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Strap regulates early follicle development in the mouse ovary

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Serine threonine kinase receptor associated protein (Strap) regulates early follicle development in the mouse ovary

Short title: Strap regulation of early follicle development

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Key words: TGFB, Strap, Smad, oocyte, granulosa cells, preantral, follicle development, follicle culture, ovary culture

Abstract:

The molecular mechanisms involved in regulating the development of small, gonadotrophin-independent follicles are poorly understood; however, many studies have highlighted an essential role for TGFB ligands. Canonical TGFB signalling is dependent upon intracellular Smad proteins that regulate transcription. Strap has been identified in other tissues as an inhibitor of the TGFB-Smad signalling pathway. Therefore, in this study we aimed to determine the expression and role of Strap in the context of early follicle development. Using qPCR, *Strap*, *Smad3* and *Smad7* revealed similar expression profiles in immature ovaries from mice aged 4-16 days containing different populations of early growing follicles. Strap and Smad2/3 proteins co-localised in granulosa cells of small follicles using immunofluorescence. Using an established culture model, neonatal mouse ovary fragments with a high density of small non-growing follicles were used to examine the effects of *Strap* knock down using siRNA and Strap protein inhibition by immuno-neutralisation. Both interventions caused a reduction in the proportion of small, non-growing follicles and an increase in the proportion and size of growing follicles in comparison to untreated controls, suggesting inhibition of Strap facilitates follicle activation. Recombinant Strap protein had no effect on small, non-growing follicles, but increased the mean oocyte size of growing follicles in the neonatal ovary model and also promoted the growth of isolated preantral follicles *in vitro*. Overall findings indicate Strap is expressed in the mouse ovary and is capable of regulating development of small follicles in a stage-dependent manner.

Introduction:

The ovarian reserve consists of a limited number of small follicles each made up of an immature oocyte surrounded by a single layer of granulosa cells (GCs) (Hirshfield 1991, Gougeon 1996). Throughout life, some of these follicles undergo developmental changes that precipitate an increased rate of oocyte growth and GC proliferation (Hirshfield 1991, Da Silva-Buttkus *et al.* 2008, Adhikari & Liu 2009). The molecular mechanisms that regulate this co-ordinated event are still unresolved; however, a number of ovarian-expressed growth factors and signalling molecules have been implicated, particularly in relation to the PI3 kinase and mTOR pathways (Adhikari & Liu 2009). The transforming growth factor beta (TGFB) pathway is also important in this context, since various mutations in specific ligands and receptors have revealed striking effects on early follicle development and consequentially, fertility (Dong *et al.* 1996, Galloway *et al.* 2000, Edson *et al.* 2009). However, very little is known about the molecular regulation of TGFB signalling in small follicles, and how other factors interacting with this pathway may influence early growth or arrest.

TGFB signalling involves ligand binding to specific Type-1 and Type-2 cell surface receptors where the kinase activity is propagated by phosphorylation of receptor-regulated Smad (R-Smad) proteins (Shi & Massague 2003). Activated R-Smads then form a complex with Smad4, which facilitates nuclear import and dwell time, leading to DNA binding and regulation of target genes (Wakefield & Hill 2013). Activins, TGFB1-3 and growth differentiation factor 9 (GDF9) tend to signal mainly via Smad2/3, while anti-Mullerian hormone (AMH) and bone morphogenetic proteins (BMPs) utilise Smad1/5/9 (Wakefield & Hill 2013). Transgenic mouse models harbouring GC-specific (conditional) mutations in each of these R-Smads have revealed fundamental roles for TGFB signalling in growing follicles (Li *et al.* 2008, Pangas *et al.* 2008, Middlebrook *et al.* 2009); however, the distinct role of this

pathway in small, single-layered (non-growing) follicles is still unclear. Recent studies have also highlighted distinct, differential expression of R-Smads in granulosa cells, with Smad1/5/8 localised in multi-layered, growing preantral follicles and Smad2/3 predominantly localised in small, single-layered follicles (Xu *et al.* 2002, Fenwick *et al.* 2013). This indicates that Smad2/3 may be an important regulator of the very early stages of follicle development.

The TGFB pathway is highly conserved across a range of species, and within species is a pivotal regulator of numerous developmental processes that influence cell phenotype (Massague 2012, Wakefield & Hill 2013). Fine control of the TGFB pathway is achieved in part by the different interacting proteins (Wakefield & Hill 2013). One example is Smad7, which is induced by R-Smads and acts to negatively regulate TGFB signalling by binding and promoting R-Smad or receptor degradation (Nakao *et al.* 1997, Itoh & ten Dijke 2007, Yan *et al.* 2016). Although the role of Smad7 during early follicle development has not been determined, recent studies have shown that Smad7 regulates TGFB signals and apoptosis of GCs from growing follicles (Quezada *et al.* 2012, Shimizu *et al.* 2012, Gao *et al.* 2013). In other cell types, the activity of Smad7, and consequently Smad2/3, is further regulated by serine threonine kinase receptor associated protein (Strap) (Datta *et al.* 1998). Strap acts by stabilising a complex between TGFBR1 and Smad7, thereby preventing Smad2 and Smad3 from access to the Type 1 receptor (Datta *et al.* 2000). Strap may therefore represent an indirect, negative regulator of TGFB signalling in the ovary.

In this study we aimed to determine if Strap plays a role in small follicles. We show that transcript levels of *Strap*, *Smad3* and *Smad7* exhibit similar expression profiles in immature mouse ovaries enriched with different proportions of non-growing and early growing follicles. Using an *in vitro* model to monitor follicle activation, we also show that Strap modulation using different interventions leads to changes in the

proportions of non-growing and growing follicles, consistent with a role for the TGFB pathway as an important regulator of early follicle development.

Materials and Methods:

Animals and tissues

All tissues used in this study were obtained from wild-type C57Bl6 mice housed under standard conditions in compliance with the Animals and Scientific Procedures Act, 1986, and also in accordance with approval from the University of Sheffield Training and Competency Officer. Ovaries were dissected from female mice at precisely 4, 8, 13 and 16 days of age (d4, d8, d13, d16) and finely dissected free of adhering tissue in isolation media consisting of Liebovtiz L-15 (Life Technologies, ThermoFisher, Cheshire, UK) with 1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, Dorset, UK). Ovaries were used immediately for culture or were rinsed in PBS and frozen in liquid nitrogen for later RNA analysis or were fixed in 10% neutral buffered formalin (NBF; Sigma) for paraffin embedding, sectioning (5µm) and staining.

RNA extraction, cDNA and quantitative PCR

Total RNA was extracted from d4, d8 and d16 ovaries using RNeasy microcolumns, which includes a DNase digestion step (Qiagen; Crawley, West Sussex, UK). The quality of all RNA samples was assessed with an Agilent 2100 Bioanalyser (Agilent Technologies, Stockport, UK) and only those with an integrity value of 9-10 were included. An equivalent amount of RNA from all samples (50ng) was converted to cDNA using random hexamer primers and SuperScript III reverse transcriptase in accordance with the manufacturer's guidelines (Invitrogen, ThermoFisher, UK). For quantitative PCR assays, a reaction mixture consisting of nuclease-free H_2O, Kapa SYBR Green and ROX dye (Kapa Biosystems Ltd., London, UK) was combined with 500nM gene-specific primers (Table 1) and added to a 384-well plate. All primers were initially confirmed for suitability for qPCR by the presence of a single band at the appropriate size by gel electrophoresis. For each gene, an equal volume (1µl) of

cDNA or H2O (control) was added to each reaction in duplicate. An initial activation step at 95°C (3 minutes) was followed by cycling (40x) at 95°C (3 seconds), 58°C (20 seconds) and 72°C (10 seconds) using an Applied Biosystems 7900HT Fast instrument (Applied Biosystems Inc., ThermoFisher, UK). Fluorescence was recorded at each cycle and also during a final DNA product melting protocol to ensure consistent and specific amplification. Final CT values were normalised against mouse *Atp5b* (PrimerDesign, Southampton, UK), which was stably expressed across all samples and used previously in similar experiments (Fenwick *et al.* 2011). Fold changes relative to d4 ovaries were calculated using $2^{-\Delta\Delta CT}$ (Livak & Schmittgen 2001).

