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Reproduction

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Journal:	*Reproduction*
Manuscript ID	REP-16-0172.R1
mstype:	Research paper
Date Submitted by the Author:	n/a
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Keywords:	Ovary, Extracellular matrix, Fetus, Developmental biology, fibrillin

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, *FBN1* is expressed during fetal ovarian development and increases significantly in the adult ovary (Hatzirodos *et al.* 2011). *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, *FBN2* is expressed during fetal ovarian development but is very low in the adult ovary (Hatzirodos *et al.* 2011). *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 *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 *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 Mg^{2+} and Ca^{2+} ($HBSS^{+/+}$; 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 -80ºC 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 37ºC 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 Mg^{2+} and Ca^{2+} ($HBSS^{-/-}$; Sigma-Aldrich) for 5 min at 37ºC 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 38.5ºC and 5% CO_2 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-mercapthoethanol (Sigma Aldrich) and stored at -80°C for RNA isolation (T_0). The remaining cells were cultured in 2 wells of a 12-well plate at 37°C and 5% CO_2 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-mercapthoethanol and stored at -80°C for RNA isolation (S_0). 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 trypsination. An aliquot of the cells was collected for RNA extraction. The

remaining cells were passaged into either a 6-well plate or 25 cm^2 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 38.5ºC and 5% CO_2 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 38.5ºC and 5% CO_2 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 38.5ºC and 5% CO_2. 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 cm^2 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 $2^{-\Delta Ct}$, where ΔCt represents the target gene Ct – *18S* Ct. The standard error of the mean (+/-SEM) for the power calculation was determined accordingly: $2^{(-\Delta Ct + SEM\Delta Ct)} - 2^{-\Delta Ct}/2^{(-\Delta Ct - SEM\Delta Ct)} - 2^{-\Delta Ct}$.

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. *TGFB1-3*, *LTBP1-4*, *FBN1-3*, *AR*, *INHBA*, *HTRA1* and *BDNF* expression levels were subsequently measured. SB431542 treatment significantly lowered *TGFB1*, *LTBP2*, *TGFBI*, *INHBA* and *BDNF* expression, whereas TGFβ-1 treatment significantly reduced *AR* expression (Fig. 5). None of the treatments significantly affected *TGFB2-3*, *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

in AR-mediated transcription as indicated by luciferase reporter activity in these cells (Hayes *et al.* 2001). 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 *TGFB1*, *LTBP2*, *TGFBI*, and *INHBA* genes when the human fetal ovarian fibroblasts were treated with the TFGβ 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/smad 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.

Funding

Funding support for this research was obtained from the National Health and Medical Research Council of Australia, the University of Adelaide, the Clive and Vera Ramaciotti Foundation, the Wellcome Trust, the National Institute of Health, and the Medical Research Council UK (G1100357).

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.

Acknowledgements

We would like to thank Mrs. Wendy Bonner for the collection of bovine fetal ovaries from the abattoir and Thomas Foods International, Murray Bridge, SA for the supply of bovine fetal ovaries. We also thank Anne Saunderson and the staff of the Bruntsfield Suite, Royal Infirmary of Edinburgh for patient recruitment.

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Table 1 Treatments for cultured bovine fetal ovarian fibroblasts.

Category	Treatments	Concentration	Distributor
Hormones	Dihydroxytestosterone	100 ng/ml	Sigma-Aldrich
	Estradiol	100 ng/ml	Sigma-Aldrich
	Progesterone	100 ng/ml	Sigma-Aldrich
	Testosterone	100 ng/ml	Sigma-Aldrich
	Prolactin	1 µg/ml	National Institute of Diabetes and Digestive and Kidney Diseases – National Institutes of Health
	Relaxin	16.7 nM	From Dr. Ross Bathgate – University of Melbourne, Australia
	Insulin-like protein 3	100 ng/ml	From Dr. Ross Bathgate – University of Melbourne, Australia
	Müllerian-inhibiting substance	10 ng/ml	Biogen Idec Australia Pty Ltd., North Ryde, NSW, Australia
	Insulin-like growth factor 1	30 ng/ml	GroPep Bioreagents Pty Ltd., Thebarton, SA, Australia
Prostaglandins	Prostaglandin E2	1 µM	Upjohn Company, Michigan, USA
	Prostaglandin F2α	1 µM	Upjohn Company

