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ORIGINAL RESEARCH

# The forkhead transcription factor FOXL2 is expressed in somatic cells of the human ovary prior to follicle formation

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**ABSTRACT:** Interactions between germ cells and surrounding somatic cells are central to ovarian development as well as later function. Disruption of these interactions arising from abnormalities in either cell type can lead to premature ovarian failure (POF). The forkhead transcription factor FOXL2 is a candidate POF factor, and mutations in the *FOXL2* gene are associated with syndromic and non-syndromic ovarian failure. Foxl2-deficient mice display major defects in primordial follicle activation with consequent follicle loss, and earlier roles in gonadal development and sex determination have also been suggested. However, despite its importance no data presently exist on its expression in the developing human ovary. Expression of *FOXL2* mRNA was demonstrated in the human fetal ovary between 8 and 19 weeks gestation, thus from soon after sex determination to primordial follicle development. Expression in the ovary was higher after 14 weeks than at earlier gestation weeks and was very low in the fetal testis at all ages examined. Immunolocalization revealed FOXL2 expression to be confined to somatic cells, both adjacent to germ cells and those located in the developing ovarian stroma. These cells are the site of action of oocyte-derived activin signalling, but *in vitro* treatment of human fetal ovaries with activin failed to reveal any regulation of *FOXL2* transcription by this pathway. In summary, the expression of FOXL2 in somatic cells of the developing human ovary before and during follicle formation supports a conserved and continuing role for this factor in somatic/germ cell interactions from the earliest stages of human ovarian development.

**Key words:** fetal ovary / forkhead / germ cell / primordial follicle / transcription factor

## Introduction

Gonadal development is preceded by germ cell formation and migration from the proximal epiblast of the embryo to the urogenital ridge. During and following gonadal colonization, germ cells proliferate: while the majority subsequently undergo apoptosis, some go on to form primordial follicles in conjunction with surrounding somatic cells (Byskov, 1986). While appropriate germ cell development is clearly essential for subsequent fertility, it is evident that the somatic cells of the gonad are critical for the formation of the microenvironment in which the germ cells can mature (McLaren, 1991; De Felici, 2000), known as the germ cell niche. These interactions between germ and somatic cells regulate germ cell proliferation, meiotic entry and arrest and formation of the primordial follicle complement, the latter of which is required in women to ensure several decades of fertile life as well as the endocrine function necessary for puberty, ovulation, fertilization and establishment of pregnancy. This period of development occurs over many weeks in the human fetal ovary with considerable overlap such that the oogonial mitosis continues beyond the onset of meiosis at 9 weeks *post coitum* (11 weeks gestation) (Gondos *et al.*, 1986; Speed, 1988; Baker and Neal, 1974; Fulton *et al.*, 2005; Bendsen *et al.*, 2006). The less mature germ cells are localized to the periphery of the ovary, with progressively more mature germ cells located deeper in the tissue (Anderson *et al.*, 2007). This spatial and temporal arrangement differs from the rodent where these stages occur in synchrony over 10–12 days, culminating in follicle formation in the immediate neonatal period.

Premature ovarian failure (POF), defined as the onset of the menopause before the age of 40 years, can broadly result from disorders of