Immunohistochemistry

Approximate midsections of d4, d8 and d16 ovaries were dewaxed in Histochoice solvent (Sigma) and re-hydrated in changes of ethanol of decreasing concentrations. Slides were immersed in 0.01M citrate buffer (pH6.0) and microwaved for 4 x 5 minutes to retrieve antigens before washing in phosphate buffered saline (PBS; pH7.4). Non-specific binding was blocked with CAS-Block (ThermoFisher) for 20 minutes before applying a mixture of rabbit anti-Smad2/3 (1:400; #5678; Cell Signalling Technology, Danvers MA) and mouse anti-Strap (0.3µg/ml; sc-136083; Santa Cruz Biotechnology, Dallas TX) diluted in CAS-Block overnight at 4°C. Primary antibodies were replaced on some sections with equivalent concentrations of non-immune mouse IgG (Vector Laboratories, Bakewell, UK) or rabbit IgG (Vector) to determine non-specific binding. After washes in PBS, all sections were incubated at room temperature for 45 minutes in a mixture of Alexa555 anti-mouse IgG (1:400; Invitrogen) and Alexa488 anti-rabbit IgG (1:400; Invitrogen) diluted in PBS. Sections were washed in PBS and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) and imaged using a Leica inverted SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Images presented in this study

were taken from sections stained in the same run, using the same laser and gain settings.

Ovary fragment culture

Ovaries from d4 mice were finely cut into 6-8 equivalent sized pieces in drops of isolation media and placed into a single well of a 24-well cell culture plate (Sigma). Each well contained 1ml of culture medium consisting of MEM-α (ThermoFisher) supplemented with 10% (v/v) foetal bovine serum (FBS; ThermoFisher), streptomycin sulphate 100µg/ml (Sigma) and penicillin 75µg/ml (Sigma). To assess the effects of exogenous Strap protein on early follicle development, ovary fragments were treated with 0 (diluent only; control), 100 or 200ng/ml Human Unrip full-length recombinant protein (designated rhStrap; ab132509; Abcam, Cambridge, UK). For immunoneutralisation experiments, media was supplemented with either 1 or 10µg/ml rabbit anti-Strap (AB1) IgG (AV48038; Sigma). Control wells were instead supplemented with 0, 1 or 10µg/ml of non-immune rabbit IgG (Vector). For the siRNA experiments, ovary fragments were initially maintained in culture media alone for three days before supplementation with either 1µM Accell mouse Strap siRNA (E-045977; Dharmacon, ThermoFisher, UK), or 1µM Accell non-targeting siRNA (D-001910-10; Dharmacon). Both siRNA contain a mixture of four oligonucleotides provided as a single reagent. Additional control wells without siRNA were also included. One well from each group was collected at the termination of culture for analysis of Strap mRNA expression by qPCR according to the procedures described above. All cultures were carried out three times (n=3 ovaries/group/experiment). After three days at 37°C and 5% CO2, ovary fragments would adhere and spread on the base of the well. This permits visualisation of oocytes with a standard inverted light microscope (Olympus CKX41 with a Nikon camera DS-Fi1). Using this approach, we were able to capture images and measure oocyte diameters using ImageJ (http://imagej.nih.gov/ij/) to monitor growth at specified time points during culture.

Only oocytes with a clearly identifiable boundary were counted and measured. At the end of culture, some ovary fragments were briefly fixed in 10% NBF (Sigma), washed in PBS, treated with 0.25% (v/v) triton X-100 (Sigma) for 15 minutes, and blocked in CAS-Block (ThermoFisher) before applying a solution of rabbit anti-Ddx4 (5µg/ml; ab13840; Abcam) and mouse anti-Amh (1:400; MCA2246; AbD Serotec, Oxford, UK) followed by overnight incubation at 4ºC. Tissues were washed in PBS before applying secondary antibodies as above. After further washing in PBS, tissues were counterstained in 10µg/ml DAPI (Sigma) for 10 minutes before being replaced by PBS. Images were recorded using an Olympus IX73 inverted microscope.

Classification of oocytes

Oocytes from ovary fragment cultures were classified as non-growing, transitional or growing using morphological parameters obtained from haematoxylin and eosin stained sections. One section was analysed from each ovary from the following ages: d4 (n=5), d8 (n=8), d13 (n=5), d16 (n=7). Digital images of each section were recorded with a light microscope and were imported to ImageJ. Oocyte diameters were measured in all follicles that had a clearly identifiable oocyte nucleus. Follicles were classified as non-growing (primordial) if it had a single layer of relatively flat granulosa cells. Transitional follicles had of a mixture of flat and enlarged/cuboidal granulosa cells in a single layer. Follicles were considered to be growing if they contained at least one complete layer of enlarged granulosa cells and a visually larger oocyte. After plotting these distributions (Suppl. Fig 1), we considered that any oocyte smaller than one standard deviation from the mean of the transitional category to be defined as non-growing (<18.3µm), and conversely any oocyte larger than one standard deviation from the mean of the transitional category to be defined as growing (>25.7µm). Any oocyte diameter between these boundaries was defined as transitional (18.3µm-25.7µm). Across all ages, a total of 992 non-growing, 292 transitional and 331 growing preantral follicles were measured.

Preantral follicle culture

Preantral follicles from d16 ovaries (n=7) were mechanically dissected in drops of isolation media using acupuncture needles as previously described (Fenwick *et al.* 2013). Follicles of similar size and appearance were transferred into 96-well plates with each well containing 100µl MEM-α (ThermoFisher) supplemented with 0.1% (w/v) BSA (Sigma), 75µg/ml penicillin (Sigma), 100µg/ml streptomycin sulphate (Sigma) and insulin-transferrin-sodium selenite (ITS; Sigma; 5µg/ml, 5µg/ml, 5ng/ml, respectively). A single follicle was placed into each well; 8 wells contained media with 200ng/ml rhStrap (Abcam), while 8 contained media with diluent only (control). Each plate contained follicles from a single ovary, which were maintained for 72 hours at 37°C and 5% CO2. Follicles were imaged daily using an Olympus CKX41 inverted microscope and diameters were determined from perpendicular measurements taken from the basement membrane of the follicle using ImageJ. Any follicles containing an oocyte that showed signs of degeneration or extrusion were excluded from the analysis.

Statistical analyses

For qPCR data, comparisons between d4, d8 and d16 samples (n=5 or 6 ovaries per age group as stated) were analysed by a Kruskal-Wallis test followed by a post-hoc Dunn's multiple comparisons test. For cultured ovarian fragments, proportions were grouped by treatment and stage and analysed using a two-way ANOVA (n=3 cultures per treatment). Post-hoc Bonferroni multiple comparisons tests were used to identify specific differences (effect of treatment) within each stage (non-growing, transitional, growing). For each experiment, growing oocytes from all three cultures were considered together (number as stated in figure legends) and analysed using a Kruskal-Wallis and Dunn's multiple comparisons test to determine effect of treatment at each time point. Preantral follicle growth was analysed by a two-way repeated

measures ANOVA (n=7 ovaries), with a Bonferroni's multiple comparisons test to determine effects at each time point. All analyses were performed using Prism (v6.0d; Graphpad) with differences considered significant if $P<0.05$.