Cytokines	Interleukin 1 beta	50 ng/ml	R&D Systems, distributed by Bio-Scientific Pty Ltd., Gymea, NSW, Australia
	Tumour necrosis factor	10 ng/ml	R&D Systems
Stimulators	Forskolin	4.1 µg/ml	Sigma-Aldrich
	Dibutyryladenosine cyclic monophosphate	1 mM	Sigma-Aldrich
Vitamin	Retinoic acid	3 µg/ml	Sigma-Aldrich
Growth Factors	Activin A	100 ng/ml	R&D Systems
	Basic fibroblast growth factor	100 ng/ml	Roche Australia Pty Ltd., Thebarton, SA, Australia
	Bone morphogenetic protein 6	100 ng/ml	R&D Systems
	Epidermal growth factor	10 ng/ml	Boehringer Ingelheim Pty Ltd., North Ryde, NSW, Australia
	Fibroblast growth factor 7	10 ng/ml	R&D Systems
	Fibroblast growth factor 9	30 ng/ml	R&D Systems
	Platelet-derived growth factor	10 ng/ml	R&D Systems
	Bone morphogenetic protein 15	100 ng/ml	R&D Systems

Connective tissue growth	25 ng/ml	Invitrogen/Life Technologies
Stem cell factor	100 ng/ml	R&D Systems
Transforming growth factor beta 1	10 ng/ml	R&D Systems
Vascular endothelial growth factor	10 ng/ml	R&D Systems
Glial-derived factor 9	100 ng/ml	R&D Systems
Glial-cell derived neurotrophic factor	100 ng/ml	R&D Systems
Leukemia inhibitory factor	10^3 U/ml	Sigma-Aldrich

Table 2 List of genes and primers used for qRT-PCR.

Gene Name	Gene Symbol	Species Specificity	Location of Amplicon	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')	Genebank Accession Number
18S rRNA	*18S*	Bovine Human	445-535	AGAAACGGCTACCA CATCCAA	CCTGTATTGTTATT TTTCGTCACTACC	AM711877.1
Fibrillin 1	*FBN1*	Bovine	280-384	GGGATGGATTTTGTT CGAGGC	CATCACTGCAGCT ACCTCCATT	NM_174053.2
Fibrillin 2	*FBN2*	Bovine	1697-1758	GGACTCCTACCAAGC AAGCATG	ACAAAGAACCCCA TTCTGGATG	NM_001278588.1
Fibrillin 3	*FBN3*	Bovine	8011-8090	GCCACAGCCTGCCTA GATGT	CTGCCCTCAGTGT TTTTGC	XM_002688838.2
60S ribosomal protein L32	*RPL32*	Human	34-186	CATCTCCTTCTCGGC ATCA	AACCCTGTTGTCA ATGCCTC	AK311909.1
Fibrillin 1	*FBN1*	Human	1822-1882	AGCACACTCACGCG ACA	AGATCCGGCCATT CTGTAAACA	BC146854
Fibrillin 2	*FBN2*	Human	7545-7646	TCCAGTCAAGTTCTT	TGCGACTACTGGA	NM_001999

				CAGGCAC	TGCCATTT	
Fibrillin 3	*FBN3*	Human	1716-1787	TGGCGGCCACTACTG CAT	TTGGTACAGTGGC CGTTCAC	NM_032447
Latent TGFβ binding protein 1	*LTBP1*	human	3195-3322	CCCCAATGTCACGAA ACAAGA	AACCTTTCCCTTTG GGACACA	BC130289
Latent TGFβ binding protein 2	*LTBP2*	human	3276-3382	CAGGAAAGGACACT GCCAAGA	CCTCACAGGCCAG ACAAGTGTA	NM_000428
Latent TGFβ binding protein 3	*LTBP3*	human	2882-2962	TCTACAGCTCAGCCG AGTTCC	TGCCGTAGTTGAC GATGTTGTT	NM_001130144.1
Latent TGFβ binding protein 4	*LTBP4*	human	4003-4068	CGCTGCGTCTCCAAC GA	CCCACTTCCTGCC AGCAC	NM_001042544.1
TGFβ1	*TGFB1*	human	1191-1253	CACCCGCGTGCTAAT GG	TGTGTACTCTGCTT GAACTTGTCAT	NM_000660.3
TGFβ2	*TGFB2*	human	1209-1287	AAAGCCAGAGTGCC TGAACA	AGCGCTGGGTTGG AGATG	NM_001135599.1
TGFβ3	*TGFB3*	human	1164-1264	AAGAAATCCATAAA TTCGACATGATC	CACATTGAAGCGG AAAACCTT	NM_003239.2
TGFbeta Induced	TGFBI	Human	1274-1533	CATCCCAGACTCAGC CAAGA	GAGTTTCCAGGGT CTGTCCA	NM_000358.2

