

Investigating the Role of TGFB Signalling in Preantral Follicle Development

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Statement of Originality

All experiments included in this thesis were performed by the author unless otherwise stated throughout the text and figure legends. Where the work of others is quoted the source is always given.

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Abstract

The neonatal ovary encompasses a finite pool of primordial follicles which serve the need of the entire reproductive lifespan. Follicle activation is therefore a tightly controlled process, the rate of which has a profound influence on fertility. Despite this the exact mechanisms responsible for maintaining the primordial follicle pool or, conversely, for activating the initiation of follicle recruitment remain unknown. Many of the extracellular signalling molecules implicated in early preantral follicle development are members of the transforming growth factor beta (TGFB) superfamily. The aim of this thesis was to examine the role of TGFB signalling in primordial follicle activation and subsequent early preantral follicle development. TGFB signalling is classically facilitated by the SMAD intracellular transcription factors. The SMAD2/3 cascade is the major TGFB signalling pathway expressed in the early preantral follicle. Pharmacological inhibition of the upstream receptors of the SMAD2/3 signalling cascade ALK4/5/7 with small molecule inhibitor SB505124 accelerated primordial follicle activation in a neonatal ovary culture while in a paradoxical manner inhibited development of the small multilayered preantral follicle in an isolated follicle culture. A screen of ligands that converge preferentially to the SMAD2/3 pathway highlighted TGFB2 as the most abundantly expressed in the early preantral follicle. TGFB2 inhibited primordial follicle activation in the neonatal ovary culture while in a differential manner promoted the growth of isolated small multilayered preantral follicles suggesting the action of TGFB2 is dependent on the developmental stage of the follicle. Pharmacological inhibition of ALK4/5/7 (SB505124) and the MAPK/ERK (U0126) signalling cascade ablated TGFB2 induced preantral follicle growth suggesting the coordinated action of SMAD and non-SMAD signalling underlies the context dependent action of TGFB2 in preantral follicle development. In conclusion these novel data suggest that TGFB signalling plays an important role in both maintenance of the primordial follicle pool and early stage preantral follicle development.

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Dedication

I dedicate this thesis to my parents, my scientific inspiration.

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List of Abbreviations

ALK	Activin-like kinase
AMH	Anti-Müllerian hormone
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CCND2	Cyclin D2
CDK	Cyclin-dependent kinase
CDKN1B	Cyclin-dependent kinase inhibitor 1B
cDNA	Complementary DNA
c-kit	Kit tyrosine kinase receptor
Co-SMAD	Common SMAD
Cyp17 α	Cytochrome P450, family 17, subfamily A, polypeptide 1
DAPI	4, 6-diamino-2-phenylindole
DDX4	Mouse vasa protein
DMSO	Dimethyl sulfoxide
dpp	Days postpartum
DSM	Dorsomorphin
ED	Embryonic day
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase

FAST	Forkhead activin signal transducer
FBS	Fetal bovine serum
FHSR	Follicle stimulation hormone receptor
FSH	Follicle stimulating hormone
FST	Follistatin
GC	Granulosa cell
GDF	Growth differentiation factor
GREL	Gonadal ridge epithelial-like
gsc	Goosecoid
HRP	Horseradish peroxidase
HTRA1	HtrA serine peptidase 1
Id	Inhibitor of DNA-binding
IgG	Immunoglobulin G
INHA	Inhibin alpha
INHBA	Inhibin beta-A
INHBB	Inhibin beta-B
I-SMAD	Inhibitory SMAD
ITS	Insulin transferrin sodium selenite
JNK	c-Jun N-terminal kinase
KGF	Keratinocyte growth factor
KL	Kit ligand
LAP	Latency associated peptide

MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
MH	Mad homology
MMP	Matrix metalloproteinase
mTORC1	Mammalian target of rapamycin complex 1
NBF	Neutral buffered formalin
Oo	Oocyte
Ose	Ovarian surface epithelium
P	Primary follicle
P ⁺	Primary plus follicle
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
Pf	Primordial follicle
PGC	Primordial germ cell
PI3K	Phosphatidylinositol-3-kinase
POI	Premature ovarian insufficiency
RGM	Repulsive guidance molecule
RIPA	Radioimmunoprecipitation assay

R-SMAD	Receptor regulated SMAD
RT-PCR	Real-time polymerase chain reaction
S	Secondary follicle
SBE	SMAD binding element
Smurf	SMAD ubiquitination-regulatory factor
SNAI	Snail family zinc finger
t	Transitional follicle
TBS	Tris-buffered saline
TGFB	Transforming growth factor beta
TGFBR	Transforming growth factor beta receptor
TIMP1	Tissue inhibitor of metalloproteinase 1
TSC	Tuberous sclerosis complex
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
α – MEM	Minimum essential medium alpha

Chapter 1 Introduction

1.1 The ovary

The mammalian ovary is the female reproductive organ within which lie the finite resource of germ cells required for the entire reproductive lifespan. The ovary is organised into an outer cortex and an inner medulla enclosed by the ovarian surface epithelium. The ovarian follicles, which constitute the functional units of the ovary, are situated in the stroma of the cortex. The principle functions of the ovary are the cyclic release of a mature oocyte capable of undergoing fertilisation and the synthesis and secretion of hormones necessary for female reproduction.

1.2 Formation of the primordial follicle

The primordial follicle represents the fundamental developmental unit of the ovary and comprises a single oocyte arrested in meiotic prophase I surrounded by flattened somatic granulosa cells (GC) (Hirshfield, 1991). Oocytes in primordial follicles are derived from primordial germ cells (PGC) that are specified in early embryogenesis (Reviewed in Ewen and Koopman, 2010). In mice, precursors of PGCs have been identified as early as embryonic day (ED) 6 – 6.5 (Ohinata et al., 2005). Following a period of proliferation and migration, PGCs arrive at the gonadal ridge between ED 10.5 – 12.5 in the mouse (Godin et al., 1990; Molyneaux et al., 2001). Further mitotic division and aggregation results in the formation of germ cell cysts (ED 10.5 – 13.5), following which germ cells cease clonal division and enter meiotic prophase I (Gomperts et al., 1994; McLaren & Southee, 1997; Pepling & Spradling, 1998). Following entry into meiosis the PGC is now termed the oocyte. Subsequent formation of the primordial follicle, which occurs from ED 17.5 – 4 days postpartum (dpp) is a consequence of somatic cell ingression and germ cell cyst breakdown, whereby individual oocytes become segregated and enveloped by flattened pre-GCs forming the primordial follicle unit (Pepling & Spradling, 2001).

The pre-GC is derived from a somatic precursor cell in the genital ridge (Reviewed in Hummitzsch et al, 2015; Liu et al, 2010). Unlike the well-established derivation of the oocyte however, the embryonic origin of the somatic pre-GC has long been a subject of debate. Although originally considered mesonephric in origin, the ovarian surface epithelium has

more recently been considered as an alternative source of the GC precursor (Juengel et al., 2002; Mork et al., 2012; Sawyer et al., 2002). Indeed, a study by Mork et al. (2012) investigating lineage tracing of the GC in the mouse provides clear evidence for a surface epithelium origin of the GC precursor. An alternative postulation however, born from a recent examination of bovine ovarian development suggests that GCs are not derived from differentiated epithelial cells but instead both the ovarian surface epithelium and the GC arise from a novel precursor population of gonadal ridge epithelial-like (GREL) cells. In this study Hummitzsch et al. (2013) postulate these cells to derive from the surface of the mesonephros. When consolidating the existing literature, it may therefore be that GCs are derived from epithelial-like precursors on the surface of the ovary rather than specifically from the classic surface epithelium. Of course, the possible contribution of multiple origins to the GC lineage cannot be excluded. The ingression of somatic GC precursors is not a unified process. Instead it is believed to occur in two temporally distinct waves, both of which are thought to be the descendent of a single progenitor source, born at different stages of development; a consequence of which is two distinct populations of follicles within the ovary. The first GC wave, which is specified early in fetal development, contributes to the specific follicle population in the medulla which activate immediately after birth, while the second wave, which arises perinatally (ED 15.5 – 4 dpp), equips the cortical follicle population which are activated throughout adult life (Hirshfield, 1992; Mork et al., 2012). Following assembly, the primordial follicle enters a developmental hiatus. The pre-GCs cease mitotic division and enter a period of quiescence. Somatic cell proliferation will not resume until the primordial follicle is activated to grow. The germ cell, which has passed through the stages of meiotic prophase in parallel to primordial follicle formation, undergoes meiotic arrest at the diplotene stage of prophase I (Borum, 1961). This is a state maintained until the mid-cycle surge of gonadotropins at ovulation. In this first stage of follicle development a large but finite reserve of quiescent primordial follicles is established in the mammalian ovary.

A central theory in mammalian ovarian biology is the prenatal establishment of the ovarian primordial follicle reserve (Zuckerman, 1951). More recent studies however have postulated the existence of a rare stem cell-like population within the postnatal ovary that could contribute to follicle development during adult life (Johnson et al., 2004; White et al., 2012; Zou et al., 2009). This idea however has proved highly controversial and in the absence of

material evidence is not yet an accepted concept. Therefore, the belief that the entire germ cell pool is endowed during the first stage of follicle development still holds true.

1.3 Follicle development

The course of follicle development encompasses the maturation of the follicle from the quiescent primordial stage through a complex series of changes that culminates in the ovulation of a mature oocyte (Reviewed in Edson et al., 2009). (**Figure 1.1**). Follicles are activated from the pool of dormant primordial follicles and develop through primary and secondary stages before acquiring an antral cavity. At the antral stages, most follicles undergo atretic degeneration. A select few however undergo further growth and reach the preovulatory stage under post-pubertal cyclic gonadotropin stimulation. In response to the preovulatory surge of gonadotropins during each cycle, the dominant Graafian follicle ovulates to release the mature oocyte for fertilisation. These major stages of follicle development can be separated into two principle phases; the gonadotropin-independent phase and the gonadotropin-dependent phase. Preantral follicle development is dependent on the action of local autocrine and paracrine regulatory growth factors in a gonadotropin independent manner. The subsequent transition from preantral to antral follicle development marks the juncture at which the regulation of follicle development switches from principally local intraovarian factors to the extraovarian gonadotropins.

As the work in this thesis is concerned solely with the events underlying early preantral follicle development, only this stage of the development sequence will be subsequently addressed.

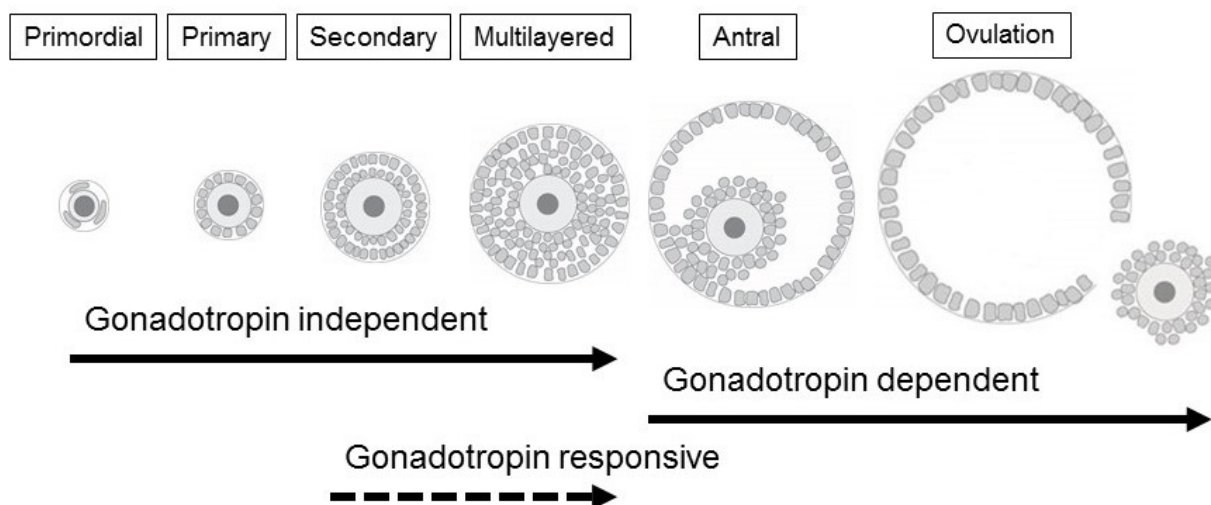


Figure 1.1. The major stages of mammalian follicle development. The primordial follicle is activated from the pool of dormant primordial follicles and develops through the primary and secondary stages before acquiring an antral cavity. Under the control of post-pubertal cyclic gonadotropin stimulation, the follicle undergoes further growth. In response to the preovulatory surge of gonadotropins during each cycle, the dominant preovulatory follicle ovulates to release the mature oocyte for fertilisation.

1.3.1 Primordial follicle activation

The first wave of primordial follicle activation occurs in the corticomedullary border, shortly after formation of the primordial follicle pool is complete (Peters, 1969). This is pattern believed to be consistent with the order in which the oocytes entered meiosis, as dictated by the two temporally distinct waves of somatic cell ingress. The primordial follicle comprises a single oocyte arrested in meiotic prophase I surrounded by flattened somatic GCs (Hirshfield, 1991). The initiation of primordial growth, and the onset of mammalian follicle development, is associated with oocyte growth, proliferation of the epithelial GCs and the transformation of GC morphology from flattened to cuboidal (**Figure 1.2**) (Lintern-Moore & Moore, 1979). The developmental sequence in which these events transpire however has not been fully characterised, nor is it clear how they are related. In 1979, the morphological and functional events that surround the initiation of oocyte and follicle growth in the mouse were described by Lintern-Moore and Moore. In this study the authors describe an increase in both

GC number and morphology before the onset of oocyte nucleolar RNA polymerase activity and subsequent oocyte growth. This observation is supported by Gougeon and Chainy (1987) who reported oocyte growth subsequent to GC proliferation and cuboidilisation in the human. The relationship underlying the developmental sequence of the GC however is slightly less coherent. Da Silva-Buttkus et al. (2008) observed that cuboidal GCs divide at a much higher rate than that of flattened GCs suggesting that morphological transition precedes GC proliferation. However Lintern-Moore and Moore (1979) report that cuboidal morphology appeared subsequently to the increase in GC number surrounding the oocyte. This sequence is supported by Oktay et al. (1995), who described expression of proliferation marker, proliferation cell nuclear antigen (PCNA) coinciding with and in some cases preceding the first sign of pre-GC growth. In this regard it appears cuboidilisation of the GC is not an absolute pre-requisite for entry into the cell cycle. In an alternative scenario, Hardy et al. (unpublished observations) suggest that cuboidilisation may in fact be a passive event. The authors consider that given the combination of increased GC number with the absence of oocyte growth, GC cuboidilisation may simply be a result of the higher packing density and subsequent GC compression on the oocyte surface.

The initiation of follicle development is accompanied by the proliferation and differentiation of the previously quiescent epithelial pre-GC into a GC proper. As previously described the developmental origin of the GC in the mouse is uncertain, however most likely to derive predominantly from the epithelial like precursors on the ovarian surface epithelium (Hummitzsch et al., 2013; Mork et al., 2012). A recent study from Mora et al. (2012) reported that the activated GC expresses morphological characteristics that are akin to mesenchymal rather than epithelial cells suggesting that a partial epithelial-to-mesenchymal transition (EMT) takes place as they activate and develop (Childs & McNeilly, 2012; Mora et al., 2012). The authors revealed a lack of classic epithelial markers such as E-cadherin, coupled with the presence of mesenchymal markers such as vimentin and N-cadherin which was upregulated in the cuboidal GC. The GC has been attributed with pluripotent stem cell properties, originally highlighted by cumulus and mural differentiation in the antral follicle (Rodgers et al., 1999). Moreover, in concert with the study by Mora et al. (2012) a mesenchymal stem cell potential of a subpopulation of antral GCs has specifically been identified (Reviewed in Hummitzsch et al., 2015). Collectively these studies suggest that the

mesenchymal stem cell potential of the GC and the putative EMT event may be important in follicle activation and subsequent follicle development.

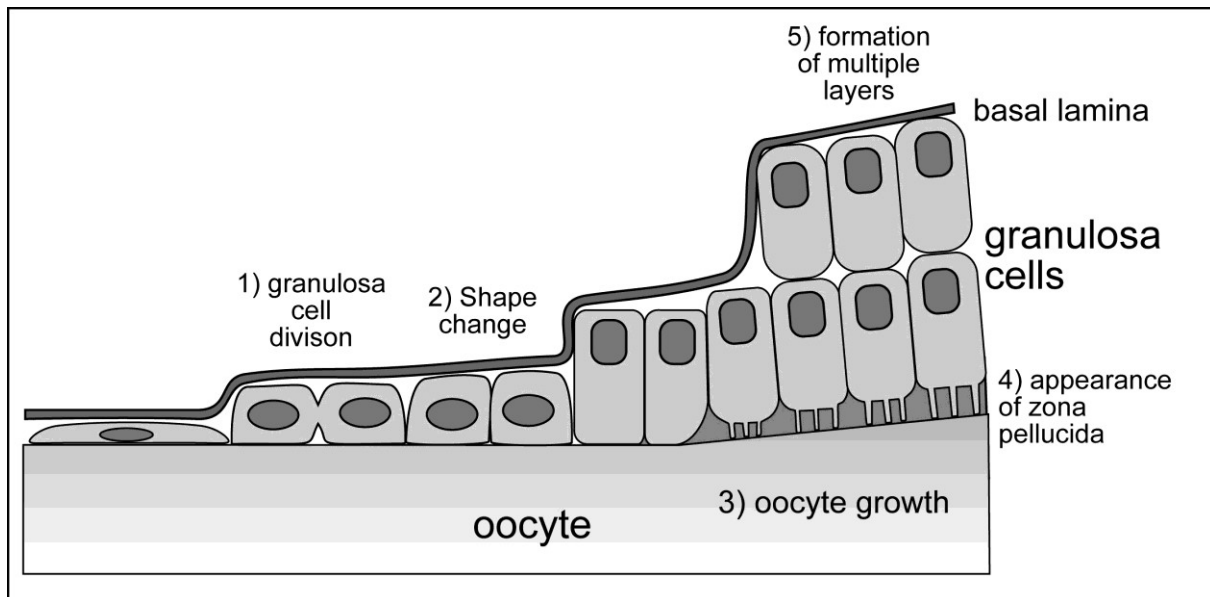


Figure 1.2. Summary of cellular changes that take place during initiation of follicle growth and early preantral follicle development. The initiation of primordial growth is associated with oocyte growth, GC proliferation and the transformation of GC morphology from flattened to cuboidal. Subsequent preantral development encompasses GC multilayering and zona pellucida development. The exact developmental sequence in which these events transpire has not been fully characterised. Image adapted from Da Silva-Buttkus et al. (2008).

1.3.2 Preantral follicle development

The activated follicle which is now termed a transitional follicle, consists of an oocyte surrounded by a combination of flattened and cuboidal cells. In parallel with the appearance of cuboidal GCs, a glycoprotein layer – the zone pellucida – forms between the GCs and the oocyte. When the oocyte is surrounded by a complete layer of cuboidal GCs it is termed a primary follicle, at which point the extensive growth phase of the oocyte begins. During follicle activation and early development in the mouse, the oocyte undergoes an almost 300-fold increase in volume during the 2 – 3 week growth phase (Wasserman & Albertini, 1994). Further GC proliferation leads to maximum packing density of the cells on the basal lamina whereby GCs become columnar and polarised with the nucleus lying adjacent to the basal lamina (Da Silva-Buttkus et al., 2008). The formation of the second layer of GCs results from basal cells changing their mitotic spindle orientation. Inward cellular division results with the

new layer forming on the inner aspect of the basal GC layer next to the oocyte. Following the acquisition of a complete second layer of GCs, the theca-layer is recruited from fibroblast-like precursor cells within the ovarian stroma to envelope the basement membrane (Peters, 1969; Young & McNeilly, 2010). Continued GC proliferation to form the multilayered follicle completes the preantral stage of follicle development.

1.3.3 Pathologies of follicle development

Initial primordial follicle reserve and rate of primordial follicle activation determine the duration of reproductive life. The rate of follicle activation therefore has a profound effect on fertility and must be tightly regulated. Too few follicles initiating growth will lead to anovulatory cycles whereas accelerated activation will lead to premature depletion of the follicle pool, exhaustion of which correlates with the onset of reproductive senescence (McGee & Hsueh, 2000). Premature exhaustion of the follicle pool is clinically termed premature ovarian insufficiency (POI). POI, defined as the onset of menopause before the age of 40, affects approximately 1% of women (Coulam et al., 1986). The vast majority of cases of POI are idiopathic however the proposed pathogenesis is failure to establish a normal supply of primordial follicles during development or accelerated depletion of the cohort during life; whether by increased activation or direct atretic depletion from the dormant state (Beck-Peccoz & Persani, 2006; Goswami & Conway, 2005). Polycystic ovary syndrome (PCOS) is the main cause of anovulatory infertility affecting women of reproductive age (Franks, 1997). The distinct polycystic ovarian morphology, a consequence of primary, preantral and small antral follicle stockpiling, is a characteristic feature of the syndrome; a phenotype postulated to arise from abnormalities in early follicle development (Maciel et al., 2004; Webber et al., 2003). While it is clear that pathologies underlying early follicle development can result in extreme alterations in reproductive outcome and female fertility, the mechanisms responsible for maintaining the primordial pool or, conversely, for activating the initiation of primordial follicle recruitment remain largely unknown.

1.4 Regulation of preantral follicle development

1.4.1 Primordial follicle activation

The cortex-medulla distribution of follicle growth has long been recognised (Peters, 1969). Formed by a rim of small non-growing follicles residing in the outer cortex of the ovary and

growing oocytes centrally situated at the cortex-medulla boundary, it is a pattern believed to be consistent with the order in which the oocytes entered meiosis (Henderson & Edwards, 1968). The initiation of follicle growth is not solely intrinsic to the follicle however but also subject to factors in the intraovarian environment. In 1969, Krarup et al proposed that the number of growing follicles was inversely correlated with the size of the quiescent primordial follicle pool. This was later corroborated by Hirshfield (1994) who reported accelerated entry of follicles into the growth phase in the rodent ovary in which the stockpile of quiescent follicles had been artificially depleted. The idea was further supported by morphometric studies in the human ovary which demonstrated accelerated follicular depletion correlating with a diminished primordial follicle pool in the perimenopausal period (Faddy & Gosden, 1995; Gougeon & Chainy, 1987; Richardson et al., 1987). In 1996 Wandji et al. hypothesised that the presence of putative local growth inhibitors may maintain unrecruited primordial follicles in the resting pool. This was in response to a body of evidence demonstrating the spontaneous activation of bovine, and later baboon, primordial follicles in ovarian cortical strips *in vitro*, suggesting that primordial follicles are subject to inhibitory control *in vivo* (Braw-Tal & Yossefi, 1997; Wandji et al., 1997; Wandji et al., 1996). It was therefore proposed that a decrease in local inhibitory factors and/or an increase in local stimulatory factors might allow initiation of follicle growth. In line with this Da Silva-Buttkus et al. (2009) provided mathematical evidence to support the existence of an inhibitory factor released by the primordial follicle that suppressed the growth of neighbouring follicles.

The role of local autocrine and paracrine growth factors in the regulation of primordial follicle activation has long been established. The pre-GCs of the primordial follicle express a number of peptide growth factors including Kit ligand (KL; also termed stem cell factor). Interactions between KL and the Kit tyrosine kinase receptor (c-kit), which is localised to the oocyte surface, appear to be critical in early folliculogenesis. Antibody neutralisation of c-kit in the neonatal mouse disturbed the onset of primordial follicle development (Yoshida et al., 1997). Similarly, c-kit neutralisation inhibited spontaneous primordial follicle development *in vitro* while recombinant KL accelerated follicle activation (Parrott & Skinner, 1999). Basic fibroblast growth factor (bFGF) is expressed by the oocyte from the primordial follicle stage (Nilsson et al., 2001). In a manner similar to KL, bFGF stimulated initiation of follicle development *in vitro* while neutralisation of endogenous ligand was reported to limit follicle activation (Nilsson et al., 2001). The transforming growth factor beta (TGFB) superfamily

has been strongly implicated in many critical aspects of follicle development. Disruption of the function or expression of members of the TGFB superfamily, receptors or key components of the downstream signalling pathway induces profound effects on the number and developmental potential of preantral follicles. The functional role of Anti-Müllerian hormone (AMH) as an inhibitor of primordial growth is well characterised in both the mouse and human (Carlsson et al., 2006; Durlinger et al., 2002; Durlinger et al., 1999). Expressed in the GC of the growing follicle, homozygous deletion of *Amh* leads to significant depletion of the murine primordial follicle pool by 4 months of age (Durlinger et al., 1999). This observation was confirmed in vitro whereby inhibition of primordial follicle activation was observed in the presence of AMH (Carlsson et al., 2006; Durlinger et al., 2002). A role in maintenance of the quiescent primordial follicle pool has additionally been described for the prototypic TGFB family member, TGFB1, expression of which is reported in the fetal germ cell onwards (Wang et al., 2014). Conversely other members of the TGFB superfamily appear to have a stimulatory effect on primordial follicle recruitment. Culture with theca derived bone morphogenetic protein (BMP)-7 or BMP4 promotes in vitro primordial follicle development in neonatal rat ovaries (Lee et al., 2004; Nilsson & Skinner, 2003). A summary of the potential autocrine and paracrine signalling interactions involved in primordial follicle activation are illustrated in **Figure 1.3**.

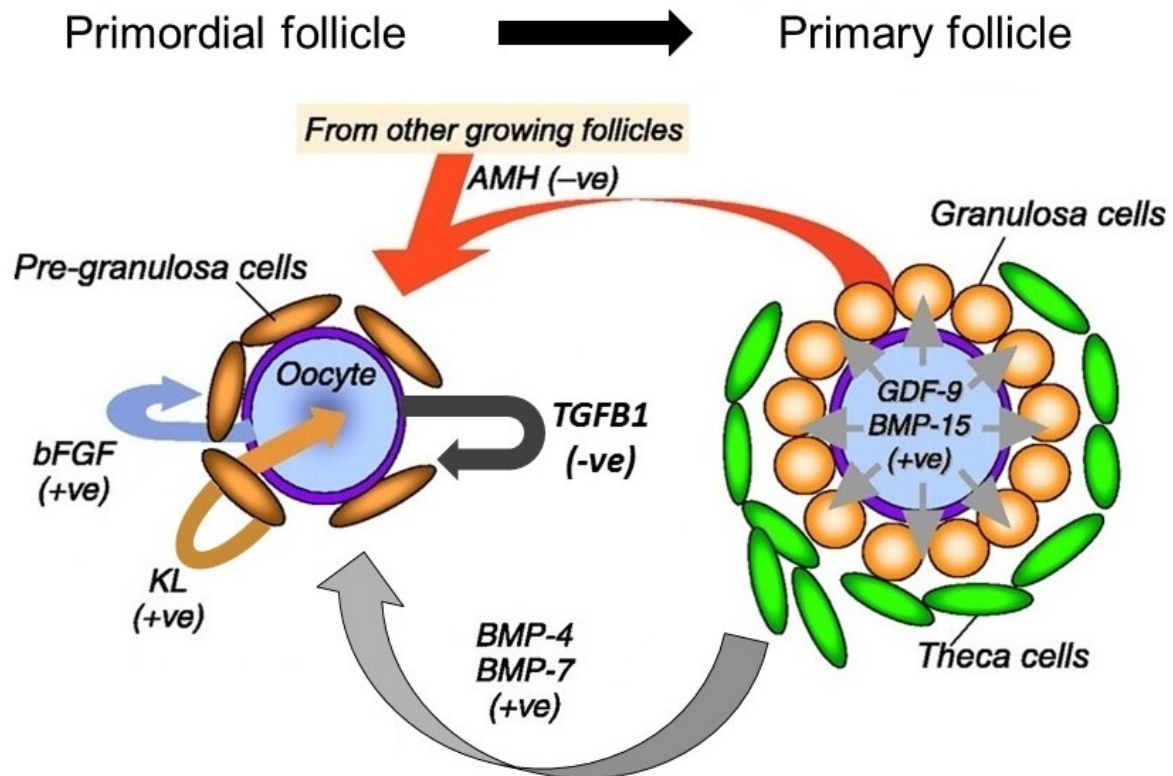


Figure 1.3. A summary of the potential autocrine and paracrine signalling interactions involved in primordial follicle activation and subsequent preantral follicle development. KL and bFGF secreted by pre-GC and oocyte respectively, promote primordial follicle activation. TGFβ1 expressed in the oocyte acts to maintain the primordial follicle pool. BMP7 and BMP4 secreted by the theca cells promote activation of the primordial follicle. GDF9 and BMP15, secreted by the oocyte of the activated follicle promote GC proliferation in the primary follicle onwards. GC of the growing follicle secrete AMH which inhibits primordial follicle recruitment. (+ve, stimulatory; -ve, inhibitory). Image adapted from Knight and Glister (2006).

1.4.2 Preantral follicle development

In comparison with the largely elusive mechanism underlying the regulation of primordial follicle activation, substantially more is known about the process of subsequent preantral follicle growth and development. During the later stages of follicle development, the necessity for bidirectional signalling between the various follicular cell types of the preantral follicle becomes apparent; indeed the cellular interaction between the germ-line and somatic cell compartment is integral for the progression of preantral follicle development beyond the primary stage (Matzuk et al., 2002). Although the precise mechanisms underlying follicle

development from the primary stage onward have not been fully elucidated the role of locally secreted factors appear crucial. The oocyte secretes potent TGFB superfamily growth factors including BMP15 and growth differentiation factor (GDF)-9 to stimulate GC proliferation in the preantral follicle (**Figure 1.3**) (Gilchrist et al., 2001; Gilchrist et al., 2006; Vanderhyden et al., 1992). GDF9 is a TGFB superfamily member selectively expressed in the oocyte of the primary staged follicle onwards (McGrath et al., 1995). Mice homozygous for a null allele at the *Gdf9* locus demonstrate arrested follicle development at the primary stage, indicating oocyte derived GDF9 is crucial for further follicle progression (Dong et al., 1996). A further regulator of preantral follicle development is BMP15, an oocyte-secreted TGFB superfamily member with a follicular staged expression pattern identical to that of GDF9. While BMP15 is not required during preantral follicle development in the murine model, an essential role in these stages has been ascribed to low ovulation rate species such as sheep and humans (Di Pasquale et al., 2004; Galloway et al., 2000). Sheep homozygous for the BMP15 mutation demonstrate an ovarian phenotype directly comparable to that of the *Gdf9* null mouse, indicating an essential role for oocyte secreted BMP15 in preantral follicle development (Braw-Tal et al., 1993).

1.4.3 Systemic factors

The initiation of primordial follicle recruitment appears to be a locally induced gonadotropin independent process, despite in vivo exposure to a complex extrafollicular milieu including branches of the systemic circulation. This is in part demonstrated by the absence of follicle stimulating hormone (FSH) receptors in primordial follicles (Oktay et al., 1997) and follicle development to the late preantral stage in ovaries of mice lacking the expression of FSH β -subunit or FSH receptor (FSHR) (Abel et al., 2000; Dierich et al., 1998; Kumar et al., 1997). Nevertheless, preantral follicles have the capacity to respond to FSH as exemplified by FSHR gene expression in the primary preantral follicle onwards (O'Shaughnessy et al., 1996; Oktay et al., 1997). However functional responses have only been reported from the multilayered secondary stage onwards disputing the functional capacity of the receptors in the early preantral follicle. Indeed, a recent study from Hardy et al. (2016) demonstrates the secondary stage of follicle development marks the onset of FSH responsiveness in the murine preantral follicle. Therefore, while it is apparent that the multilayered preantral follicle is gonadotropin responsive, follicle development can progress to the antral stage in the absence of systemic

factors suggesting gonadotropins are unlikely to be the primary physiological signalling mechanism underlying preantral follicle development.

It is therefore evident that gonadotropin independent early follicle development is regulated by intraovarian growth factors that mediate communication between different ovarian cell types (oocyte granulosa theca stroma) and neighbouring follicles. While it has become clear that many of the extracellular signalling molecules implicated in this dialogue are members of the TGFB superfamily both the identity and source of the regulatory factor(s) responsible for maintaining the primordial follicle pool or, conversely, activating the initiation of follicle development remain largely unknown.

1.5 The TGFB superfamily

The TGFB superfamily comprises over 30 members including the prototypic isotypes TGFB1, TGFB2 and TGFB3, the BMPs, GDFs, activins and nodal (Reviewed in Moustakas & Heldin, 2009). At the cellular level, the TGFB superfamily members regulate cell growth, differentiation, adhesion, migration and apoptosis, in a context-dependent and cell-type specific manner, to govern events underlying embryonic development and adult tissue homeostasis. The family largely constitutes homodimeric or heterodimeric secreted polypeptides, structurally characterised by a specific three-dimensional fold and a conserved number and spacing of cysteine residues in the C terminus of the mature polypeptide. The ligands are synthesised as precursors, with a longer N-terminal pro-peptide (known as latency associated peptide (LAP)) and a C-terminal mature polypeptide. Intermolecular disulphide linkages pair dimers of these precursors via conserved cysteine residues in the pro-peptide and mature peptide sequence. Members of the proprotein convertase family facilitate cleavage of the pro-peptide to release the biologically active mature peptide (Dubois et al., 1995). Subsequent peptide signalling is facilitated in an autocrine or paracrine manner.

1.5.1 TGFB signal transduction

1.5.1.1 SMAD signal transduction

Members of the TGFB superfamily signal through heterotetrameric receptor complexes composed of two types of membrane-bound serine threonine kinases. Currently, seven type I receptors (activin receptor-like kinase (ALK) 1 – 7) and five type II receptors (activin

receptor (ACVR)2A, ACVR2B, BMPR2, TGFBR2 and AMHR2) have been identified (Heldin & Moustakas, 2016). Both receptor types are organised sequentially into an N-terminal extracellular ligand binding domain, a transmembrane region, and a C-terminal serine/threonine kinase domain (Shi & Massague, 2003). Each member of the TGFB superfamily binds to a characteristic combination of type I and type II receptors (ligand-receptor interactions for select TGFB superfamily members are detailed in **Table 1.1**). For some ligands, additional co-receptors are required for optimal ligand binding to the type I-type II receptor complex. The membrane-bound type III receptor TGFBR3 binds the TGFB isoforms with high affinity, greatly enhancing presentation to the type II receptor on the cell surface thus potentiating their actions (Lewis et al., 2000; Lopez-Casillas et al., 1993). This is a mechanism most evident with TGFB2 which has lower intrinsic binding affinity for signalling receptors than the other TGFB isoforms, the biological potency of which however is equalised in the presence of TGFBR3 (Lopez-Casillas et al., 1993). Nodal signalling is facilitated through ACVR2A/ACVR2B and ALK4 however efficient ligand binding and signalling require association of fucosylated transmembrane co-receptor Cripto with ALK4 (Yan et al., 2002; Yeo & Whitman, 2001). Co-receptor interaction can additionally limit receptor signalling, TGFBR3 binding to the activin antagonist inhibin facilitates recruitment to the activin type II receptor preventing activin binding (Lewis et al., 2000). While proteolytic processing of TGFBR3 results in the secretion of a soluble ectodomain which is believed to bind and sequester TGFB, functioning in effect as a receptor antagonist (Andres et al., 1989; Lopez-Casillas et al., 1994; Philip et al., 1999). Transduction of TGFB signalling is initiated by ligand binding to the extracellular domains of the transmembrane type I and type II receptors on the cell surface. Two modes of ligand binding have been observed; sequential and cooperative. Sequential binding is characteristic of the TGFB receptors whereby the type II receptor binds TGFB and subsequently recruits the type I receptor into the complex where it becomes tethered at the ligand-type II receptor interface (Groppe et al., 2008; Laiho et al., 1991; Massague, 2008; Wrana et al., 1992; Wrana et al., 1994). The exception of which is TGFB2 which requires the pre-formation of a type I and type II receptor hetero-oligomer to enable ligand binding with high affinity to either receptor (Rodriguez et al., 1995). In contrast the BMP receptors demonstrate cooperative receptor complex formation whereby the type I and type II receptors bind independently to different sites on the ligand, the affinity of which is higher when receptor types are expressed together (Greenwald et al., 2003; Liu et al., 1995; Rosenzweig et al., 1995). Following ligand binding,

the type II receptor kinase, which is considered constitutively active, phosphorylates multiple serine and threonine residues in the characteristic juxtamembrane TTSGSGSG sequence, (termed the GS domain) in the cytoplasmic region of the type I receptor leading to its activation (Souchelnytskyi et al., 1996; Wieser et al., 1995; Wrana et al., 1994). The type I receptor, in turn, recruits and phosphorylates the SMAD proteins which facilitate subsequent signalling (Kretzschmar, Liu, et al., 1997; Macias-Silva et al., 1996).

Table 1.1. Ligand-receptor-SMAD cascade interactions for select TGFB superfamily members.

Ligand	Type I receptor	Type II receptor	Co-receptor	SMAD cascade
Activin/Nodal	ALK4/7	ACVR2A/B	Cripto 1 Cripto 3	SMAD2/3
TGFB	ALK5, ALK1*	TGFB2	TGFB3 Endoglin Cripto 3	SMAD2/3 SMAD1/5/8*
BMP	ALK1/2/3/6	ACVR2A/B, BMP2	RGM a, b, c	SMAD1/5/8
GDF	ALK3/6 ALK4/5/7	ACVR2A/B, BMP2	Cripto 3	SMAD2/3 SMAD1/5/8
AMH	ALK2/3	AMH2	ND	SMAD1/5/8

ND, not determined. Repulsive guidance molecule (RGM). *ALK1-SMAD1/5/8 is endothelial specific.