Results:

Expression of Strap and Smads during early follicle development

Ovaries from 4, 8 and 16 day old mice (d4, d8, d16) enriched with increasing proportions of developing preantral follicles were used to initially determine relative levels of mRNA of the BMP signalling Smads - *Smad1*, *Smad5* and *Smad9* (also known as Smad8), the TGFB signalling Smads – *Smad2* and *Smad3*, the co-Smad – *Smad4*, the inhibitory Smads - *Smad6* and *Smad7,* and *Strap* (Fig 1). Transcript levels of *Smad1* and *Smad5* were significantly higher in d16 ovaries relative to d4 ($P<0.01$ and $P<0.001$, respectively), while *Smad9*, *Smad2* and *Smad4* did not vary between age groups. Conversely, *Smad3*, *Smad7* and *Strap* were significantly lower in d16 ovaries relative to d4 ($P<0.05$ each). The level of *Smad6* mRNA was increased in d8 ovaries relative to d4 only ($P<0.05$).

To further evaluate the relationship between Strap and the TGFB receptor regulated Smads during early follicle development; proteins were immunolocalised in sections of d4, d8 and d16 ovaries (Fig 2). In d4 ovaries densely populated with primordial follicles, Smad2/3 staining was evident in the flattened granulosa cells of single layered follicles, being more intense in cells of primordial and transitional than early growing (primary staged) follicles. Strap was also detectable in granulosa cells of small, single layered follicles and was strong in primordial oocytes. In d8 ovaries, Smad2/3 and Strap were both detectable in granulosa cells of primary follicles and those that had begun to develop a second layer. In d16 ovaries, Smad2/3 and Strap remained co-localised in granulosa cells of multi-layered preantral follicles, although the intensity of Smad2/3 staining was weaker in comparison to smaller follicles. Strap was also detectable in extra-follicular stromal cells, although some of this could be accounted for by non-specific binding of rabbit IgG (Fig 2).

Effect of Strap supplementation on early follicle development

To reconcile the expression of intracellular TGFB pathway components in small follicles, we used a culture model to assess the effect of exogenous Strap on early growth. Ovaries from d4 mice were initially dissected and into fragments and maintained *in vitro* for 3 days to allow them to adhere and spread across the base of the well. This permits visualisation of the oocytes by light microscopy, which can be used as an indicator of follicle growth. After supplementation with 0, 100 or 200ngml recombinant human (rh) Strap protein, oocyte diameters were measured 4 and 7 days later (Fig 3). At day 4, the proportion of oocytes classified as non-growing was similar across the three groups. The proportion of oocytes classified as transitional was slightly reduced in ovary fragments exposed to 200ng/ml rhStrap vs control ($P<0.01$). Conversely, the proportion of oocytes classified as growing was slightly elevated in ovary fragments exposed to 200ngml rhStrap vs control ($P<0.001$) (Fig 3a). Similar trends were evident after 7 days of treatment: the proportion of non-growing oocytes was equivalent, whereas a reduction in the proportion of transitional oocytes was evident for ovary fragments exposed to 200ng/ml rhStrap relative to both 0 and 100ng/ml groups ($P<0.05$ and 0.01, respectively). The proportion of oocytes classified as growing was higher in ovary fragments exposed to 200ng/ml vs 100ng/ml rhStrap ($P<0.05$) (Fig 3b).

When only growing oocytes were considered (i.e. >25.7μm), the median diameter was greater in wells exposed to 200ng/ml rhStrap at both 4 and 7 days ($P<0.01$ vs 0ng/ml). The median diameter of growing oocytes was also increased in fragments exposed to 100ng/ml vs 0ng/ml ($P<0.05$) at 7 days (Fig 3c).

To determine whether an increase in oocyte diameter could be associated with markers of follicle development, some wells were stained with antibodies to clearly delineate oocytes (Ddx4) and to identify granulosa cells from growing preantral follicles (Amh) (Fig 3d). Large Ddx4-positive oocytes were mostly surrounded by Amh positive cells, indicating that the culture system was able to support primordial follicle activation and early preantral development. Although not quantified, Amh staining appeared to be more prevalent in the 200ng/ml rhStrap treated in comparison to controls.

Effect of Strap inhibition on early follicle development

The same ovary fragment model was then used to assess the effects of Strap protein neutralisation on oocyte growth (Fig 4). Fragments were exposed to rabbit anti-Strap IgG (A-S) or non-immune rabbit IgG (N-I) or no IgG (Control) for 7 days. For those treated with 1µg/ml A-S, there was no effect on the proportion of oocytes classified as non-growing, transitional or growing after 4 days (Fig 4a). However, after 7 days, there was a significant reduction in the proportion of non-growing oocytes with a corresponding increase in the proportion of transitional and growing oocytes when compared with N-I or Control ($P<0.001$ each) (Fig 4b). When ovary fragments were treated with 10µg/ml A-S, a reduction in the proportion of non-growing follicles was evident at 4 days, with a corresponding increase in the proportion of growing oocytes ($P<0.001$ vs N-I or Control) (Fig 4c). The same trend was also found at 7 days (Fig 4d).

When only growing oocytes were considered, there was no detectable difference in the median diameter of oocytes treated with 1µg/ml A-S after 4 days; however, after 7 days the median diameter was increased relative to Control ($P<0.05$) and N-I ($P<0.01$) (Fig 4e). By comparison, the median diameter of growing oocytes was increased at 4 days when exposed to a higher concentration (10µg/ml) of A-S

relative to Control (P<0.05) or N-I (P<0.05). This increase was also evident at 7 days (P<0.01 vs Control or P<0.001 vs N-I) (Fig 4f).

In addition to protein inhibition, we then evaluated the effect of *Strap* mRNA inhibition using small interfering RNA (siRNA) in the same culture model (Fig 5). Ovarian fragments were exposed to 1μM oligonucleotides targeting *Strap* mRNA, or 1μM non-targeting oligonucleotides (NT siRNA), or control (0μM). After 4 days of treatment (7 days in culture), *Strap* mRNA was reduced by approximately 50% in the targeting vs the non-targeting groups and control groups; however, the difference was only significant between the targeting and non-targeting groups (P<0.05; Suppl. Fig 2). Based on image analysis, there was a reduction in the proportion of non-growing oocytes in fragments exposed to *Strap* siRNA relative to both control and NT siRNA (P<0.001). This corresponded with an increase in the proportion of growing oocytes in those fragments exposed to *Strap* siRNA relative to controls and NT siRNA (P<0.001) (Fig 5a). The diameter of growing oocytes was also significantly increased relative to control (P<0.05) and NT siRNA groups (P<0.01) (Fig 5b). The increase in oocyte size in fragments exposed to *Strap* siRNA was also associated with many Amh positive granulosa cells (Fig 5c).

Effect of Strap supplementation on preantral follicle growth

We then looked at the effect of exogenous Strap on isolated growing follicles. Preantral follicles from d16 mice were cultured either in the absence (Control) or presence of 200ng/ml rhStrap for 72 hours (Fig 6). Follicles exposed to rhStrap grew at a faster rate than controls; meaning treated follicles were significantly larger in diameter at 48 hours (P<0.01) and 72 hours (P<0.001) relative to untreated follicles (Fig 6a). The morphological appearance of these cultured follicles was similar regardless of treatment group (Fig 6b).

Discussion

In this study we show that Strap, a TGFB pathway-regulating protein, is expressed in small follicles in the mouse ovary and is capable of influencing early follicle development. To examine the role of Strap on follicle growth we used an *in vitro* system where pieces of neonatal (d4) mouse ovaries enriched with small, non-growing follicles were monitored in conditions that aimed to reduce or increase Strap activity. In this context, inhibition of the protein by antibody neutralisation, or the transcript by RNA interference, caused a reduction in the proportion of non-growing follicles and a corresponding increase in the proportion of growing follicles. This shows the primary effect of Strap inhibition is to promote the activation of non-growing follicles. By comparison, when d4 ovaries were treated with exogenous Strap protein, there was no effect on the proportion of non-growing follicles. Interestingly, Strap protein seemed to promote the growth of follicles that had already begun to grow.