Androgen Receptor	AR	Human	763-961	GACCAGATGGCTGTCATTCA	GGAGCCATCCAAACTCTTGA	NM_001011645.2
Activin Beta A Subunit	INHBA	Human	530-652	AAGTCGGGGAGAACGGGTATGTGG	TCTTCCTGGCTGTTCCTGACTCG	NM_002192.2
HtrA Serine Peptidase 1	HTRA1	Human	1054-1119	TCCGCAACTCAGACATGGAC	GGCCTCCCGAGTTTCCATAG	NM_002775.4
Brain Derived Neurotrophic Factor	BDNF	Human	531-752	AACAATAAGGACGCAGACTT	TGCAGTCTTTTTGTCTGCCG	X91251.1

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*, *LTBP1-4*, *FBN1-2*, *TGFBI*, *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.

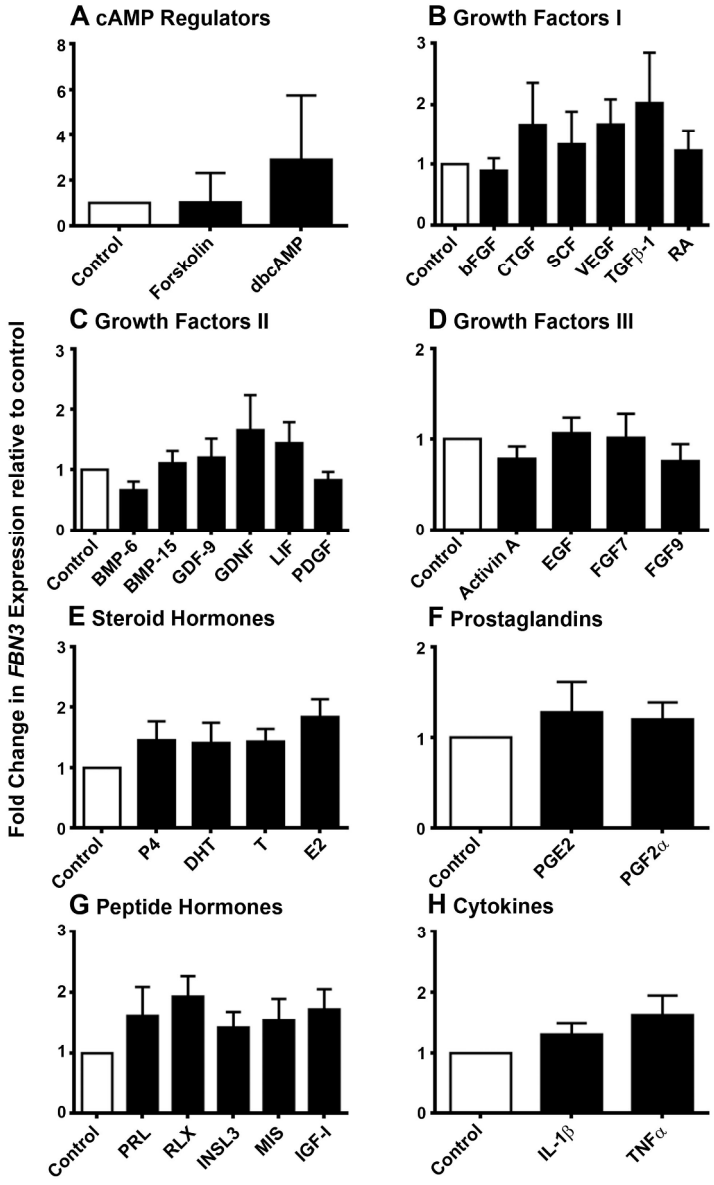

Figure 1
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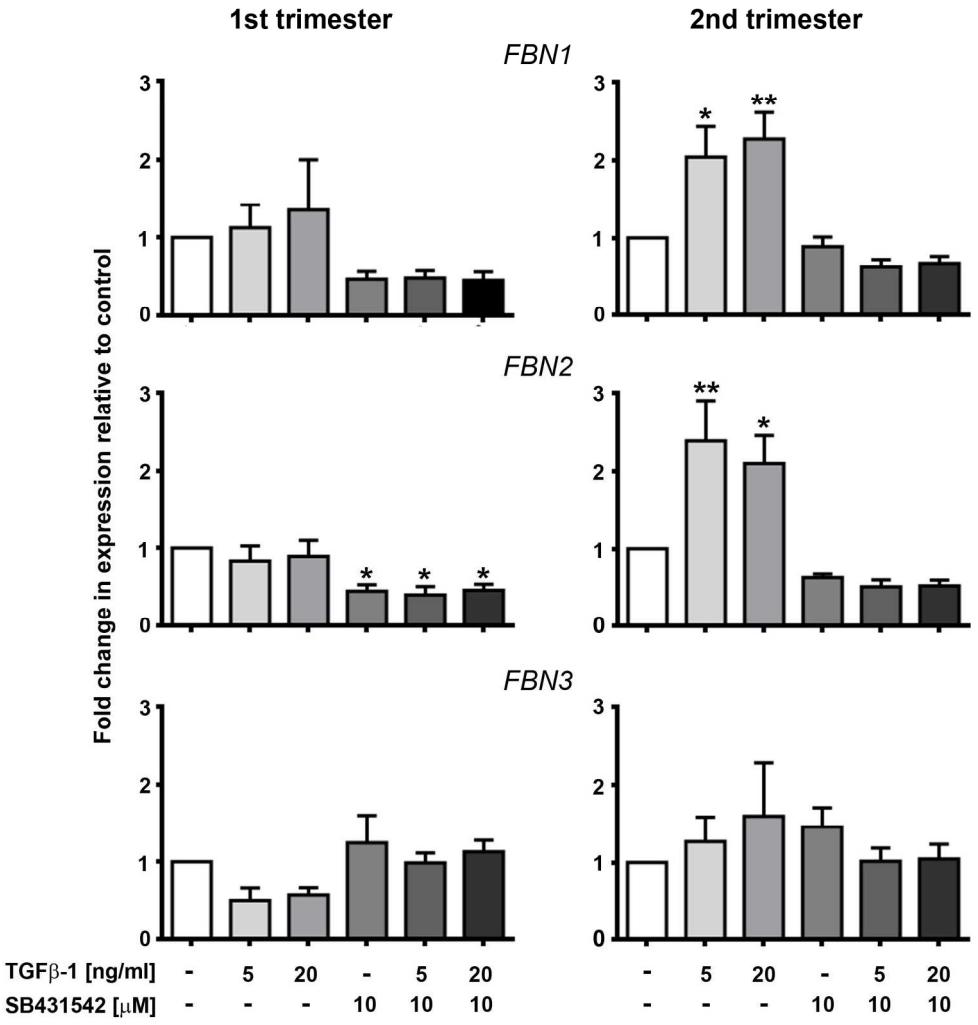

Figure 2
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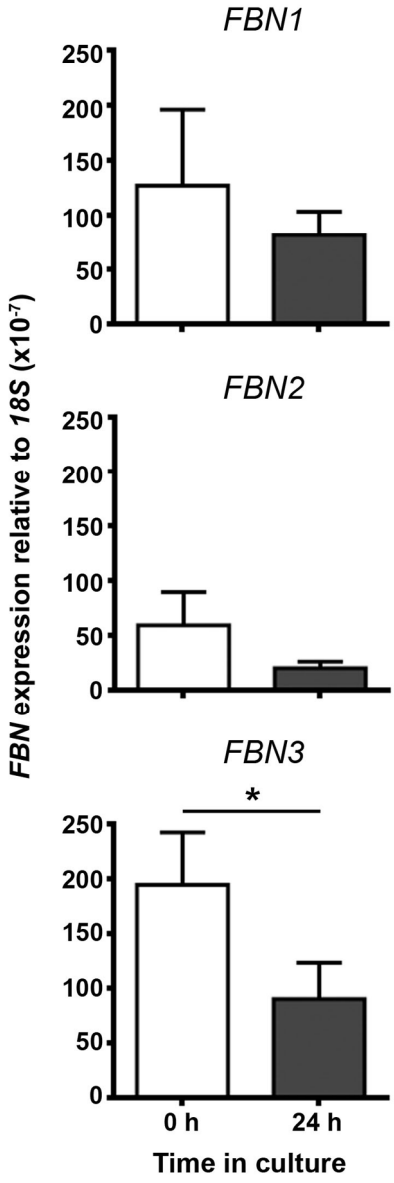