follicle formation or premature depletion. It is relatively common, affecting 1% of women (Goswami and Conway, 2005). A range of causes of POF have been identified, including genetic, metabolic, autoimmune, infectious and iatrogenic mechanisms; however, many cases are currently idiopathic. Genetic mechanisms include X chromosome abnormalities and mutations in specific autosomal genes, both dominant and recessive: among these dominant phenotypes is the gene encoding the Forkhead box L2 protein (FOXL2). Mutations in *FOXL2* result in blepharophimosis/ptosis/epicanthus inversus syndrome (BPES). Type I BPES is associated with ovarian failure (although males are fertile) and *FOXL2* mutations producing truncated proteins. Type II BPES is a hypomorphic phenotype associated with the production of abnormally long mutant proteins arising from expansions in a polyalanine tract of unknown function (although correlation between genotype and phenotype is imprecise) (Crisponi *et al*., 2001; De Baere *et al*., 2001, 2003). FOXL2 is a member of the winged helix/forkhead transcription factor family, of which there are 39 known members in the human and mouse genomes with a variety of functions acting as transcriptional activators and repressors (Carlsson and Mahlapuu, 2002). Consistent with the phenotypes seen in BPES, murine *Foxl2* has been localized to Rathke's pouch in the developing pituitary, the developing eyelids and ovarian follicles (Crisponi *et al*., 2001; Cocquet *et al*., 2003). Expression in ovarian somatic cells is detectable from early stages of ovarian development through to adulthood (Cocquet *et al*., 2003), with expression from embryonic day (e)13 in mice (Loffler *et al*., 2003; Pannetier *et al*., 2003).

Female mice that are homozygous for targeted disruptions of the *Foxl2* gene are infertile, due to a failure of granulosa cells to progress from squamous to cuboidal, preventing the formation of primary follicles (Schmidt *et al*., 2004; Uda *et al*., 2004). Defects in primordial follicle formation are also evident (Uda *et al*., 2004) indicating an earlier onset of critical Foxl2 action. Subsequent studies have indicated that *Foxl2* is necessary for gonadal differentiation and that it is one of only a few genes identified as necessary for female gonadal specification (Ottolenghi *et al*., 2005, 2007).

While FOXL2 is known to be expressed by granulosa cells of the adult human ovary (Crisponi *et al*., 2001) and is necessary for human ovarian function, it may also be involved in sex determination and normal formation of primordial follicles. We have therefore explored its expression in the developing human ovary up to the time of primordial follicle formation.

# Methods

## Tissue samples

Human fetal gonads were obtained after medical termination of pregnancy at a range of gestations (8–19 weeks). Gestational age was determined by ultrasound during pregnancy and, for second trimester specimens, confirmed by direct measurement of foot length. Termination of pregnancy was induced by treatment with mifepristone (200 mg orally) followed by misoprostol (200 mg per vaginum every 3 h). None of the terminations was carried out for reasons of fetal abnormality, and all fetuses studied appeared morphologically normal. Collection of fetal samples was approved by the Lothian Research Ethics Committee, and written consent was obtained. Gonads were dissected and snap frozen at −80°C or fixed in Bouin's fluid, transferred into 70% ethanol and processed into paraffin using standard histological methods. Sex of first trimester specimens was determined by detection of *SRY* in males by PCR (Friel *et al*., 2002). A total of 25 fetal specimens were used in these studies.

## RNA extraction and cDNA synthesis

RNA extraction was performed using RNeasy Micro (1st trimester specimens, mouse ovaries) or Mini (2nd trimester specimens and cultures) Kits (Qiagen, Crawley, UK), and concentration and purity of extracted RNA were assessed using a NanoDrop 1000 (NanoDrop Products, Wilmington, DE, USA). RNA was reverse transcribed with Expand Reverse Transcriptase as described previously (Coutts *et al*., 2008; gestation analyses) or using the Superscript III First Strand Synthesis Supermix Kit (Invitrogen, Paisley, UK; cultures).

## Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using ImmoMix Red (Bioline, London, UK). For PCR amplification, 0.5 μl of each cDNA sample was added to a 25 μl reaction containing 1× ImmoMix Red PCR Master Mix and 500 nM forward and reverse primers suitable for amplifying both the human and mouse genes for *FOXL2*: F: 5′-TACTCGTACGTGGCGCTCAT-3′, R: 5′-CTCGTTGAGGCTGAG GTTGT-3′ or Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Hartley *et al*., 2002) as a control. PCR cycling conditions were as follows: 10 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 53°C, and 30 s at 72°C; 10 min at 72°C. Negative controls included RT- samples and nuclease-free water. PCR products were run on a 2.5% agarose gel with a 100 bp ladder (Promega, Southampton, UK) for confirmation of predicted fragment size (*FOXL2*:162 bp; *GAPDH*: 212 bp) and stained with GelRed.