In other cell types, Strap is known to act by stabilising a complex between TGFBR1 and Smad7 to effectively inhibit downstream R-Smad signalling (Datta & Moses 2000) (Fig 7A). We therefore looked at R-Smad expression in relation to early follicle development. Initial results in this study confirmed previous observations that Smad2/3 are found in GCs of small, single-layered follicles (Xu *et al.* 2002, Fenwick *et al.* 2013). Smad2/3 protein expression is weak in growing follicles and similarly, the level of *Smad3* mRNA is relatively reduced in d16 ovaries containing many more growing follicles than younger d4 ovaries. We also previously showed that Smad1/5/8 protein is undetectable in small non-growing follicles and is very clearly expressed in GCs of growing preantral follicles (Fenwick *et al.* 2013). This is consistent with levels of *Smad1* and *Smad5* mRNA, which are increased in d16 ovaries relative to d4. Together, the expression patterns of these R-Smads suggest a

stage-specific role for a sub-group of TGFB ligands and receptors, with those that activate the Smad2/3 pathway as potentially key during the earliest stages. Since TGFBs often promote the expression of factors that negatively regulate Smad signalling (Nakao *et al.* 1997, Stopa *et al.* 2000, Shi & Massague 2003, Gao *et al.* 2013), we also looked at *Smad7* and *Strap* in immature mouse ovaries. In this model, *Smad7* and *Strap* transcript levels both followed a similar trend to *Smad3,* but not *Smad1*, *Smad5* or *Smad9* in d4-16 ovaries. In other cell types, Smad7 can inhibit the Type 1 receptors upstream of both Smad2/3 and Smad1/5/9 (Hanyu *et al.* 2001, Kamiya *et al.* 2010). The suggestion from the expression data is that Strap and Smad7 may be associated with the Smad2/3 pathway rather than Smad1/5/9.

Using immunohistochemistry the Strap protein appeared to localise to GCs of single and multi-layered preantral follicles consistent with a role for Strap in modulating Smad2/3 signalling. We did not see a reduction in Strap protein in growing follicles in the same way that Smad2/3 was reduced, although others have reported that the Strap protein is highly stable (Reiner & Datta 2011). The functional role of Strap in follicle development and fertility has not been determined since knocking out the gene in mice causes embryonic lethality (Chen *et al.* 2004). Experimental reduction of Strap in our culture model resulted in an increased proportion of follicles initiating growth. Thus, it is possible that a loss of Strap permits increased Smad2/3 signalling in GCs of small follicles. The role of Smad2/3 signalling in small single-layered follicles is not clear, although mice lacking exon 8 of *Smad3* have significantly more non-growing follicles and fewer growing follicles than wild-type mice at 3 months of age (Tomic *et al.* 2004). In human cultured human ovarian cortical tissues, treatment of with a low concentration of activin inhibited follicle activation, while a higher concentration increased activation (Ding *et al.* 2010). Increased Smad2/3 signalling may therefore be an important prerequisite for follicles to initiate growth.

In the same culture model we observed a positive influence of recombinant Strap protein on growing follicles. Interestingly, there was no effect of exogenous Strap on the small, non-growing follicles. The mechanism is not clear; however, the observed reduction in Smad2/3 expression in follicles with multiple layers of granulosa cells provides some suggestion that loss of this signalling pathway may be important for growing follicles. A recent study in mice showed that inhibition of TGFBR1 in a similar ovary culture model resulted in accelerated oocyte growth and GC proliferation (Wang *et al.* 2014). Therefore, the differential actions of Strap in small follicles may reflect the change in Smad2/3 expression observed between non-growing and growing follicles.

Strap also binds numerous proteins independent of the TGFB-Smad pathway (Fig 7A). For example, Strap can interact with factors that promote proliferation (Seong *et al.* 2005, Kashikar *et al.* 2011, Reiner *et al.* 2011), regulate the epithelial/mesenchymal phenotype (Kashikar *et al.* 2010) and inhibit apoptosis (Jung *et al.* 2010). For these reasons, elevated levels of Strap have been associated with tumourigenesis (Kim *et al.* 2007). Activation of the PI3 kinase/Akt pathway is known to promote early follicle development (Reddy *et al.* 2005, Liu *et al.* 2007, Reddy *et al.* 2008, Adhikari & Liu 2009). Within this pathway, Strap is capable of binding PDK1, where it enhances phosphorylation of target substrates (Seong *et al.* 2005), including mTORC1, the activation of which is positively associated with early follicle development (Adhikari *et al.* 2010, Zhang *et al.* 2014). The observed increase in the proportion and size of growing follicles, as well as the increased rate of growth of isolated preantral follicles treated with recombinant Strap protein may reflect these interactions with Smad-independent pathways. Considering the stage-dependent effects that we observed by either increasing or decreasing Strap activity, we propose a model that explains how this factor may influence early follicle development, in accordance with the expression of TGFB-Smads (Fig 7B).

In this study we used a novel model of ovary culture to study the effects of Strap *in vitro*. Cutting the ovary into fragments causes loss of the original architecture but still allows follicles to grow within a heterogeneous environment of other follicles and ovarian cells. A similar model has been used to evaluate the effects of chemotherapeutics on small follicles (Maiani *et al.* 2012). Culturing whole rodent ovaries has been carried out for some time (Eppig & O'Brien 1996, O'Brien *et al.* 2003); however, the ability of this system to support follicles developing multiple layers of GCs is sometimes limited. In addition, the density of whole ovaries prohibits clear visualisation of individual follicles *in vitro*. In our cultures, we were able to observe oocytes during culture and also show that growing oocytes were associated with GC-specific Amh expression, an additional marker of follicle growth. Intriguingly, this model was amenable to various treatments including siRNA, as well as other macromolecules introduced to the culture media, such as recombinant protein and antibodies. The mechanism that allows the entry of these substances into the cells to interact with target proteins is not clear, but may involve non-specific receptor-independent endocytotic processes, such as clathrin-independent endocytosis or macropinocytosis (Maldonado-Baez *et al.* 2013, Mayor *et al.* 2014). Regardless of the mechanism involved, we observed clear dose-dependent effects of these macromolecules on oocyte growth in this system. Since these outcomes were evaluated in relation to controls indicates that the observed differences were specific to the macromolecule introduced to the system, and may therefore be an invaluable model for testing the effects other exogenous compounds on early follicle development *in vitro*.

In summary we have shown that Strap is expressed and is capable of influencing small follicle growth in the mouse ovary *in vitro*. The mechanism of action is not clear but the co-expression of Smads during the early stages imply interactions with the

TGFB pathway might be important, while the effect of Strap on growing follicles could be via alternative signalling pathways. The subtle influences of proteins that interact with these pathways potentially lead to more substantial effects on the rate and number of follicles that develop to more advanced stages.

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652 **Table 1.** Primer sequences used for PCR assays in mouse ovaries.

Gene Symbol	Primer Sequence (5'→3')	GenBank Accession	Product Size (bp)
Smad1	Fwd: ACCTGCTTACCTGCCTCCT Rev: GCCTGAACATCTCCTCTGCT	NM_008539.3	114
Smad2	Fwd: CGTCCATCTTGCCATTCAC Rev: GTCCATTCTGCTCTCCACCA	NM_001252481.1	102
Smad3	Fwd: GTCAAAGAACACCGATTCCA Rev: TCAAGCCACCAGAACAGAAG	NM_016769.4	154
Smad4	Fwd: CGGCGATTGTGCATTCTCAG Rev: CCTGGAAATGGTTAGGGCGT	NM_008540.2	209
Smad5	Fwd: CCTTGCTCATCTCCCTGTCT Rev: CCGTGAATCTCCTTTCTGTG	NM_001164041.1	173
Smad6	Fwd: TGCAACCCCTACCACTTCA Rev: GCTGGCATCTGAGAATTCA	NM_008542.3	180
Smad7	Fwd: AGTCAAGAGGCTGTGTTGCTGT Rev: CATTGGGTATCTGGAGTAAGGA	NM_001042660.1	130
Smad9	Fwd: GTCTGACCTTGCAGATGGCT Rev: TAGGTGCCAGGCTGAGAGAT	NM_019483.5	235
Strap	Fwd: GGCTACTTTCTGATCAGCGC Rev: CTGAGACCGCATCCCATACT	NM_011499.3	187

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Figure 1:

Relative mRNA expression of *Smads* and *Strap* in juvenile mouse ovaries. Transcripts were amplified from cDNA derived from whole mouse ovaries by qPCR and normalised with endogenous *Atp5b* as described in the *materials and methods*. Fold changes (mean ±SEM) in d8 and d16 ovaries are shown relative to d4. *Smad1-3*, *5*, *7*, n=6 ovaries each age; *Smad4*, *Smad6*, *Smad9* and *Strap*, n=5 ovaries each age; *P<0.05 vs d4, **P<0.01 vs d4, ***P<0.001 vs d4, Dunn's multiple comparisons test.