The SMAD proteins are intracellular transcription factors which classically facilitate the transduction of TGFB superfamily signalling (Reviewed in Heldin & Moustakas, 2012). There are eight members of the SMAD family, classified according to intracellular function; the receptor regulated (R-SMADs) SMAD1, SMAD2, SMAD3, SMAD5, SMAD8, the common (co-SMAD) SMAD4 and the inhibitory (I-SMADs) SMAD6 and SMAD7. The R-SMAD and co-SMAD proteins are composed of two conserved structural domains, the N-terminal Mad homology (MH)-1 domain and the C-terminal MH2 domain connected by a proline rich linker region (**Figure 1.4**) (Derynck & Zhang, 2003; Moustakas & Heldin, 2009;

Shi & Massague, 2003). The MH1 domain of the co-SMAD and most R-SMADs, exhibits sequence-specific DNA binding activity, with the exception of SMAD2 which has an inserted sequence in this region (Shi et al., 1998). The N-terminal domain of the structurally divergent I-SMADs displays weak homology to the MH1 domain of R-SMADs, a consequence of which is an absence of DNA binding. In contrast the MH2 domain is highly conserved among all SMAD proteins and confers R-SMAD specific receptor interaction via a characteristic SSXS motif at the C terminal.

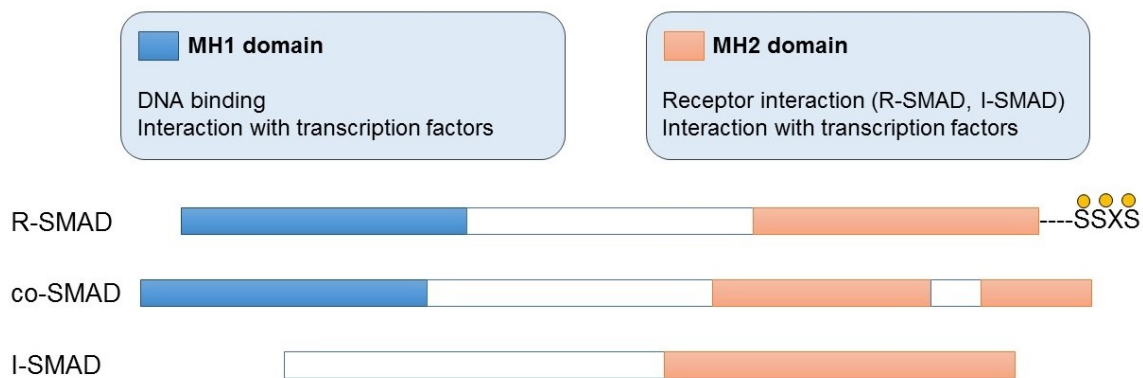


Figure 1.4. Structural organisation and the role of the SMAD domains. The MH1 domain exhibits sequence specific DNA binding activity. The MH2 domain confers R-SMAD specific receptor interaction via a characteristic SSXS motif at the C terminal. Both the MH1 and MH2 domains interact with a large number of nuclear sequence-specific transcription factors. Image adapted from Derynck and Zhang (2003).

There are two distinct R-SMAD signalling cascades, activation of which is determined by the TGFB ligand. The TGFBs, activins, GDF9 and nodal classically activate SMAD2 and SMAD3 whereas the BMPs activate SMAD1, SMAD5, SMAD8 (**Table 1.1**). The phosphorylation of the C-terminal two serine residues in the SSXS motif of the R-SMAD MH2 domain drives the activation of the R-SMAD (Abdollah et al., 1997; Souchelnytskyi et al., 1997). The selective interaction between the type I receptor and corresponding R-SMAD is determined by discrete structural elements, whereby a cluster of residues in the L45 loop of the type I receptor kinase domain and a corresponding set of residues in the L3 loop of the SMAD C-terminal domain establish the specificity of receptor-SMAD interactions (Chen et al., 1998; Lo et al., 1998). Activated R-SMADs then form a heteromeric complex with the

co-SMAD and translocate into the nucleus. The stoichiometry of the oligomeric complex may comprise either a heterotrimeric formation, two R-SMADs (which themselves may be of homomeric or heteromeric stoichiometry) and SMAD4 or a heterodimeric (Chacko et al., 2001; Inman & Hill, 2002; Wu et al., 2001). In the basal state R-SMADs are predominantly cytoplasmic whereas SMAD4 is distributed throughout the cytoplasm and the nucleus. The SMADs are not static however but undergoing continual nucleocytoplasmic shuttling; a phenomenon that appears to be a key event in TGFB signalling (Inman et al., 2002; L. Xu et al., 2002). Following TGFB receptor stimulation, SMAD complexes are formed and accumulate in the nucleus. Nucleocytoplasmic shuttling ensures that, in the presence of signal, the SMADs continuously monitor levels of receptor activation thus ensuring that the level of nuclear SMADs continuously reflect the level of activated receptors in the cytoplasm. Following nuclear accumulation, the SMAD complexes regulate target gene expression (via both transcriptional repression or activation) by means of specific promoter elements. SMAD3 and SMAD4 recognise the sequence AGAC or its reverse complement, known as the Smad-binding element (SBE) (Dennler et al., 1998; Zawel et al., 1998). In contrast the BMP regulated R-SMADs preferentially bind GC rich-elements with less conserved motifs, often referred to as BMP response elements (Brugger et al., 2004; Ishida et al., 2000; Kim et al., 1997). Such elements are commonly found in close proximity to SBE motifs however suggesting the simultaneous requirement of two independent DNA binding elements permits functional cooperativity of both BMP-regulated SMADs and SMAD4 (Lopez-Rovira et al., 2002). The R-SMADs and co-SMAD exhibit sequence-specific DNA binding activity via a β hairpin structure in the MH1 domain although their affinity for DNA is very low (Shi et al., 1998). Because of this they require cooperation with a multitude of sequence specific binding transcription factors in addition to cell specific coactivators and corepressors to regulate transcription (Reviewed in Ross & Hill, 2008). To summarise, the TGFB-SMAD signalling cascade is illustrated in **Figure 1.5**.

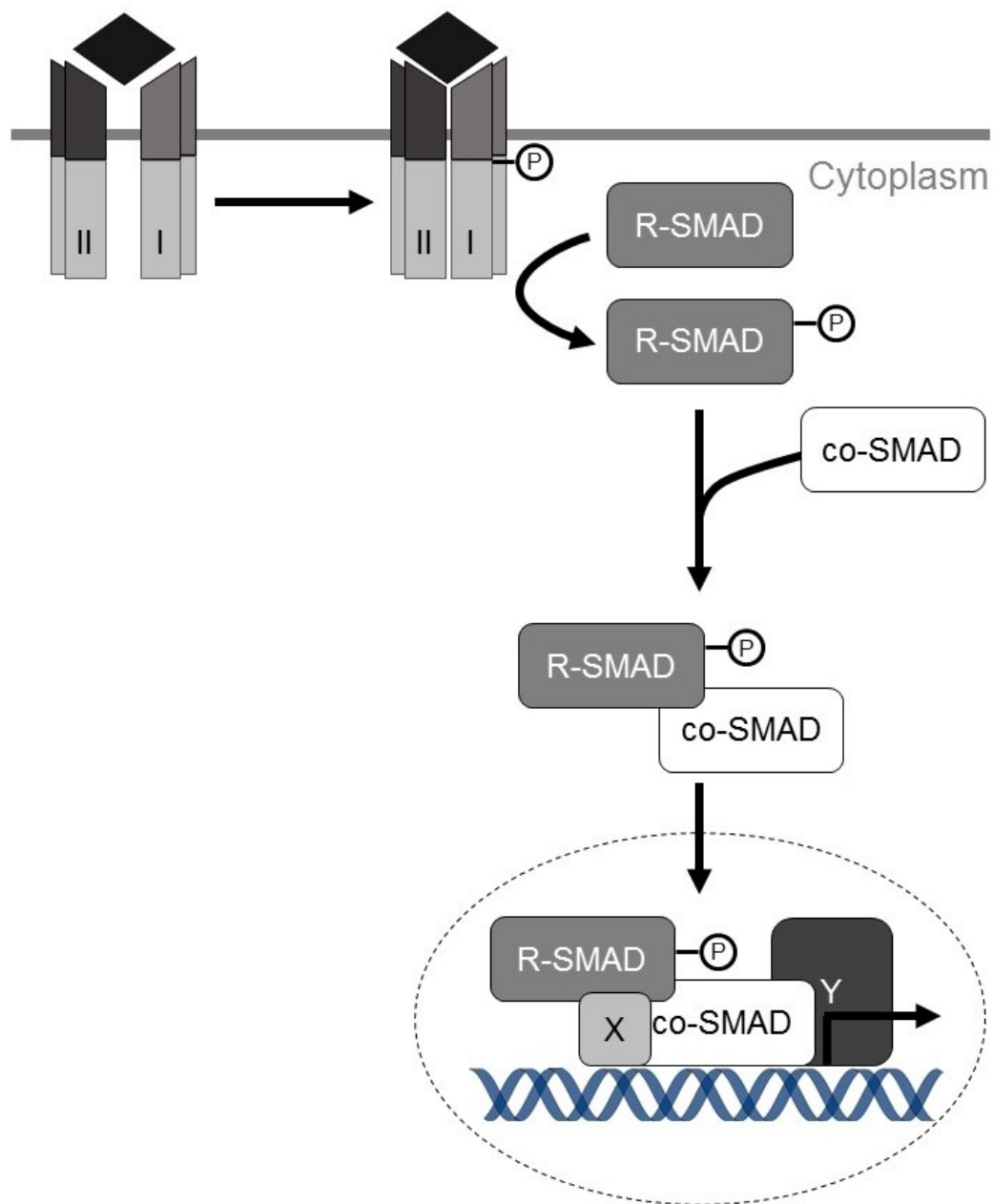


Figure 1.5. The TGFβ-SMAD signalling cascade. TGFβ signalling is initiated by ligand binding to the extracellular domains of the transmembrane type I and type II receptors on the cell surface. The constitutively active type II receptor transphosphorylates and activates the type I receptor. The type I receptor consequently recruits and phosphorylates selected R-SMADs which form a complex with the co-SMAD. Activated SMAD complexes translocate to the nucleus where they regulate target gene transcription via interaction with DNA binding transcription factors (X), coactivators and corepressors (Y).

1.5.1.2 Regulation of SMAD signalling

The TGFB signalling pathway is terminated through ubiquitination-proteasome-mediated degradation of the type I receptors. In response to TGFB or BMP stimulation, the E3 ubiquitin ligases, Smad-ubiquitination-regulatory factor (Smurf) – 1 and Smurf2, which are recruited in complex with the nuclear I-SMAD, SMAD7, target the activated receptor complex mediating ubiquitination and receptor turnover (Ebisawa et al., 2001; Kavsak et al., 2000; Tajima et al., 2003). In addition, consistent with the common MH2 domain which confers SMAD specific receptor interaction, the Smurf-SMAD7 complex further modulates TGFB signalling via SMAD7 binding to the activated type I receptor inhibiting subsequent phosphorylation of the R-SMADs (Hayashi et al., 1997; Nakao et al., 1997; Suzuki et al., 2002). At the nuclear level, SMAD7 inhibits transcriptional activity, disrupting the formation of the TGFB induced SMAD-DNA complex (Zhang et al., 2007). Unlike SMAD7, the selective BMP signalling inhibition elicited by SMAD6 is mediated by competition with SMAD4 for binding to receptor-activated SMAD1 yielding an inactive SMAD1-SMAD6 complex (Hata et al., 1998). As direct downstream gene targets of TGFB and BMP, SMAD6 and SMAD7 signalling regulation is perpetuated as a negative regulatory feedback loop (Ishida et al., 2000; Nagarajan et al., 1999). The R-SMADs are further targets of signal regulation. Ubiquitin mediated degradation targets both unstimulated basal levels of R-SMADs, regulating the sensitivity of cells to incoming signals and ligand activated nuclear SMADs, terminating signalling (Lo & Massague, 1999; Zhu et al., 1999). Smurf2 likely mediates ubiquitination-proteasome-mediated degradation of SMAD2, while ubiquitination of nuclear SMAD3 appears to be mediated by the E3 ligase complex, SCF/Roc1 (Fukuchi et al., 2001; Lin et al., 2000; Zhang et al., 2001). The vast majority of R-SMAD regulation however appears to be regulated by nuclear dephosphorylation by as yet unidentified phosphatases, permitting export to the cytoplasm (Inman et al., 2002; Pierreux et al., 2000).

1.5.1.3 Non-canonical signal transduction

Further to the canonical SMAD signalling cascade, the cellular response to TGFB can be regulated by an array of non-SMAD pathways including the extracellular-signal-regulated kinase (ERK) branch of the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway (Reviewed in Derynck & Zhang, 2003). This regulation can occur in response to direct activation of non-SMAD proteins by the

TGFB receptor complex initiating a parallel signalling cascade or be elicited by cross talk with parallel pathways (Moustakas & Heldin, 2005). The TGFB receptors have both serine/threonine kinase activity and weaker tyrosine kinase activity (Lawler et al., 1997). The type II receptor undergoes autophosphorylation on three tyrosine residues, albeit at a much lower level than the well-defined serine and threonine residues. Tyrosine phosphorylation of the type I receptor can also occur in response to TGFB stimulation, whether this is due to autophosphorylation or type II receptor transphosphorylation however remains unclear (Lawler et al., 1997; Lee et al., 2007). TGFB induced phosphorylation of tyrosine residues on both the TGFB type I and type II receptor enables recruitment of signal transduction adaptor protein complex SchA/Grb2/Sos (Lee et al., 2007). Subsequent Ras activation leads to sequential activation of Raf, mitogen-activated protein kinase kinase (MEK) and ERK which culminates in a variety of cellular events via ERK1/2 phosphorylation of an array of transcription factors (Qi & Elion, 2005). The TGFB receptors also have the capacity to associate with p85, the regulatory subunit of PI3K, enabling kinase induced PI3 activation and subsequent activation of downstream effector Akt (Yi et al., 2005). In addition to facilitating SMAD-independent signalling, non-SMAD signalling proteins can modulate SMAD activity via pathway cross talk. The linker region of the SMAD domains is a focal point for the integration of regulatory inputs. In this regard it is known that ERK MAP kinases have the capacity to phosphorylate serine residues in the linker domain of R-SMADs, regulating nuclear translocation and transcriptional output (Kretzschmar, Doody, et al., 1997). A further level of pathway modulation comprises interaction of downstream MAPK/ERK components with nuclear SMADs to regulate gene expression. In this manner MAPK/ERK substrate AP-1 functions in conjunction with SMADs to regulate gene expression (Davies et al., 2005).

1.6 SMAD signalling in follicle development

TGFB superfamily signalling is classically facilitated by two distinct branches of SMAD signalling; SMAD2/3 and SMAD1/5/8. Recent investigation by Fenwick et al. (2013) into the spatiotemporal expression patterns of the SMAD proteins in the postnatal ovary localised both signalling cascades in the GC of the preantral follicle in a stage specific manner (**Figure 1.6**). Fenwick et al. (2013) reported abundant SMAD2/3 staining in the small single-layered follicle (primordial, transitional and primary) in the absence of SMAD1/5/8, whereas both

SMAD2/3 and SMAD1/5/8 were observed in the small preantral follicle (1 – 3 layers of GCs). Prolific SMAD1/5/8 staining was described in the larger multilayered follicle (>3 layers GCs) while observed SMAD2/3 staining was considerably weaker. With reference to these observations it is clear that SMAD2/3 is the major signalling pathway in the early preantral follicle. As previously described the capacity for the TGFB related ligands to signal requires the co-SMAD (SMAD4), that partners with the R-SMADs to form the transcriptional complex which regulates target gene expression. In accordance with SMAD2/3 expression SMAD4 has been localised in the GCs of all follicle stages from primordial to antral and the oocytes of preantral follicles (Pangas et al., 2006).

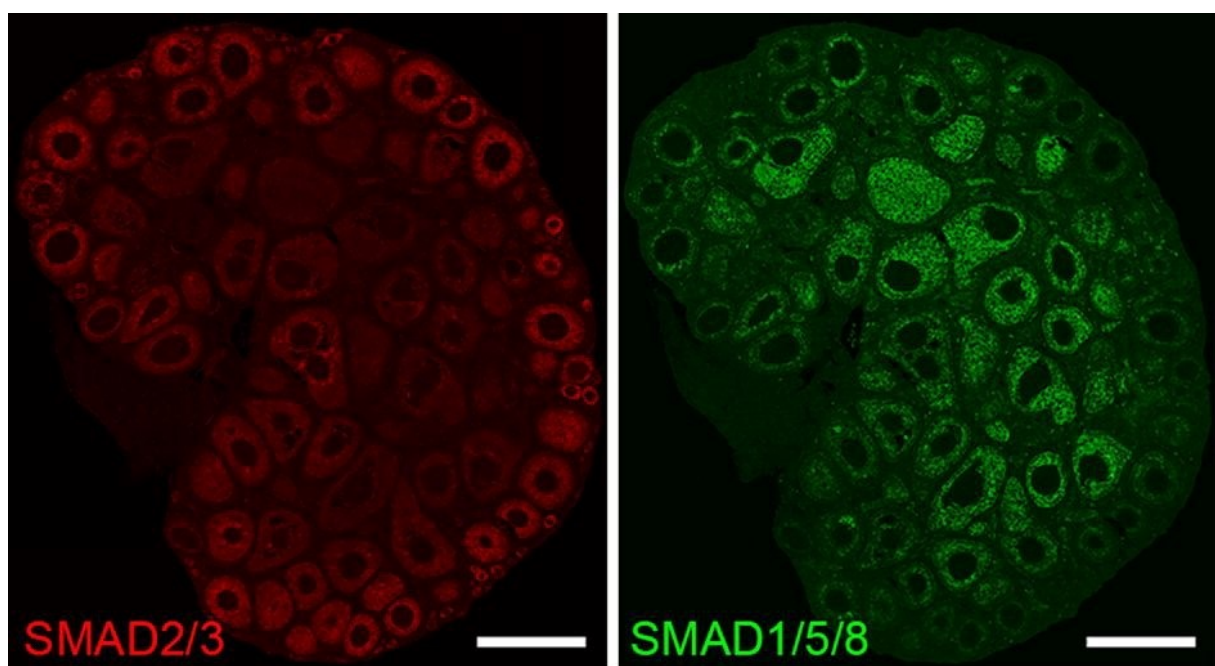


Figure 1.6. The SMAD2/3 and SMAD1/5/8 signalling cascades are expressed in a stage specific manner in the day 16 mouse ovary. SMAD2/3 (red) is abundant in the small single-layered follicle (primordial, transitional and primary). Small preantral follicles (1 – 3 layers of GCs) are positive for SMAD2/3 and SMAD1/5/8 (green). SMAD1/5/8 is prolific in the larger multilayered follicle (>3 layers GCs) while SMAD2/3 is weaker. Image adapted from Fenwick et al. (2013). Image reproduced with permission from the copyright holder, Endocrine Society.

SMAD2/3 expression in the preantral follicle is characterised by stage specific expression perhaps reflective of the functional role of the pathway in preantral follicle development (Fenwick et al., 2013; J. Xu et al., 2002). The abundant localisation observed in the GC of early preantral follicles (primordial transitional and primary) and low expression in large

multilayered follicles is indicative of a role in both the maintenance of the primordial pool and early preantral follicle development. Recent observations by Hardy et al. (unpublished) further corroborate this. The authors describe abundant nuclear GC expression of SMAD2/3 in the primordial and transitional follicle, an observation not mirrored in the primary and small multilayering preantral follicle. In this manner, the authors suggest that active SMAD2/3 signalling in the GC of the primordial follicle, as demonstrated by nuclear localisation, is indicative of a role in maintaining the primordial follicle in a quiescent state. Hardy et al. further demonstrate an association between nuclear GC SMAD2/3 expression and proliferation marker Ki67 whereby nuclear SMAD2/3 and Ki67 expression are mutually exclusive in the majority of nuclei (**Figure 1.7**). The expression of Ki67 in nuclei lacking SMAD2/3 suggests that the pathway is associated with the maintenance of GCs in cell cycle arrest and that SMAD2/3 export from the nucleus may be a prerequisite for the onset of GC proliferation and follicle activation.

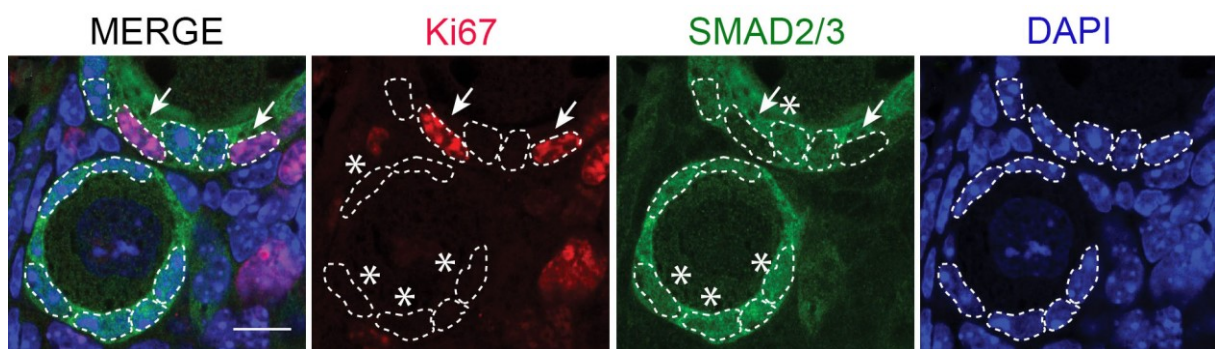


Figure 1.7. Mutually exclusive staining of nuclear SMAD2/3 and Ki67. Unilaminar preantral follicle demonstrating nuclei that are positive for Ki67 (red) and negative for SMAD2/3 (green) (white arrows) and conversely negative for Ki67 and positive for SMAD2/3 (*). Image adapted from Hardy et al. (unpublished).

Targeted disruption of the SMAD family has provided further insight into the role of the SMAD proteins in follicle development however detailed knowledge of the functional role of SMAD2 and SMAD3 in the postnatal ovary remains incomplete. This is due in part to the embryonically lethal phenotype of the *Smad2* null mouse which precludes direct examination of the reproductive role of SMAD2, while homozygous disruption of *Smad3* results in animals predisposed to colorectal cancer with reduced lifespan (Nomura & Li, 1998; Waldrip et al., 1998; Zhu et al., 1998). Despite the reduced lifespan, mice that harbour a deletion of the *Smad3* gene are viable, however discrepancies have been reported in the reproductive

phenotype of the two *Smad3* knockout lines published, both of which target distinct exons. Zhu et al. (1998) who reported a homozygous deletion of *Smad3* in exon 2, described female mice that were fertile and produced homozygous litters. This is in contrast to mice with a targeted deletion of *Smad3* in exon 8 in which reduced fertility was demonstrated due to impaired follicle development whereby significantly higher numbers of primordial follicles were observed in comparison to wild-type animals with a concomitant reduction in numbers of large preantral and antral follicles (Tomic et al., 2002). Li et al. (2008) later employed a conditional murine knockout of both *Smad2* and *Smad3* to circumvent the deleterious effects of universal gene disruption. The combined loss of SMAD2/3 in GCs led to dramatically reduced female fertility via disrupted follicle development and reduced ovulation efficiency. Ovarian histological examination of *Smad2/3* cKO mice identified fewer antral follicles, and, while significant changes of secondary and preantral follicle counts were not observed, there was a trend toward reduction in preantral follicle counts. Histological analysis however failed to address the extent of the primordial follicle pool. Furthermore the nature of the *Amhr2* promotor used, which exhibits low endogenous expression in primordial and primary follicles as previously described by Jorgez et al. (2004), resulted in the circumvention of SMAD2/3 ablation at the single layered follicle stage, thus the role of the SMAD2/3 pathway in primordial follicle activation remains unexamined.

The spatiotemporal expression pattern of SMAD2/3 and data from knockout models therefore highlight the signalling cascade as an integral regulatory pathway in the postnatal ovary. Despite this the functional role of the SMAD2/3 pathway during the window of early follicle development remains unexamined.

1.6.1 The differential roles of SMAD2 and SMAD3

Although considered within the same R-SMAD subgroup it would be an oversimplification to consider SMAD2 and SMAD3 as functionally equivalent molecules. While SMAD2 and SMAD3 share 92% amino acid sequence similarity and are often considered functionally interchangeable in biological systems, as exemplified by the previously described redundancy demonstrated in ovarian GCs in addition to other cellular contexts such as zebrafish mesoderm induction, the dramatically contrasting phenotypes of global *Smad2* and *Smad3* KO mice provide evidence to suggest differential biological roles (Brown et al., 2007; Jia et al., 2008; Li et al., 2008). Indeed, the embryonic lethal phenotype observed in mice deficient

in *Smad2* contrasts *Smad3* null mice which are both viable and fertile (Nomura & Li, 1998; Waldrip et al., 1998; Zhu et al., 1998). The R-SMADs and SMAD4 exhibit sequence-specific DNA binding activity via a β hairpin structure in the MH1 domain with the exception of SMAD2 which has an inserted 30 amino acid sequence in this region (Shi et al., 1998). The insert prevents direct contact of SMAD2 with DNA and is the singularity likely to underlie the functional biological differences between SMAD2 and SMAD3. It is proposed that SMAD2 and SMAD3 are differentially regulated in the nucleus by their interaction with a unique subset of transcriptional regulators (transcription factors, transcriptional co-repressors and transcriptional co-activators) which confer distinct interaction with different target genes (Brown et al., 2007). Indeed, several profiling studies have revealed distinct nonoverlapping target genes for SMAD2 and SMAD3 (Dzwonek et al., 2009; Kretschmer et al., 2003; LaGamba et al., 2005). An example of which is demonstrated by SMAD3/4 unique SMAD7 transcriptional regulation whereby transfection assays in mouse embryonic fibroblasts, with targeted deletions of either *Smad2* or *Smad3* revealed that both SMAD3 and SMAD4, but not SMAD2, were absolutely required for induction of the SMAD7 promoter reporter gene by TGFB (von Gersdorff et al., 2000). Further evidence for the selective roles of SMAD2 and SMAD3 is demonstrated by opposing regulation of a common target gene. The SMAD2/4 complex associates with forkhead activin signal transducer (FAST)-2 to induce the goosecoid (*gsc*) promoter, whereas the SMAD3/4 complex induces repression of the *gsc* promoter (Labbe et al., 1998). Moreover, transcriptional profiling studies have demonstrated SMAD2 repression of SMAD3-activated genes (Yang et al., 2003).

1.7 Summary and Hypothesis

The neonatal ovary encompasses a finite pool of primordial follicles which serve the need of the entire reproductive lifespan (Zuckerman, 1951). Follicle activation is therefore a tightly controlled process, the rate of which has a profound influence on fertility. Despite this the exact mechanisms responsible for maintaining the primordial follicle pool or, conversely, for activating the initiation of primordial follicle recruitment remain unknown. Many of the extracellular signalling molecules implicated in early preantral follicle development are members of the TGFB superfamily. The SMAD2/3 cascade is the major TGFB signalling pathway expressed in the preantral follicle, characterised by stage specific expression in the GCs of early preantral follicles (Fenwick et al., 2013; J. Xu et al., 2002). The onset of GC

proliferation is believed to be the first event in the developmental sequence underlying primordial follicle activation (Hardy et al. unpublished; Lintern-Moore & Moore, 1979; Oktay et al., 1995). Evidence suggests that the presence of an inhibitory factor growth factor may maintain unrecruited primordial follicles in the resting pool (Da Silva-Buttkus et al., 2009; Wandji et al., 1996). Nuclear GC expression of SMAD2/3 is abundant in the primordial follicle, indicative of active SMAD2/3 signalling by a putative inhibitor of primordial follicle activation. Candidate ligands which signal via the SMAD2/3 pathway include TGBF1, TGFB2, TGFB3, Activin A, Activin B and Nodal.

It is therefore hypothesised that the action of an inhibitory factor through the SMAD2/3 pathway in the GC of the primordial follicle acts to maintain the follicle in an inactive state.

1.8 Project aims

- Investigate the role of the SMAD2/3 pathway in primordial follicle activation
- Identify candidate ligands of the SMAD2/3 pathway in the early preantral follicle
- Using candidate(s) identified examine the role in early preantral follicle development, specifically primordial follicle activation and subsequent preantral follicle development

Chapter 2 Materials and Methods

2.1 Tissue collection

Murine ovaries (4, 8, 12, 16, 21, 42 dpp) and organs (muscle, heart and oviduct (42 dpp)) were collected from C57BL/6 mice (day of birth 0 dpp) supplied by Harlan (Olac Ltd, UK). All animals were sacrificed by cervical dislocation. Whole ovaries and organs were fixed in 10% neutral buffered formalin (NBF; Cat. No HT5014, Sigma-Aldrich) for immunohistochemical analysis, snap-frozen in liquid nitrogen for RNA isolation or protein extraction or collected in minimum essential medium alpha (α – MEM; Cat. No 22561-021, Gibco) for tissue culture or follicle isolation. Prior to processing the oviduct, fat and ovarian bursa were dissected from the ovaries using 29G insulin needles (Terumo) in α – MEM. All mice were housed in accordance with the Animals (Scientific Procedures) Act of 1986 and associated Codes of Practice.

2.2 Follicle isolation

Preantral follicles (2-3 layers of GCs (~ 90 μ m)) were mechanically isolated from ovaries (12, 16 and 21 dpp) using acupuncture needles (AcuMedic) in Liebovitz's L-15 medium (Cat. No 11415-049, Gibco) with 1% (w/v) bovine serum albumin (BSA; Cat. No A7030, Sigma-Aldrich) and either processed for culture or snap frozen in liquid nitrogen for RNA isolation and subsequent gene expression studies.

2.3 Tissue culture

2.3.1 Ovary culture

Ovaries 4 dpp were collected and prepared for culture as previously described (section 2.1). Ovaries were cultured on Costar Transwell Membranes (24 mm Transwell with 3.0 μ m pore polycarbonate membrane insert) in 6-well plates (Cat. No 3414, Corning) above 1.5 ml of Waymouth's medium (Cat. No 31220-023, Gibco) supplemented with 0.23 mM sodium pyruvate (Cat. No 11360-070, Gibco), 75 μ g/ml penicillin (Cat. No P7794, Sigma-Aldrich), 10 μ g/ml streptomycin sulphate (Cat. No S6501, Sigma-Aldrich) and 10% (v/v) fetal bovine serum (FBS; Cat. No 12359802, Fisher Scientific) such that the tissue was covered with a thin film of medium. Cultures were maintained in humid conditions at 37°C (5% CO₂) for 6

days and 750 μ l medium was replaced after 72 hours. The details of treatments are given in the relevant chapter. Experimental compounds are detailed in section 2.12. Following culture ovaries were fixed in 10% NBF for immunohistochemical analysis.

2.3.2 Follicle culture

Ovaries 16 dpp were collected and prepared for culture and preantral follicles were mechanically isolated as previously described (sections 2.1 and 2.2). Single follicles were cultured in individual wells in 96-well plates (Cat. No 10212811, Fisher Scientific) containing 100 μ l α - MEM supplemented with 0.1% (w/v) BSA, 75 μ g/ml penicillin, 100 μ g/ml streptomycin sulphate and a solution of insulin (5 μ g/ml), transferrin (5 μ g/ml) and sodium selenite (5 ng/ml) (ITS; Cat. No I1884, Sigma-Aldrich). Cultures were maintained in humid conditions at 37°C (5% CO₂) for up to 48 hours. The details of treatments and time points are given in the relevant chapter. Experimental compounds are detailed in section 2.12. Following culture follicles were fixed in 10% NBF for immunohistochemical analysis or snap-frozen in liquid nitrogen for RNA isolation or protein extraction.

2.4 Immunohistochemistry

2.4.1 Tissue fixation, embedding and sectioning

Cultured ovaries and follicles were fixed in 10% NBF (3 hours and 30 minutes respectively) before being set in droplets of 2% (w/v) low-melting-point agarose (Cat. No A9045, Sigma-Aldrich) and transferred to 70% ethanol. Follicle samples were stained with nuclear fast red (Cat. No N3020, Sigma-Aldrich) prior to ethanol transfer. Non-cultured ovaries were fixed overnight. All tissue was subsequently processed and embedded in paraffin according to standard protocols. Tissue was processed through graded alcohol (70% (1 hour), 90% (1 hour), 100% (3 $\times$ 1 hour)) and then placed in histoclear (Cat. No HS-200, National Diagnostics) overnight. Samples were then embedded in paraffin wax for 2 hours at 65°C. Tissue was serial sectioned (5 μ m) (Leica RM 2135 microtome) and one section mounted per Superfrost glass slide (Cat. No 631-0108, VWR) and dried overnight at 37°C.

2.4.2 Immunofluorescence

Sections were dewaxed in histoclear (2×5 minutes), rehydrated in graded alcohol (100%, 95%, 70% for 3 minutes each) and washed in dH₂O. Antigen retrieval was performed by boiling slides in 0.01 M citrate buffer (1.92 g citric acid, 1 L dH₂O; pH 6.0) or 0.1 M tris buffer (12.11 g tris base, 1 L dH₂O; pH 10.0) with 5% (w/v) urea. After washing in phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 2 mM KH₂PO₄, 10 mM Na₂HPO₄; pH 7.4) (2×5 minutes) non-specific binding was blocked using 10% (v/v) serum with 4% BSA in PBS for 30 minutes. Slides were then incubated with primary antibody overnight at 4°C. After 3×10 washes in PBS sections were incubated in the appropriate Alexa Fluor conjugated secondary antibody for 60 minutes. Slides to be examined for terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) were incubated in 10% (v/v) TUNEL enzyme (Cat. No 11767305001, Roche) and 90% (v/v) TUNEL label (Cat. No 11767291910, Roche) for 60 minutes at 37°C. After washing in PBS (2×5 minutes) slides were counterstained with 4, 6-diamino-2-phenylindole (DAPI; Cat. No D8417, Sigma-Aldrich) for 3 minutes. All sections were mounted in Prolong Gold Antifade Mountant with DAPI (Cat. No P-36931, Molecular Probes). Corresponding species-specific nonimmune immunoglobulin G (IgG) isotype controls were applied to all tissue types at an equivalent concentration as the primary antibody. Details of the primary and secondary antibodies and IgG isotype controls used in this protocol can be found in **Table 2.1**, **Table 2.2** and **Table 2.3**. Digital images were recorded using a Leica inverted SP5 confocal laser-scanning microscope (Leica Microsystems).

Table 2.1. Primary antibodies used for immunofluorescence.

Primary antibody	Species	Concentration ($\mu\text{g/ml}$)	Supplier
Vasa (DDX4)	Rabbit polyclonal	3	Abcam (ab13840)
Laminin	Rabbit polyclonal	7	Abcam (ab11575)
Ki67	Rabbit monoclonal	0.84	Cell Signalling (12202)
Cleaved Caspase-3 (Asp175)	Rabbit monoclonal	0.4	Cell Signalling (9664)
ALK-5	Rabbit polyclonal	1	Abcam (ab31013)
TGFB2	Mouse monoclonal	2.5	Abcam (ab36495)
SMAD2/3	Mouse monoclonal	0.25	Santa Cruz (sc-133098)

Table 2.2. Secondary antibodies used for immunofluorescence.

Secondary antibody	Concentration ($\mu\text{g/ml}$)	Supplier
Donkey anti-Rabbit IgG (H+L) Alexa Fluor555	8	Invitrogen (A-31572)
Goat anti-Mouse IgG (H+L) Alexa Fluor488	8	Invitrogen (A-11029)
Goat anti-Rabbit IgG (H+L) Alexa Fluor633	8	Invitrogen (A-21071)
Goat anti-Rabbit IgG (H+L) Alexa Fluoro488	8	Invitrogen (A-11008)

Table 2.3. IgG controls used for immunofluorescence.

IgG	Concentration (µg/ml)	Supplier
Mouse IgG1	Equivalent to primary antibody concentration	Dako (X0931)
Mouse IgG2a	Equivalent to primary antibody concentration	Dako (X0943)
Rabbit IgG	Equivalent to primary antibody concentration	Sigma-Aldrich (I8140)

2.5 Culture analysis

2.5.1 Ovary culture

Immunohistochemical co-localisation of mouse vasa protein (DDX4) and laminin enabled clear definition of morphological characteristics by highlighting oocytes and basement membranes respectively. Antibodies are summarised in **Table 2.1**. Follicle classification was performed on three central ovarian sections, 25 µm apart, and all sections were assessed blind as to treatment. For each section, multiple confocal images (40x oil immersion objective) were combined into a digital image using DoubleTake (version 2.2.8; Echo One Software). Initially follicle development was defined by GC shape and extent of multilayering as described previously (Fenwick et al., 2011). Follicles were classified according to the following morphology: primordial follicle (oocyte surrounded by a flattened layer of GCs), transitional follicle (at least one, but not all, GCs cuboidal), primary follicle (oocyte surrounded by a complete layer of cuboidal GCs), primary plus follicle (incomplete second layer of GCs developing), secondary follicle (two complete layers of GCs), secondary plus follicle (further GC multilayering but no atrum formation). Later analysis used oocyte area as a surrogate marker of follicle growth. Only oocytes with a clear germinal vesicle were included to ensure oocytes were measured at their widest cross section. A threshold area that defined the point at which a follicle was classified as growing was chosen based on data obtained from a set of control ovaries analysed by both GC morphology and oocyte area. This will be covered in detail in Chapter 3. Oocytes that were larger than 275 µm² were defined as

growing. The number of growing follicles was then presented as a proportion of the sum of follicles within each section.