## Quantitative RT–PCR

Quantification of *FOXL2* mRNA expression relative to that for *GAPDH* (gestation analyses) or the housekeeping gene *RPL32* (F: 5′-CATCTCCTTCTCGGCATCA-3′, R: 5′-AACCCTGTTGTCAATGCC TC-3′; cultures) was measured in duplicate on 1/10 dilutions of each cDNA in 10 μl reactions using the Quantitect SYBRGreen Kit (Qiagen) on the the Roche LightCycler 1.0 instrument (gestation analysis) or PowerSYBR Master Mix on a ABI7900HTFast instrument (both Applied Biosystems, Warrington, UK; cultures) each with appropriate 200 nM forward and reverse primers. LightCycler runs consisted of a hot start at 95°C for 15 min, followed by 45 cycles of 15 s at 95°C, 20 s at 56°C and 20 s at 72°C, with fluorescence detection at the end of each extension step. ABI7900HT runs used the manufacturer's default two-step protocol in standard mode for 40 cycles. On both instruments, melt curve analysis was also performed to confirm specific products and standard curves (using increasing dilutions of second trimester ovary cDNA and P2 mouse cDNA) were performed to confirm efficient amplification of each gene before analysis of all samples using the ΔCt method.

## Immunofluorescence

Paraffin-embedded gonads were cut into 5 μm sections and mounted onto electrostatically charged slides (VWR, Leicestershire, UK). After dewaxing and rehydration, antigen retrieval was performed by pressure cooking for 5 min in 0.01 M sodium citrate (pH 6.0). Slides were incubated in 3% hydrogen peroxide in methanol to quench endogenous peroxidases, washed in phosphate-buffered saline (PBS), and blocked in PBS containing 5% Bovine Serum Albumin and 20% Normal Goat Serum (NGS, single immunofluorescence) or 20% normal chicken serum (CS, dual staining). Slides were washed in PBS and incubated with anti-FOXL2 primary antibody (1/500; provided by L. Crisponi; Uda *et al*., 2004) overnight at

4°C. Slides were washed twice in PBS and incubated with secondary antibody (goat anti-rabbit, 1/200 dilution, Dako, Cambridge, UK). Following two washes in PBS, slides were incubated with Tyramide Cy3 Green (Perkin Elmer Life Sciences, Beaconsfield, Bucks, UK, diluted 1:50) for 10 min at room temperature in a covered slide tray then washed in PBS, and counterstained with propidium iodide for 10 min. For co-localizing FOXL2 and OCT4, after the tyramide step slides were washed in 0.05% PBS Tween (PBST), rinsed in PBS, incubated in EnVision Peroxidase Block (Dako), washed in PBS and blocked again in 20% CS. OCT4 antibody was applied overnight at 4°C. Following PBS washes, slides were incubated with secondary antibody (chicken anti-rabbit for FOXL2, chicken anti-goat for OCT4 (both Santa Cruz Biotechnology, Santa Cruz, CA USA, 1/200 dilution), washed again in PBS then incubated in Tyramide Cy3 Red (TSA Plus Cyanine 3 System, diluted 1:50) for 2 min at room temperature in a covered slide tray. Slides were rinsed in PBST then PBS and counterstained with DAPI (4',6-diamidino-2-phenylindole, Sigma). In each case, glass coverslips were mounted using PermaFluor Aqueous Mounting Medium (Beckman Coulter, High Wycombe, UK), and slides visualized and imaged using an LSM510 meta-Confocal microscope (Carl Zeiss, Welwyn Garden City, Herts, UK). Negative controls were achieved by substituting blocking serum for primary antibodies.

### Culture of disaggregated ovaries with activin and follistatin

Human fetal ovaries (17–19 weeks, $n = 5$) were disaggregated and cultured in serum-free medium exactly as described previously (Coutts *et al*., 2008). Cultures were performed in the presence or absence of 10 ng/ml human recombinant activin A or 400 ng/ml human recombinant follistatin (both R&D Systems, Abingdon, UK) for 4 or 24 h. After culture, cells were collected and RNA extracted as described above.

