<td></td>
<td>Progesterone</td>
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<td>Sigma-Aldrich</td>
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<td></td>
<td>Testosterone</td>
<td>100 ng/ml</td>
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<tr>
<td></td>
<td>Prolactin</td>
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<td>Relaxin</td>
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<td>From Dr. Ross Bathgate – University of Melbourne, Australia</td>
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<tr>
<td>Müllerian-inhibiting substance</td>
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<tr>
<td>Insulin-like growth factor 1</td>
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<td>GroPep Bioreagents Pty Ltd., Thebarton, SA, Australia</td>
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<tr>
<td>Prostaglandins</td>
<td>Prostaglandin E2</td>
<td>1 µM</td>
<td>Upjohn Company, Michigan, USA</td>
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<tr>
<td></td>
<td>Prostaglandin F2α</td>
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<td>Cytokines</td>
<td>Interleukin 1 beta</td>
<td>50 ng/ml</td>
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<td>Stimulators</td>
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<td>Basic fibroblast growth factor</td>
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<td>Bone morphogenetic protein 6</td>
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**Table 2** List of genes and primers used for qRT-PCR.

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<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Species Specificity</th>
<th>Location of Amplicon</th>
<th>Forward Primer Sequence (5’→3’)</th>
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<td>18S rRNA</td>
<td>18S</td>
<td>Bovine Human</td>
<td>445-535</td>
<td>AGAAACGGCTACCA CATCCAA</td>
<td>CCTGTATTGTATT TTTGTCACCTACC</td>
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<td>Fibrillin 1</td>
<td>FBN1</td>
<td>Bovine</td>
<td>280-384</td>
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<td>CATCACTGCAGCT ACCTCCATT</td>
<td>NM_174053.2</td>
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<td>Fibrillin 2</td>
<td>FBN2</td>
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<td>1697-1758</td>
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<td>Fibrillin 3</td>
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<td>8011-8090</td>
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<td>60S ribosomal protein L32</td>
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<td>34-186</td>
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<td>Fibrillin 1</td>
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<td>TGFβ1</td>
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<td>Androgen Receptor</td>
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<td>HTRA1</td>
<td>Human</td>
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<td>Brain Derived Neurotrophic Factor</td>
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<td>AACAAATAAGGACGCTAGACCTT</td>
<td>TGCAGTCTTTTTGCTGCCG</td>
<td>X91251.1</td>
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Figure Legends

Figure 1 Expression of $FBN3$ in bovine fetal fibroblasts from different gestational ages cultured in the presence of 31 different chemical agents for 18 hours. The data are shown as mean ± SEM of fold change in $FBN3$ expression relative to the untreated control (n = 5 ovaries, each from 13, 14, 17, 19 and 33 weeks of gestation, respectively).

Figure 2 Expression of $FBN1-3$ in bovine fetal fibroblasts from the first and second trimester of gestation cultured in the presence of 5 and 20 ng/ml TGFβ-1 with and without 10 µM SB431542 for 18 hours. The data are shown as mean ± SEM of fold change in $FBN1-3$ expression relative to the untreated control (n = 5 ovaries from weeks 9-15 in the first trimester, n = 6 ovaries from weeks 19-26 in the second trimester). Significantly different results for qRT-PCR were determined by one-way ANOVA with Dunnet’s post-hoc test. All values which were statistically different from the control are indicated by asterisk symbols in the graphs. *$P < 0.05$, **$P < 0.01$, significant differences.

Figure 3 Expression of $FBN1-3$ in bovine fetal ovarian tissue slices before and after 24 h culture. The data are shown as the mean ± SEM of $FBN1-3$ expression relative to 18S (n = 1 ovary from 12 weeks of gestation and n = 3 ovaries from weeks 16-18). Significantly different results for qRT-PCR were determined by unpaired $T$-tests. All values which were statistically different are indicated by asterisk symbols in the graphs. *$P < 0.05$, **$P < 0.01$, significant differences.

Figure 4 Expression levels of $FBN1-3$ (pmol/nmol $RPL32$) in human fetal ovarian somatic cell/ fibroblast cultures from different gestational ages from tissue digestion up to the eighth passage. T0 represents disaggregated ovarian tissue before culture and P0 represents adherent ovarian cells before the first passage. The different coloured symbols represent cells from single ovaries at different gestational ages: (*) represents a 9 week ovary, (▲) represents a 15 week ovary, (●) represents a 16 week fetal ovary, and (▲) represents a 17 week fetal ovary.

Figure 5 Expression levels of $TGFB1-3$, $LTBPI-4$, $FBN1-2$, $TGFB1$, $AR$, $INHBA$, $HTRA1$, and $BDNF$ in primary human fetal ovarian fibroblast cultures in the presence or absence of 5 ng/ml TGFβ-1, 100 ng/ml activin A and 10 µM SB431542 for 24 hours. The data shown are
mean ± SEM of target gene expression relative to the untreated control from 15-17 weeks gestation human fetal ovarian fibroblast cultures (n = 3 ovaries). Significantly different results for qRT-PCR were determined by one-way ANOVA with Dunnet’s post-hoc test. All values which were statistically different from the control are indicated by asterisk symbols in the graphs. *$P < 0.05$, **$P < 0.01$, significant differences.
Figure 1
174x279mm (300 x 300 DPI)
Figure 2
181x187mm (300 x 300 DPI)
Figure 3

68x185mm (300 x 300 DPI)
Figure 4
93x199mm (300 x 300 DPI)
Figure 5
178x281mm (300 x 300 DPI)