2.5.2 Follicle Culture

Follicles were imaged (Eclipse E600; Nikon) and digital images obtained (Digital Eclipse DXM 1200; Nikon) every 24 hours throughout the culture and the area of each follicle was measured using ImageJ (1.45s; <http://imagej.nih.gov/ij>) to track growth in response to treatments. Follicles were excluded from the analysis if i. the oocyte was misshapen or extruded during culture, and ii. the follicle was atretic, had two oocytes or the basal lamina was damaged at the start of the culture. The follicle area data measured at 24 hour intervals were calculated relative to the follicle area at time 0 and presented as the mean for each treatment.

2.5.3 Immunofluorescent staining quantification

Staining for Ki67, caspase-3 and TUNEL was quantified in cultured ovaries and follicles using ImageJ. When obtaining digital images for quantification of immunofluorescence intensity both the confocal gain and exposure time were kept consistent between samples. To quantify immunofluorescent staining in cultured ovaries positive somatic cell staining was determined by the auto threshold function (ensuring non-specific oocyte background staining was omitted) and represented as the proportion of pixels from the total number of pixels (area calculator plug-in) within the ovarian section. For immunofluorescent staining quantification in cultured follicles the number of somatic cell nuclei positive for DAPI were totalled and then those positive for the marker of interest counted and represented as the proportion of positive cells from the total number of cells. A single central section from each ovary/follicle was analysed and mean proportions were obtained for each treatment.

2.6 RNA extraction

2.6.1 RNA extraction from whole ovaries and adult tissue

Total RNA was extracted from whole ovaries and organs using the RNeasyMini Kit (Cat. No 74104, Qiagen) according to the manufacturer's protocol. Tissue which had been snap frozen in liquid nitrogen was thawed on ice and lysed in the appropriate volume of RLT buffer (200

µl for 12 dpp ovary) prepared with β-mercaptoethanol (Cat. No M3148, Sigma-Aldrich) (10 µl β-mercaptoethanol per 1 ml of RLT buffer). Large tissue samples were homogenised using a mortar and pestle. Following lysis, one volume of 70% ethanol was added to each sample. Samples were transferred to RNeasy spin columns provided with the kit and centrifuged (Mikro 20; Hettich) at 10000 rpm for 15 seconds. The RNA was then purified using a series of ethanol-based buffers. First 350 µl of RW1 was added to each column and samples centrifuged at 10000 rpm for 15 seconds. On-column DNase digestion was then performed by incubating each sample with a mixture of 10 µl DNase I and 70 µl of RDD buffer (Cat. No 79254, Qiagen) for 15 minutes at room temperature. Columns were then washed with 350 µl RW1 and 550 µl RPE and centrifuged at 10000 rpm for 15 seconds. A final wash was performed using 500 µl RPE and samples centrifuged at 10000 rpm for 2 minutes followed by centrifugation without the addition of any buffer for 1 minute at maximum speed to dry the membrane. The RNA was eluted by the addition of 30 µl of RNase-free water directly onto the column membrane followed by centrifugation at 10000 rpm for 1 minute. RNA was used immediately for complementary DNA (cDNA) synthesis.

2.6.2 RNA extraction from isolated follicles

Total RNA was extracted from isolated follicles using the RNeasyMicro Kit (Cat. No 74004, Qiagen) according to the manufacturer's protocol. Isolated follicles which had been snap frozen in liquid nitrogen were thawed on ice and lysed in 75 µl RLT buffer prepared with β-mercaptoethanol as described in section 2.6.1. Samples were vortexed to homogenise the tissue. Following lysis 5 µl (4 µg/µl) carrier RNA was added to each sample. One volume of 70% ethanol was then added to each sample. Samples were transferred to RNeasy MiniElute spin columns provided with the kit and centrifuged at 10000 rpm for 15 seconds. The RNA was then purified using a series of ethanol-based buffers in a similar methodology to that described in section 2.6.1. First the RNA was washed using RW1 and on-column DNase digestion performed as described in section 2.6.1. Columns were then washed with 350 µl RW1 and 550 µl RPE and centrifuged at 10000 rpm for 15 seconds. A final wash was performed using 500 µl 80% ethanol and samples centrifuged at 10000 rpm for 2 minutes followed by centrifugation without the addition of any buffer for 1 minute at maximum speed to dry the membrane. The RNA was eluted by the addition of 14 µl of RNase-free water

directly onto the column membrane followed by centrifugation at 10000 rpm for 1 minute. RNA was used immediately for cDNA synthesis.

2.7 Determination of RNA quantity and quality

The concentration and quality of RNA extracted from adult tissue was determined with a Nanodrop spectrophotometer (Nanodrop ND-1000; Thermo Scientific). RNA extracted from juvenile whole ovaries and follicles was of immeasurable quantity for spectroscopy. Instead PCR amplification (detailed in section 2.9.1) of abundant oocyte gene *Gdf9* was used as an indicator of adequate yield and purity of RNA.

2.8 cDNA synthesis

The Superscript®III First-Strand Synthesis System (Cat. No 18080-051, Invitrogen) was used to generate cDNA using random hexamer primers and SuperScript®III reverse transcriptase in accordance with the manufacturer's guidelines. For adult tissue 500 ng of RNA was reverse transcribed. For juvenile ovaries and follicles each RNA sample was concentrated to 5 µl using a speed vac concentrator (Savant DNA120 SpeedVac Concentrator; Thermo Scientific) and the entire sample was used to prepare the RNA/primer mixture described in **Table 2.4**. This was then incubated at 65°C for 5 minutes and immediately placed on ice for 1 minute. The reverse-transcription mastermix for each reaction was prepared as shown in **Table 2.5** and added to the RNA/primer mix to make up a final reaction volume of 20 µl. Reactions were incubated in a thermal cycler (TC-3000; Techne) at 25°C for 10 minutes, 50°C for 50 minutes then 85°C for 5 minutes. cDNA samples were diluted in dH₂O if necessary and stored at -20°C.

Table 2.4. Components of RNA/primer mixture per reaction.

Component	1× reaction (μl)
Sample RNA	5
dNTP mix (10 mM)	1
Random hexamers (50 ng/μl)	1
H ₂ O	3

Table 2.5. Reagents for cDNA synthesis mixture per reaction.

Reagent	1× reaction (μl)
10× RT buffer	2
MgCl ₂ (25 mM)	4
DTT (0.1 M)	2
RNaseOUT (40 U/μl)	1
Superscript III RT (200 U/μl)	1

2.9 Polymerase chain reaction (PCR) and quantitative real-time PCR (RT-PCR)

2.9.1 PCR

PCR was employed to perform an initial presence/absence screen of juvenile ovary and adult tissue for transcripts encoded by genes of interest. Primers were designed to detect the mouse transcripts listed in **Table 2.6**. PCR was performed using KAPA2G Fast Readymix (Cat. No KK5102, Kapa Biosystems). A reaction mix containing the primer set for the gene of interest was prepared as shown in **Table 2.7**. PCR reactions were carried out in a thermal cycler. Following a 2 minute 95°C hot start, amplification consisted of 30 cycles of denaturation (95°C for 20 seconds), primer specific annealing (15 seconds) and extension (72°C for 1 second). PCR products were then separated by 2% (w/v) agarose gel electrophoresis (100 ml TAE buffer (40 mM Tris Base, 20 mM glacial acetic acid, 1 mM EDTA (pH 8.0), 2 g

agarose, 0.2 µg/ml ethidium bromide). Electrophoresis was performed at 100 V for 1 hour. PCR products were visualised under ultra violet light (AlphaImager Mini System, Protein Simple).

Table 2.6. Primer sequences for PCR and quantitative RT-PCR.

Gene symbol	Primer sequence		GenBank Accession	Product size* (bp)
	Forward	Reverse		
<i>Acvr2a</i>	ttcgccgtctttcttatctctt	gtcgccgtttatctttatcacc	NM_007396.4	151
<i>Acvr2b</i>	ctggctgttcggtttgag	cgtctctctggaagtgtgatgg	NM_007397.2	115
<i>Alk4</i>	caacatgaagcactttgactcc	tcacatacaaccttcgcatct	NM_007395.3	177
<i>Alk5</i>	attgctccaaaccacagagtag	caccaatagaacagcgtcgag	NM_009370.2	157
<i>Ccnd2</i>	agaaggacatccaaccgtacat	gctgaagatgggtcttaggagt	NM_009829.3	156
<i>Cdkn1b</i>	cgggtgcctttaattgggtct	cttcttgggcgtctgctc	NM_009875.4	227
<i>Fst</i>	ctttgtggaaatgatggagtga	aggacacagctcatcgaga	NM_008046.2	207
<i>Htra1</i>	ggaactccgatatggactacatt	ggtgttaatcccaatcacctc	NM_019564.3	104
<i>Id1</i>	aaccgcaaagtgagcaaggt	gcctcagcgacacaagatg	NM_010495.2	216
<i>Id2</i>	aattccctctgagcttatgtcg	ctggtgaaatggctgataaaa	NM_010496.3	160
<i>Id3</i>	agaggagcttttgccactga	agggctgggttaagatcgaag	NM_008321.2	149
<i>Inha</i>	ctcccaggctatcctttcc	tggccggaatacataagtga	NM_010564.4	112
<i>Inhba</i>	atcatcaccttgccgagt	tgactttggtcctggttctg	NM_008380.1	148
<i>Inhbb</i>	aaataatgttctcccgtttg	tggctatgcgtgtctatgtg	NM_008381.3	102
<i>Mmp-2</i>	atcgcccatcatcaagtcc	gcagccatagaaagtgttcag	NM_008610.2	88
<i>Snai1</i>	acacgctgccttgtgtctg	cttcacatccgagtggtgtt	NM_011427.3	176

<i>Snai2</i>	ctacagcgaactggacacaca	gggtaaaggagagtgaggagg	NM_011415.2	160
<i>Tgfb1</i>	atacgcctgagtggtgt	gtggagtttggtatcttgctg	NM_011577.1	128
<i>Tgfb2</i>	ttggatgctgcctactgct	ctgaggactttggtgtgtga	NM_009367.3	185
<i>Tgfb3</i>	tgggtctggaaatcagca	atcaggtgtgggtgtgg	NM_009368.3	181
<i>Tgfb2</i>	ggaccctactctgtctgtgg	atccgtctgctgaacgact	NM_009371.3	127
<i>Tgfb3</i>	atggctccctgtgttgc	gcggagggttaggatgtga	NM_011578.3	195
<i>Timp-1</i>	gcatctggcatcctctgtt	aggtggtctcgttgattctg	NM_001044384.1	148
<i>Amh</i>	ggggcacacagaacctct	gcacctctctgcttggtg	NM_007445	124
<i>Cyp17a</i>	gatcggtttatgcctgagcg	tccgaagggaataactgg	NM_007809	81
<i>Gdf9</i>	tcacctctacaataccgtccg	gagcaagtgtccatggcagtc	NM_008110	139

*Primers were designed such that amplified products span at least one adjacent exon boundary.

Table 2.7. PCR mastermix per reaction.

Reagent	1× reaction (μl)
Mastermix	12.5
Template cDNA	1
Primer mix	1
H ₂ O	10.5

2.9.2 Quantitative RT-PCR

SYBR Green-based detection quantitative RT-PCR was performed to determine the concentrations of transcripts encoded by genes of interest in isolated follicles and juvenile ovary. Two different methods were employed. The $\Delta\Delta C_t$ method was used to assess relative gene expression in cultured follicles and juvenile ovary. The standard curve method was used

to determine absolute transcript expression in isolated follicles. Primers are summarised in **Table 2.6**.

2.9.2.1 $\Delta\Delta C_t$ method

Quantitative RT-PCR was performed to examine gene expression in cultured follicles and juvenile ovary. Reaction mixes containing 500 nM primers were prepared as detailed in **Table 2.8**, with ROX and KAPA SYBR Fast Universal qPCR Master Mix (Cat. No KK4602, Kapa Biosystems). An equal volume (1 μ l) of cDNA was added in duplicate to each 19 μ l reaction in each well of a 384-well plate (Cat. No 4309849, Applied Biosystems) alongside no-template (H_2O) controls. Plates were sealed using an adhesive film (Cat. No 4311971, Applied Biosystems) and centrifuged at 1400 rpm for 1 minute. Cycling was carried out using the 7900HT Fast Real-Time PCR system with SDS v2.4 software (Applied Biosystems). Amplification consisted of an initial activation step at 95°C for 3 minutes followed by 40 cycles of denaturation (95°C for 3 seconds), primer specific annealing (20 seconds), extension (72°C for 1 second), and an amplicon-specific fluorescence acquisition reading. A melting curve analysis was performed for every sample to ensure specific amplification. *Atp5b* (Cat. No ATP5B, Primer Design) was deemed a suitable internal reference gene for both juvenile ovary and cultured follicles respectively because mean expression did not vary significantly between experimental groups. Expression levels of genes of interest were normalised to the internal reference gene and then calculated as fold change relative to the relevant experimental control group using the formula $2^{(-\Delta\Delta C_t)}$ (Livak & Schmittgen, 2001). $\Delta\Delta C_t$ values were used for statistical analysis and data was plotted as fold change compared to the relevant control sample.

Table 2.8. Quantitative RT-PCR mastermix per well.

Reagent	1× reaction (μ l)
KAPA SYBR Fast mastermix	10
Primer mix	0.5
ROX high	0.4
H_2O	8.1

2.9.2.2 Standard curve method

Quantitative RT-PCR was performed to quantify absolute transcript levels of selected genes in isolated follicles. Standards, identical to quantitative RT-PCR products, were prepared from a cDNA reference sample (12 dpp ovary) for each gene of interest and purified using the MinElute PCR purification kit (Cat. No 28004, Qiagen). The concentration of each purified standard was determined (Nanodrop) and dilutions made up to 10 orders of magnitude in nuclease-free water (10^{-2} , 10^{-4} , 10^{-6} , 10^{-8} , 10^{-10}). Quantitative RT-PCR was performed on the experimental and reference standards using the StepOnePlus Real-Time PCR System (Applied Biosystems) employing the same methodology as described in section 2.9.2.1. All quantitative RT-PCR reactions were performed in triplicate (standards) or duplicate (samples) alongside no-template (H_2O) controls. Absolute transcript values (femtograms per microgram reverse transcribed RNA) for each sample were extrapolated from the standard curve generated from the relevant reference standard. The linearity of all standard curves was 0.995 or greater.

2.10 Protein extraction

Protein was extracted from pooled follicles. Pooled follicles were washed in ice cold PBS and snap-frozen in liquid nitrogen in 30 μ l Radioimmunoprecipitation assay (RIPA) lysing buffer (Cat. No 89900, Thermo Scientific) containing protease and phosphatase inhibitor cocktail (1 μ l/100 μ l RIPA buffer; Cat. No 78441, Thermo Scientific). Samples were thawed on ice and homogenised by sonication (Bioruptor sonication device, Diagenode) for 3 cycles of 30 seconds on, 30 seconds off. Following homogenisation, the lysates were centrifuged at 13000 RPM for 10 minutes at 4°C. Supernatants were collected and stored at -80°C.

2.11 Western blotting

Western immunoblotting was used to identify pathways activated in response to treatments in cultured follicles. As protein extracted from isolated follicles was of immeasurable quantity for protein assay, the entire sample (30 μ l) was used for western blotting. Samples were prepared as described in **Table 2.9** and heated to 70°C for 10 minutes to denature proteins. Reduced samples were loaded into individual wells in precast Bolt 4 - 12% Bis-Tris (1.0 mm $\times$ 10 well) gels (Cat. No NW04120BOX, Invitrogen) and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V for 30 minutes in 1 $\times$ Bolt

MES SDS running buffer (Cat. No B0002, Novex) in the Mini Gel Tank (Invitrogen). Following separation, proteins were transferred onto a nitrocellulose blotting membrane (Cat. No 10600002, GE Healthcare Life Sciences) using the Mini-PROTEAN III Electrophoresis System (Bio-Rad) equilibrated in transfer buffer (100 ml 10 × TB (250 mM Tris Base, 1.92 M Glycine), 700 ml dH₂O, 200 ml methanol; pH 8.3) at 100 V, 350 mA at 4°C for 2 hours. Complete and even transfer was assessed by Ponceau stain (0.1% (w/v) Ponceau S Red powder, 5% (v/v) acetic acid) of the membrane. The membrane was then blocked for 1 hour at room temperature using 5% BSA or skim milk powder (Cat. No LP0031, Oxoid) in Tris-buffered saline (TBS; 20 mM Tris Base, 137 mM NaCl; pH 7.6) with 1% Tween-20 (v/v) before incubation with primary antibody overnight at 4°C in 1% BSA. After 3 × 5 washes in TBST the membrane was incubated in the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody for 1.5 hours. Details of the primary and secondary antibodies used in this protocol can be found in **Table 2.10** and **Table 2.11**. Following 3 × 10 minute TBST washes, protein was visualised by chemiluminescent detection. Briefly the membrane was incubated with chemiluminescent detection reagent (Cat. No 32209, Thermo Scientific) and the light signal detected using x-ray film (Cat. No 28906837, GE Healthcare Life Sciences) or digital imaging (ImageQuant LAS 4000; GE Healthcare Life Sciences). Levels of calnexin were used as a loading control. The bands were quantified using Image Studio Lite software (v5.25; LI-CORE Biosciences). The band signal of treatments were calculated relative to the experimental control group and then normalised to the loading control. The relative adjusted signal was then used for statistical analysis and data was plotted as fold change compared to the relevant control sample.

Table 2.9. Reagents per reduced sample.

Reagent	1× (μl)
Bolt LDS sample buffer (4×)	11.5
Bolt reducing agent (10×)	4.6
Protein	30
Total	46

Table 2.10. Primary antibodies used for western immunoblotting.

Primary antibody	Species	Concentration (µg/ml)	Supplier
Phospho-SMAD3 (Ser423/425)	Rabbit monoclonal	1.4	Cell Signalling (9520)
SMAD3	Rabbit monoclonal	0.075	Cell Signalling (9523)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Rabbit monoclonal	0.501	Cell Signalling (4370)
p44/42 MAPK (Erk1/2)	Mouse monoclonal	1.2	Cell Signalling (9107)
Calnexin	Rabbit polyclonal	1	AbCam (ab22595)

Table 2.11. Secondary antibodies used for western immunoblotting.

Secondary antibody	Concentration (µg/ml)	Supplier
Goat anti-Rabbit IgG	0.25	Dako (P0448)
Goat anti-Mouse IgG	1	Dako (P0447)

2.12 Experimental compounds

The suppliers and a brief overview of the mode of action of the experimental compounds used are detailed in **Table 2.12**. A comprehensive explanation of the mechanisms underlying the ligand and small molecule inhibitors are detailed below and in the relevant results chapters.

Table 2.12. Details of experimental compounds.

Compound	Mechanism	Supplier
Recombinant mouse TGF-beta 2 protein	Ligand for ALK5/TGFBR2 receptors	R&D Systems (7346-B2-005)
SB505124	Competitive inhibitor of ALK4, 5, 7 receptors	Sigma-Aldrich (S4696)
Dorsomorphin	Inhibitor of ALK 2, 3, 6 receptors	Sigma-Aldrich (P5499)
U0126	Inhibitor of MEK1 and MEK2	Calbiochem (662005)
LY294002	Inhibitor of PI3K	Sigma-Aldrich (L9908)

2.12.1 Ligands

2.12.1.1 TGFB2

Recombinant mouse TGF-beta 2 protein was reconstituted at 100 µg/ml in 4 mM HCl containing 0.1% BSA as per supplier's instructions. Working solutions were prepared in the relevant culture medium and the vehicle used as the control at a concentration equivalent to the highest treatment dose. A dose response was initially performed in both the whole ovary culture (1, 10, 100 ng/ml) and isolated preantral follicle culture (0.01, 0.1, 1, 10, 100 ng/ml). Based on this, the dose of 0.1 ng/ml was selected for all subsequent isolated follicle studies.

2.12.2 Small molecule inhibitors

2.12.2.1 SB505124

SB505124 was reconstituted to 7.5 µM in dimethyl sulfoxide (DMSO; Cat No D12345, Thermo Scientific) as per supplier's instructions. A treatment concentration of 1 µM was selected based on previously published data demonstrating efficacy of the dose and an unpublished dose response (DaCosta Byfield et al., 2004; Fenwick et al. unpublished). Working solutions were prepared in the relevant culture medium and DMSO used as the

vehicle control at a concentration equivalent to that present in the treatment dose. SB505124 is a selective inhibitor of the TGF β type I receptors ALK4, ALK5 and ALK7, and therefore blocks phosphorylation of SMAD2/3. SB505124 was developed as a competitive inhibitor of the ATP binding site of the ALK5 receptor, inhibiting kinase activity, however the closely related sequence homology of the kinase domains of the type I receptors, ALK4, ALK5 and ALK7, confers specificity of the inhibitor for all three (DaCosta Byfield et al., 2004).

2.12.2.2 Dorsomorphin

Dorsomorphin (DSM) was reconstituted to 10 mM in DMSO as per supplier's instructions. A treatment concentration of 1 μ M was selected based on a previously published dose response (Fenwick et al., 2013). Working solutions were prepared in the relevant culture medium and DMSO used as the vehicle control at a concentration equivalent to that present in the treatment dose. DSM is an inhibitor of BMP type I receptor (ALK2, ALK3 and ALK6), inhibiting kinase activity and therefore blocks phosphorylation of SMAD1/5/8 (Yu et al., 2008).

2.12.2.3 U0126

U0126 was reconstituted at 10 mM in DMSO as per supplier's instructions. A treatment concentration of 10 μ M was selected based on previously published data demonstrating efficacy of the dose (Favata et al., 1998). Working solutions were prepared in the relevant culture medium and DMSO used as the vehicle control at a concentration equivalent to that present in the treatment dose. U0126 is a specific inhibitor of the MAPK kinase family members, MEK1 and MEK2 and therefore a selective MAPK signalling cascade inhibitor (Favata et al., 1998).

2.12.2.4 LY294002

LY294002 was reconstituted to 10 mM in DMSO as per supplier's instructions. A treatment concentration of 50 μ M was selected based on previously published use of the inhibitor (Brunn et al., 1996; Vlahos et al., 1994). Working solutions were prepared in the relevant culture medium and DMSO used as the vehicle control at a concentration equivalent to that present in the treatment dose. LY294002 hydrochloride is an inhibitor of PI3K and therefore blocks PI3K-dependent Akt phosphorylation and kinase activity (Vlahos et al., 1994).

2.13 Statistical analysis

All analysis was performed using the GraphPad Prism v6.0 (Graphpad Inc, San Diego, CA). An initial analysis to assess whether the data were of Gaussian distribution was conducted using the Di'Agostino-Pearson normality test. Normally distributed data were analysed by unpaired *t* test or analysis of variance (ANOVA), and where differences were detected the appropriate post hoc test was used. Data not normally distributed were analysed by the non-parametric Kruskal-Wallis or Mann-Whitney test. N denotes the experimental unit and represents the number of ovaries, ovarian sections, oocytes or follicles as stated. Data are presented as the mean and standard error of the mean (SEM) with statistical significance considered to be $p < 0.05$.

Chapter 3 Analysis of an in vitro whole ovary culture

3.1 Introduction

The aim of this thesis was to examine the role of TGFB signalling in primordial follicle activation. In this chapter the background to the culture used to investigate primordial follicle activation will be discussed and validation of the analysis and sampling method employed presented.

The onset of follicle development is notoriously challenging to investigate. The innate nature of the primordial follicle not only lends to the technical difficulty in isolation and manipulation, but the survival in culture. Indeed, Eppig and O'Brien (1996) demonstrated that the degeneration of individual primordial oocyte-granulosa cell complexes in culture led to the failure of oocyte development due to the loss of vital GC-oocyte gap junctional communication (Eppig, 1979). To counter this a neonatal whole ovary organ culture system was employed to investigate primordial follicle activation. The culture system used was one based on that developed by Eppig and O'Brien (1996) in which neonatal mouse ovaries were grown in organ culture on transwell inserts. The model maintains normal in vivo ovarian anatomy preserving both the structural support required for primordial follicle development and the tissue interactions between different ovarian cell types (oocyte granulosa theca stroma) and neighbouring follicles that are essential for follicle growth and development. Furthermore, this in vitro model demonstrates accurate replication of the window of early follicle development to be examined in the current thesis. This is exemplified by both the spatio-temporal pattern of early follicle development, whereby the first wave of follicle development was demonstrated in the medullary region, and the unimpaired development of small multilayered preantral follicles as would be observed in the equivalent in vivo stage ovary (Eppig & O'Brien, 1996). As the current thesis is concerned with the development of the primordial follicle stage onwards, the 4 dpp neonatal mouse ovary, which is populated almost exclusively with primordial follicles, provides an ideal model with which to investigate follicle activation (Pepling & Spradling, 2001).

The classification of follicle stage is classically based on either granulosa cell number or granulosa cell morphology and extent of multilayering (Fenwick et al., 2013; Pedersen & Peters, 1968). However a preliminary examination of tissue morphology in the cultured ovary

led to the observation that follicle oocyte-GC complexes were often not circumscribed completely by a basal lamina, an observation also reported by Eppig and O'Brien (1996). The decreased clarity or indeed absence of a basal lamina therefore presented problems in ascertaining whether or not a GC belonged to a given follicle, thus introducing a level of subjectivity into the culture analysis. As the onset of mammalian follicle development is marked by both GC proliferation and cuboidalisation and oocyte growth, a study was undertaken to determine whether oocyte area could be employed as a surrogate marker of follicle activation (Lintern-Moore & Moore, 1979).

The method used to examine follicle distribution and number in the ovary is dependent on the required outcome: whether absolute values or simply a representative sample from the population without the need for absolute numbers. The most widely used approach for estimating follicle number is one based on histological sampling of the total ovarian mass for the number of primordial and growing follicles, often followed by the application of a 'correction factor' to account for the proportion of the ovary not explicitly sampled. Other commonly used techniques include follicle counts of every section or averaging follicle counts from two adjacent central sections (Lee et al., 2001; Nilsson & Skinner, 2003). In this regard studies were conducted to determine the most appropriate sampling method, indeed one that was both time efficient however allowed for a large enough sample to reduce inter-ovarian variability.

3.1.1 Specific aims

- Validate oocyte area as a surrogate marker of primordial follicle activation in a neonatal ovary culture
- Establish an effective sampling method for analysing an in vitro neonatal ovary culture

3.2 Results

3.2.1 Validating oocyte area as a surrogate marker of primordial follicle activation

In order to determine whether oocyte area could be employed as a surrogate marker of follicle activation, data were obtained from a set of three cultured control ovaries. Following

a 6-day culture, ovaries were fixed and sectioned. A 6-day culture period was selected as it permits the development of oocytes enclosed by 1 – 3 layers of GCs thus allowing the early stage of preantral follicle development to be examined. A total of 636 follicles from three central sections of each ovary were classified morphologically and oocyte area measured. Only follicles with a clear germinal vesicle were included to ensure classification and measurement at the widest cross section. On average nuclear diameter ranges from 9.3 μm for primordial follicles to 18.4 μm for secondary follicles (based on 1289 direct measurements of nuclear size from 8 dpp and 12 dpp ovaries; Thorne (2005)). As the largest follicle stage observed in the cultures was primary plus, selecting central sections 25 μm apart ensured that double counting was avoided during sampling. Morphological classification of follicle development was defined by GC shape and extent of multilayering (**Figure 3.1**). In brief follicles were classified according to the following morphology: primordial follicle (oocyte surrounded by a flattened layer of GCs), transitional follicle (at least one, but not all, GCs cuboidal), primary follicle (oocyte surrounded by a complete layer of cuboidal GCs), primary plus follicle (incomplete second layer of GCs developing). Follicles were considered growing once cuboidal GCs were present. The number of morphologically classified primordial or growing follicles were then presented as a percentage of follicles within each section. Oocyte area was then explored as a surrogate marker of follicle growth. The oocyte area data from each section were ranked in ascending order and multiple area cut offs were examined (from 250 μm^2 to 350 μm^2) to determine a threshold area that defined the point at which a follicle was classified as primordial or growing. For each examined oocyte area cut off, the number of oocytes equal to or greater than this were considered growing and presented as a percentage of the total number of oocytes measured while the number of oocytes less than this defined area were considered primordial. The most appropriate ratio (and therefore oocyte area threshold) was then selected based on similarity to the ratio obtained from the morphological classification. Based on this oocytes that were larger than 275 μm^2 were defined as growing as the ratio obtained from this size (61.4:38.6) demonstrated most similarity to the ratio obtained by morphological classification (62.5:37.5) (**Figure 3.2**).

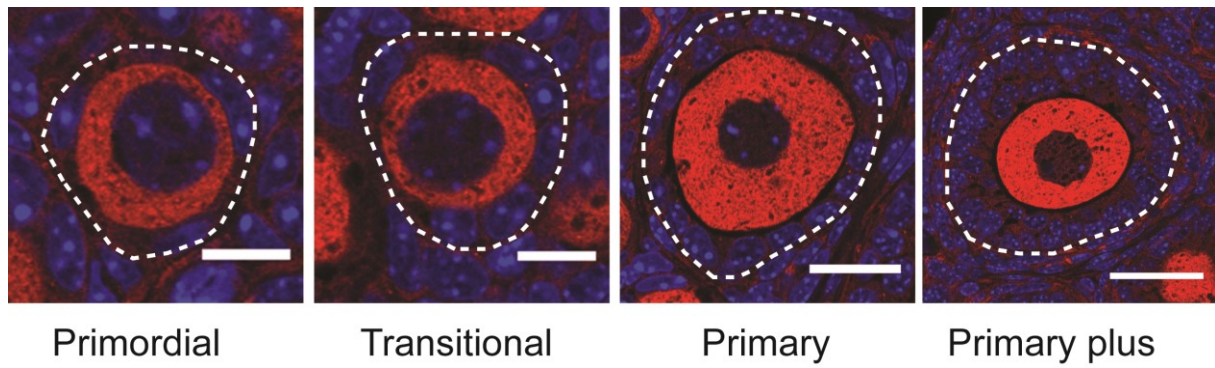


Figure 3.1. Morphological classification of follicle stage. Mouse ovaries (4 dpp) were cultured for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) and basement membrane marker laminin (both red) and nuclear DAPI (blue) for morphological classification. Follicles were classified according to the following morphology: primordial follicle (oocyte surrounded by a flattened layer of GCs), transitional follicle (at least one, but not all, GCs cuboidal), primary follicle (oocyte surrounded by a complete layer of cuboidal GCs) and primary plus follicle (incomplete second layer of GCs developing). Representative images of primordial, transitional, primary and primary plus follicles. Dashed lines represent basal lamina. Scale bar, primordial and transitional 10 μ m, primary 20 μ m and primary plus 35 μ m.

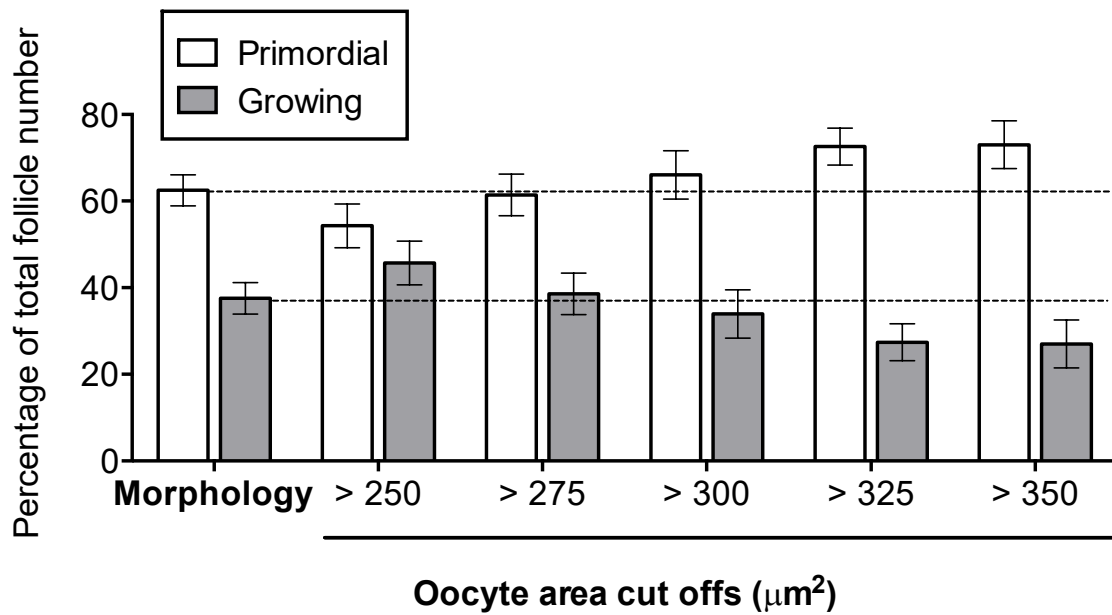


Figure 3.2. Validating oocyte area as a surrogate marker of primordial follicle activation. Mouse ovaries (4 dpp) (3 ovaries; n = 9 sections) were cultured for 6 days. Cultured ovaries were fixed and sectioned for immunolocalisation of oocyte-specific mouse vasa protein (DDX4) and basement membrane marker laminin and nuclear DAPI for morphological classification and measurement of oocyte area. Three central sections, 25 μm apart, from each ovary were used for analysis. A total of 636 follicles were classified morphologically and oocyte area measured. Follicles were considered growing once cuboidal GCs were present. The number of morphologically classified primordial or growing follicles were presented as a percentage of follicles within each section. The oocyte area data from each section were then ranked in ascending order and multiple area cut offs were examined (from 250 μm^2 to 350 μm^2). For each examined oocyte area cut off, the number of oocytes equal to or greater than this were considered growing and presented as a percentage of the total number of oocytes measured while the number of oocytes less than this defined area were considered primordial. The most appropriate ratio (and therefore oocyte area threshold) was then selected based on similarity to the ratio obtained from the morphological classification. Oocytes that were larger than 275 μm^2 were defined as growing as the ratio obtained from this size (61.4:38.6) demonstrates most similarity with the ratio obtained by morphological classification (62.5:37.5) (demonstrated by dashed line). Data presented as mean $\pm$ SEM.

3.2.2 Establishing a sampling method for an in vitro neonatal ovary culture

Oocyte area data were then examined to determine an effective sampling method for analysing an in vitro neonatal ovary culture. An observed feature of the culture system was the flattening of ovarian tissue onto the membrane (**Figure 3.3A**). This led to a subsequent

loss of normal ovarian follicle distribution as demonstrated in **Figure 3.3 B - B''**. The morphological examination of three central sections demonstrated larger growing follicles had a tendency to become situated in the upper region of the cultured ovary (**Figure 3.3 B**) while primordial follicles were more often observed at the base of the ovary adjacent to the membrane (**Figure 3.3 B''**). Oocyte area data illustrating the variation between three central sections from three individual ovaries confirmed morphological observations (**Figure 3.4**). Oocyte area data were then examined to determine the sample size required for analysis of the neonatal ovary culture. Given the variable extent of tissue flattening during the culture period (which both minimises the depth of tissue from which to sample and contributes to the variable distribution of oocyte area) and the necessity to sample from complete sections 25 μm apart, three central sections were selected as the sample size required for analysis of the neonatal ovary culture. When the distribution of oocyte area was plotted the results demonstrated that data sampling from three central sections was sufficient to ensure consistent oocyte area distribution between ovaries **Figure 3.5**.