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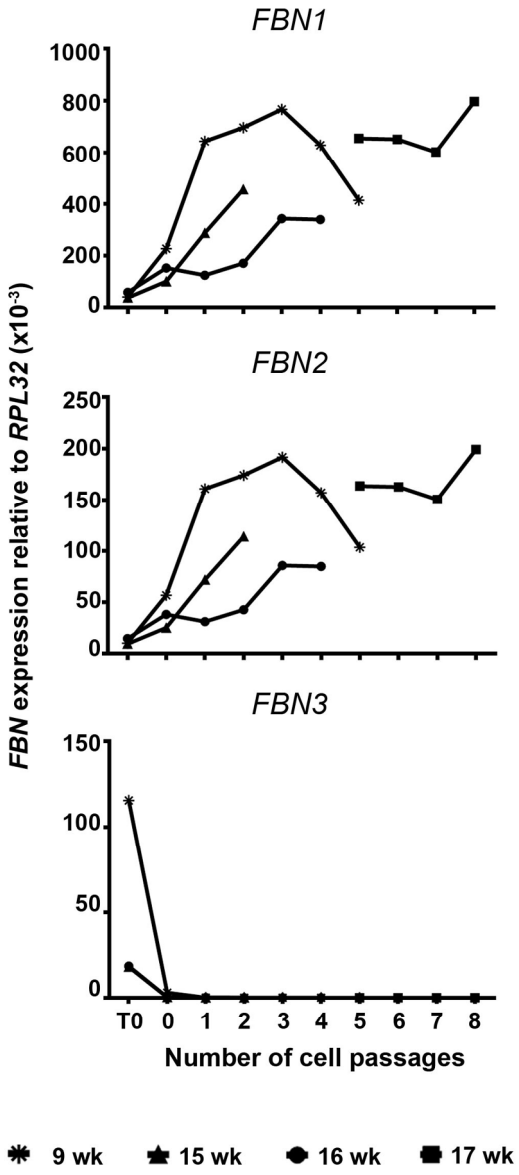

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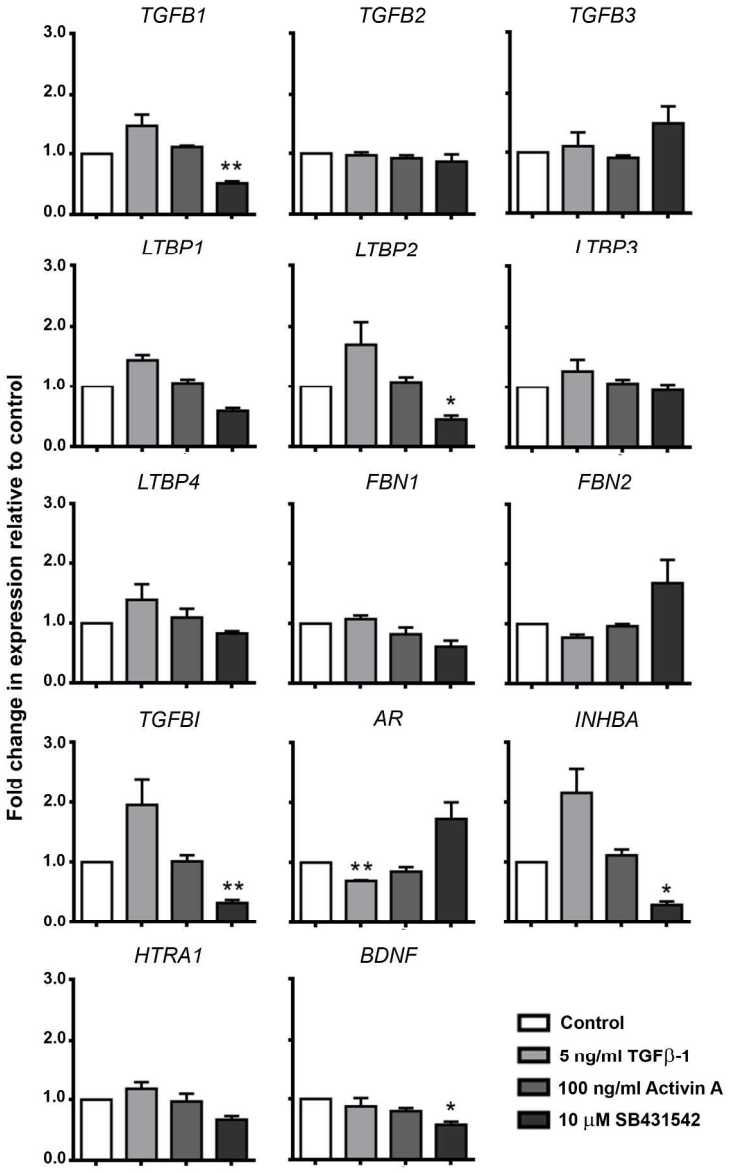

Figure 5
178x281mm (300 x 300 DPI)