Figure 2:

Co-localisation of Smad2/3 and Strap in juvenile mouse ovaries highlighting relationship with these proteins and early follicle development. Smad2/3 is labelled in green (a,d,g) and Strap is labelled in red (b,e,h). Merged images (c,f,i) represent ovaries from mice at d4 (a-c), d8 (d-f) and d16 (g-i). Arrows, arrowheads and asterisks indicate primordial, primary and multi-layered preantral follicles, respectively. Control sections from d16 mice were incubated with mouse IgG (j), rabbit IgG (k) or dilution buffer (l) in place of the primary antibodies. All sections were counterstained with DAPI (blue). Scale = 100μm.

Figure 3:

Effect of Strap supplementation on small follicle growth *in vitro*. Ovaries from d4 mice were cut into small fragments and maintained under standard culture conditions (Control) or supplemented with 100ng/ml (100) or 200ng/ml (200) recombinant human Strap (rhStrap). Oocytes were classified as non-growing, transitional or growing and proportions in each category are shown at day 3 (a) and day 6 of culture (b). Mean ±95% CI; n=3 cultures; *P<0.05, **P<0.01, Bonferroni's multiple comparisons test. At day 3 and 6 of culture, the median diameters (horizontal bars) of growing oocytes (>25.7μm) in each treatment group are plotted (c) *P<0.05,

**P<0.01, Dunn's multiple comparisons test (Day 3 n=101, 117, 164 and Day 6 n=169, 221, 263 oocytes for C, 100 and 200 groups, respectively). At day 6 some wells were co-stained with Ddx4 (green) to label oocytes and Amh (red) to label granulosa cells of growing follicles (d). Cell nuclei were counterstained with DAPI (blue). Scale bar = 50μm.

Figure 4:

Effect of Strap immuno-neutralisation on small follicle growth *in vitro*. Ovaries from d4 mice were cut into small fragments and maintained under standard culture conditions (Control) or supplemented with non-immune IgG (N-I) or anti-Strap IgG (A-S). Oocytes were classified as non-growing, transitional or growing and proportions in each category are shown at day 4 (a) and day 7 (b) of culture with 1ug/ml supplementation and day 4 (c) and day 7 (d) of culture with 10μg/ml supplementation. Mean ±95% CI; n=3 cultures; ***P<0.001 vs Control and N-I groups, Bonferroni's multiple comparisons test. At day 4 and 7 of culture, the median diameters (horizontal bars) of growing oocytes (>25.7μm) in the presence of 1μg/ml (Day 4 n=65, 73, 89 and Day 7 n=81, 86, 101 oocytes for C, 1μg N-I and 1μg A-S groups, respectively) (e) and 10μg/ml (f) IgG are plotted (Day 4 n=96, 97, 117 and Day 7 n=102, 98, 123 oocytes for C, 10μg N-I and 10μg A-S groups, respectively). *P<0.05, **P<0.01, ***P<0.001, Dunn's multiple comparisons test.

Figure 5:

Effect of Strap mRNA knock down on small follicle growth *in vitro*. Ovaries from d4 mice were cut into small fragments and maintained under standard culture conditions (Control) or supplemented with non-targeting siRNA (NT siRNA) or siRNA complementary to Strap mRNA (Strap siRNA). Oocytes were classified as non-growing, transitional or growing and proportions in each category are shown for each treatment after 4 days (7 days of culture) (a). Mean ±95% CI; n=3 cultures;

***P<0.001 vs Control and NT siRNA groups, Bonferroni's multiple comparisons test. At day 7 of culture, the median diameters (horizontal bars) of growing oocytes (>25.7µm) for the different groups are plotted (b). *P<0.05, **P<0.01, Dunn's multiple comparisons test (n=89, 80, 93 oocytes for C, NT siRNA and Strap siRNA groups, respectively). At day 7 some wells were co-stained with Ddx4 (green) to label oocytes and Amh (red) to label granulosa cells of growing follicles (c). Scale = 100µm.

Figure 6:

Effect of Strap supplementation on preantral follicle growth *in vitro*. Preantral follicles were isolated from d16 mice and maintained under standard culture conditions (Control) or supplemented with 200ng/ml recombinant human Strap (rhStrap). 6-8 follicles were included per group for each ovary and diameters measured daily. Only morphologically healthy follicles were included in the analysis. Growth is plotted as the difference in diameter relative to 0h and represents the mean (±95% CI) of 7 ovaries (n=7) at each time point (a). **P<0.01, ***P<0.001 vs control at the indicated time point. Bonferroni's multiple comparisons test. Examples of cultured preantral follicles are shown (b). Scale = 50µm.

Figure 7:

Regulation of the TGFB signalling pathway by Strap in the context of early follicle development. (A) Canonical TGFB signalling involves ligands such as Gdf9, TGFB1-3 or activins associating with Type I and II receptors, leading to recruitment and activation of R-Smads (Smad2/3) and Smad4 complexes that enter the nucleus to regulate gene transcription in association with other co-factors (triangle, pentagon). Strap inhibits the canonical pathway by forming a complex with the Type I receptor and Smad7. Strap can also interact with other pathways such as PI3K and MAPK to promote cell proliferation. (B) Granulosa cells of small single-layered follicles, which

are relatively slow growing, express Smad2/3. A reduction in Strap may facilitate canonical TGFB signalling and promote the transition of growth through the early follicle stages. Further follicle growth is associated with a reduction in Smad2/3, which may be facilitated by the ascribed role of Strap in inhibiting TGFB signalling and driving TGFB independent pathways associated with cell proliferation and follicle growth.

Supplemental Figure 1

Distributions of oocyte sizes in small follicles from sections of mouse ovaries. Follicles were classified and corresponding diameters were measured from H&E stained sections. One section was analysed from each ovary from the following ages: d4 (n=5), d8 (n=8), d13 (n=5), d16 (n=7). *Non-growing* oocytes were measured from primordial staged follicles consisting of a small oocyte surrounded by a single layer of relatively flat granulosa cells. *Transitional* oocytes were measured from follicles that also had a single layer of granulosa cells but some of which were no longer flat in appearance. These follicles were named as such because their growth status is ambiguous. *Growing* oocytes were measured from follicles that were clearly larger in appearance and had at least one complete layer of cuboidal granulosa cells - these included follicles classified as primary staged and larger. Individual points represent a single oocyte (n=992 non-growing, n=292 transitional, n=331 growing) (mean ±s.d.). Considering the overlap in in oocyte size distributions between the three groups, our criteria for classifying oocytes in the culture experiments were based on the mean ± 1 s.d. of the transitional group (i.e. above and below the shaded region indicated on the graph). Oocytes with a diameter of <18.3μm were assigned to the *non-growing* category, 18.3-25.7μm were assigned as *transitional*, and oocyte diameters >25.7 were assigned as *growing*.