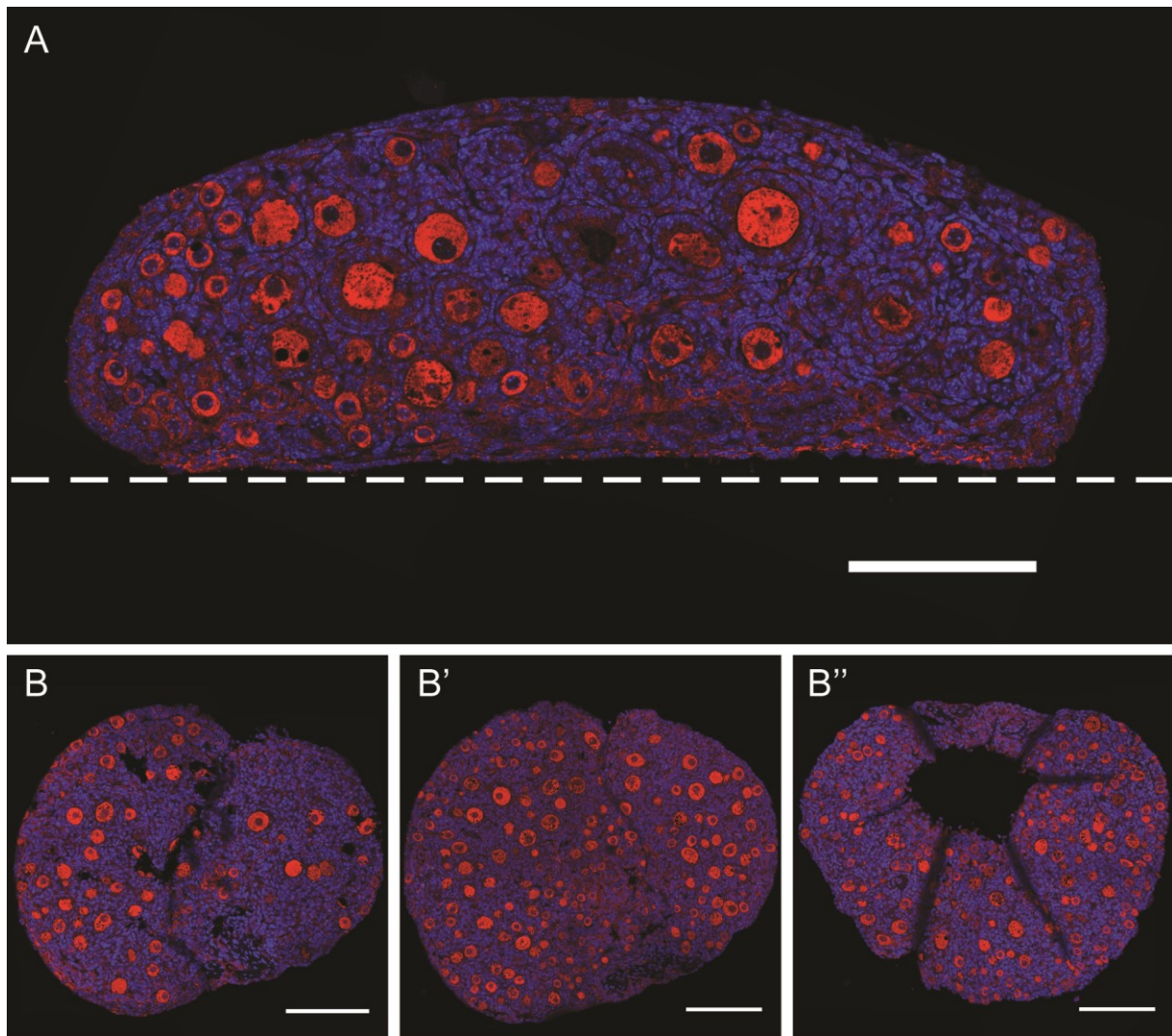


Figure 3.3. Morphology of the cultured ovary. Mouse ovaries (4 dpp) were cultured for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) (red) and nuclear DAPI (blue). A feature of cultured whole ovary tissue is the flattening of tissue onto the membrane and subsequent loss of normal ovarian follicle distribution. Representative images of a sagittal sectioned ovary (A) and a transverse sectioned ovary (B, B', B'') demonstrating flattening of the tissue and variation of follicle distribution between three central sections 25 μ m apart. Dashed line demonstrates where tissue was in contact with membrane in culture. Scale bar, 100 μ m (A), 150 μ m (B, B', B'').

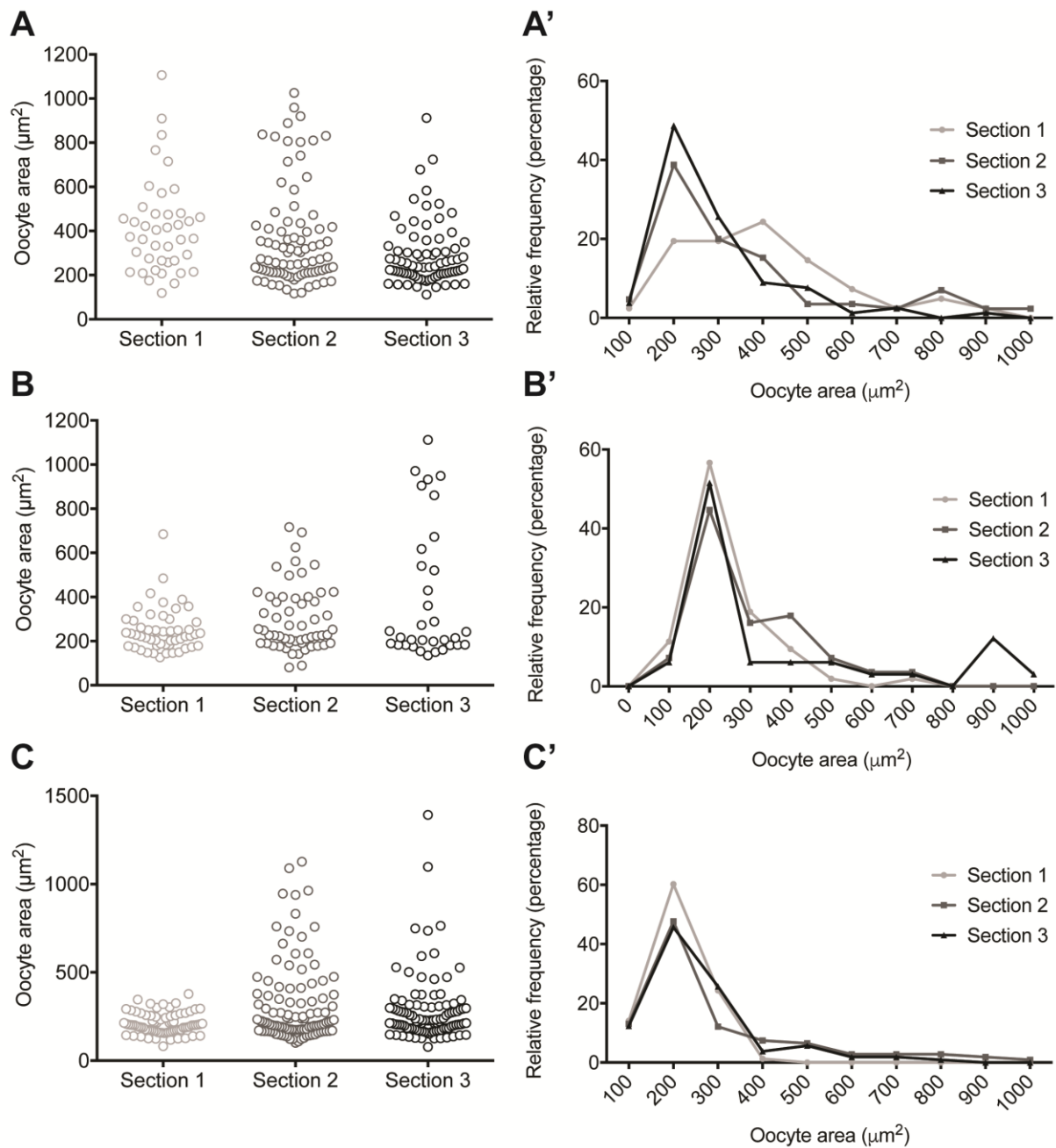


Figure 3.4. The distribution of oocyte area in three central sections from three cultured ovaries. Mouse ovaries (4 dpp) were cultured for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) and nuclear DAPI for measurement of oocyte area. Three central sections, 25 μm apart, from each ovary were used for analysis. Oocyte area was measured in every follicle with a clear nucleus. Oocyte measurements from three central sections from three representative ovaries. Data presented as oocyte area distribution (A, B, C) or percentage of oocytes within area bins (A', B', C').

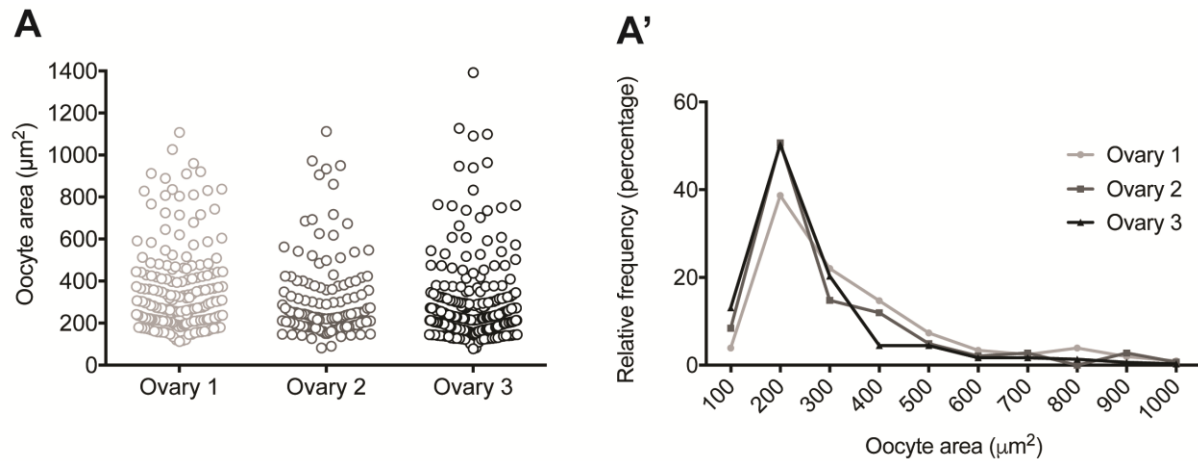


Figure 3.5. Data sampling from three central sections ensures consistent oocyte area distribution between ovaries. Mouse ovaries (4 dpp) were cultured for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific vasa and nuclear DAPI for classification. Three central sections, 25 μm apart, from each ovary were used for analysis. Oocyte area was measured in every follicle with a clear nucleus. Oocyte measurements from three representative ovaries from a single culture experiment. Data presented as oocyte area distribution (A) or percentage of oocytes within area bins (A').

3.3 Discussion

The first objective of this chapter was to validate oocyte area as a surrogate marker for primordial follicle activation in a neonatal ovary culture. An examination of culture morphology led to the observation that oocyte GC complexes were often not circumscribed completely by the basal lamina, an observation also reported by Eppig and O'Brien (1996). A consequence of which was the inability to define whether or not a GC belongs to a given follicle thereby introducing a level of subjectivity into the culture analysis. The use of oocyte area as a surrogate marker of follicle activation therefore provides a methodology in which to circumvent this issue. Based on a simultaneous study of both morphological classification and a measure of oocyte area, an oocyte area greater than 275 μm^2 was defined as the threshold for a growing follicle. Oocyte area provided a quantitative, less subjective, time efficient measure of follicle activation. For this reason, this sampling method was used for the analysis of all subsequent ovary cultures.

The second objective of the chapter was to determine the most appropriate ovarian culture sampling method, indeed one that was both time efficient but which allowed for a large

enough sample to reduce inter-ovarian variability. An observed feature of cultured whole ovary was the flattening of tissue onto the membrane. This led to a subsequent loss of normal ovarian follicle distribution as demonstrated. Given the variable extent of tissue flattening between individual ovaries (which both minimises the depth of tissue from which to sample and contributes to the variable distribution of oocyte area) and the necessity to sample from complete sections 25 μm apart, three central sections were selected as the sample size required for analysis of the neonatal ovary culture. Indeed, the current data demonstrated that the analysis of three central sections, 25 μm apart, from each ovary led to a consistent oocyte area distribution between ovaries. This sampling method ensures that when using the ovary culture in an experimental paradigm, data will be comparable between treatment groups without intending to represent absolute follicle numbers, thereby allowing for time efficient analysis.

This chapter therefore outlines the culture model used to investigate primordial follicle activation and defines both the analysis and sampling method by which subsequent investigations throughout the thesis will be performed.

Chapter 4 Investigating the role of SMAD2/3 signalling in primordial follicle activation

4.1 Introduction

The SMAD2/3 proteins have been immunolocalised in the GC of the mammalian postnatal ovary and have more recently been established as the major TGFB signalling pathway expressed in early preantral follicles (Drummond et al., 2002; Fenwick et al., 2013; Kano et al., 1999; J. Xu et al., 2002). Furthermore, stage specific expression has been demonstrated with abundant localisation observed in the GC of early preantral follicles (primordial transitional and primary) and low expression in large multilayered follicles suggesting that signalling through the SMAD2/3 pathway may play a role both in the maintenance of the primordial pool and early preantral follicle development (Fenwick et al., 2013).

Targeted disruption of the SMAD family has provided further insight into the role of the SMAD proteins in follicle development however detailed knowledge of the functional role of SMAD2 and SMAD3 in the postnatal ovary remains incomplete. This is due in part to the embryonically lethal phenotype of the *Smad2* null mouse which precludes direct examination of the reproductive role of SMAD2, while homozygous disruption of *Smad3* results in animals predisposed to colorectal cancer with reduced lifespan (Nomura & Li, 1998; Waldrip et al., 1998; Zhu et al., 1998). Despite the reduced lifespan, mice that harbour a deletion of the *Smad3* gene are viable, however discrepancies have been reported in the reproductive phenotype of the two *Smad3* knockout lines published, both of which target distinct exons. Zhu et al. (1998) who reported a homozygous deletion of *Smad3* in exon 2, described female mice that were fertile and produced homozygous litters. This is in contrast to mice with a targeted deletion of *Smad3* in exon 8 in which reduced fertility was demonstrated due to impaired follicle development (Tomic et al., 2002). Li et al. (2008) later employed a conditional murine knockout of both *Smad2* and *Smad3* to circumvent the deleterious effects of universal gene disruption. The combined loss of SMAD2/3 in GCs led to dramatically reduced female fertility via disrupted follicle development and reduced ovulation efficiency. The nature of the promotor used in the study however led to the circumvention of SMAD2/3 ablation at the single layered follicle stage, thus the role of the SMAD2/3 pathway in primordial follicle activation remains unexamined.

Despite accumulating evidence highlighting the signalling cascade as an integral regulatory pathway in the postnatal ovary the functional role of SMAD2/3 during the window of early follicle development remains unexamined. The spatiotemporal expression patterns of SMAD2/3 and data from knockout models therefore provide the rationale for a detailed examination of both the expression and functional role of the SMAD2/3 pathway in early follicle development in the juvenile ovary.

4.1.1 Specific aims

- Examine the spatiotemporal expression patterns of the SMAD2/3 transcription factors in the juvenile ovary
- Assess the effect of SMAD2/3 signalling inhibition on primordial follicle activation in a neonatal ovary culture
- Identify upstream ligands of the SMAD2/3 signalling cascade in the juvenile ovary likely to contribute to early follicle development

4.2 Results

4.2.1 The expression of SMAD2/3 in the juvenile ovary

To investigate the role of the SMAD2/3 pathway in early follicle development the spatiotemporal localisation of the SMAD2/3 transcription factors was first explored in the juvenile mouse ovary by immunofluorescence. SMAD2/3 protein was expressed in all age groups (4, 8, 12, 21 dpp) (**Figure 4.1**), however a differential pattern of staining was clearly observed within follicle stages. SMAD2/3 was predominantly expressed in the GCs of small single-layered follicles (primordial, transitional and primary) and, to a lesser extent, small preantral follicles (1-3 layers of GCs) as illustrated in **Figure 4.2**. Staining was considerably less prevalent in large multilayered follicles as shown in the 21 dpp ovary. Finally, on a cellular level, SMAD2/3 protein was localised solely in the nucleus and cytoplasm of GCs, no staining in the oocyte was noted. The IgG control demonstrated negligible background staining confirming antibody specificity (**Figure 4.1**).

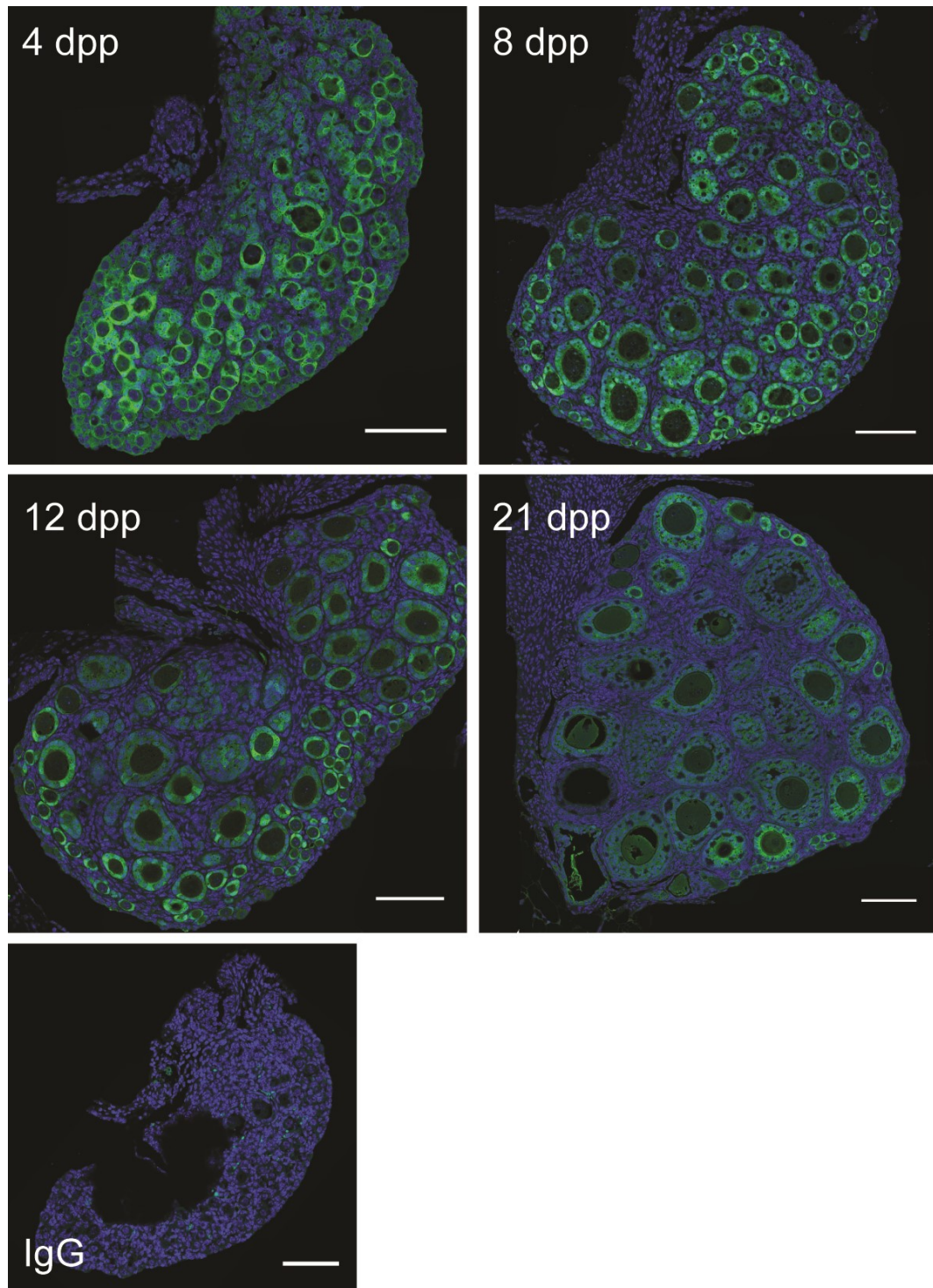


Figure 4.1. SMAD2/3 immunolocalisation in the juvenile mouse ovary. Approximate mid-section of ovaries (4, 8, 12, 21 dpp) were labelled with SMAD2/3 (green) or nonimmune IgG and counterstained with DAPI (blue). SMAD2/3 protein was expressed in all age groups (4, 8, 12, 21 dpp). Staining was predominantly localised to the small single layered follicles. Staining was less prevalent in large multilayered follicles. The IgG control demonstrated negligible background staining confirming antibody specificity. Scale bar, 100 μ m.

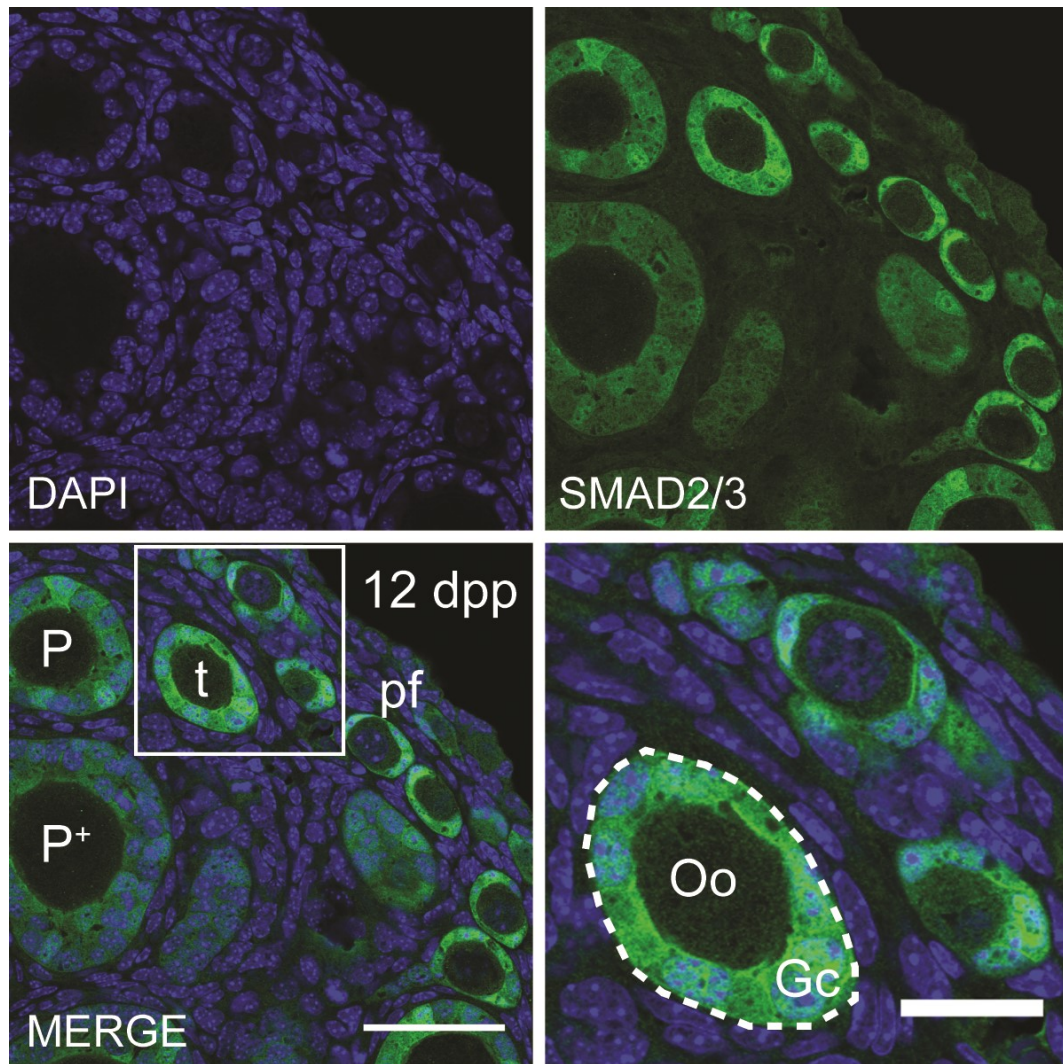


Figure 4.2. Follicle stage and cell specific immunolocalisation of SMAD2/3 in the day 12 mouse ovary. Approximate mid-section of 12 dpp ovary labelled with SMAD2/3 (green) and counterstained with DAPI (blue). SMAD2/3 was predominantly expressed in the GCs of small single layered follicles and to a lesser extent small preantral follicles (1-3 layers of GCs). SMAD2/3 staining was localised solely in the nucleus and cytoplasm of GCs, no staining in the oocyte was noted. Pf, primordial follicle; t, transitional follicle; P, primary follicle; P⁺, primary plus follicle; Oo, oocyte; Gc, granulosa cells. Area indicated by white box magnified in bottom right panel. Dashed line represents basal lamina. Scale bar, 50 μ m, 20 μ m (zoom).

4.2.2 The role of SMAD2/3 signalling in primordial follicle activation

4.2.2.1 Inhibition of ALK4/5/7 signalling promotes primordial follicle activation

The clear stage-specific expression of the SMAD2/3 transcription factors in early stage follicles in the juvenile ovary highlights the signalling cascade as a potential regulatory pathway in early follicle development. In order to further examine the role of SMAD2/3 signalling in early follicle development a neonatal ovary culture was employed to model primordial follicle activation (detailed in Chapter 3). Ovaries (4 dpp) were cultured with vehicle or SB505124 (1 μ M) for 6 days. SB505124 is a competitive inhibitor of the ATP binding site of the TGF β type I receptors ALK4, ALK5 and ALK7, which act upstream of SMAD2/3, inhibiting kinase activity and therefore blocking phosphorylation of SMAD2/3 and the signalling cascade (**Figure 4.3**). Following culture, ovaries were fixed and sectioned and ovarian morphology was examined. After 6 days in culture ovaries treated with vehicle control were predominantly populated by primordial follicles as well as some growing follicles composed mostly of transitionary and primary follicles (**Figure 4.4 A, B**). A few larger follicles surrounded by GCs beginning to multilayer were observed (**Figure 4.4 B'**). In contrast the ovaries treated with SB505124 appeared to be populated with more growing follicles than the control ovaries (**Figure 4.4 C, D**). The growing cohort however was composed solely of transitional and primary follicles. No GC multilayering was observed, even in the visibly larger follicles (**Figure 4.4 D'**). To confirm morphological observations follicle counts were performed on three central sections of each ovary whereby follicles were classified as either primordial or growing. SB505124 treatment resulted in a reduction in the percentage of primordial follicles per section with a concomitant increase in the percentage of growing follicles when compared to the vehicle control ($p = 0.0424$) (**Figure 4.5**). This was further confirmed by an increase in mean oocyte area in response to SB505124 treatment when compared to vehicle control ($p < 0.0001$) (**Figure 4.6**).

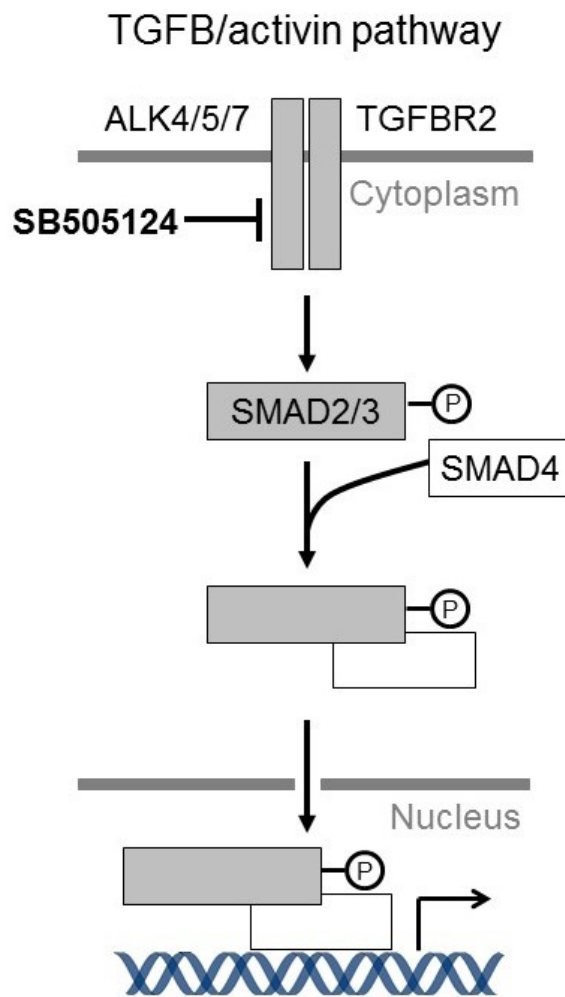


Figure 4.3. SB505124 mechanism of action. SB505124 is a selective inhibitor of the TGFB type I receptors ALK4, ALK5 and ALK7 which act upstream to phosphorylate SMAD2/3.

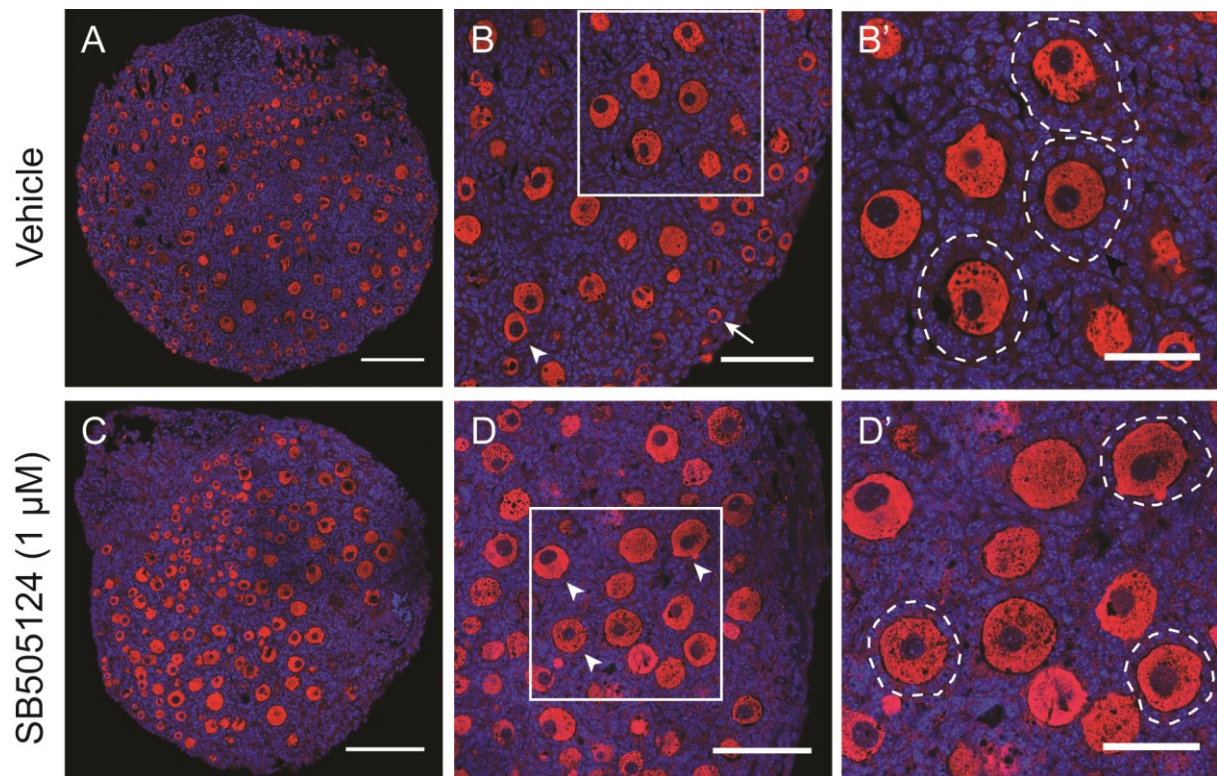


Figure 4.4. The effect of SB505124 on ovarian morphology in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control or 1 μ M SB505124 for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) (red) and nuclear DAPI (blue) for morphological examination and classification. Representative images of vehicle control (A, B, B') and SB505124 treated ovary (C, D, D'). Vehicle control treated ovaries were populated predominantly by primordial follicles and some growing follicles (B). A few larger follicles surrounded by GCs beginning to multilayer were observed (B'). SB505124 treated ovaries contained more growing follicles than the vehicle control (D), however no GC multilayering was observed, even in the visibly larger follicles (D'). Arrows indicate primordial follicles and arrowheads indicate growing follicles. Areas indicated by white box magnified in far right panels. Dashed lines represent basal lamina. Scale bar, 150 μ m (A, C), 100 μ m (B, D), 50 μ m (B', D').

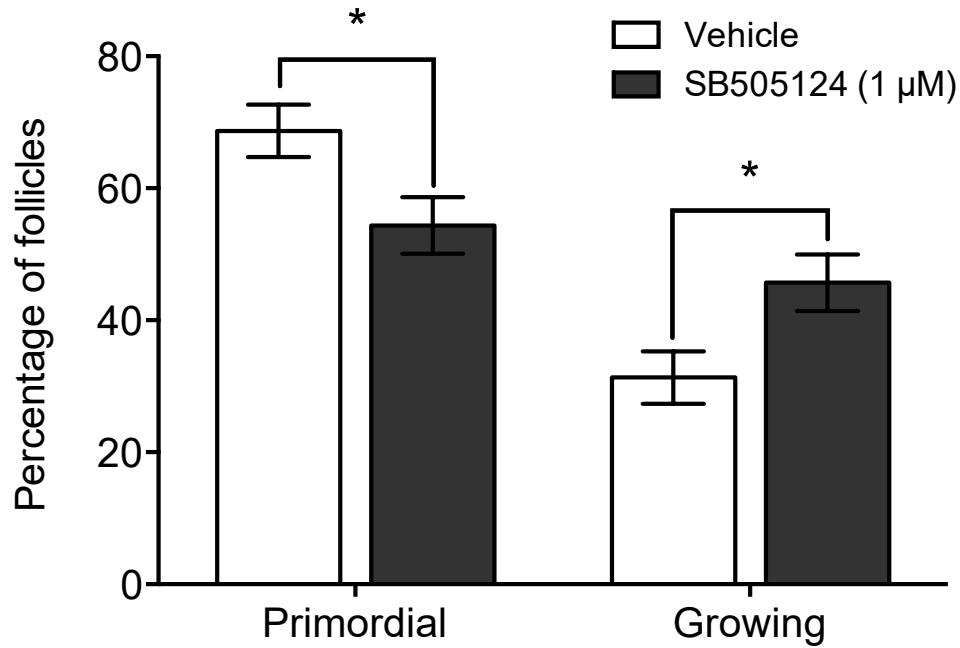


Figure 4.5. SB505124 promotes primordial follicle activation in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (4 ovaries; n = 12 ovarian sections) or 1 μ M SB505124 (6 ovaries; n = 18 ovarian sections) for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) and nuclear DAPI for measurement of oocyte area. Three central sections, 25 μ m apart, from each ovary were used for analysis. Oocytes that were larger than 275 μ m² were classified as growing. The number of primordial or growing follicles were then presented as a percentage of follicles within each section. Data presented as mean $\pm$ SEM. Comparisons between treatment and vehicle were performed using an unpaired t test where * p < 0.05.

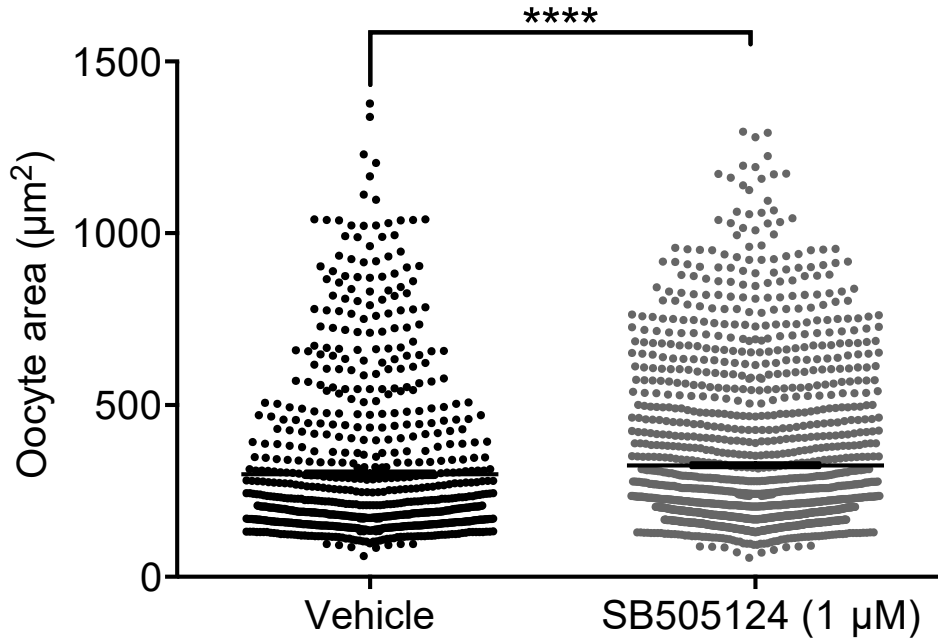


Figure 4.6. SB505124 promotes an increase in mean oocyte area in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (4 ovaries; n = 817 oocytes) or 1 µM SB505124 (6 ovaries; n = 1537 oocytes) for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) and nuclear DAPI for measurement of oocyte area. Three central sections, 25 µm apart, from each ovary were used for analysis. Data presented as mean (horizontal bar) ± SEM. Comparisons between treatment and vehicle were performed using a Mann-Whitney where **** p < 0.0001.

4.2.2.2 Increased follicle activation is not attributable to a reduction in follicle atresia

Once the initial follicle pool is established following germ cell cyst breakdown, the prepubertal ovary undergoes a significant loss of primordial follicles (Bristol-Gould et al., 2006; Pepling & Spradling, 2001). Failure of early stage follicle regression due to a reduction in follicle atresia may explain the observed increased proportion of activated follicles. The apoptotic activity of GCs was therefore assessed by expression of cleaved caspase-3, a marker of cellular apoptosis, and follicle counts were examined to investigate whether the observed increase in follicle activation was accompanied by decreased levels of follicle atresia. There was no significant difference in the mean follicle count per section between the vehicle treated ovaries (90.78 ± 12.81) and the SB505124 treated ovaries (85.39 ± 11.52) (Figure 4.7). Furthermore, the amount of cleaved caspase-3 (positive staining expressed as a

percentage area of total ovary area) was not altered in SB505124 compared with control ovaries (**Figure 4.8**). Together these findings suggest that the observed increase in follicle activation was not attributable to a reduction in follicle atresia.