## Results

### *FOXL2* mRNA is present in fetal gonads throughout development

RT–PCR amplification of *FOXL2* mRNA revealed it to be expressed in both first and second trimester ovaries and testes (Fig. 1A). Expression in fetal testis was very low at all gestations however, and consistently below the limit of quantification. In contrast, expression in both first and second trimester ovary was readily quantifiable, and showed higher expression in the second trimester than the first with expression at 14–15 weeks being 2.9-fold that at 8–9 weeks ($P < 0.05$, Fig. 1B). There was no difference in expression between early second trimester (14–15 weeks, germ cell proliferation and entry into meiosis) and later specimens (18–19 weeks: onset of primordial follicle formation).

Expression of the paralogous gene *Foxl2*, in the embryonic and neonatal mouse ovary over the equivalent period of development was also investigated. This demonstrated a progressive increase in *Foxl2* expression between embryonic day 14.5 and post-natal day 2, with an overall increase of 6.4-fold ($P < 0.001$, Fig. 1C).

### FOXL2 is expressed by somatic cells of the human fetal ovary

Immunofluorescent staining localized FOXL2 expression to somatic cells of the human fetal ovary in both the first and second trimesters (Fig. 2). Expression was predominantly nuclear, in keeping with FOXL2 being a transcription factor. FOXL2 was notably present in the somatic cell streams surrounding germ cell clusters (Fig. 2A–C) but it was also expressed by somatic cells intermingled with germ cells (Fig. 2B and C): this population will become the pre-granulosa cells of primordial follicles. These cells also displayed intense staining by immunofluorescence after primordial follicle formation (Fig. 2D, 19 weeks gestation). Somatic cell specificity was confirmed by the absence of colocalization of FOXL2 with the germ cell-specific marker OCT4 (Fig. 2E, 9 weeks gestation; F, 19 weeks): OCT4 labels all germ cells in the first trimester and less mature germ cells, located at the periphery of the ovary, in the second trimester (Anderson *et al*., 2007). FOXL2 expression was not detected in the fetal testis between 8 and 19 weeks gestation (not shown).

### Activin does not regulate *FOXL2* expression

The somatic cells shown here to express FOXL2 are the same population that are the site of action of activin within the second trimester ovary (Coutts *et al*., 2008). The possible regulation of *FOXL2* expression by activin was therefore investigated. Treatment of disaggregated ovarian tissue with activin A (10 ng/ml, $n = 5$) for 4 and 24 h resulted in a small but non-significant reduction in expression of *FOXL2* (78 ± 12% of control at 4 h, Fig. 3). Treatment with the activin antagonist follistatin (400 ng/ml, $n = 5$, 24 h) had no effect on *FOXL2* expression (103 ± 14% of control).

## Discussion

An increasing number of genes associated with POF are being identified (Goswami and Conway, 2005), largely based on laboratory studies investigating the key regulatory factors and pathways in ovarian development (Matzuk and Lamb, 2002; Pangas and Rajkovic, 2006). One such is the forkhead transcription factor FOXL2, mutations in which are associated with POF and BPES (Crisponi *et al*., 2001). Ovarian biopsies in BPES type I show unstimulated primordial follicles ('resistant ovary'), or no follicles (Fraser *et al*., 1988; Nicolino *et al*., 1995). Non-syndromic POF has also been described in association with a polyalanine deletion in FOXL2 (Gersak *et al*., 2004). Mouse models with targeted disruptions of the *Foxl2* locus have been generated (Schmidt *et al*., 2004; Uda *et al*., 2004), indicating a major defect in the activation of primordial follicles due to a failure of granulosa cells to progress from squamous to cuboidal. Although the ovaries of *Foxl2* null mice have a normal oocyte and follicle number in the neonatal period (Schmidt *et al*., 2004; Uda *et al*., 2004), defects in primordial follicle formation are evident (Uda *et al*., 2004). Insect FoxL has also been implicated in oocyte production (Hansen *et al*., 2007). Here we have demonstrated the expression of FOXL2 in the developing human ovary, by somatic but not germ cells, throughout the stages of ovarian development from germ cell proliferation in the first trimester through the onset of meiosis to primordial follicle formation.