Supplemental Figure 2

Expression of *Strap* mRNA in samples of cultured neonatal mouse ovary fragments after treatment with siRNA. Ovaries from d4 mice were cut into small fragments and were cultured for 3 days (6-8 equivalent sized pieces per well). Cultures were then either maintained under standard conditions (Control) or supplemented with 1μM non-targeting siRNA (NT siRNA) or 1μM siRNA complementary to Strap mRNA (Strap siRNA). After 4 days of treatment, samples were pooled from 3 wells to obtain sufficient RNA from each group and processed for cDNA synthesis. Expression of *Strap* mRNA was determined in relation to the internal reference gene *Atp5b* and expressed as fold change relative to Control group using the formula $2^{-\Delta\Delta CT}$ as described in *Materials and Methods*. Fold changes (mean ±SEM) are shown for 4 technical replicates (n=4). *P<0.05, One-way ANOVA and Bonferroni's multiple comparisons test.

Figure 1

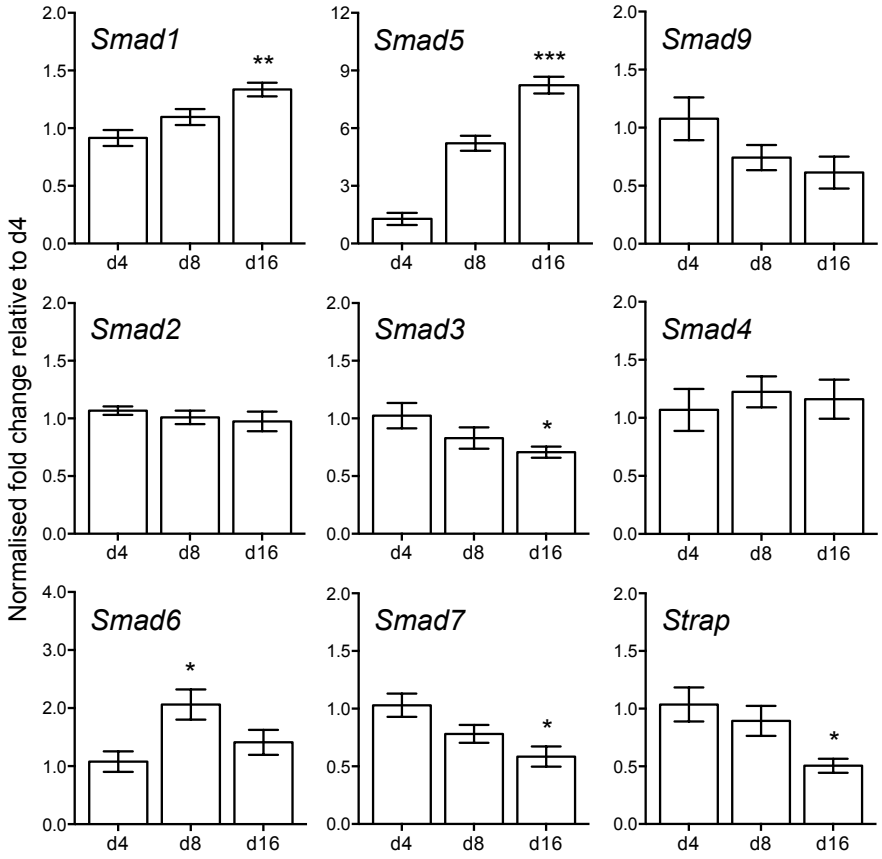

Normalised fold change relative to d4
Smad1
**
Smad5
***
Smad9
Smad2
Smad3
*
Smad4
Smad6
*
Smad7
*
Strap
*
d4
d8
d16