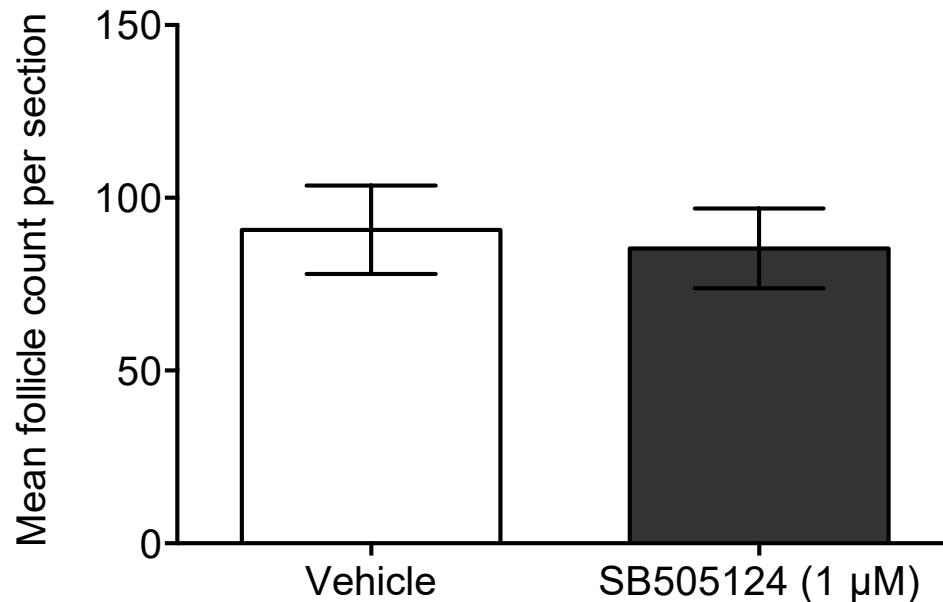


Figure 4.7. SB505124 does not affect mean follicle count in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (4 ovaries; n = 12 sections) or 1 µM SB505124 (6 ovaries; n = 18 sections) for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) and nuclear DAPI for classification. Three central sections, 25 µm apart, from each ovary were used for analysis. The number of follicles per section were quantified. Data presented as mean ± SEM. Comparisons between treatment and vehicle were performed using an unpaired t test.

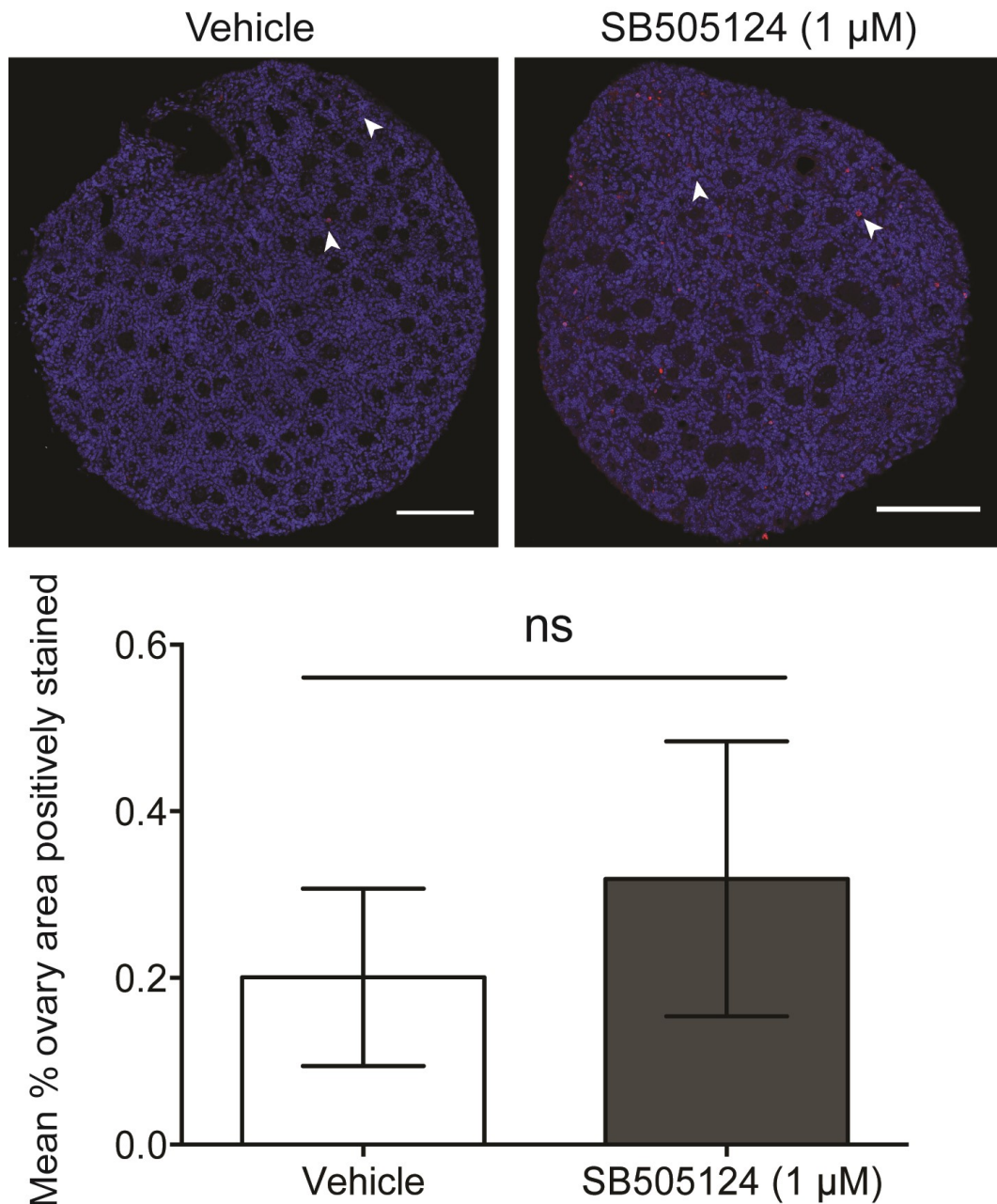


Figure 4.8. SB505124 does not affect GC apoptosis in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (n = 4) or 1 μ M SB505124 (n = 6) for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of apoptotic marker cleaved caspase-3 (red) and nuclear DAPI (blue). Cleaved caspase-3 staining was quantified in a single central section from each ovary. Positive staining was determined and represented as the proportion of pixels from the total number of pixels within the ovarian section. Representative images of vehicle control and 1 μ M SB505124 treated ovary. Arrowheads indicate staining. Scale bar, 150 μ m. Data presented as mean $\pm$ SEM. Comparisons between treatment and vehicle were performed using an unpaired t test.

4.2.2.3 Increased follicle activation is not accompanied by an increase in GC proliferation

The onset of mammalian follicle development is marked by GC proliferation and cuboidalisation and oocyte growth (Lintern-Moore & Moore, 1979). The mitotic activity of GCs was therefore assessed by expression of Ki67, a marker of cellular proliferation, to investigate whether the observed increase in follicle activation was accompanied by increased levels of GC proliferation. The amount of Ki67 (positive staining expressed as a percentage area of total area) was not altered in SB505124 treated ovaries as compared with control ovaries (**Figure 4.9**). This finding suggests that the observed increase in follicle activation in response to SB505124 was not accompanied by an increase in GC proliferation.

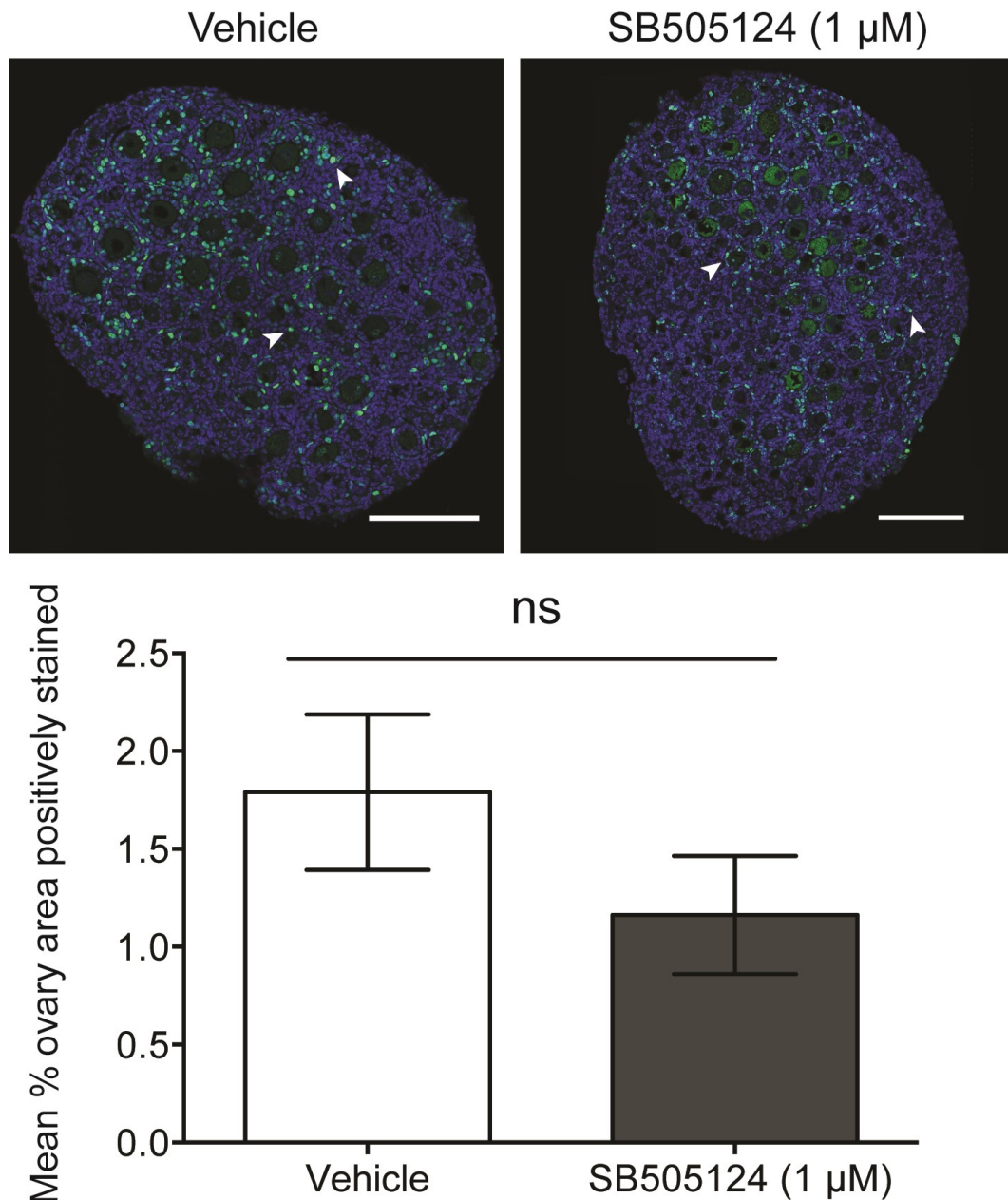


Figure 4.9. SB505124 does not affect GC proliferation in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (n = 4) or 1 μ M SB505124 (n = 6) for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of proliferation marker Ki67 (green) and nuclear DAPI (blue). Ki67 staining was quantified in a single central section from each ovary. Positive staining was determined and represented as the proportion of pixels from the total number of pixels within the ovarian section. Representative images of vehicle control and 1 μ M SB505124 treated ovary. Arrowheads indicate staining. Scale bar, 150 μ m. Data presented as mean $\pm$ SEM. Comparisons between treatment and vehicle were performed using an unpaired t test.

4.2.3 The expression of upstream components of the SMAD2/3 pathway in the juvenile mouse ovary

The observed perturbation of follicle development in response to ALK4/5/7 signalling inhibition suggests a role for the SMAD2/3 pathway in early follicle development. In light of this the expression of upstream components of the SMAD2/3 signalling cascade (**Figure 4.10**) were examined in the juvenile ovary to determine which ligand(s) would most likely contribute to the regulation of early follicle development.

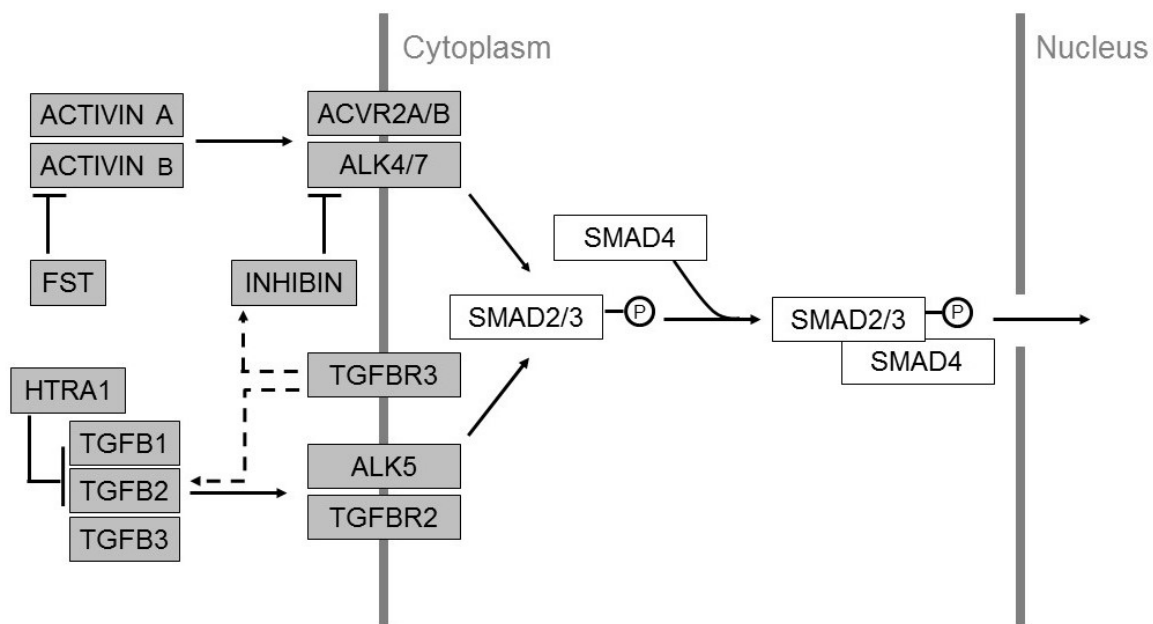


Figure 4.10. Overview of upstream signalling components of the SMAD2/3 signalling cascade.

An RT-PCR screen of juvenile (4, 8, 12 dpp) and adult (42 dpp) mouse ovary was performed and demonstrated the presence of key ligands (**Figure 4.11**), receptors (**Figure 4.12**) and antagonists (**Figure 4.13**) of the SMAD2/3 pathway. *Tgfb1*, *Tgfb2* and *Tgfb3* were detected in the juvenile ovary at 4, 8 and 12 dpp along with their receptors, *Alk5* and *Tgfb2* and co-receptor *Tgfb3*. Expression of the activin and inhibin subunits (*Inha*, *Inhba*, *Inhbb*) were demonstrated in the juvenile ovary in addition to receptors *Alk4* and *Acvr2a*. *Acvr2b* was very weakly detected at all ages. *Fst* and *Htra1* were highly expressed at all ages. Finally, to confirm the presence or absence of specific cell types within the ovarian samples the control

genes *Amh*, *Cyp17 α* and *Gdf9* were used to demonstrate GC, theca-cell and oocyte respectively in growing follicles (**Figure 4.14**).

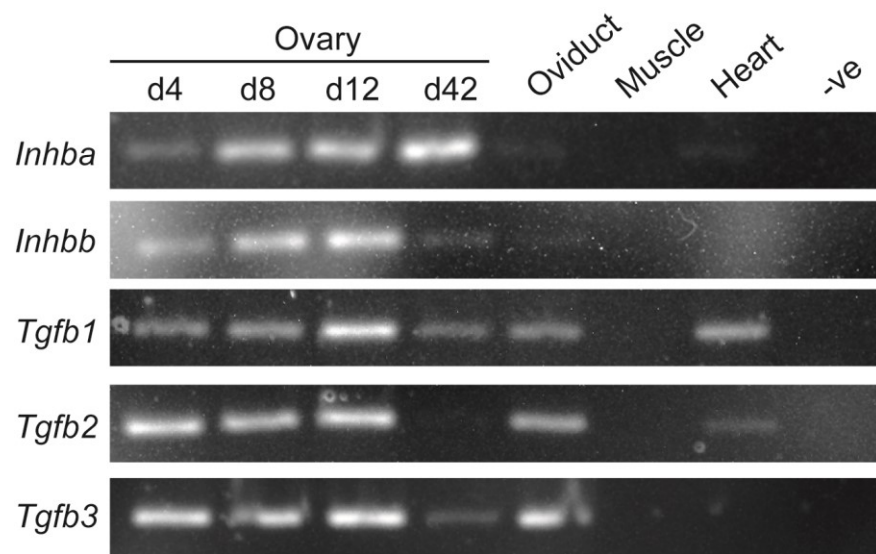


Figure 4.11. RT-PCR screen of mouse tissue demonstrating presence or absence of transcripts for upstream ligands of the SMAD2/3 pathway. An equivalent amount of input cDNA was used for each sample and subjected to 30 cycles of amplification. -ve samples contained no cDNA.

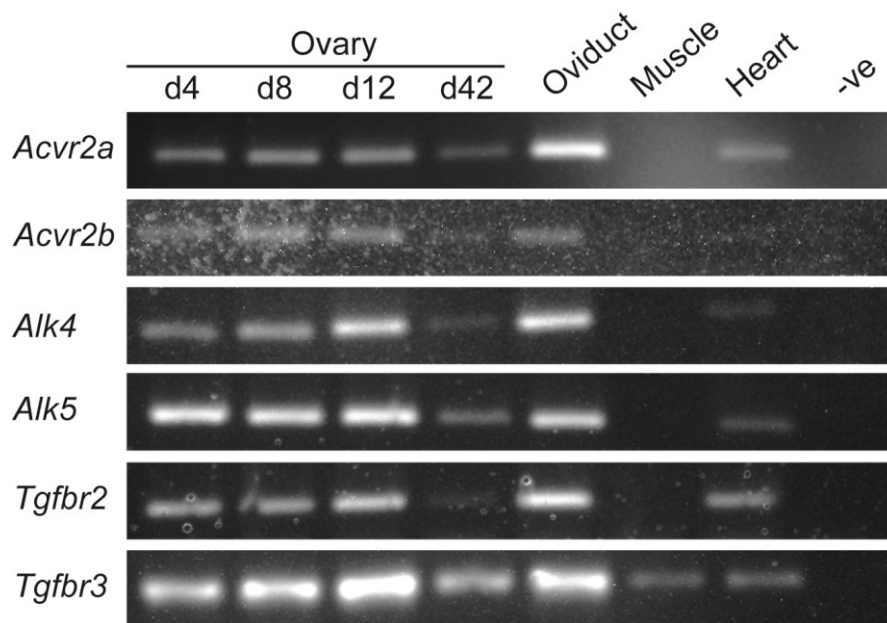


Figure 4.12. RT-PCR screen of mouse tissue demonstrating presence or absence of transcripts for upstream receptors of the SMAD2/3 pathway. An equivalent amount of input cDNA was used for each sample and subjected to 30 cycles of amplification. -ve samples contained no cDNA.

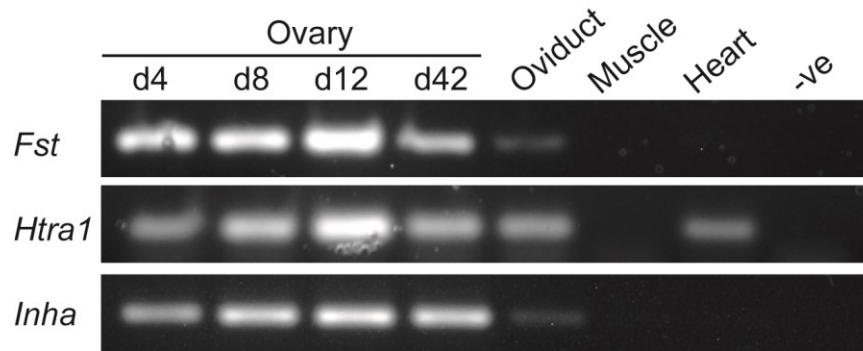


Figure 4.13. RT-PCR screen of mouse tissue demonstrating presence or absence of transcripts for upstream antagonists of the SMAD2/3 pathway. An equivalent amount of input cDNA was used for each sample and subjected to 30 cycles of amplification. -ve samples contained no cDNA.

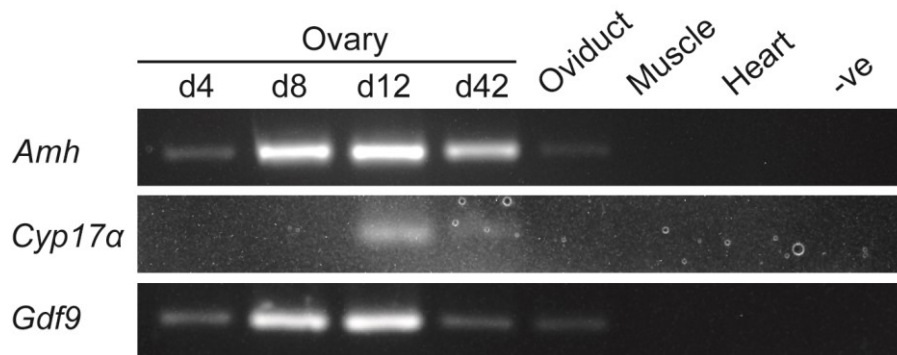


Figure 4.14. RT-PCR screen of mouse tissue demonstrating control genes to confirm the presence or absence of specific cell types. *Amh*, *Cyp17α* and *Gdf9* are GC, theca-cell and oocyte specific genes respectively. An equivalent amount of input cDNA was used for each sample and subjected to 30 cycles of amplification. -ve samples contained no cDNA.

To determine whether follicular expression contributes to overall ovarian expression of upstream components of the SMAD2/3 pathway, isolated preantral follicles (100 – 130 μ m diameter; 2 – 3 layers GCs) from 12 dpp (**Figure 4.15 A**) and 21 dpp (**Figure 4.15 B**) ovaries were used to compare absolute transcript abundance by quantitative RT-PCR. Overall a similar pattern of candidate gene expression was observed between both follicle groups. *Tgfb2* and *Tgfb3* were the most highly expressed ligands examined in both groups of follicles. Consistent with the RT-PCR ovary screen *Alk5* was the most highly expressed receptor while *Acvr2a*, *Acvr2b* and *Alk4* receptors were slightly less abundant. *Fst* and *Inha* were the most highly expressed antagonists. By comparison *Tgfb1* and *Tgfb2* were generally expressed at a low level, whereas *Inhba* was almost undetected in both groups of follicles.

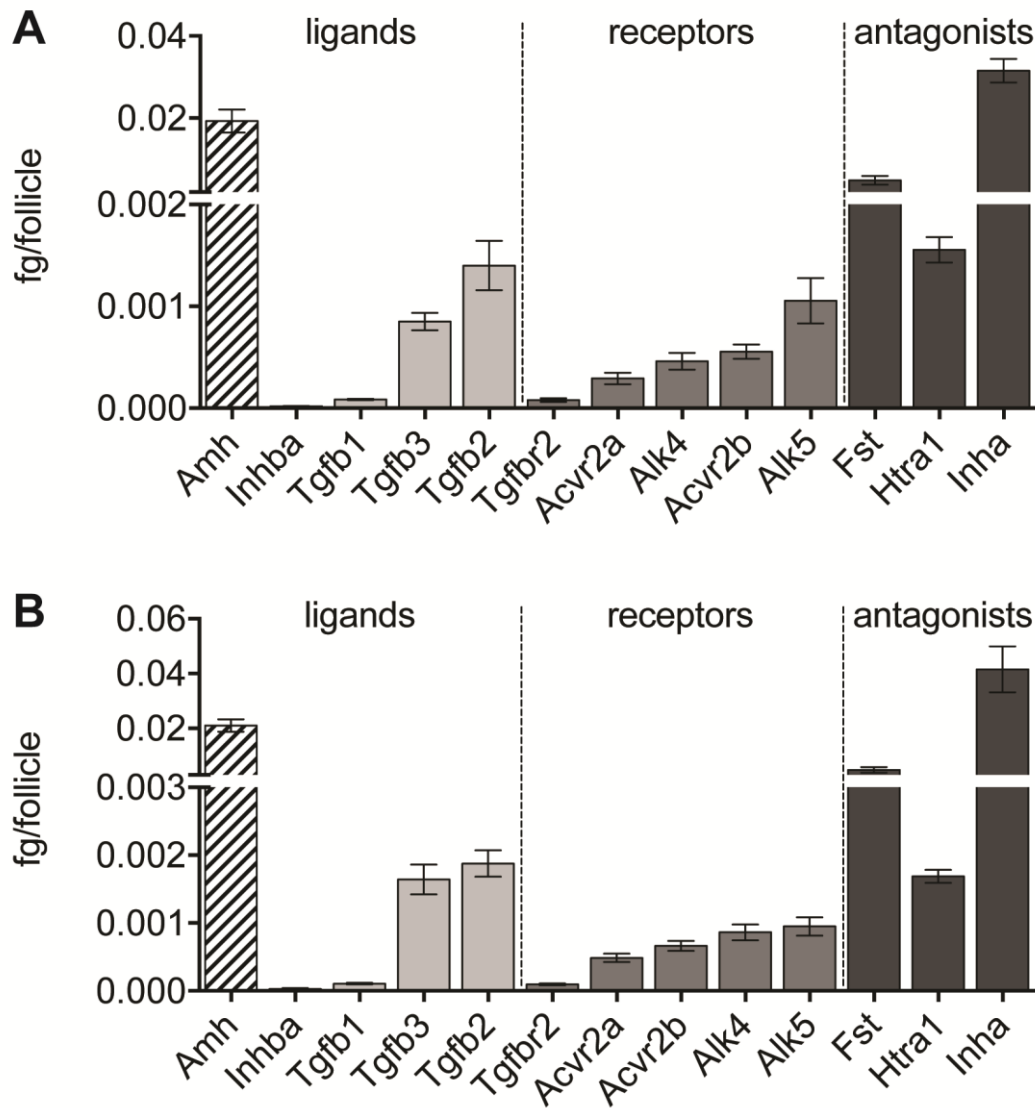


Figure 4.15. Upstream ligands, receptors and antagonists of the SMAD2/3 pathway ordered by absolute transcript expression level in isolated preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 12 (A) and 21 (B) dpp mouse ovaries. Transcript expression was quantified by RT-PCR. Values were derived from standard curves. Data presented as mean $\pm$ SEM (20 follicles from a single ovary (n = 5 ovaries)). *Amh* represents positive follicle control (hashed bar).

Table 4.1. Summary of expression of upstream components of the SMAD2/3 pathway.

Gene	Juvenile Ovary^a	Adult Ovary^a	Preantral Follicle^b
Inhba	+	+	Absent
Inhbb	+	-	*
Tgfb1	+	+/-	Low
Tgfb2	+	-	Medium
Tgfb3	+	+/-	Medium
Alk4	+	-	Low
Acvr2a	+	+/-	Low
Acvr2b	+	-	Low
Alk5	+	+/-	Medium
Tgfbr2	+	-	Low
Tgfbr3	+	+	*
Fst	+	+	High
Htra1	+	+	Medium
Inha	+	+	High

Expression is scored as undetectable (-), weak (+/-) or strong (+). *indicates unknown expression.

^a based on information from RT-PCR of juvenile and adult ovaries

^b based on information from absolute expression level quantitative PCR assays of isolated preantral follicles

Finally, to investigate changes in the expression of upstream components of the SMAD2/3 pathway during early follicle development juvenile ovaries of 4, 8, 12 and 21 dpp enriched with follicles at successive developmental stages were used to assess changes in candidate gene transcript levels by quantitative RT-PCR. To illustrate the dominant structures present within the ovaries, ovarian sections from each age group were labelled for oocyte-specific

mouse vasa protein (DDX4) and basement membrane marker laminin as illustrated in **Figure 4.16**. Ovaries from 4 dpp mice were densely populated with primordial follicles throughout the cortical region with a few larger transitional and primary follicles located within the ovarian medulla. At 8 dpp, primary plus follicles that had developed an incomplete second layer of GCs around a larger oocyte were observed within the medulla in addition to transitional and primary follicles and a primordial rich cortex. Theca cells were also found to be associated with these later stage developing follicles. By 12 dpp secondary follicles were apparent while 21 dpp ovaries were characterised by all the preceding follicle stages, with the emergence of large multilayered preantral follicles. Juvenile ovaries were then used to assess changes in candidate gene transcript levels of upstream ligands (**Figure 4.17**), receptors (**Figure 4.18**) and antagonists (**Figure 4.19**) of the SMAD2/3 pathway by quantitative RT-PCR. When transcript expression levels across age groups were compared with that of 4 dpp ovaries in terms of fold change a significant increase was observed for activin and inhibin subunits, *Inha*, *Inhba*, *Inhbb*, and *Tgfb3* and *Htra1*. The increased expression of these factors therefore coincides with the emergence of larger follicles and/or associated structures (**Figure 4.16**). *Alk4* and *Tgfb3* exhibited elevated expression at 8 dpp relative to all other ages which is consistent with the emergence of transitional and primary follicles. *Acvr2a*, *Acvr2b*, *Tgfb1*, *Tgfb2* and *Tgfb2* were consistently expressed throughout each of the age groups, suggesting either relatively constant follicular expression or expression from an extrafollicular component such as the stroma. Finally, *Alk5* demonstrated a significantly reduced expression at 12 dpp in comparison to 4 dpp.

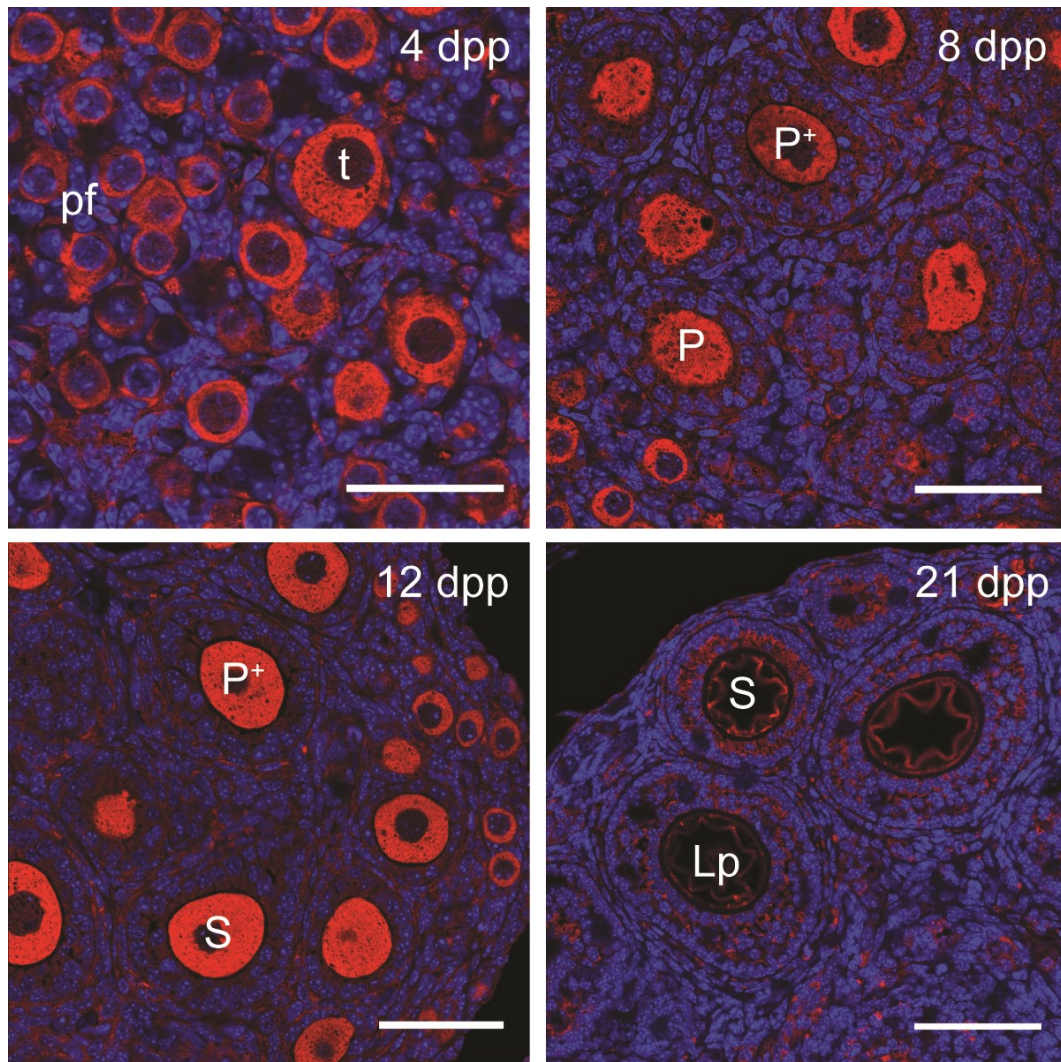


Figure 4.16. The juvenile mouse ovary illustrating emerging follicular stages. Approximate midsection of ovaries (4, 8, 12, 21 dpp) were labelled with oocyte-specific mouse vasa protein (DDX4) and basement membrane marker laminin (both red) and counterstained with DAPI (blue) to highlight the emergence of follicles at successive developmental stages. Ovaries from 4 dpp mice were densely populated with primordial follicles (pf) and a few transitional (t) and primary staged follicles (P). 8 dpp ovaries illustrate the emergence of primary plus follicles (P⁺) that had developed an incomplete second layer of GCs. Secondary follicles (S) were present in 12 dpp ovaries. Large multilayered preantral follicles (Lp) were evident in 21 dpp ovaries. Scale bars, 50 μ m, 60 μ m, 60 μ m, 70 μ m.

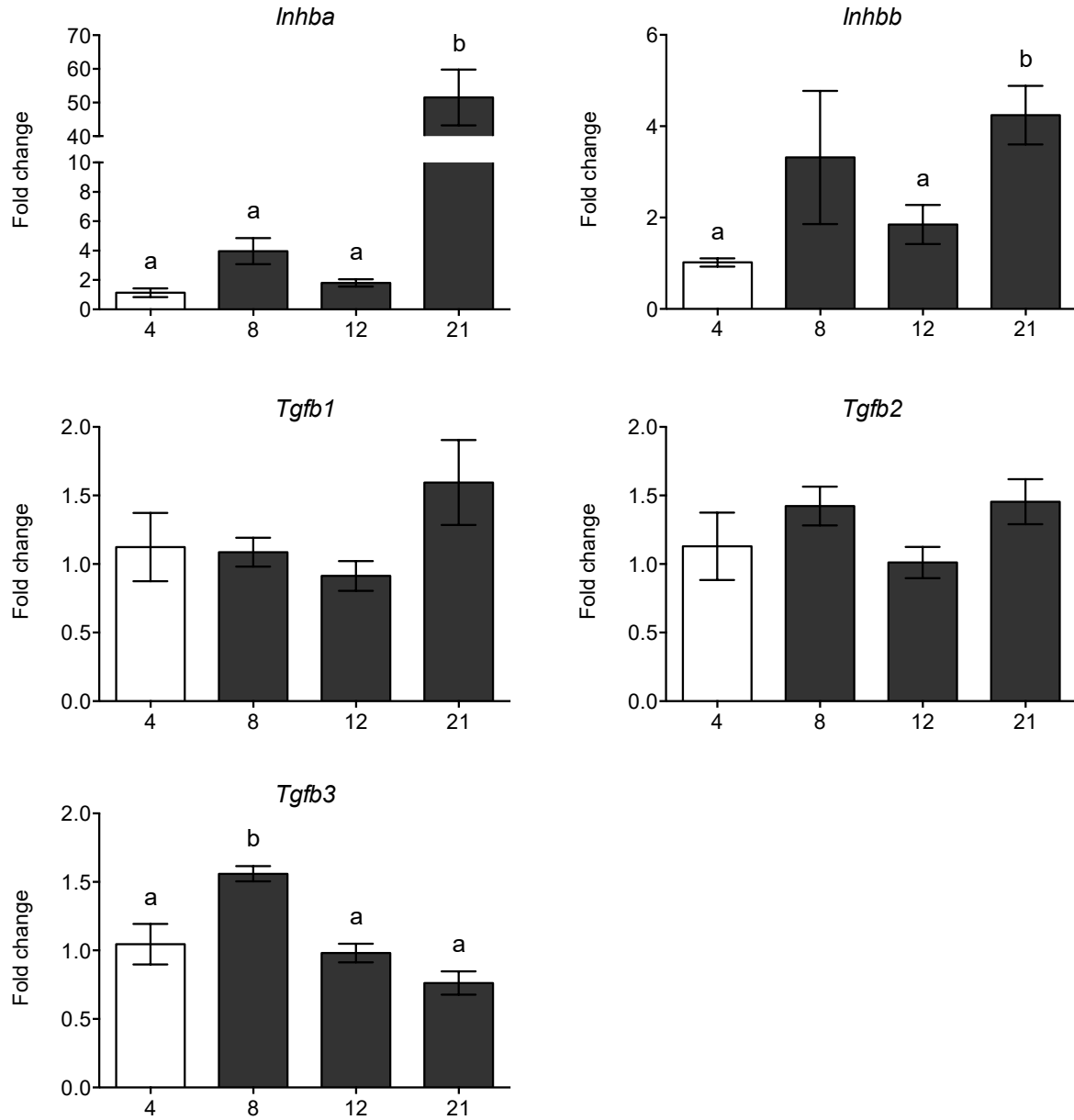


Figure 4.17. Relative transcript expression of upstream ligands of the SMAD2/3 pathway in the juvenile mouse ovary. Transcript expression was quantified by RT-PCR in the 4, 8 12 and 21 dpp ovary. Each transcript was normalised to ATP5B, and fold changes calculated relative to 4 dpp expression. Data presented as mean $\pm$ SEM (n = 5 ovaries per age group). Comparisons between age groups were made by one-way ANOVA and Tukey's post hoc test. Groups with different letters indicate statistical differences ($p < 0.05$).