Expression of *FOXL2* mRNA was readily detectable in the earliest gestation specimens investigated (8 weeks gestation, 6 post-ovulatory weeks). It therefore remains to be determined how early FOXL2 expression commences in the human ovary, but it clearly precedes the onset of meiosis. In the mouse, expression is first

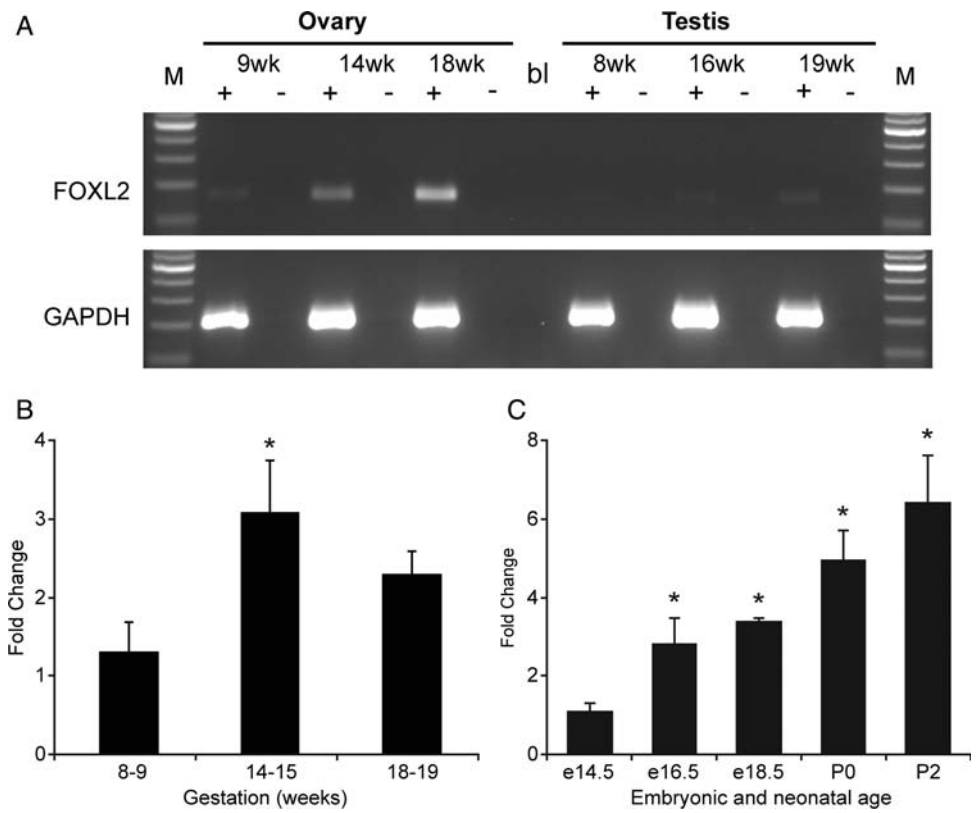


**Figure 1** Detection and quantification of *FOXL2* gene expression in the human fetal gonad. (**A**) Expression of *FOXL2* and *GAPDH* mRNA in the human fetal ovary and testis. Samples at indicated gestational age were analysed by RT–PCR. '+' and '−'indicate the presence or absence of reverse transcriptase during cDNA synthesis. M: 100 bp ladder, bl: water blank in PCR reaction. (**B**) Quantitative PCR measurement of *FOXL2* expression in the human fetal ovary at gestations of 8–9 weeks, 14–15 and 18–19 weeks as indicated, $n = 4–5$ per group. (**C**) Quantitative PCR measurement of *Foxl2* expression in embryonic and post-natal mouse ovary at ages indicated, $n = 3$-5 per group. Both human and mouse data were analysed by ANOVA with Duncan's post hoc test using earliest gestation as comparator, $^*P < 0.05$.