Figure 2

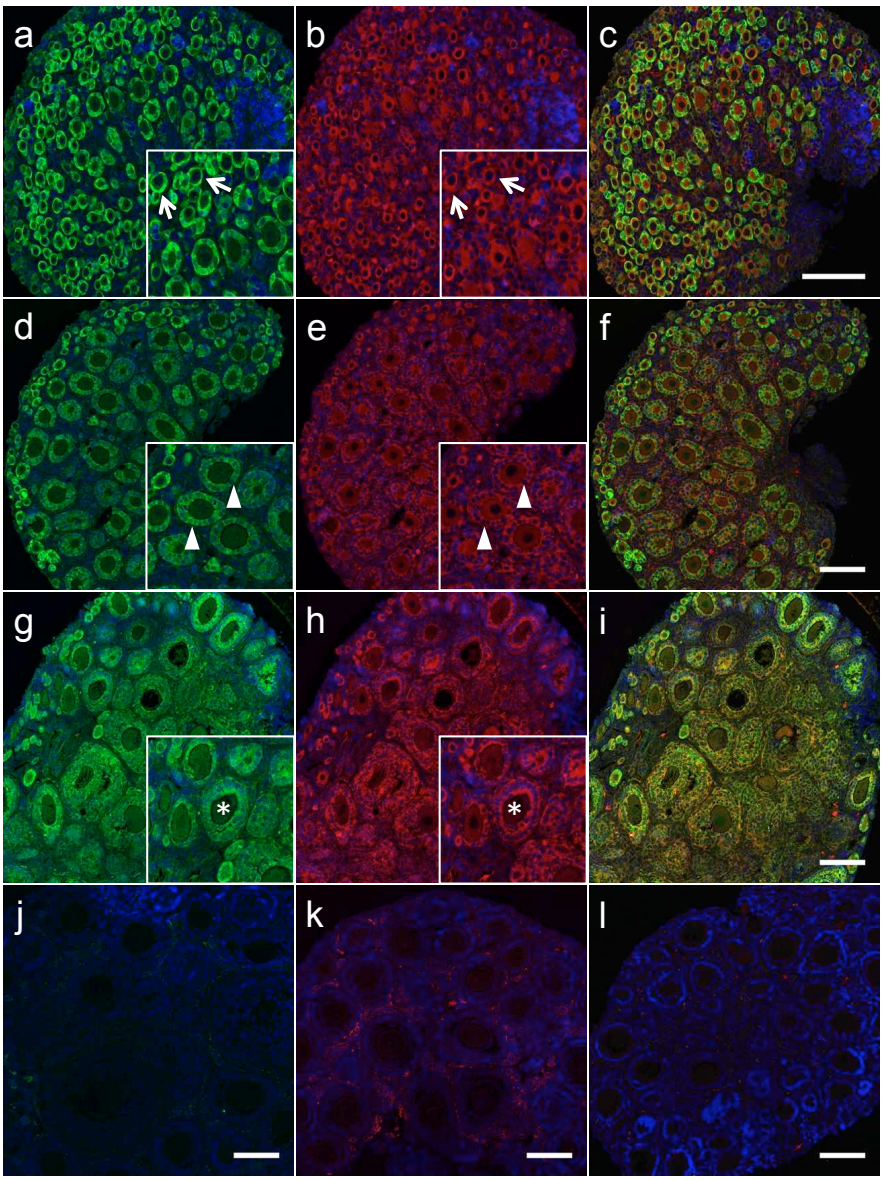


Figure 3

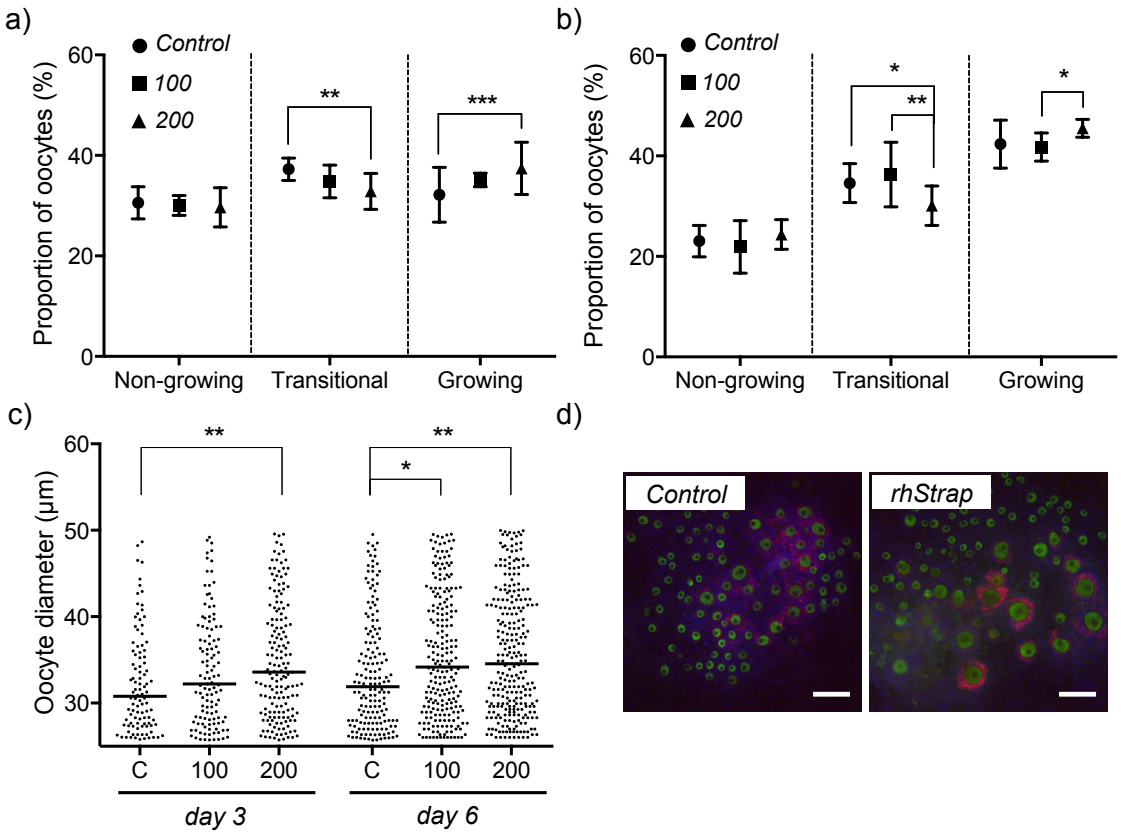

a)
Control
100
200
Proportion of oocytes (%)
60
40
20
0
**
***
Non-growing
Transitional
Growing
b)
Control
100
200
Proportion of oocytes (%)
60
40
20
0
*
**
*
Non-growing
Transitional
Growing
c)
Oocyte diameter (µm)
60
50
40
30
**
**
*
C
100
200
C
100
200
day 3
day 6
d)
Control
rhStrap

Figure 4

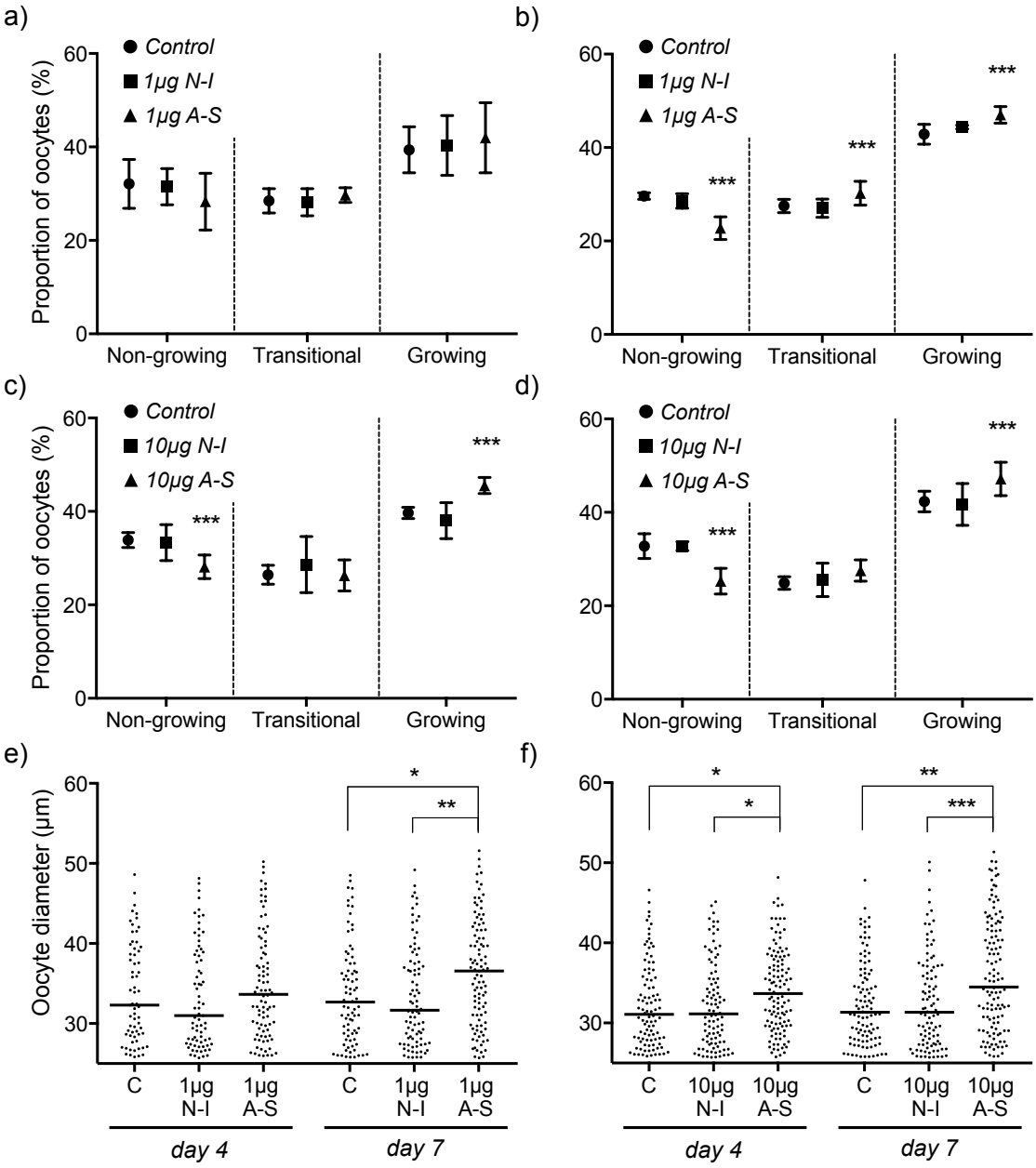

a)
b)
c)
d)
e)
f)
Control
1µg N-I
1µg A-S
10µg N-I
10µg A-S
Proportion of oocytes (%)
Non-growing
Transitional
Growing
Oocyte diameter (µm)
C
1µg N-I
1µg A-S
10µg N-I
10µg A-S
day 4
day 7