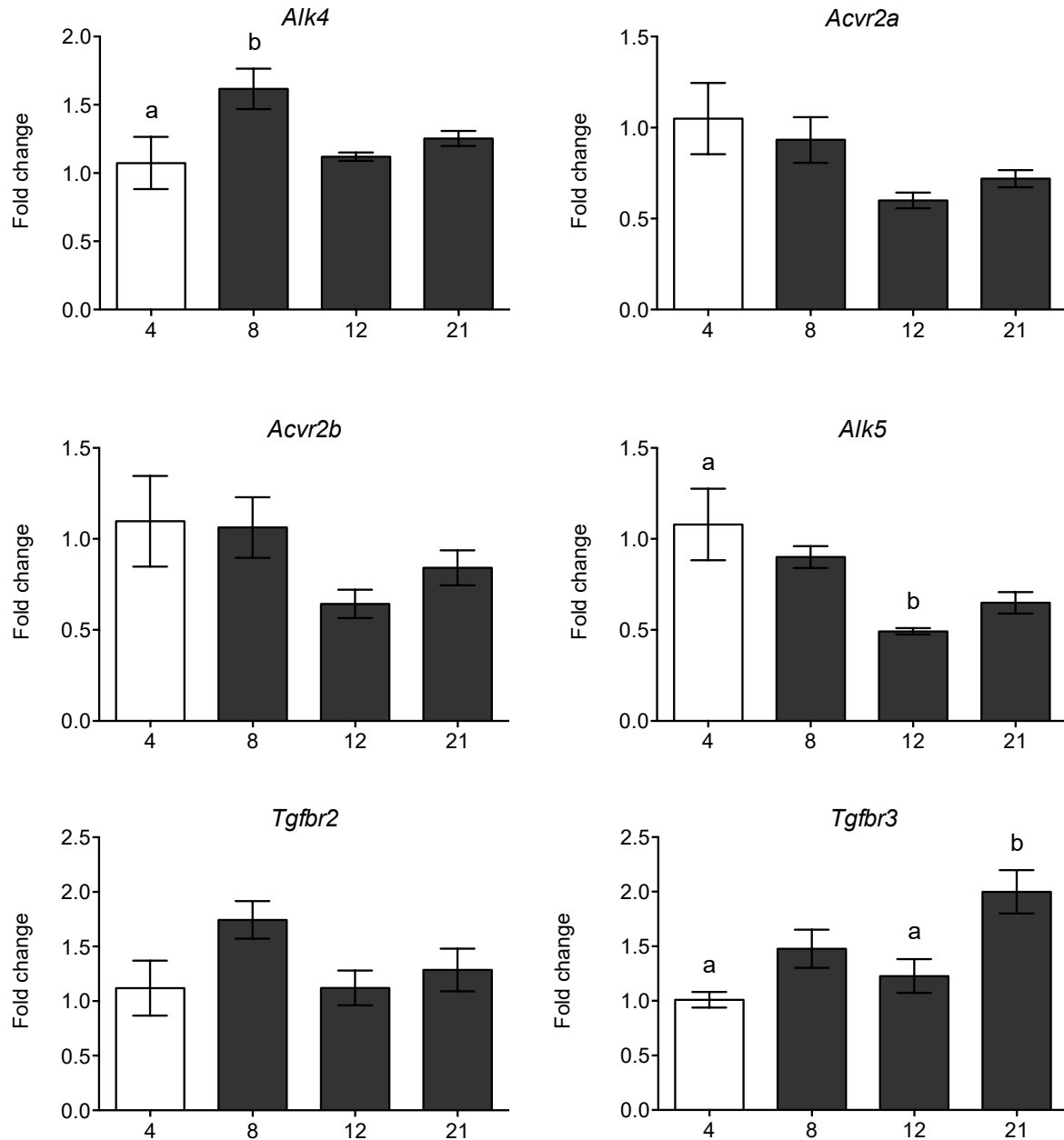


Figure 4.18. Relative transcript expression of upstream receptors of the SMAD2/3 pathway in the juvenile mouse ovary. Transcript expression was quantified by RT-PCR in the 4, 8 12 and 21 dpp ovary. Each transcript was normalised to ATP5B, and fold changes calculated relative to 4 dpp expression. Data presented as mean $\pm$ SEM (n = 5 ovaries per age group). Comparisons between age groups were made by one-way ANOVA and Tukey's post hoc test. Groups with different letters indicate statistical differences (p < 0.05).

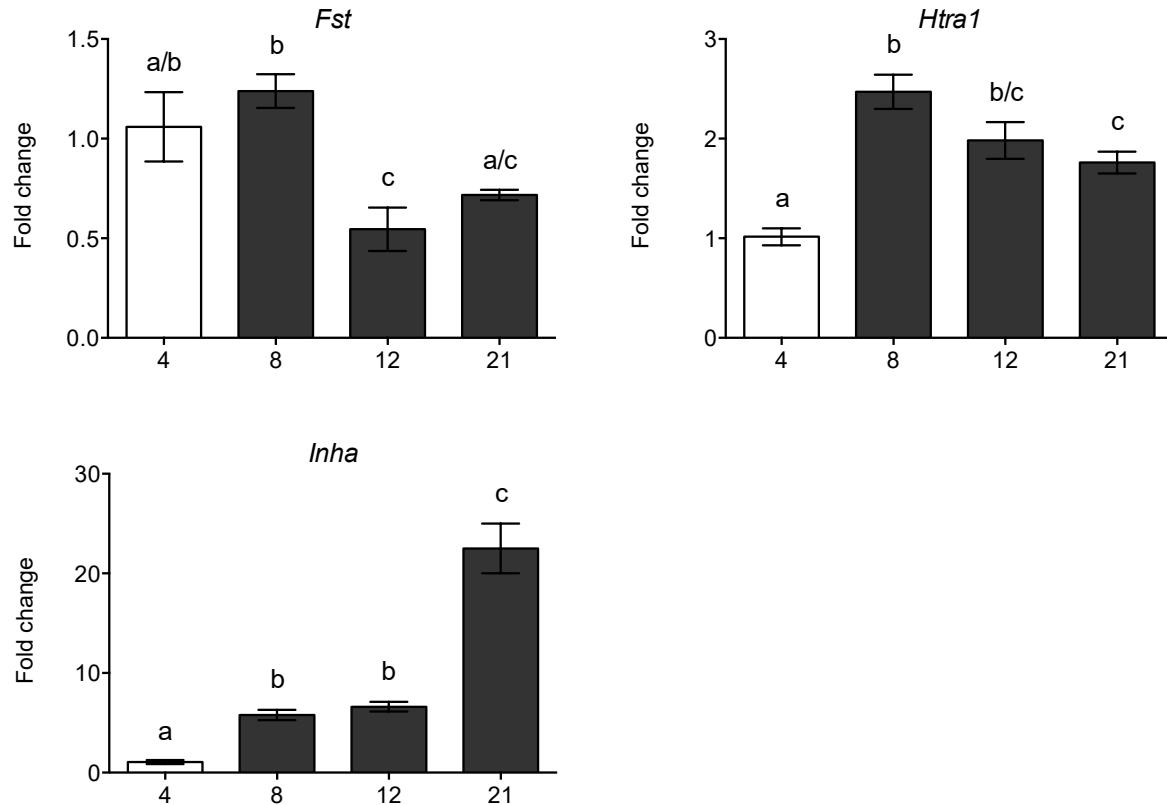


Figure 4.19. Relative transcript expression of upstream antagonists of the SMAD2/3 pathway in the juvenile mouse ovary. Transcript expression was quantified by RT-PCR in the 4, 8 12 and 21 dpp ovary. Each transcript was normalised to ATP5B, and fold changes calculated relative to 4 dpp expression. Data presented as mean $\pm$ SEM (n = 5 ovaries per age group). Comparisons between age groups were made by one-way ANOVA and Tukey's post hoc test. Groups with different letters indicate statistical differences ($p < 0.05$).

4.3 Discussion

Despite accumulating evidence highlighting the SMAD2/3 signalling cascade as an integral regulatory pathway in the postnatal ovary the functional role during the window of early follicle development remains unexamined. The current study reveals ALK4/5/7 signalling inhibition led to accelerated activation of the primordial pool therefore suggesting a role for the SMAD2/3 signalling cascade in regulation of early follicle development.

A previous role for SMAD2/3 signalling in ovarian physiology was proposed following the localisation of high levels of SMAD in the GCs of the mouse ovary (Kano et al., 1999). In addition, a study by J. Xu et al. (2002) in the rat described the expression of SMAD2/3 in the

oocytes of primordial and primary follicles and GCs of preantral follicles whereas levels of expression were low in large antral and preovulatory follicles. The clear stage-specific expression of the SMAD2/3 transcription factors in early stage follicles in the juvenile ovary demonstrated in the current study therefore confirms previous work and further highlights the signalling cascade as a potential key regulatory pathway in early follicle development. Indeed, the abundant expression in the GCs of small single layered follicles and low expression in large multilayered follicles suggests a role both in the maintenance of the primordial pool and early preantral follicle development.

In line with the stage-specific expression pattern of SMAD2/3, it was demonstrated that the pharmacological inhibition of the type I receptors ALK4/5/7 led to perturbed early follicle development in vitro. The accelerated activation of follicles in response to inhibition of the ALK4/5/7, with a concomitant decrease in the percentage of non-growing primordial follicles, suggests a regulatory role for SMAD2/3 signalling cascade in maintenance of the primordial pool. Previous studies investigating the role of SMAD2/3 in the ovary have made use of multiple KO models. A cKO of both *Smad2* and *Smad3* demonstrated loss of SMAD2/3 signalling in GCs led to disrupted follicular development and reduced ovarian efficiency (Li et al., 2008). Indeed, a trend towards reduced numbers of early preantral follicles preceded premature ovarian failure after 4 months of breeding. It is therefore plausible that the current study provides evidence for the mechanism underlying the adult ovarian phenotype whereby the observed disruption in follicle distribution and premature ovarian failure is a result of accelerated follicle activation in the juvenile ovary. The current study however reports accelerated activation of the primordial follicle pool, a phenotype not explicitly observed in the cKO model. The reason for this discrepancy is likely explained by the nature of the *Amhr2* promoter used by Li et al. (2008) whereby low endogenous expression in primordial and primary follicles resulted in the circumvention of SMAD2/3 ablation at the single layered follicle stage. The current results therefore provide the impetus for a targeted in vivo study encompassing the early preantral follicle to fully elucidate the role of the SMAD2/3 cascade in maintenance of the primordial follicle pool.

In contrast to the current study however Zhu et al. (1998), who reported a homozygous deletion of *Smad3* in exon 2, described female mice that were fertile and produced homozygous litters. A further discrepancy was demonstrated by a study describing targeted deletion of *Smad3* in exon 8 in which female mice displayed reduced fertility due to impaired

follicle development (Tomic et al., 2002). In this case targeted deletion of *Smad3* appeared to slow the growth of primordial follicles to the antral stage. Regardless it is important to acknowledge that the current project employed the use of a small molecule inhibitor to investigate the regulatory role of the SMAD2/3 pathway. SB505124 is a competitive inhibitor of the ATP binding site of the TGFB type I receptors ALK4, ALK5 and ALK7, which act upstream of SMAD2/3, inhibiting kinase activity and therefore blocking phosphorylation of SMAD2/3 and the signalling cascade (DaCosta Byfield et al., 2004). It is therefore likely that discrepancies in ovarian morphology are a result of the different approaches employed to inhibit the action of SMAD2/3 signalling (KO vs inhibitor). The inconsistencies may reflect the involvement of non-canonical signalling pathways in the TGFB cascade. The TGFB receptors in particular, have the capacity to activate both SMAD-dependent and SMAD-independent pathways (Derynck & Zhang, 2003). Alternatively, the disparities observed may reflect the operational redundancy in the SMAD2/3 signalling system. Li et al. (2008) describe GC ablation of individual *Smad2* or *Smad3* resulted in insignificant changes in female fertility compared to the dramatic reduction in ovarian function observed in the double KO. In this regard pharmacological ablation of the ALK4/5/7 type I receptors undertaken in the current project allows for a more complete examination of SMAD2/3 function. The current results however are in accordance with work by Wang et al. (2014) who report an accelerated activation of the primordial pool in response to treatment with SD208, an inhibitor of the ALK5 receptor. Moreover, given ALK5 was a common target of both small molecule inhibitors it is likely that upstream signalling via the ALK5 receptor is an essential step of the signalling cascade that regulates primordial follicle activation.

Once the initial follicle pool is established following germ cell cyst breakdown, the prepubertal ovary undergoes a significant loss of primordial follicles (Bristol-Gould et al., 2006; Pepling & Spradling, 2001). To ensure the observed increase in follicle activation was not in fact due to a failure of early stage follicle regression, levels of GC apoptosis and follicle counts were assessed. The findings from this study demonstrate no difference in either follicle counts or apoptotic activity in ovaries treated with SB505124 and indicate a true increase in follicle activation.

The activation of the primordial follicle is associated with morphological and proliferative changes in the GCs and growth of the oocyte (Lintern-Moore & Moore, 1979). Ensuing follicle development is then characterised by further proliferation and multilayering of GCs.

Despite the accelerated recruitment of follicles from the primordial pool increased levels of GC proliferation were not observed, furthermore it was noted that SB505124 treatment resulted in the absence of multilayered follicles compared to the follicle distribution observed in the vehicle control. It appeared that inhibition of ALK4/5/7 signalling resulted in failure of growing follicles to undergo normal GC proliferation and multilayering. The TGFB signalling cascade is complex and promiscuous in nature whereby multiple ligands converge on various combinations of heteromeric receptor complexes and transduce their signals via the SMAD transcription factors (Moustakas & Heldin, 2009). The observed ovarian morphology is therefore a manifestation of the loss of numerous ligands that signal via the SMAD2/3 pathway. It is likely that the failure of follicles to proceed past the primary stage (single layer of cuboidal GCs) of follicle development is due to the concurrent inhibition of oocyte specific GDF9 signalling in the ovary (McGrath et al., 1995). A study by Dong et al. (1996) demonstrated a block in follicular development after the primary one-layer follicle stage in GDF-9 deficient mice, whereby oocyte growth proceeded as normal however somatic cell proliferation was compromised. Regardless it is highly likely that if the expression of GC Ki67 had been examined earlier, an increase would have been observed coinciding with the initial onset of GC proliferation associated with primordial follicle activation. Finally, while it was possible to exclude the non-specific oocyte background staining from the threshold analysis, staining present in non-GC somatic cells was unable to be omitted. In this regard, it would be more accurate to instead total the number of Ki67 (and cleaved caspase-3) positive follicles to ensure GC specific quantification in future analysis.

Recent observations by Hardy et al. (unpublished) describe abundant nuclear GC expression of SMAD2/3 in the primordial and transitional follicle, an observation not mirrored in the primary and small multilayering preantral follicle. Given this it could be postulated that the active SMAD2/3 pathway in the GC of the primordial follicle is necessary for maintaining the primordial follicle in a quiescent state. To further support this hypothesis, Hardy et al. demonstrate an association between nuclear GC SMAD2/3 expression and Ki67 whereby nuclear SMAD2/3 and Ki67 protein expression were mutually exclusive. The expression of Ki67 in nuclei lacking SMAD2/3 suggests that the pathway is indeed associated with the maintenance of GCs in cell cycle arrest and that SMAD2/3 export from the nucleus may be a prerequisite for the onset of GC proliferation and follicle activation.

The clear expression of SMAD2/3 in the juvenile ovary in addition to the observed perturbation of follicle development in response to ALK4/5/7 signalling inhibition suggests a role for the SMAD2/3 pathway in early follicle development. In light of this the expression of upstream components of the SMAD2/3 signalling cascade were examined to determine which ligand(s) would most likely contribute to the regulation of early follicle development. SMAD2 and SMAD3 specifically transduce signals from activated TGFB/activin receptors. The juvenile ovary was therefore used to investigate changes in transcript levels of TGFB and activin signalling machinery during preantral follicle development. Transcripts of upstream elements from both the activin and TGFB pathways were present in the juvenile ovary (Summarised in **Table 4.1**). Of the ligands examined, *Tgfb2* and *Tgfb3* were the most abundantly expressed, localised in both the juvenile ovary and the preantral follicle. In contrast the activin A subunit, *Inhba* was almost undetectable in the preantral follicle. *Alk5*, the type I receptor for the TGFB ligands, was the most abundantly expressed receptor in isolated preantral follicles while the type II receptor, *Tgfb2*, was expressed at lower levels. This is consistent with work by Schmid et al. (1994) who report *Tgfb1*, *Tgfb2*, *Tgfb3* and *Tgfb2* localisation by in situ hybridisation in mouse preantral follicles. Expression of *Tgfb3*, a membrane bound co-receptor for the TGFB isoforms and inhibin, was also observed in the juvenile ovary. TGFBR3 binds to select ligands with high affinity, greatly enhancing their presentation to the type II receptor on the cell surface thus potentiating their actions (Lewis et al., 2000; Lopez-Casillas et al., 1993). *Tgfb3* expression was not quantified in preantral follicles however previous findings have localised TGFBR3 in the oocytes of preantral follicles and GCs undergoing cuboidilisation in growing follicles (Sarraj et al., 2007).

In conclusion, in vitro studies clearly demonstrate accelerated activation of the primordial pool in response to ALK4/5/7 signalling inhibition. The results are indicative of the action of an inhibitory factor through the SMAD2/3 cascade that maintains the primordial follicle in an inactivated state. Following a screen of upstream SMAD2/3 signalling components *Tgfb2* was identified as a potential candidate, in concert with *Alk5*, the TGFB type I receptor. The following chapter will therefore examine the role of the TGFB2 ligand in primordial follicle activation.

Chapter 5 Investigating the role of TGFB2 in primordial follicle activation

5.1 Introduction

The neonatal ovary encompasses a finite pool of primordial follicles which serve the need of the entire reproductive lifespan (Zuckerman, 1951). Follicle activation is therefore a tightly controlled process, the rate of which has a profound influence on fertility. The previous chapter demonstrated that inhibition of ALK4/5/7 signalling in the juvenile ovary led to increased activation of the primordial follicle pool. Based on this it was hypothesised that the action of an, as yet unidentified, inhibitory factor through the SMAD2/3 cascade maintains the primordial follicle in an inactivated state. Following a screen of upstream SMAD2/3 signalling components in the juvenile ovary and early preantral follicle, as detailed in Chapter 4, TGFB2 was identified as a potential candidate.

The effects of TGFB2 are mediated through activation of the membrane receptors ALK5 and TGFBR2 with the presence of the membrane co-receptor TGFBR3 necessary for TGFB2 binding (Lopez-Casillas et al., 1993). Expression of the components required for TGFB2 signalling have previously been localised in the ovary however reports are highly variable regarding species, animal maturity and stage of follicular development (Ghiglieri et al., 1995; Gueripel et al., 2004; Juneja et al., 1996; Sarraj et al., 2007; Schmid et al., 1994). Nevertheless, previous reports of TGFB2, ALK5 and TGFBR3 expression in the early preantral follicle of the prepubertal mouse ovary are indicative of a role in early follicle development (Ghiglieri et al., 1995; Sarraj et al., 2007; Wang et al., 2014).

Knowledge of the functional role of TGFB in the postnatal ovary is limited, perhaps in part due to the deleterious effects of universal *Tgfb* gene deletion. Homozygous disruption of both *Tgfb2* and *Tgfb3* results in perinatal lethality, while targeted disruption of *Tgfb1* produces animals with severe inflammatory pathology and a maximum lifespan of 3 weeks (Proetzel et al., 1995; Sanford et al., 1997; Shull et al., 1992). Ingman et al. (2006) however more recently employed an immunocompromised mouse model to circumvent the lethal effect of gene disruption and demonstrated severely impaired fertility in the *Tgfb1* null mouse. The knowledge obtained from functional in vitro studies is similarly limited and often conflicting. In rodents, TGFB1 has been demonstrated as a potent stimulator of granulosa cell

proliferation (Dorrington et al., 1988; Saragueta et al., 2002). Furthermore, TGFB1 has been shown to increase the growth of isolated murine preantral follicles (Liu et al., 1999). In addition both TGFB1 and TGFB2 significantly increase follicular [³H]thymidine incorporation in a dose dependent manner in the hamster (Roy, 1993). In contrast to this however TGFB was shown to inhibit EGF induced DNA synthesis in both porcine and bovine GCs (Mondschein et al., 1988; Skinner et al., 1987). In 2008, Rosairo et al. demonstrated that TGFB1 does not affect the activation of the primordial pool unless in combination with FSH, however a more recent in vitro study reported that TGFB1 inhibited primordial follicle activation (Wang et al., 2014).

The current literature concerning both the localisation and functional role of TGFB in early follicle development is both limited and conflicting and focusses almost entirely on the TGFB1 isoform. As the most abundantly expressed ligand in the preantral follicle, identified by the ligand screen in Chapter 4, a detailed examination of both the expression and role of the TGFB2 ligand in early follicle development in the juvenile ovary was undertaken.

5.1.1 Specific aims

- Examine the spatiotemporal expression patterns of TGFB2 and ALK5 in the juvenile ovary
- Assess the effect of TGFB2 on primordial follicle activation in a neonatal ovary culture

5.2 Results

5.2.1 The expression of TGFB2 and ALK5 in the juvenile ovary

To investigate the role of TGFB2 in early follicle development the localisation of TGFB2 and receptor ALK5 was first explored in the juvenile mouse ovary by immunofluorescence.

5.2.1.1 TGFB2 expression in the juvenile ovary

TGFB2 protein was localised in all age groups (4, 8, 12 dpp) (**Figure 5.1 - Figure 5.3**). TGFB2 was clearly localised to the oocyte compartment of all stages of follicles observed (small single-layered follicles (primordial, transitional and primary) and small preantral follicles (1-3 layers of GCs)) in addition to the ovarian surface epithelium. In contrast TGFB2

expression within somatic cells was observed only in the flattened granulosa cells of the primordial follicle whereas expression was absent in the cuboidal GCs of growing follicles. The IgG control demonstrated negligible background staining confirming antibody specificity (Figure 5.1).

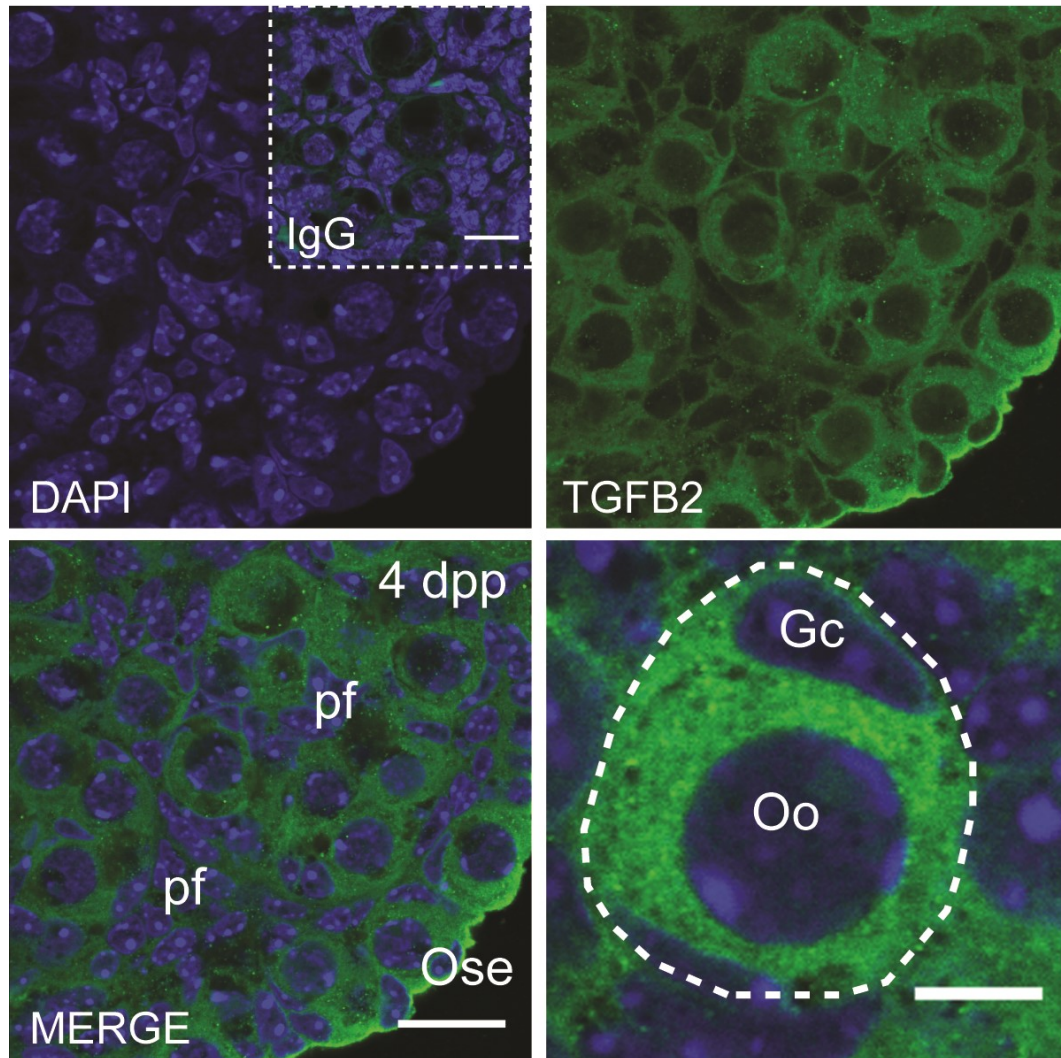


Figure 5.1. Immunolocalisation of TGFB2 in the day 4 mouse ovary. Approximate mid-section of a 4 dpp ovary labelled with TGFB2 (green) or nonimmune IgG and counterstained with DAPI (blue). TGFB2 was localised in the oocytes and the ovarian surface epithelium. Staining was also observed in the flattened GCs of primordial follicles. The IgG control demonstrated negligible background staining confirming antibody specificity. Pf, primordial follicle; Ose, ovarian surface epithelium; Oo, oocyte; Gc, granulosa cells. Magnified area in bottom right panel selected from an alternative image. Dashed inset represents IgG control. Dashed line represents basal lamina. Scale bar, 20 μ m, 6 μ m (zoom).

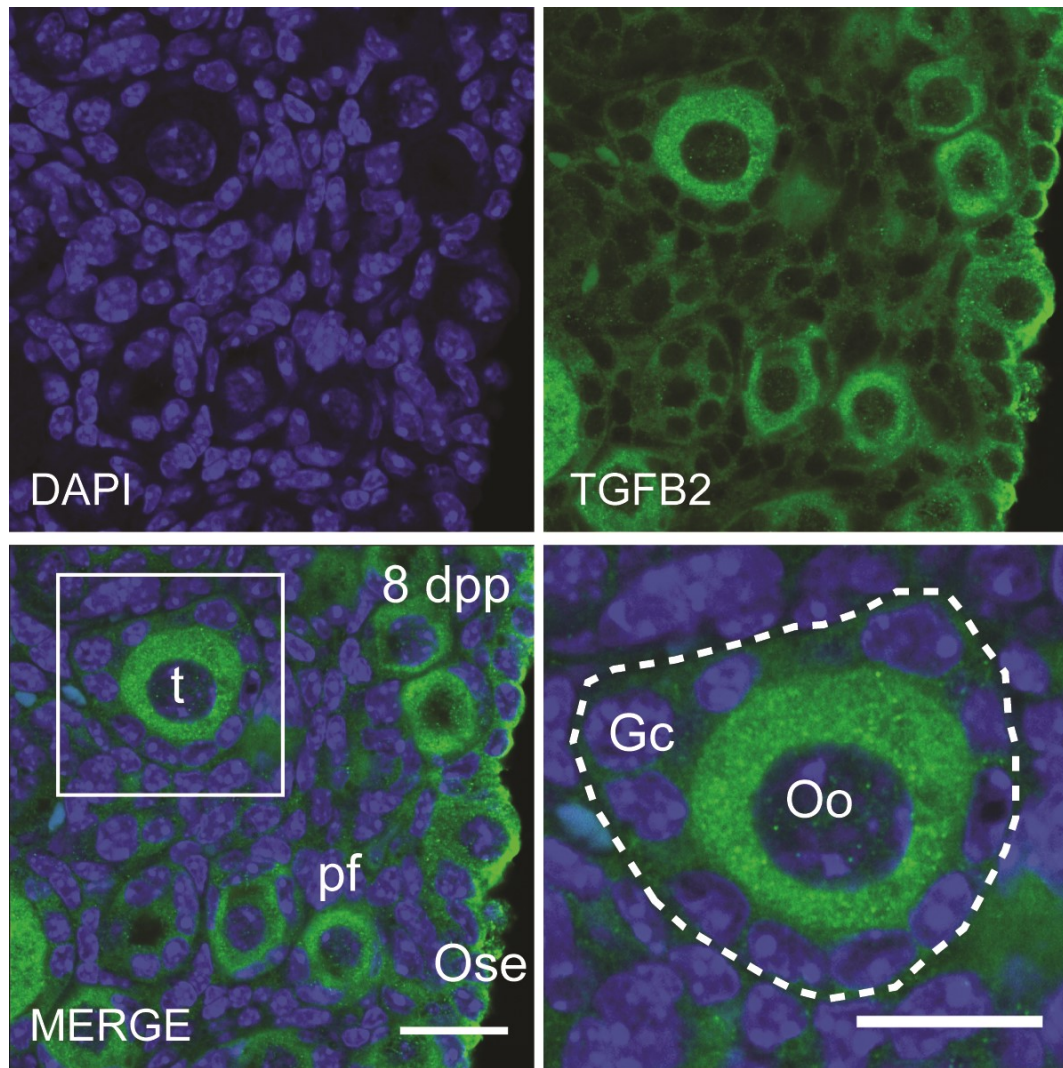


Figure 5.2. Immunolocalisation of TGFB2 in the day 8 mouse ovary. Approximate mid-section of an 8 dpp ovary labelled with TGFB2 (green) and counterstained with DAPI (blue). TGFB2 was localised in the oocytes of all follicle stages and the ovarian surface epithelium. Staining was also observed in the flattened GCs of primordial follicles. Pf, primordial follicle; t, transitional follicle; Ose, ovarian surface epithelium; Oo, oocyte; Gc, granulosa cells. Area indicated by white box magnified in bottom right panel. Dashed line represents basal lamina. Scale bar, 20 μm , 15 μm (zoom).

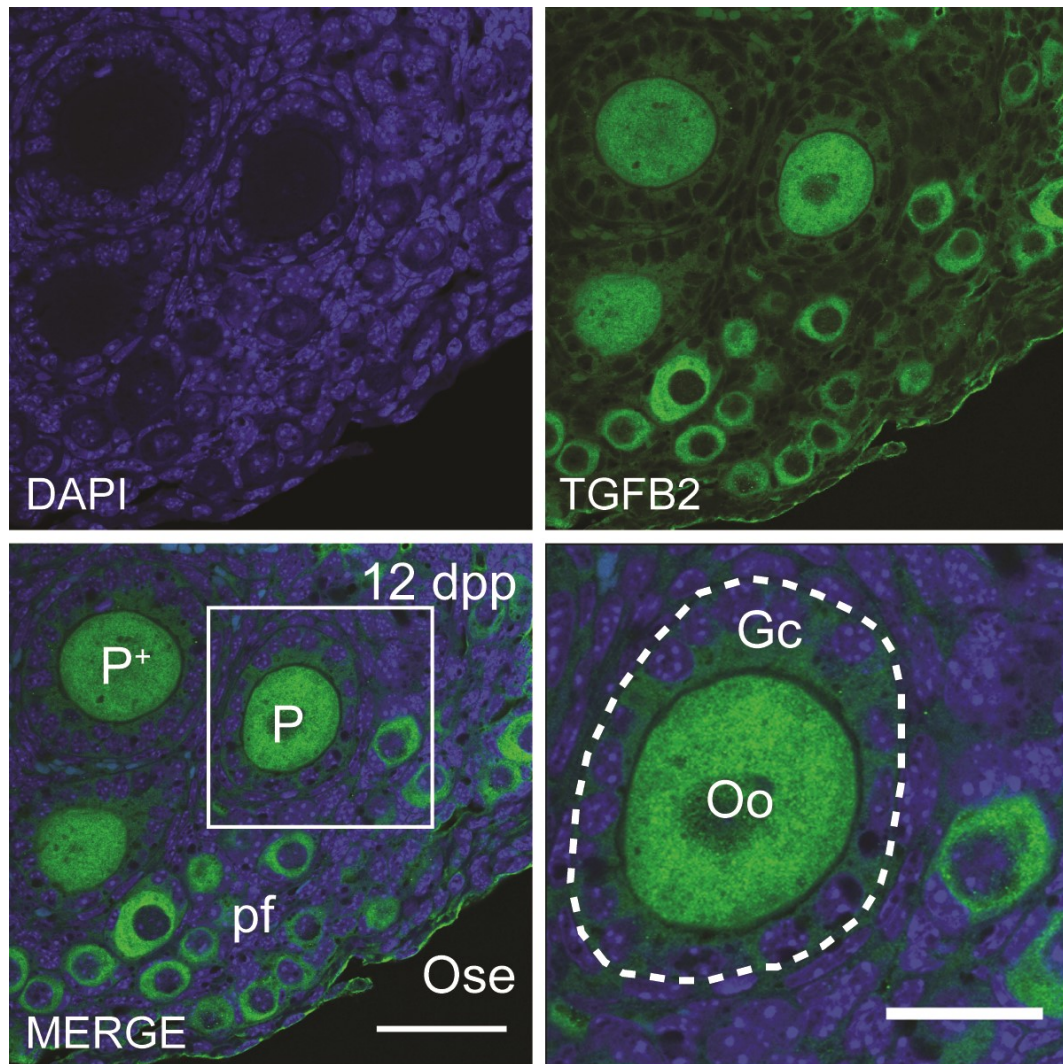


Figure 5.3. Immunolocalisation of TGFB2 in the day 12 mouse ovary. Approximate mid-section of a 12 dpp ovary labelled with TGFB2 (green) and counterstained with DAPI (blue). TGFB2 was localised in the oocytes of all follicle stages and the ovarian surface epithelium. Staining was also observed in the flattened GCs of primordial follicles. Pf, primordial follicle; t, transitional follicle; P, primary follicle; P⁺, primary plus follicle; Ose, ovarian surface epithelium; Oo, oocyte; Gc, granulosa cells. Area indicated by white box magnified in bottom right panel. Dashed line represents basal lamina. Scale bar, 50 μ m, 25 μ m (zoom).

5.2.1.2 ALK5 expression in the juvenile ovary

ALK5 protein was localised to the oocyte compartment of all stages of follicles observed (small single-layered follicles (primordial, transitional and primary) and small preantral follicles (1-3 layers of GCs)) in the 12 dpp ovary (**Figure 5.4** and **Figure 5.5**). Staining was predominantly observed as a punctate pattern on the oocyte membrane however some oocytes

also demonstrated strong staining within the ooplasm. Staining was less prominent in the primordial follicle but could be observed within the ooplasm and the germinal vesicle. Protein expression was completely absent in the somatic cells. The IgG control demonstrated negligible background staining confirming antibody specificity (**Figure 5.5**).