detected at e13 (Loffler *et al.*, 2003; Pannetier *et al.*, 2003), coincident with the onset of meiosis. Expression increased approximately 3-fold between 8–9 and 14–15 weeks gestation, but there was no further increase at weeks 18–19, at which stage primordial follicle formation has started. This therefore differs from the progressive rise demonstrated in the mouse, and also in other factors associated with primordial follicle formation reported by ourselves and other groups (Bayne *et al.*, 2004; Fowler *et al.*, 2009). An additional key role for FOXL2 in earlier developmental processes than follicle formation has been suggested (Ottolenghi *et al.*, 2007; Garcia-Ortiz *et al.*, 2009). This was based on the demonstration that mice with targeted deletions of *Foxl2* show only subtle defects in primordial follicle formation, whilst those doubly deficient for both *Foxl2* and *Wnt4* show a severe ovarian phenotype at birth (i.e. prior to follicle formation) distinct from the *Wnt4*-only null (Ottolenghi *et al.*, 2007). These double-null ovaries showed evidence of sex reversal with tubule formation and markedly increased expression of the male-specific gene *Sox9*, normally expressed only in Sertoli cells. Recent data indicate that Sox9 actively represses Foxl2 expression in Sertoli cells (Wilhelm *et al.*, 2009). The goat polled/intersex syndrome (PIS), characterized by the absence of horns (polledness) and XX female to male sex reversal (Vaiman *et al.*, 1996), is also associated with mutations in *Foxl2* (Pailhoux *et al.*, 2001). These and the present data are consistent with a sex-determining role for Foxl2 in large mammals as well as rodents.

A low level of expression of *FOXL2* mRNA was detected in the human testis in both the first and second trimester. This is in agreement with detection of *Foxl2* mRNA in the developing goat testis and in the mouse testis between e13.5 and e15.5 (Pailhoux *et al.*, 2001; Loffler *et al.*, 2003). We were unable to detect FOXL2 protein in the testis by immunofluorescence, thus the low level of expression may not be of functional importance, in keeping with the preserved fertility of men with BPES and *FOXL2* mutations (Crisponi *et al.*, 2001; De Baere *et al.*, 2003).

Immunofluorescence demonstrated that expression of FOXL2 was confined to the somatic cells of the developing ovary. Foxl2 has been localized previously to the somatic cells of the neonatal and adult rodent ovary (Cocquet *et al.*, 2003; Loffler *et al.*, 2003; Pisarska *et al.*, 2004), and to follicles of the adult human ovary (Crisponi *et al.*, 2001), thus this selective pattern of expression is conserved. A search for downstream targets of Foxl2 identified that expression of the *steroidogenic acute regulatory* (*StaR*) gene was directly repressed by Foxl2 in the adult mouse ovary (Pisarska *et al.*, 2004) but it is uncertain whether this is involved in the primordial follicle activation seen in