Figure 5

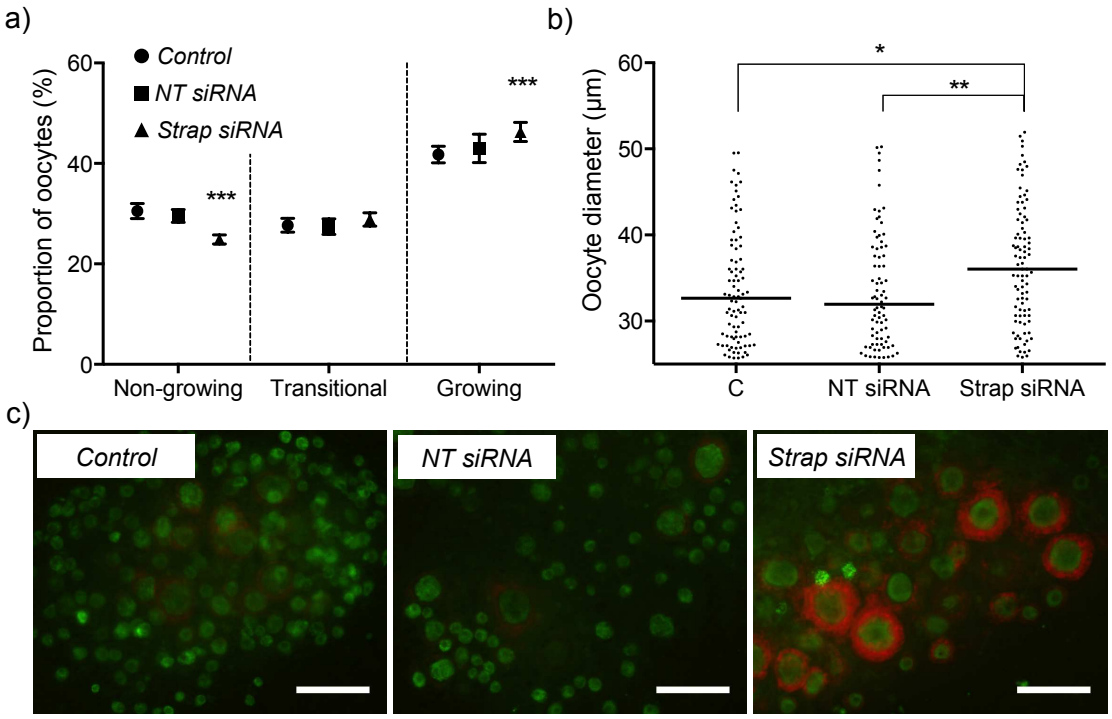

a)
Control
NT siRNA
Strap siRNA
Proportion of oocytes (%)
60
40
20
0
***
***
Non-growing
Transitional
Growing
b)
Oocyte diameter (µm)
60
50
40
30
*
**
C
NT siRNA
Strap siRNA
c)
Control
NT siRNA
Strap siRNA

Figure 6

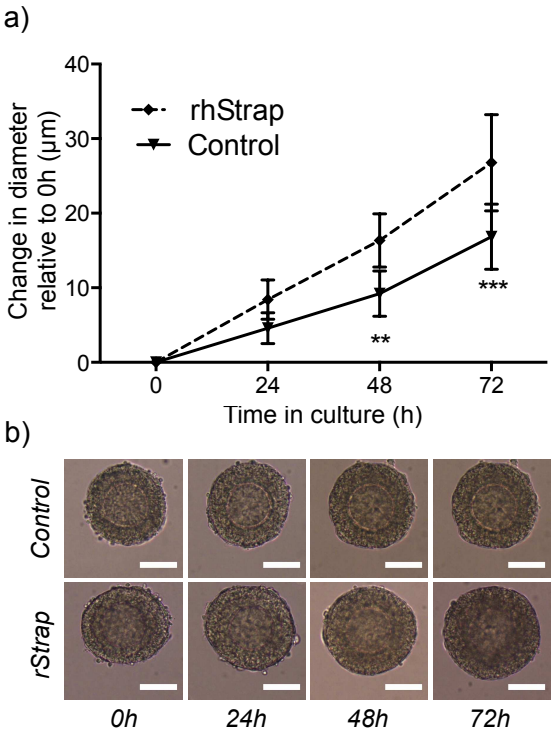
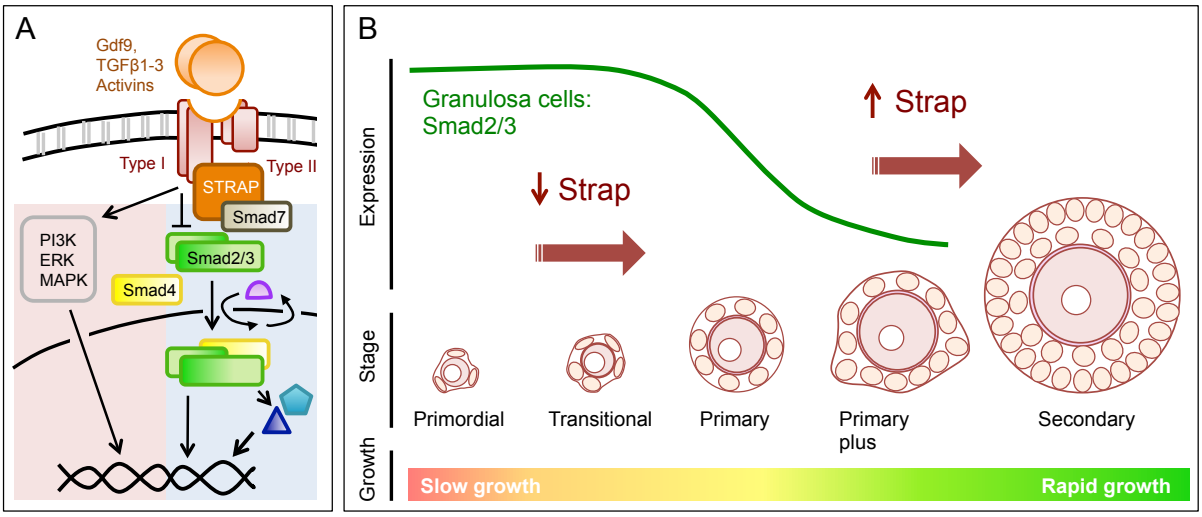
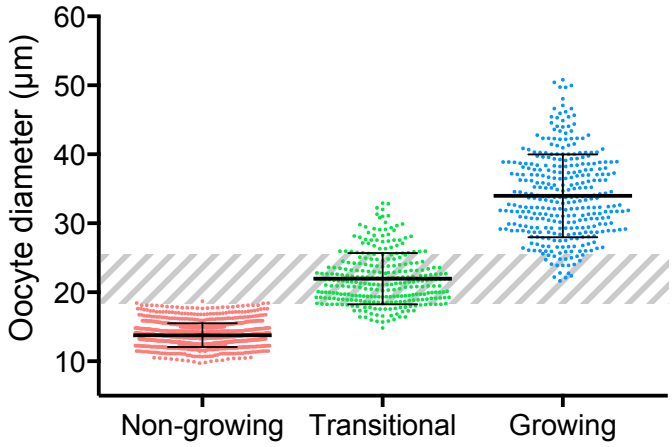

a)
Change in diameter relative to 0h (µm)
40
30
20
10
0
rhStrap
Control
**
***
0
24
48
72
Time in culture (h)
b)
Control
rStrap
0h
24h
48h
72h

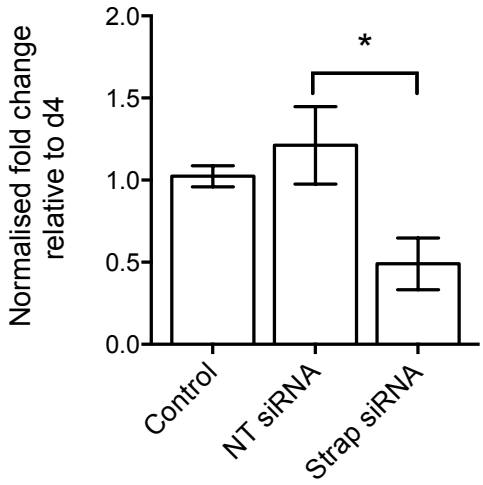
Figure 7



Supplementary Figure 1



Supplementary Figure 2



Supplementary Table 1 Characteristics of qPCR assays

Gene	Primer efficiency (CT range)*	Intra-assay variation (%CV)**	Range of CT values across all ovary samples (d4, d8, d16)	
			Min	Max
Smad1	98% (26.5-36)	1.71	25.0	29.3
Smad2	109% (26.5-31)	2.70	25.6	29.7
Smad3	99% (27-36)	0.75	23.7	27.9
Smad4	105% (24-33)	4.88	25.7	28.8
Smad5	103% (27-34.5)	0.91	24.1	30.1
Smad6	89% (30-35)	0.84	28.3	33.0
Smad7	109% (27.5-34)	1.00	29.0	32.6
Smad9	103% (29-35.5)	1.82	30.1	33.0
Strap	95% (24.5-32)	0.68	25.6	27.9

*Primer efficiency was evaluated using a dilution series of adult mouse ovary cDNA in triplicate. Mean efficiency (%) was determined from the slope of the log-linear portion of the curve (in parentheses) using the equation $(10^{-1/slope}-1)*100$.

**Intra-assay variation was calculated from CT values from each of the dilution standards and is presented as mean %CV across the curve.