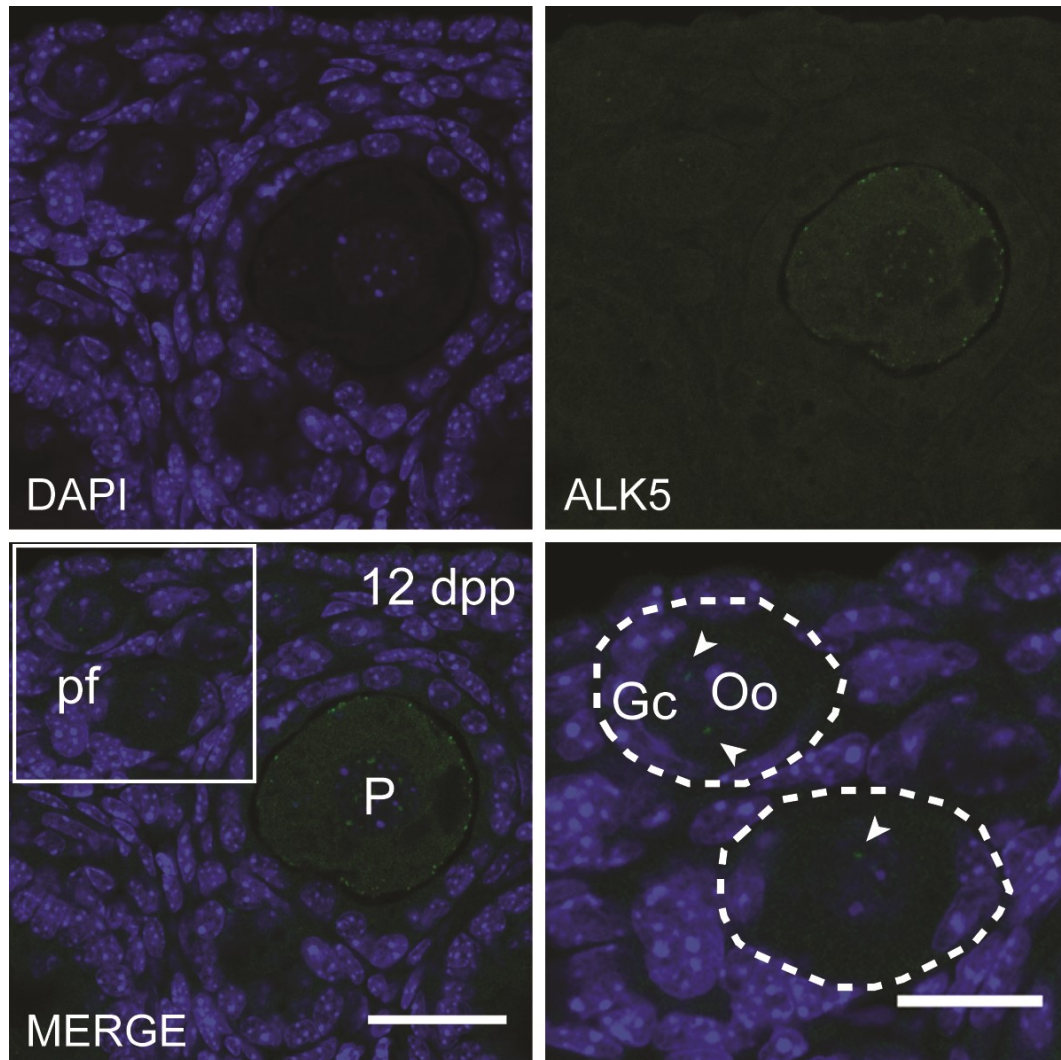


Figure 5.4. Immunolocalisation of ALK5 in the single-layered preantral follicle. Approximate mid-section of a 12 dpp ovary labelled with ALK5 (green) and counterstained with DAPI (blue). ALK5 was localised to the oocyte membrane of small single layered follicles. Staining was observed as punctate spots within the ooplasm and germinal vesicle of primordial follicles and as a punctate pattern on the oocyte membrane of primary follicles. Pf, primordial follicle; P, primary follicle; Oo, oocyte; Gc, granulosa cells. Area indicated by white box magnified in bottom right panel. Dashed line represents basal lamina. Arrowheads indicate staining. Scale bar, 30 μ m, 15 μ m (zoom).

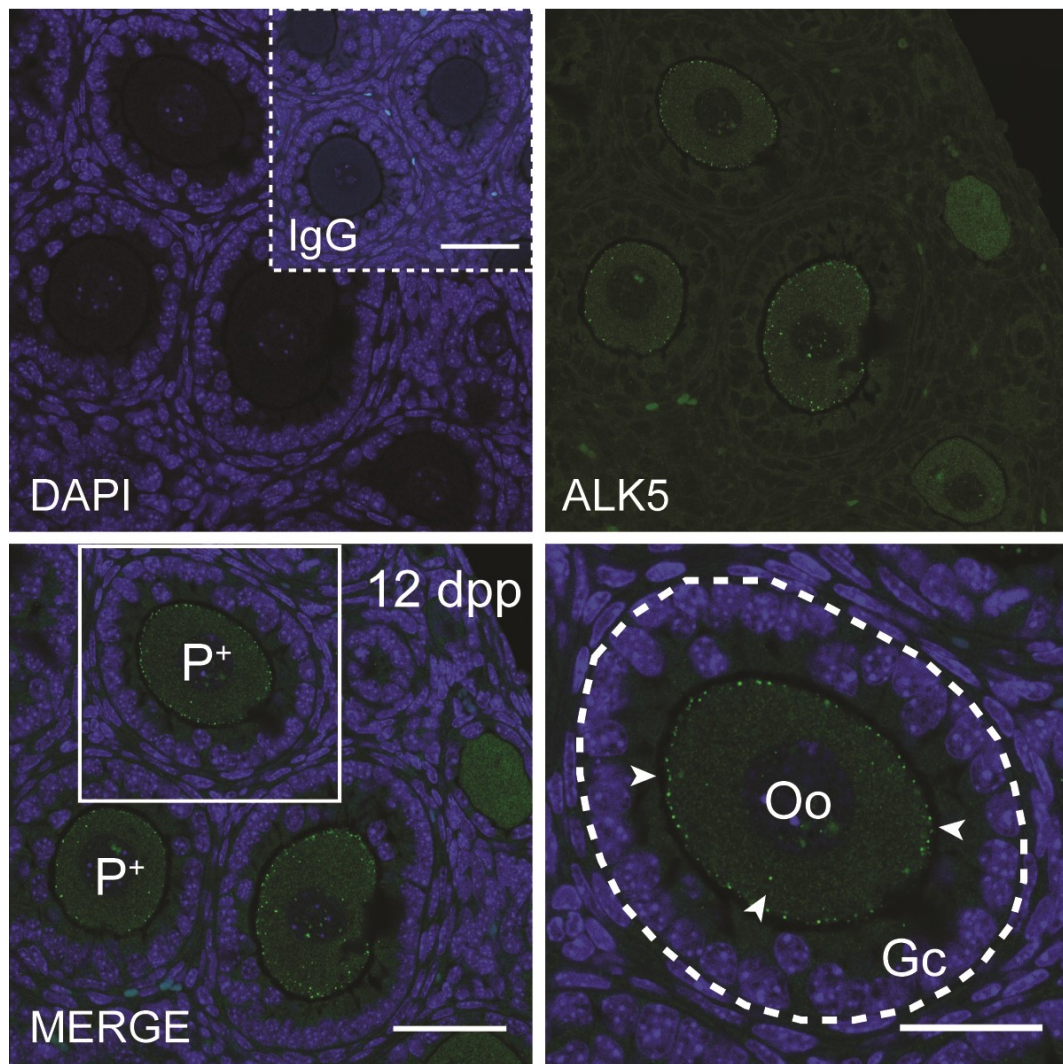


Figure 5.5. Immunolocalisation of ALK5 in the small preantral follicle. Approximate mid-section of a 12 dpp ovary labelled with ALK5 (green) or nonimmune IgG and counterstained with DAPI (blue). ALK5 was localised to the oocyte membrane of small preantral follicles (1-3 layers of GCs). Staining was observed as a punctate pattern on the oocyte membrane with some staining within the ooplasm. The IgG control demonstrated negligible background staining confirming antibody specificity. P⁺, primary plus follicle; Oo, oocyte; Gc, granulosa cells. Dashed inset represents IgG control. Area indicated by white box magnified in bottom right panel. Dashed line represents basal lamina. Arrowheads indicate staining. Scale bar, 50 μ m, 30 μ m (zoom).

5.2.2 The role of TGFB2 in primordial follicle activation

5.2.2.1 TGFB2 inhibits primordial follicle activation *in vitro*

Following the observed immunolocalisation of TGFB2 and receptor ALK5 in small preantral follicles within the juvenile ovary the role of TGFB2 in primordial follicle activation was investigated using a neonatal ovary culture (detailed in Chapter 3). Ovaries (4 dpp) were treated with vehicle or TGFB2 (1, 10 100 ng/ml) for 6 days. Following culture, ovaries were fixed and sectioned and ovarian morphology was examined. After 6 days in culture ovaries treated with vehicle control were populated predominantly by primordial follicles with some growing follicles composed mostly of transitionary and primary follicles (**Figure 5.6 A, A'**). A few larger follicles surrounded by GCs beginning to multilayer were observed. In contrast ovaries treated with TGFB2 (10 ng/ml and 100 ng/ml) appeared to be populated with fewer growing follicles than the control ovaries (**Figure 5.6 B, B'**). To confirm morphological observations follicle counts were performed on three central sections of each ovary whereby follicles were classified as either primordial or growing. TGFB2 treatment (10 ng/ml and 100 ng/ml) resulted in a decrease in the percentage of growing follicles per section with a concomitant increase in the number of primordial follicles when compared to the vehicle control (10 ng/ml $p = 0.04$; 100 ng/ml $p = 0.03$) (**Figure 5.7**). This was further confirmed by a decrease in mean oocyte area in response to TGFB2 treatment when compared to the vehicle control (1 ng/ml $p = 0.02$; 10 ng/ml $p < 0.0001$; 100 ng/ml $p < 0.0001$) **Figure 5.8**.

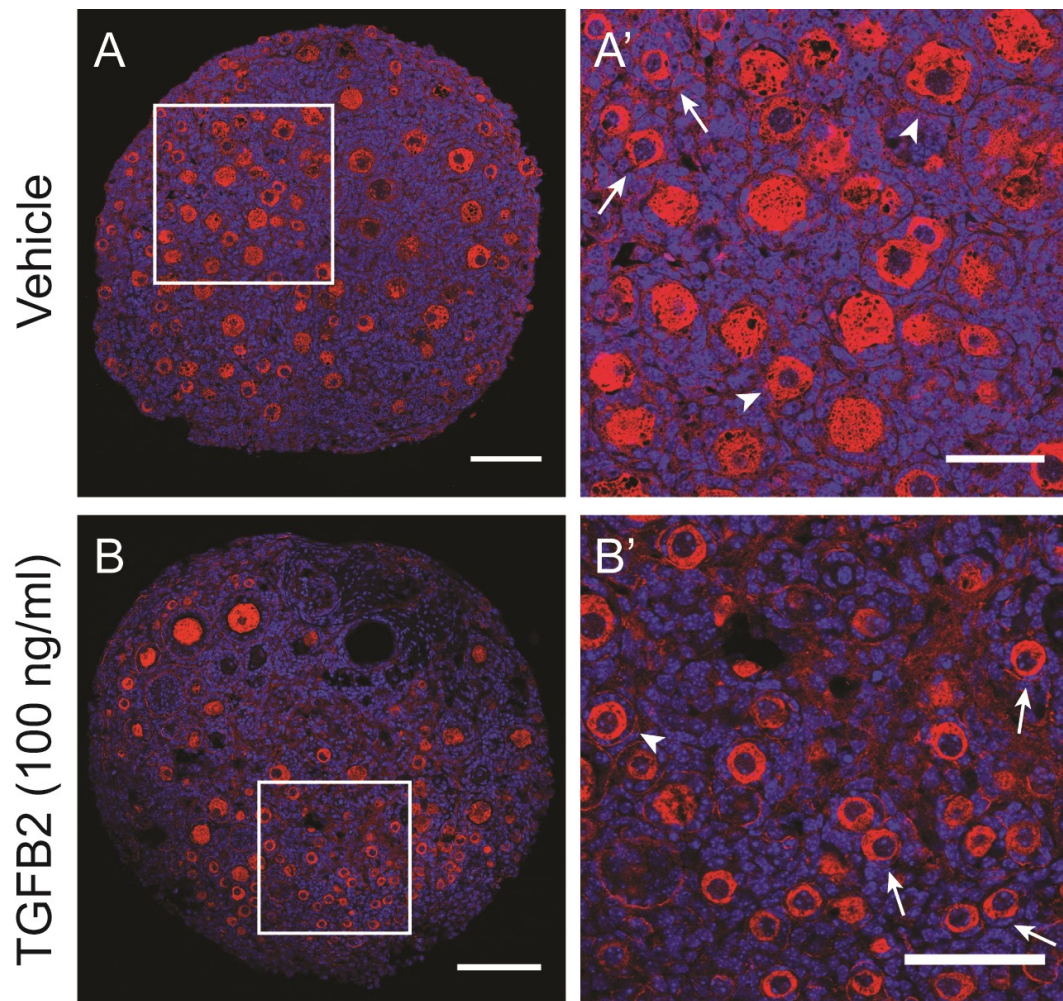


Figure 5.6. The effect of TGFB2 on ovarian morphology in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control, 1 ng/ml, 10 ng/ml or 100 ng/ml TGFB2 for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) (red) and counterstained with DAPI (blue) for morphological examination and classification. Representative images of vehicle control (A, A') and 100 ng/ml TGFB2 treated ovary (B, B'). Vehicle control treated ovaries were populated predominantly by primordial follicles and some growing follicles (A'). TGFB2 (10 ng/ml and 100 ng/ml) treated ovaries contained fewer growing follicles than the vehicle control. Areas indicated by white box magnified in far right panels. Arrows indicate primordial follicles and arrowheads indicate growing follicles. Scale bar, 100 μ m (A, B), 50 μ m (A', B').

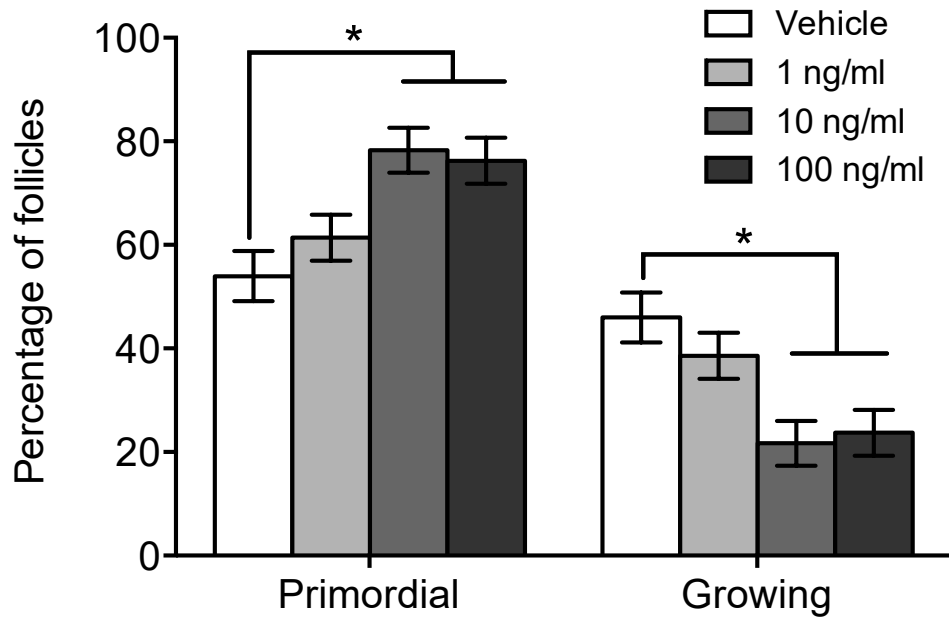


Figure 5.7. TGFB2 inhibits primordial follicle activation in a neonatal ovary culture. Mouse ovaries were treated with vehicle control (9 ovaries; n = 27 sections), 1 ng/ml (9 ovaries; n = 27 sections), 10 ng/ml (4 ovaries; n = 12 sections) or 100 ng/ml (6 ovaries; n = 18 sections) TGFB2 for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific vasa (DDX4) and counterstained with DAPI for measurement of oocyte area. Three central sections, 25 μm apart, from each ovary were used for analysis. Oocyte area was used as a marker of follicle activation. Oocytes that were larger than 275 μm^2 were classified as growing. The number of primordial or growing follicles were then presented as a percentage of follicles within each section. Data presented as mean $\pm$ SEM. Comparisons between treatment groups were performed using Kruskal-Wallis with Dunn's post hoc test where * $p < 0.05$.

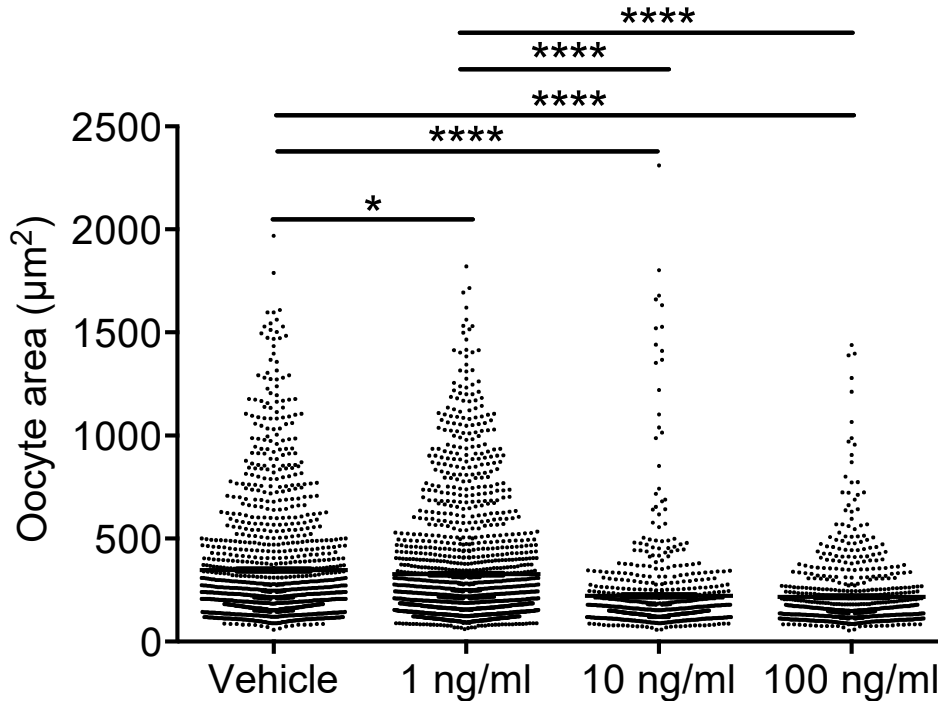


Figure 5.8. TGFB2 promotes a decrease in mean oocyte area in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (9 ovaries; n = 1210 oocytes), 1 ng/ml (9 ovaries; n = 1517 oocytes), 10 ng/ml (4 ovaries; n = 647 oocytes) or 100 ng/ml (6 ovaries; n = 636 oocytes) TGFB2 for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific mouse vasa protein (DDX4) and nuclear DAPI for measurement of oocyte area. Three central sections, 25 µm apart, from each ovary were used for analysis. Data presented as mean (horizontal bar) ± SEM. Comparisons between treatment groups were performed using Kruskal-Wallis with Dunn's post hoc test where * p < 0.05, **** p < 0.0001.

5.2.2.2 TGFB2 treatment does not induce follicle atresia

The exposure of cells to TGFB can induce a variety of cellular responses including the inhibition of cell growth, migration, differentiation and apoptosis (Heldin et al., 2009). The cellular mechanism underlying the observed decrease proportion of activated follicles was investigated. It was initially proposed that follicle loss due to increased follicular atresia may explain the observed decreased proportion of activated follicles. The apoptotic activity of GCs was therefore assessed by expression of cleaved caspase-3, a marker of cellular apoptosis, and follicle counts were examined to investigate whether the observed decrease in follicle activation was accompanied by increased levels of follicle atresia. There was no significant difference in the mean follicle count per section between the vehicle treated

ovaries (60.5 ± 6.7) and the TGFB2 treated ovaries (1 ng/ml 75.9 ± 7.5 ; 10 ng/ml 80.9 ± 11.8 ; 100 ng/ml 53 ± 6.6) (**Figure 5.9**). However, the amount of cleaved caspase-3 (positive staining expressed as a percentage area of total ovary area) was increased in response to treatment with 100 ng/ml TGFB2 when compared with control ovaries ($p = 0.04$) (**Figure 5.10**). Cleaved caspase-3 staining was not altered in ovaries treated with 1 ng/ml and 10 ng/ml TGFB2. The increased apoptotic activity in response to 100 ng/ml TGFB2 is therefore likely reflective of a toxic effect induced by the supraphysiological treatment dose. Together these findings suggest that the observed decrease in follicle activation is not attributable to an increase in follicle atresia.

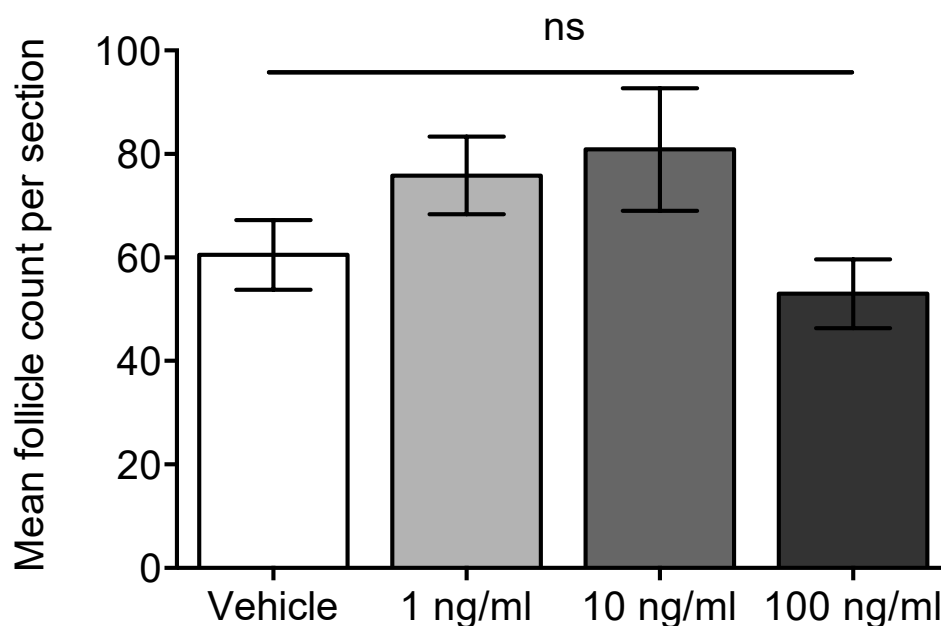


Figure 5.9. TGFB2 does not affect mean follicle count in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (9 ovaries; $n = 27$ sections), 1 ng/ml (9 ovaries; $n = 27$ sections), 10 ng/ml (4 ovaries; $n = 12$ sections) or 100 ng/ml (6 ovaries; $n = 18$ sections) TGFB2 for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of oocyte-specific vasa and nuclear DAPI for classification. Three central sections, 25 μm apart, from each ovary were used for analysis. The number of follicles per section were quantified. Data presented as mean $\pm$ SEM. Comparisons between treatment and vehicle were performed using one-way ANOVA with Tukey's post hoc test.

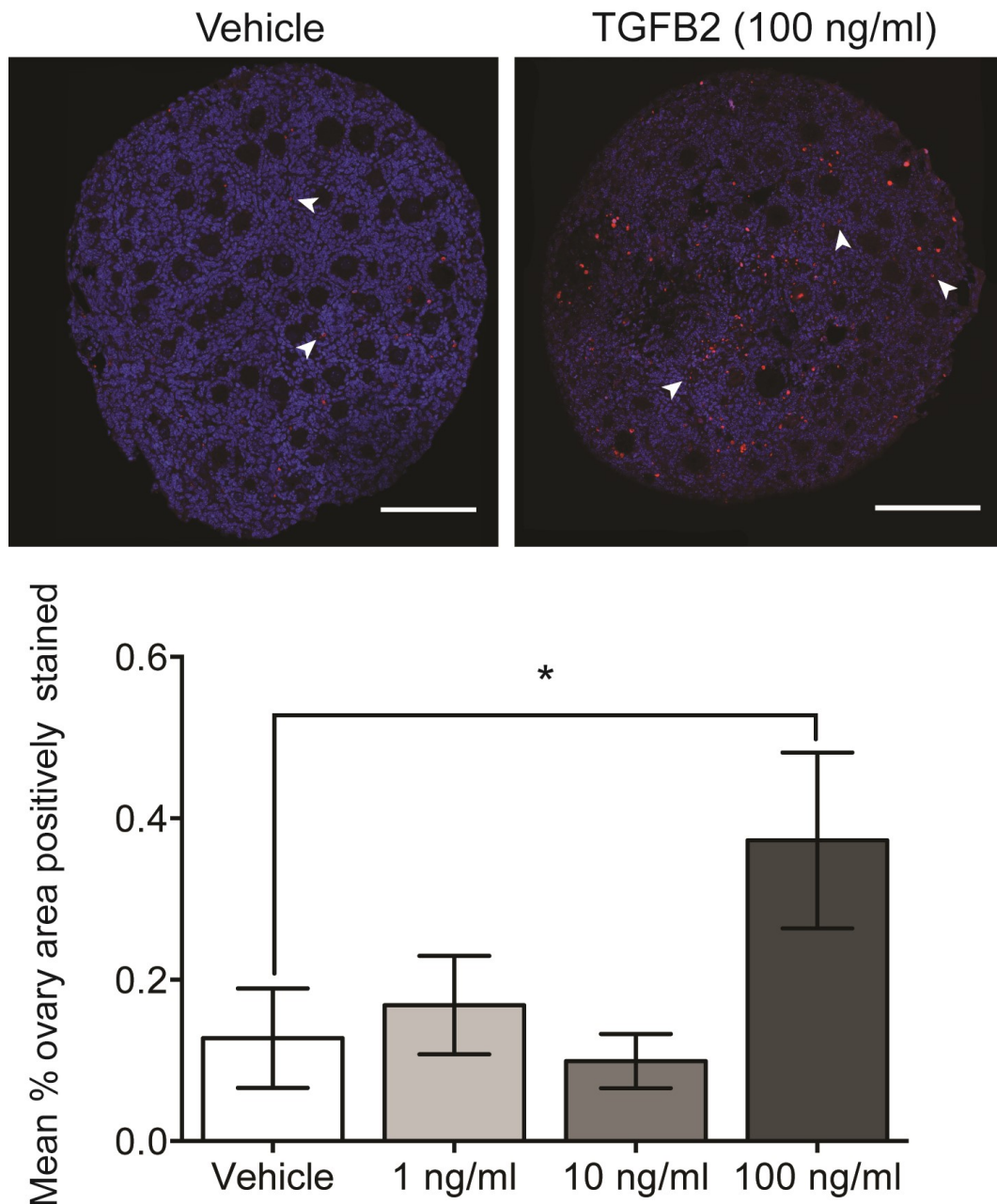


Figure 5.10. TGFβ2 promotes GC apoptosis in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (n = 9) or TGFβ2 (1 ng/ml n = 9; 10 ng/ml n = 4; 100 ng/ml n = 6) for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of apoptotic marker cleaved caspase-3 (red) and nuclear DAPI (blue). Cleaved caspase-3 staining was quantified in a single central section from each ovary. Positive staining was determined and represented as the proportion of pixels from the total number of pixels within the ovarian section. Representative images of vehicle control and 100 ng/ml TGFβ2 treated ovary. Arrowheads indicate staining. Scale bar, 150 μm. Data presented as mean ± SEM. Comparisons between treatment and vehicle were performed using Kruskal-Wallis with Dunn's post hoc test where * p < 0.05.

5.2.2.3 Decreased follicle activation is not accompanied by a decrease in GC proliferation

As lower concentrations of TGFB2 were enough to inhibit follicle activation without promoting apoptosis it was proposed that the inhibition of GC proliferation was the cellular mechanism underlying the observed inhibition of primordial follicle activation by TGFB2. The mitotic activity of GCs was therefore assessed by expression of Ki67, a marker of cellular proliferation, to investigate whether the observed decrease in follicle activation was accompanied by decreased levels of GC proliferation. The amount of Ki67 (positive staining expressed as a percentage area of total area) was not altered in TGFB2 treated ovaries as compared with control ovaries (**Figure 5.11**). This finding suggests that the observed decrease in follicle activation in response to TGFB2 was not accompanied by a decrease in GC proliferation.

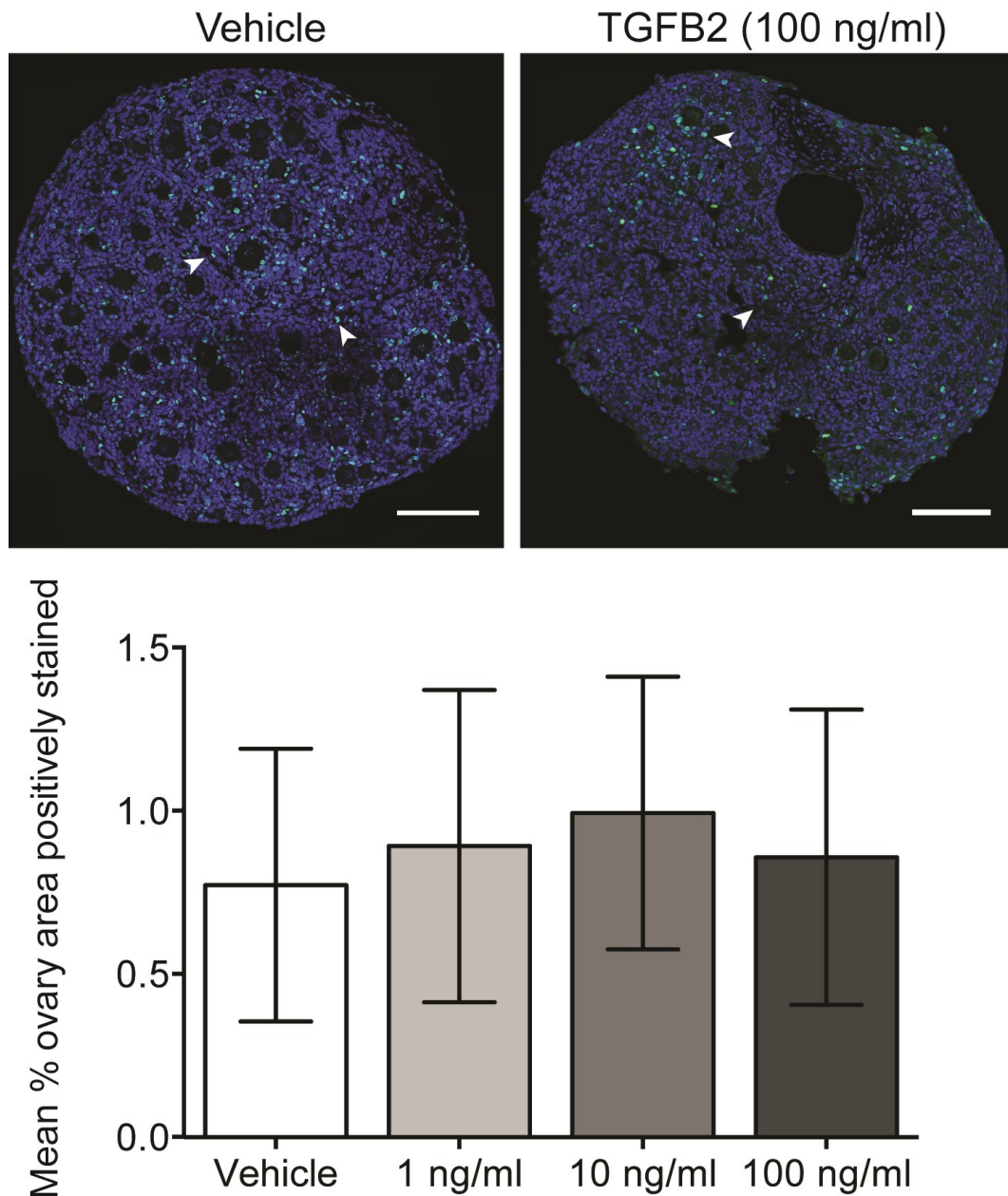


Figure 5.11. TGFβ2 does not affect GC proliferation in a neonatal ovary culture. Mouse ovaries (4 dpp) were treated with vehicle control (n = 9) or TGFβ2 (1 ng/ml n = 9; 10 ng/ml n = 4; 100 ng/ml n = 6) for 6 days. Cultured ovaries were fixed and sectioned for immunohistochemical localisation of proliferation marker Ki67 (green) and nuclear DAPI (blue). Ki67 staining was quantified in a single central section from each ovary. Positive staining was determined and represented as the proportion of pixels from the total number of pixels within the ovarian section. Representative images of vehicle control and 100 ng/ml TGFβ2 treated ovary. Arrowheads indicate staining. Scale bar, vehicle 200 μm, TGFβ2 (100 ng/ml) 100 μm. Data presented as mean ± SEM. Comparisons between treatment and vehicle were performed using Kruskal-Wallis with Dunn's post hoc test.

5.3 Discussion

The current study demonstrates, for the first time, a role for TGFB2 in maintaining the primordial follicle pool by inhibiting primordial follicle activation.

Consistent with previously published work in the mouse, TGFB2 was localised within the oocyte compartment of both primordial and growing preantral follicles (Gueripel et al., 2004). TGFB2 expression within somatic cells was observed only in the flattened GCs of the primordial follicle whereas expression was absent in the cuboidal GCs of growing follicles. In contrast ALK5 was localised only to the oocyte membrane of growing preantral follicles (both single-layered and multilayered), and was not detected in somatic cells at any stage. Primordial follicle expression was limited to the ooplasm and germinal vesicle. This finding contrasts previous reports of immunolocalisation of ALK5 in which Gueripel et al. (2004) and Juneja et al. (1996) both describe strong ALK5 immunostaining in oocytes from primordial and growing follicles and, to a lesser extent, in GCs at all stages of follicle development. A more recent examination of ALK5 localisation in the ovary describes expression in the oocytes of primordial and primary follicles in addition to the cuboidal cells of growing follicles (Wang et al., 2014). The disparity in reported staining patterns of ALK5 is unclear, however it is likely that the considerable variation in the spatiotemporal expression may simply reflect a very low abundance of ALK5 in the preantral follicle. Collectively the expression of both TGFB2 and signalling receptor ALK5 within the oocyte of early stage follicles is indicative of a role in early preantral follicle development. Indeed, the localisation of the ligand specifically in the flattened GCs of the primordial follicle highlights TGFB2 as a potential regulator of primordial follicle activation.

In correlation with the localisation of TGFB2 in the juvenile ovary, it was demonstrated that TGFB2 inhibited primordial follicle activation *in vitro*. The decreased percentage of growing follicles in response to TGFB2, with a concomitant increase in the percentage of non-growing primordial follicles, suggests a regulatory role in maintaining the primordial follicle pool. Despite the differential biological activity displayed by the TGFB isoforms, previous studies investigating the role of TGFB in the ovary have focussed primarily on TGFB1. The current results however are comparable with a recent study by Wang et al. (2014) who describe the inhibition of primordial follicle activation in a neonatal ovary culture by TGFB1. Knowledge of the functional role of TGFB in the postnatal ovary is limited, perhaps in part due to the

deleterious effects of universal TGFB gene deletion. Indeed, homozygous disruption of TGFB2 results in an embryonically lethal phenotype which precludes direct examination of the role in reproduction (Sanford et al., 1997). The current results however demonstrate, for the first time, a role for TGFB2 in maintaining the follicle pool and justify further targeted examination of the role of TGFB2 in follicle activation *in vivo*.

The exposure of cells to TGFB can induce a variety of cellular responses including the inhibition of cell growth, migration, differentiation and apoptosis (Heldin et al., 2009). Wang et al. (2014) describe a loss of follicles following treatment of neonatal ovaries with TGFB1 accompanied by an increased expression of cleaved caspase-3. A similar apoptotic response to high levels of TGFB2 was observed in the current study however this was not accompanied by a loss of follicles. More importantly, lower concentrations of TGFB2 were enough to inhibit follicle activation without promoting apoptosis. Collectively this suggests that an atretic response to TGFB2 is unlikely to be underlying the observed reduction in follicle activation. Additionally, given the differential effect of TGFB1 and TGFB2 on germ cell loss, as indicated by the current work and that of Wang et al. (2014), it could be postulated that the TGFB isoforms employ different cellular mechanisms to regulate follicle development in the neonatal ovary.

The primordial follicle is composed of an oocyte surrounded by a single layer of non-dividing flattened GCs arrested in G₀ phase of the cell cycle. Exit from this quiescent state, or follicle activation, is accompanied by GC entry into the cell cycle. Indeed, GC expression of PCNA, a cellular marker of proliferation, marks the initiation of follicle growth (Oktay et al., 1995). The antimitogenic role of TGFB is well recognised, inducing cell cycle arrest in the late G1 phase via upregulation of cyclin-dependent kinase (CDK) inhibitors (Derynck et al., 2001). Furthermore, the role of SMAD signalling in facilitating the induction of CDK inhibitors and subsequent TGFB induced growth inhibition is well documented (Pardali et al., 2000). Given this it is highly plausible that inhibition of GC cycle progression is the cellular mechanism underlying TGFB2 inhibition of primordial follicle activity. Despite the observed decrease in primordial follicle activation however decreased levels of mitotic activity were not observed. The onset of mammalian development is a protracted process whereby primordial follicles leave the resting pool and initiate a phase of slow growth in which the granulosa cells enter the cell cycle but proliferation is exceedingly slow (Hirshfield, 1991). This is reflected by the very low proportion (<10%) of GCs that are positively labelled for proliferation marker Ki67

in primordial and early growing transitional follicles (Da Silva-Buttkus et al., 2008). In retrospect the low level of proliferative activity in the early preantral follicle warrants the use of a proliferation marker with a longer half-life which, given the longer persistence of the label, would allow for more accurate detection of GCs that are in the process of, or have recently undergone, cell division. In this regard, PCNA with a biological half-life of 20 hours, would likely prove a more suitable marker than Ki67 as employed in the current study, which has an estimated half-life of one hour (Bravo & Macdonald-Bravo, 1987; Scholzen & Gerdes, 2000). Finally, as discussed in Chapter 4, while it was possible to exclude the non-specific oocyte background staining from the threshold analysis, staining present in non-GC somatic cells was unable to be omitted. In this regard, it would be more accurate to instead total the number of Ki67 (and cleaved caspase-3) positive follicles to ensure GC specific quantification in future analysis. Whether GC cycle inhibition is the regulatory mechanism underlying TGFB2 induced inhibition of primordial follicle activity requires further investigation, however given that TGFB is a potent antimitogen in a multitude of cell types, it is like that this is the manner in which regulation of the primordial follicle pool is being effected.