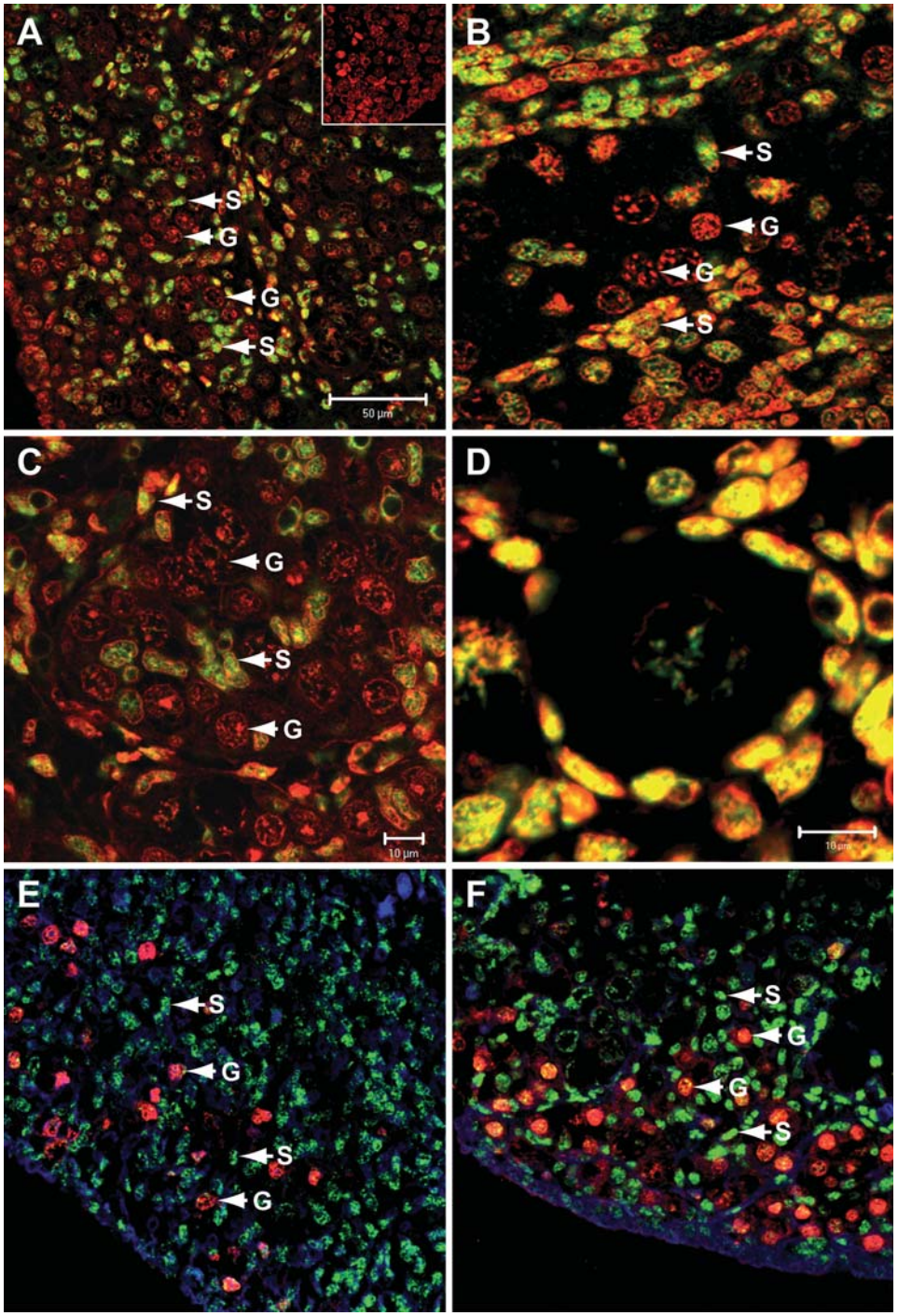


**Figure 2** Immunolocalization of FOXL2 in the human fetal ovary. **A** and **B**, 14 weeks; **C** and **D**, 19 weeks. Positive immunofluorescence for FOXL2 is green, counterstain is propidium iodide (red), hence nuclear FOXL2 is yellow. Inset in (A) is negative control. (**E**, **F**) Double immunofluorescence for FOXL2 (green) and OCT4 (red) in human fetal ovary showing absence of colocalization, with FOXL2 expressed by somatic cells and OCT4 by germ cells. E, 9 weeks gestation; F, 19 weeks. Blue nuclear stain is DAPI. G: germ cell, S: somatic cell. Scale bars are 50 μm (A, applies to E and F) or 10 μm (B, C, D).

Foxl2-deficient mice. In this study, FOXL2 was found to be expressed by both main populations of somatic cells within the developing ovary in the second trimester. One population is intermingled with the germ cells within germ cell cysts or nests: these are syncitial groups of synchronously dividing germ cells, which subsequently break down yielding individual germ cells that are either lost through apoptosis or other pathways, or go on to form the oocytes within primordial follicles. This population of somatic cells is therefore intimately

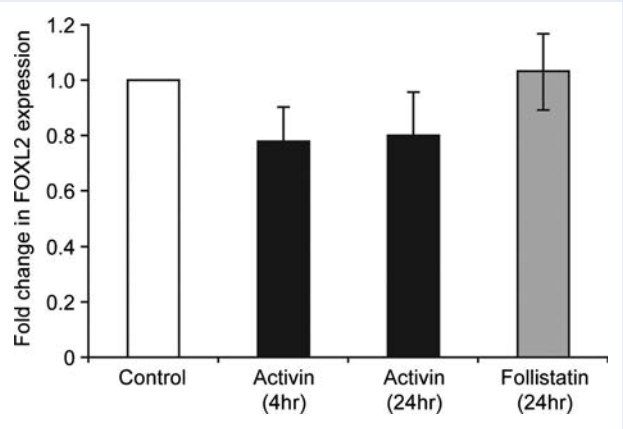


**Figure 3** Expression of *FOXL2* in disaggregated human fetal ovary (17–19 weeks gestation) cultured with activin A (10 ng/ml) for 4 or 24 h as indicated, or with follistatin (400 ng/ml, 24 h) versus untreated control cultures. Mean ± SEM, $n = 5$.

associated with germ cells across a range of developmental stages, regulation of which is essential for the formation of the primordial follicle complement. The second population of somatic cells constitute what have been termed the 'cell streams' (McNatty *et al.*, 2000) which make up a meshwork between and around the germ cell nests, but are separated from them by a basement membrane (Sawyer *et al.*, 2002). This is incomplete at the periphery, where these somatic cells intermingle with the less mature germ cells which are at a similar stage of development to those in the first trimester, on the basis of expression of the pluripotency-associated factor OCT4 (Anderson *et al.*, 2007). The widespread expression of FOXL2 by both populations of somatic cells is consistent with multiple roles during ovarian development, both related to sex determination possibly in conjunction with WNT4 and in germ cell/somatic cell interactions leading to primordial follicle formation.

There is emerging evidence that the transforming growth factor TGFβ family member activin A is a key regulator of ovarian development. Activin βA is specifically expressed by more mature germ cells within the human ovary in the second trimester (Martins da Silva *et al.*, 2004), acting on surrounding somatic cells (Coutts *et al.*, 2008) which are here shown to also be the site of expression of FOXL2. In both humans (*in vitro*) and mice (*in vivo*), activin treatment of the fetal ovary increases the number of germ cells and, in mice, of primordial follicles (Martins da Silva *et al.*, 2004; Bristol-Gould *et al.*, 2006). There are data from a number of developmental systems of interactions between TGFβ members and forkhead transcription factors, including mutual regulation of expression (Zhou *et al.*, 2002; Sommer *et al.*, 2006) and interaction with Smad signalling pathways (Attisano *et al.*, 2001), also demonstrated for Foxl2 (Ellsworth *et al.*, 2003; Blount *et al.*, 2009). The present data suggest that activin does not directly regulate *FOXL2* expression in the human, but it remains possible that FOXL2 and activin-regulated Smad signalling interact to regulate target gene expression in fetal ovarian somatic cells. Additionally it is possible that other oocyte-expressed members of the TGFβ family such as growth and differentiation factor GDF9 or bone morphogenic protein BMP15 which are known to exert effects on ovarian somatic cells (Li *et al.*, 2008) might interact with FOXL2.

In conclusion, FOXL2 is expressed in the somatic cells of the human ovary from early in development. Only very low levels of expression were found in the testis. Expression precedes the onset of meiosis and is consistent with multiple roles for FOXL2 in ovarian function, possibly including sex determination as well as follicle growth.

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