In the previous chapter it was postulated that the activated SMAD2/3 pathway in the primordial follicle inhibits release from the primordial pool. Given the localisation of TGFB2 in the flattened GCs of the primordial follicle and the demonstrated inhibitory role in primordial follicle activation it is therefore plausible that TGFB2 acts in an autocrine manner via SMAD2/3 to inhibit GC proliferation and maintain the primordial pool. Indeed the autocrine action of TGFB is a long established signalling paradigm (Sporn & Todaro, 1980). When considering this model the localisation of the ALK5 receptor to the oocyte membrane and absence in the somatic GCs was an intriguing finding. Given this it is therefore speculated that the spatial pattern of ALK5 expression reflects either a low receptor abundance in the GC or an alternative signalling paradigm whereby TGFB2 is signalling solely via the oocyte in a SMAD independent mechanism. This will be considered in more detail in the final discussion.

In 1996 Wandji et al. hypothesised that the presence of putative local growth inhibitors may maintain unrecruited primordial follicles in the resting pool. This was in response to a body of evidence demonstrating the spontaneous activation of bovine, and later baboon, primordial follicles in ovarian cortical strips *in vitro*, suggesting that primordial follicles are subject to

inhibitory control in vivo (Braw-Tal & Yossefi, 1997; Wandji et al., 1997; Wandji et al., 1996). In line with this Da Silva-Buttkus et al. (2009) provided mathematical evidence to support the existence of an inhibitory factor released by the primordial follicle that suppressed the growth of neighbouring follicles. The data presented in the current chapter strongly indicate that TGFB2 is a primary factor underlying the inhibitory maintenance of the primordial follicle pool.

In conclusion, in vitro studies clearly demonstrate the expression of TGFB2 and receptor ALK5 in the early preantral follicle and, for the first time, a role for TGFB2 in maintaining the primordial follicle pool.

Chapter 6 Investigating the role of TGFB2 in preantral follicle growth

6.1 Introduction

The context-dependent role of TGFB is a well-documented phenomenon. Initially identified as a stimulator of mesenchymal fibroblast growth, TGFB was subsequently proven to also function as an epithelial cell antimitogen (Roberts et al., 1981; Tucker et al., 1984). As described in the previous chapter, accumulated evidence has demonstrated a bifunctional role for TGFB1 in the ovary. In rodents, TGFB1 has been described as a potent stimulator of granulosa cell proliferation (Dorrington et al., 1988; Saragueta et al., 2002). Furthermore, TGFB1 has been shown to increase both follicular [³H]thymidine incorporation and the growth of isolated murine preantral follicles (Liu et al., 1999). In contrast however TGFB1 is reported to inhibit primordial follicle activation (Wang et al., 2014).

The regulatory effect of TGFB within the ovary is undoubtedly context dependent and likely depends on the developmental and functional stage of the follicle. The previous chapter demonstrated an inhibitory role for TGFB2 in the maintenance of the primordial follicle pool. A detailed examination of the role of the TGFB2 ligand in preantral follicle development was therefore undertaken. It was hypothesised that the functional role of TGFB2 may differ depending on the developmental stage of the follicle.

6.1.1 Specific aims

- Assess the effect of TGFB2 on preantral follicle growth using an isolated follicle culture system
- Investigate the mechanism underlying the TGFB2 effect by examining TGFB target gene expression

6.2 Results

6.2.1 The role of TGFB2 in preantral follicle development

To model preantral follicle development an isolated follicle culture system was employed. Unlike the 4 dpp ovary used previously, the culture of older ovaries containing more

developmentally advanced follicles is of limited use due to the inefficient nutrient, gas and waste exchange in the larger tissue mass leading to central areas of necrosis (Murray & Spears, 2000). The current model is an established culture method, based on a mechanical isolation protocol first developed by Nayudu and Osborn (1992). The model maintains the normal three-dimensional relationship and signalling loop between the GCs and oocyte, preserving the intrinsic GC-GC and GC-oocyte junctional complexes, while separating the granulosa-oocyte unit from the stroma/theca cells at the basement membrane interface (Fenwick et al., 2013).

6.2.1.1 TGFB2 promotes preantral follicle growth

In order to investigate the role of TGFB2 in the development of the preantral follicle, isolated follicles (100 – 130 μm diameter; 2 – 3 layers GCs) were cultured with vehicle or a dose range of TGFB2 for 48 hours. Follicles were imaged every 24 hours and follicle area was quantified. An initial TGFB2 dose response was performed. Exposure of follicles to TGFB2 promoted a rapid increase in follicle growth relative to vehicle controls at all concentrations after 24 and 48 hours (1 ng/ml ($p = 0.0001$, < 0.0001), 10 ng/ml ($p < 0.0001$) and 100 ng/ml ($p < 0.0001$, 0.0001)), with the exception of 10 ng/ml TGFB2 at 24 hours (**Figure 6.1 A**). Given the increase in follicle growth at all concentrations a second lower dose response was performed. Exposure of follicles to TGFB2 promoted a rapid increase in follicle growth relative to vehicle controls at all concentrations after 24 and 48 hours (0.01 ng/ml ($p < 0.0001$, 0.0001), 0.1 ng/ml ($p < 0.0001$, 0.0001), 1 ng/ml ($p < 0.0001$, 0.0001)) (**Figure 6.1 B**). Representative images of a vehicle control and 0.1 ng/ml TGFB2 treated follicle are illustrated in **Figure 6.2**. Based on the dose response data, 0.1 ng/ml TGFB2 was chosen as the minimum dose required to elicit a maximal TGFB2-induced growth response for all subsequent investigations unless otherwise stated.

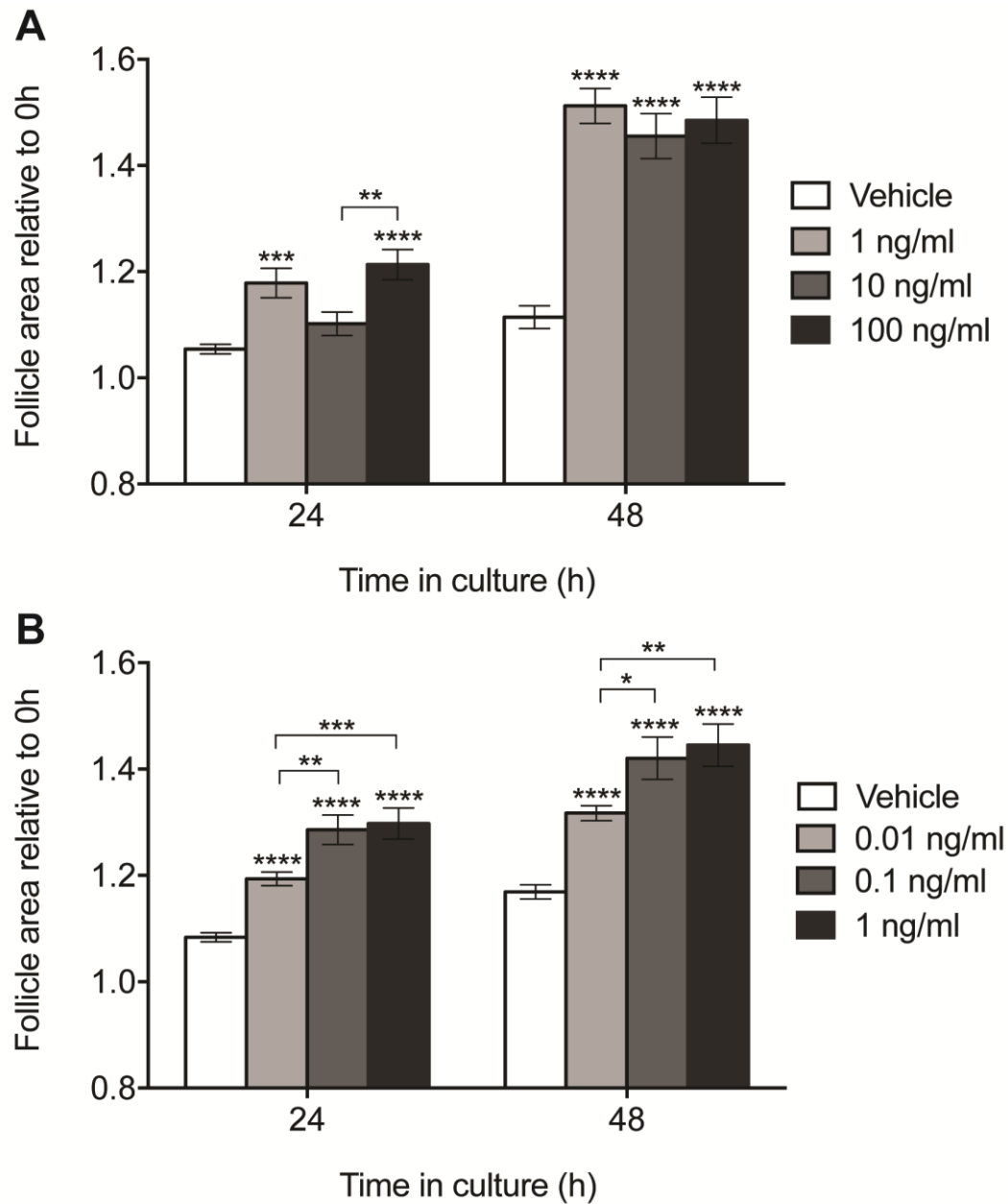


Figure 6.1. TGFβ2 promotes preantral follicle growth in vitro. Follicles (2 - 3 layers of GCs (100 – 130 μm diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control (n = 47), 1 ng/ml (n = 31), 10 ng/ml (n = 29) or 100 ng/ml (n = 30) TGFβ2 (**A**) or vehicle control (n = 71), 0.01 ng/ml (n = 80), 0.1 ng/ml (n = 42) or 1 ng/ml (n = 38) TGFβ2 (**B**) for 48 hours. Follicle area was quantified every 24 hours and calculated relative to the area at time 0 (0h). Data presented as mean ± SEM. Comparisons between treatment groups were performed within each time point by one-way ANOVA with Tukey's post hoc test where * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001. In the case of not normally distributed data comparisons were confirmed by Kruskal-Wallis with Dunn's post hoc test.

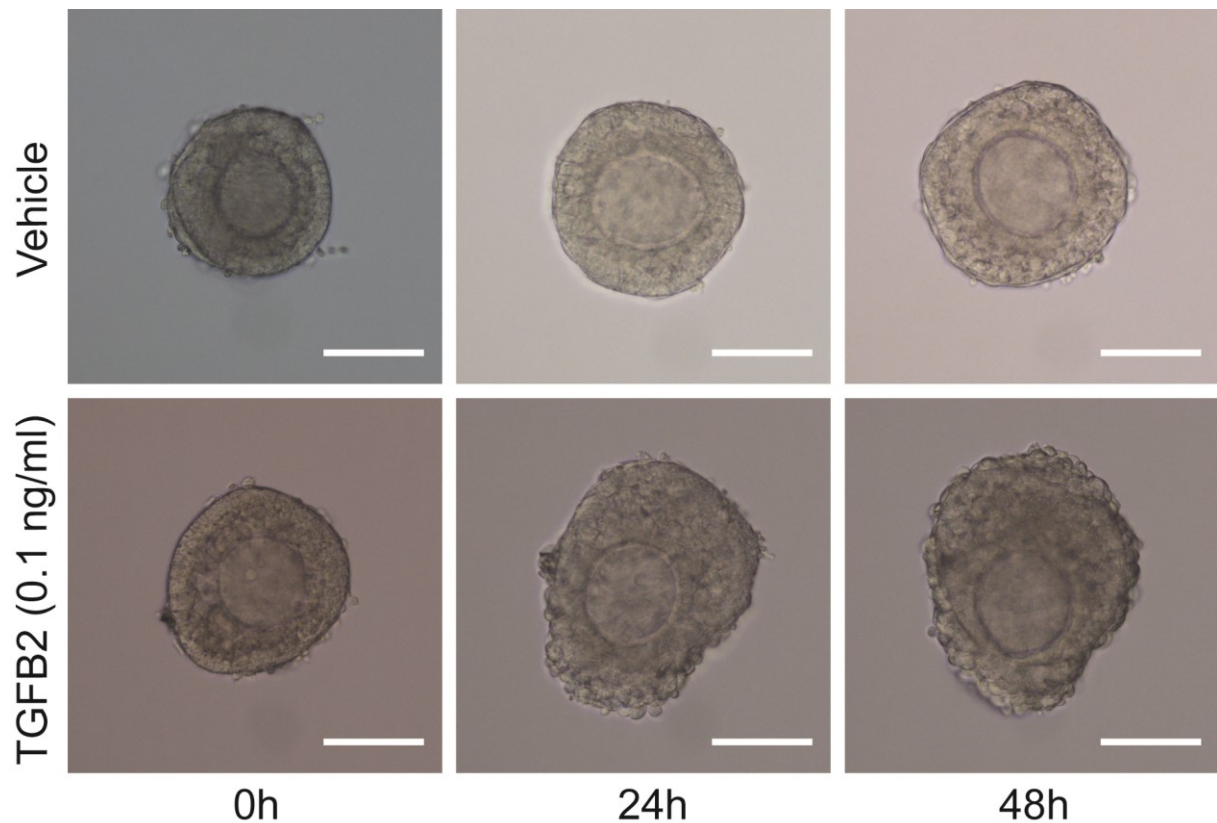


Figure 6.2. The effect of TGFB2 on follicle morphology in vitro. Follicles (2 - 3 layers of GCs (100 – 130 μm diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or a range of concentrations of TGFB2 (0.01, 0.1, 1, 10 or 100 ng/ml) for 48 hours. Follicles were imaged at 0, 24 and 48 hours. Representative images of a vehicle control and 0.1 ng/ml TGFB2 treated follicle are shown. TGFB2 promotes a rapid increase in follicle growth relative to vehicle. Scale bar, 70 μm .

6.2.1.2 TGFB2 treatment often leads to loss of follicle integrity

In addition to the observed increase in follicle growth, TGFB2 treatment often resulted in the loss of structural integrity of the follicle resulting in oocyte and GC expulsion (**Figure 6.3**). If this was observed, follicle data were excluded from the analysis as it was not possible to obtain an accurate measurement of follicle area.

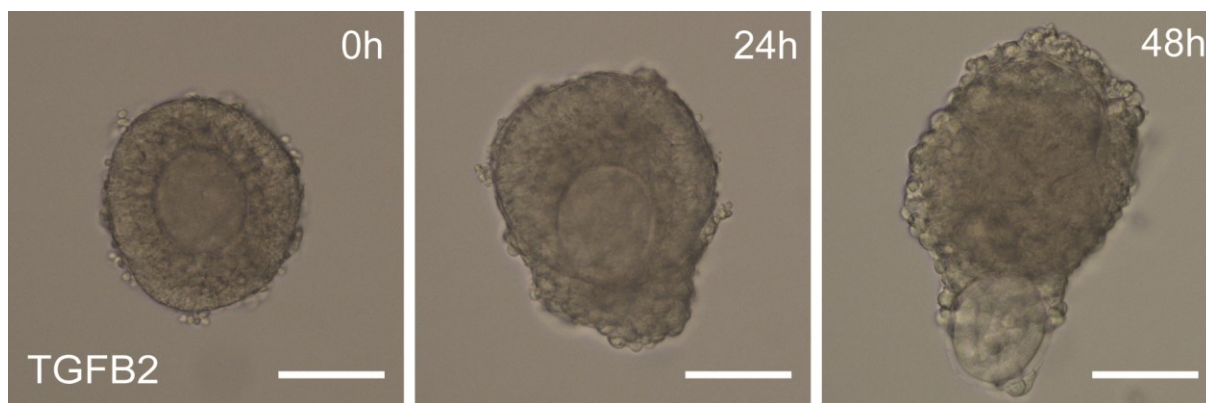


Figure 6.3. The effect of TGFB2 on follicle morphology in vitro. Follicles were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or a range of concentrations of TGFB2 (0.01, 0.1, 1, 10 or 100 ng/ml) for 48 hours. Follicles were imaged at 0, 24 and 48 hours. Representative images of a 0.1 ng/ml TGFB2 treated follicle are shown. TGFB2 treatment often results in the loss of structural integrity of the follicle. Scale bar, 70 μ m.

6.2.2 Cellular mechanisms underlying TGFB2 induced preantral follicle growth

6.2.2.1 Increased follicle growth in response to TGFB2 is accompanied by regulation of multiple target genes

TGFB typically mediates its effects by regulation of target gene expression (Massague, 2012). Quantitative RT-PCR was used to examine changes in the expression of downstream targets of the TGFB pathway in follicles exposed to a dose range of TGFB2 for 24 hours. Initially follicle response to TGFB2 was confirmed to ensure the culture replicated the growth curve previously observed (**Figure 6.4**). Four families of regulatory genes were examined; cell cycle, cell differentiation, EMT and tissue remodelling. Transcript levels of the cell-cycle inhibitor, *Cdkn1b* were significantly reduced at 24 hours in response to TGFB2 (0.1 ng/ml) relative to control ($p = 0.02$), while levels of the opposing stimulatory cell-cycle regulator *Ccnd2* remained unaffected (**Figure 6.5**). Downstream transcription regulators of proliferation and differentiation, *Id2* and *Id3*, were downregulated in response to TGFB2 (1 ng/ml and 0.1 ng/ml respectively), relative to control ($p = 0.03$, $p = 0.04$ respectively; **Figure 6.6**). Following 24 hour treatment with TGFB2, EMT transcriptional regulators *Snai1* and *Snai2*, and tissue remodelling regulators *Mmp2* and *Timp1* remained unchanged (**Figure 6.7** and **Figure 6.8**).

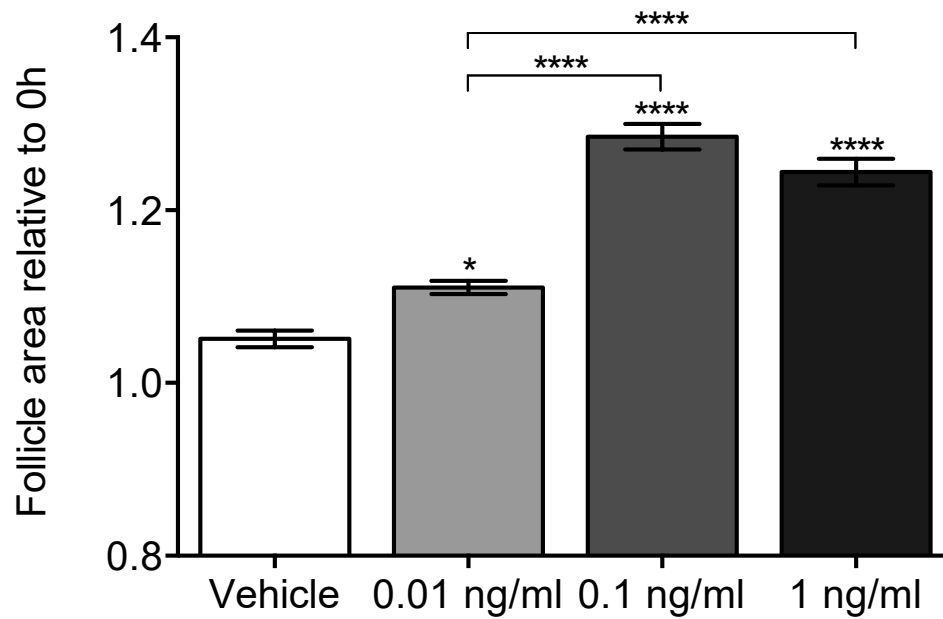


Figure 6.4. TGFB2 promotes preantral follicle growth in vitro. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control (n = 50), 0.01 ng/ml (n = 48), 0.1 ng/ml (n = 45) or 1 ng/ml (n = 43) TGFB2 for 24 hours. Follicle area was quantified every 24 hours and calculated relative to the area at time 0 (0h). Data presented as mean $\pm$ SEM. Comparisons between treatment groups were performed by Kruskal-Wallis with Dunn's post hoc test where * $p < 0.05$, **** $p < 0.0001$.

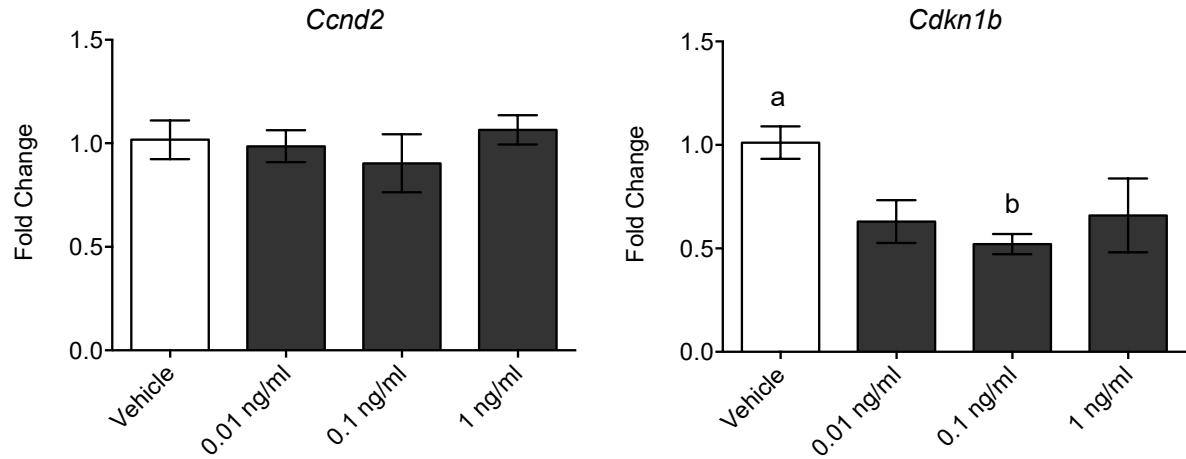


Figure 6.5. The effect of TGFB2 on the expression of cell cycle regulators in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control, 0.01 ng/ml, 0.1 ng/ml or 1 ng/ml TGFB2 for 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment groups were made by one-way ANOVA and Tukey's post hoc test. Groups with different letters indicate statistical differences ($p < 0.05$).

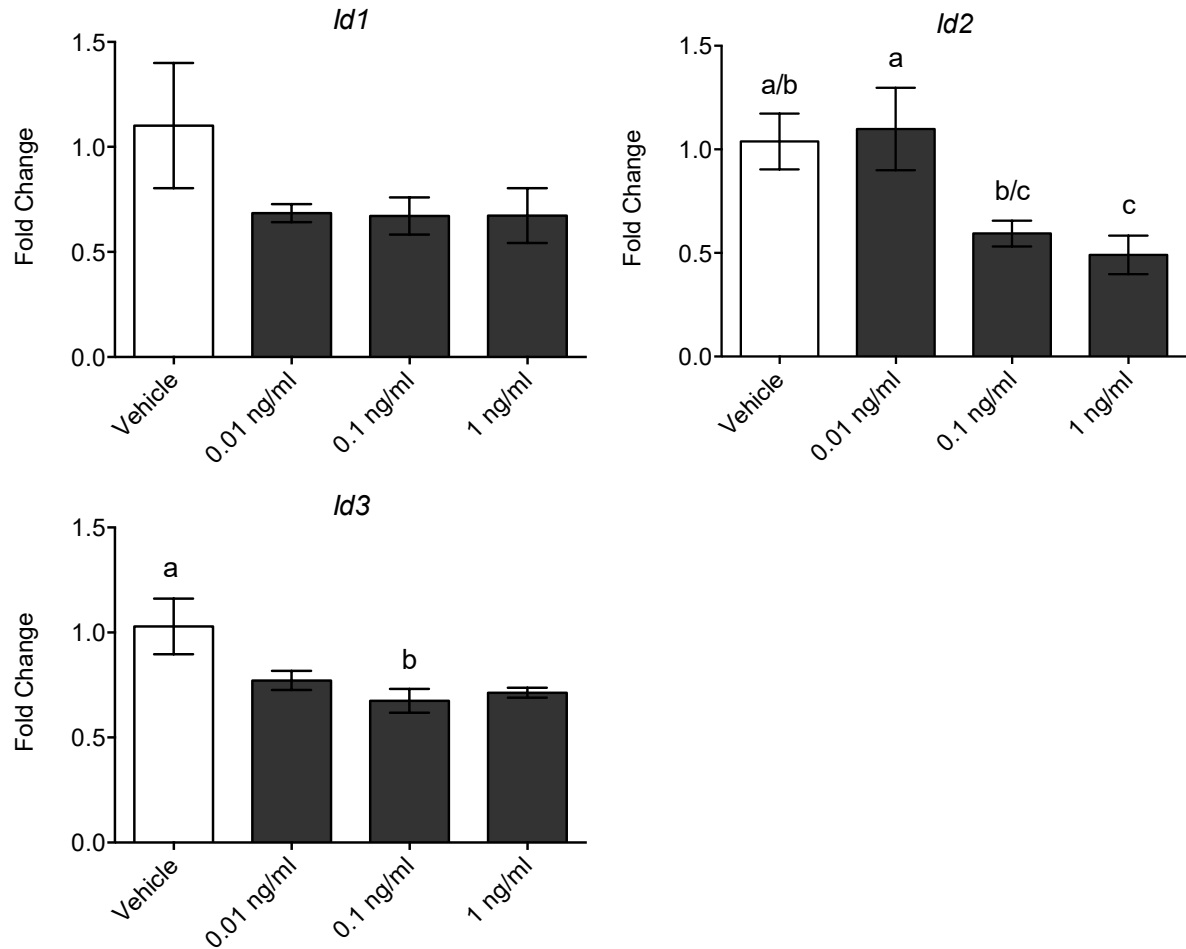


Figure 6.6. The effect of TGFB2 on the expression of regulators of cell proliferation and differentiation in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control, 0.01 ng/ml, 0.1 ng/ml or 1 ng/ml TGFB2 for 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment groups were made by one-way ANOVA and Tukey's post hoc test. Groups with different letters indicate statistical differences ($p < 0.05$).

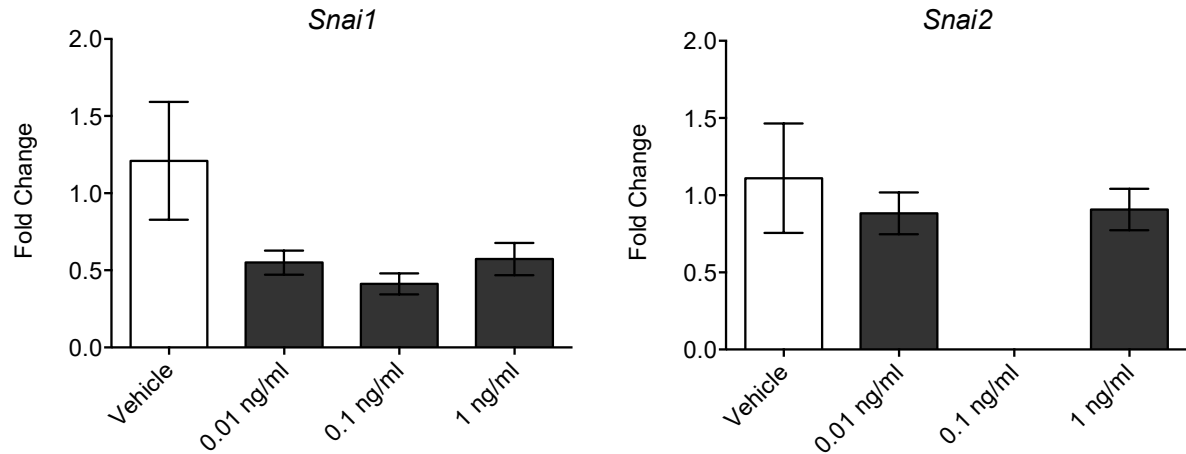


Figure 6.7. The effect of TGFB2 on the expression of regulators of EMT in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control, 0.01 ng/ml, 0.1 ng/ml or 1 ng/ml TGFB2 for 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment groups were made by one-way ANOVA and Tukey's post hoc test.

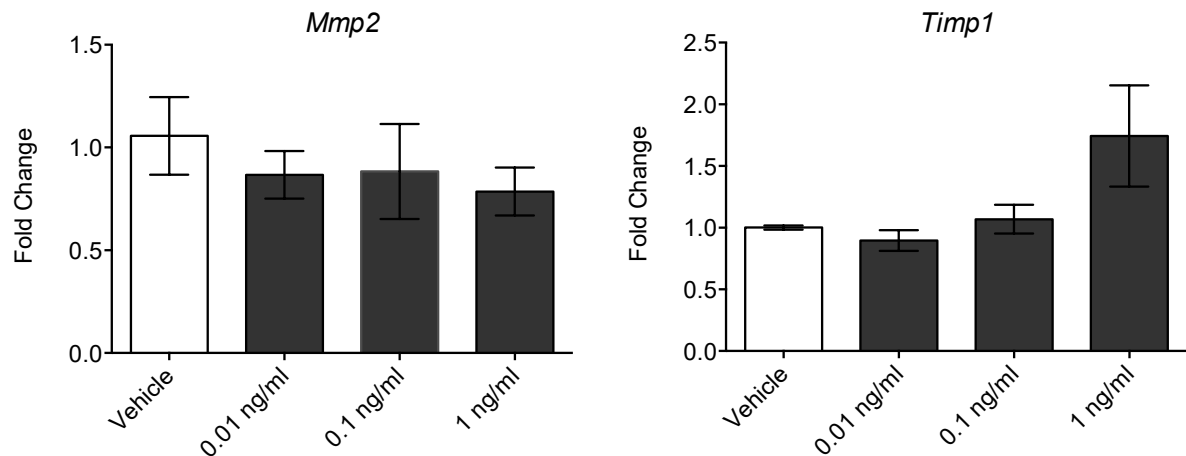


Figure 6.8. The effect of TGFB2 on the expression of regulators of tissue remodelling in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control, 0.01 ng/ml, 0.1 ng/ml or 1 ng/ml TGFB2 for 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment groups were made by one-way ANOVA and Tukey's post hoc test.

6.2.2.2 Regulation of multiple target genes occurs rapidly after TGFB2 treatment

It was postulated that due to the temporal nature of gene expression, examination of transcripts at 24 hours may not reflect any prior influence of TGFB2. Accordingly, candidate gene expression was examined at 6 hour intervals following the onset of culture. Again the follicle response to TGFB2 was confirmed to ensure the culture replicated the growth curve previously observed. The exposure of follicles to TGFB2 promoted a rapid increase in follicle growth relative to vehicle controls at 24 hours but not at 12 hours, which suggests that any changes in gene expression would occur before this (**Figure 6.9**). Transcript levels of the cell-cycle inhibitor *Cdkn1b* were significantly reduced at all time points (6 ($p = 0.0006$), 12 ($p = 0.008$) and 24 hours ($p = 0.0001$)) in response to TGFB2 relative to control, while levels of the opposing stimulatory cell-cycle regulator *Ccnd2* were significantly increased at 6 and 12 hours ($p = 0.0005$, $p = 0.0032$, respectively; **Figure 6.10**). Downstream regulators of proliferation and differentiation, *Id1*, *Id2* and *Id3*, were downregulated at 6 hours in response to TGFB2 ($p = 0.004$, 0.0001 , 0.02 respectively; **Figure 6.11**). *Id2* was additionally downregulated at 12 ($p = 0.002$) and 24 hours ($p = 0.0005$). Expression of *Snai2*, a transcriptional regulator of EMT, initially decreased at 6 hours ($p = 0.01$) before expression increased at 24 hours ($p = 0.006$) compared to vehicle control (**Figure 6.12**). Finally, transcript levels of tissue remodelling regulator, *Mmp2*, were significantly increased at 24 hours ($p = 0.008$), whereas levels of MMP inhibitor *Timp1* remained unchanged at all time points (**Figure 6.13**).

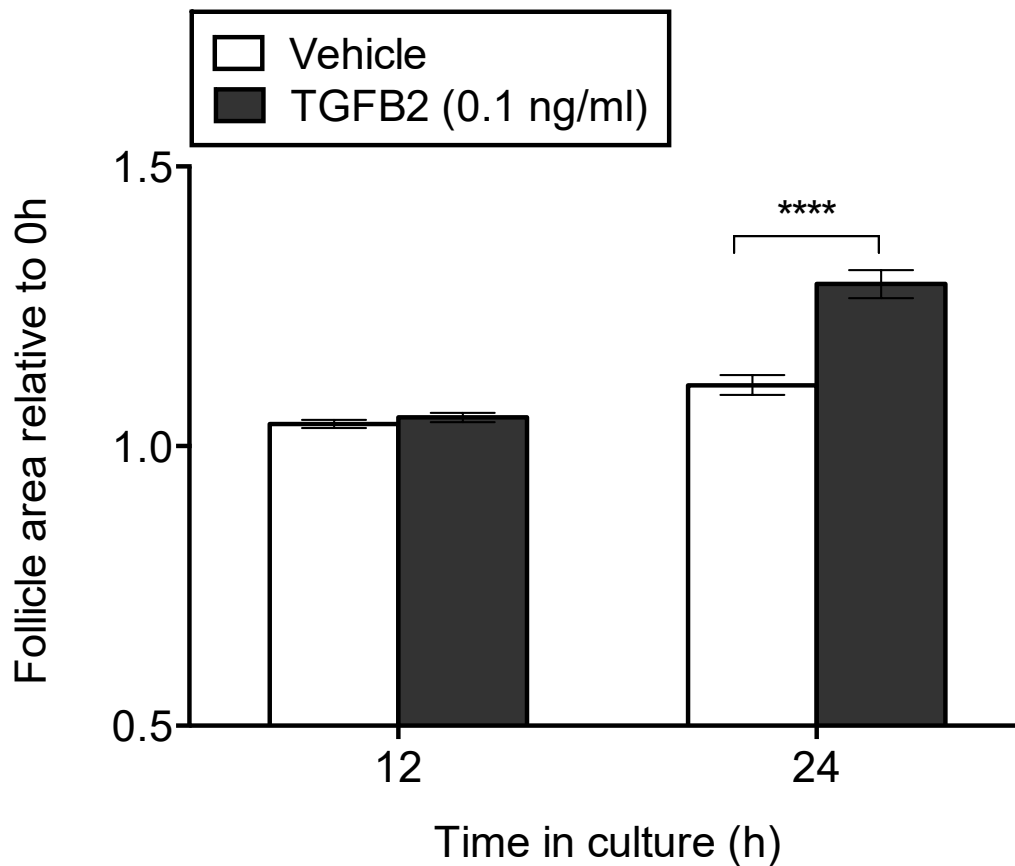


Figure 6.9. TGFB2 promotes preantral follicle growth in vitro. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control (n = 72) or 0.1 ng/ml TGFB2 (n = 70) for 24 hours. Follicle area was quantified every 12 hours and calculated relative to the area at time 0 (0h). Data presented as mean $\pm$ SEM. Comparisons between treatment groups were performed by unpaired t test where **** p < 0.0001. In the case of not normally distributed data a Mann-Whitney test was used to confirm the comparison.

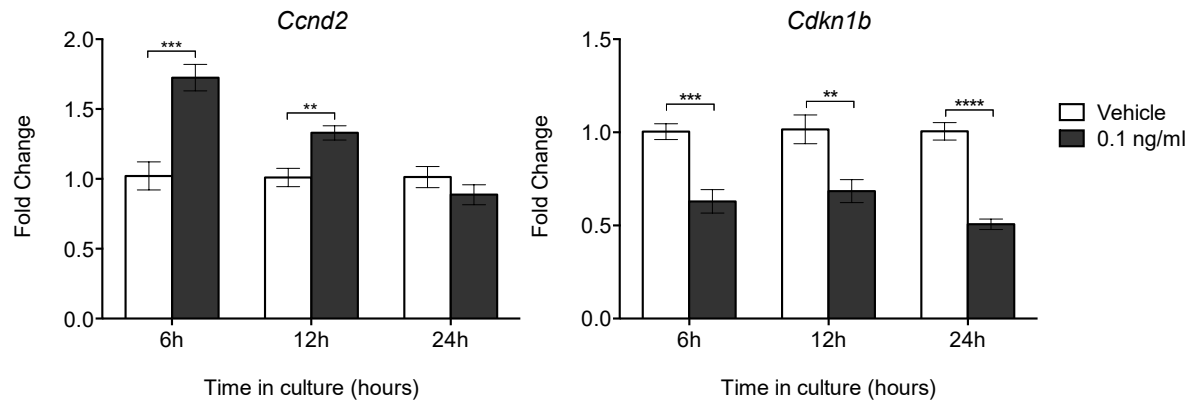


Figure 6.10. The effect of TGFB2 on the expression of cell cycle regulators in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or 0.1 ng/ml TGFB2 for 6, 12 or 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression within each time point. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment and vehicle were made by unpaired t test within each time point where ** p < 0.01, *** p < 0.001, **** p < 0.0001.

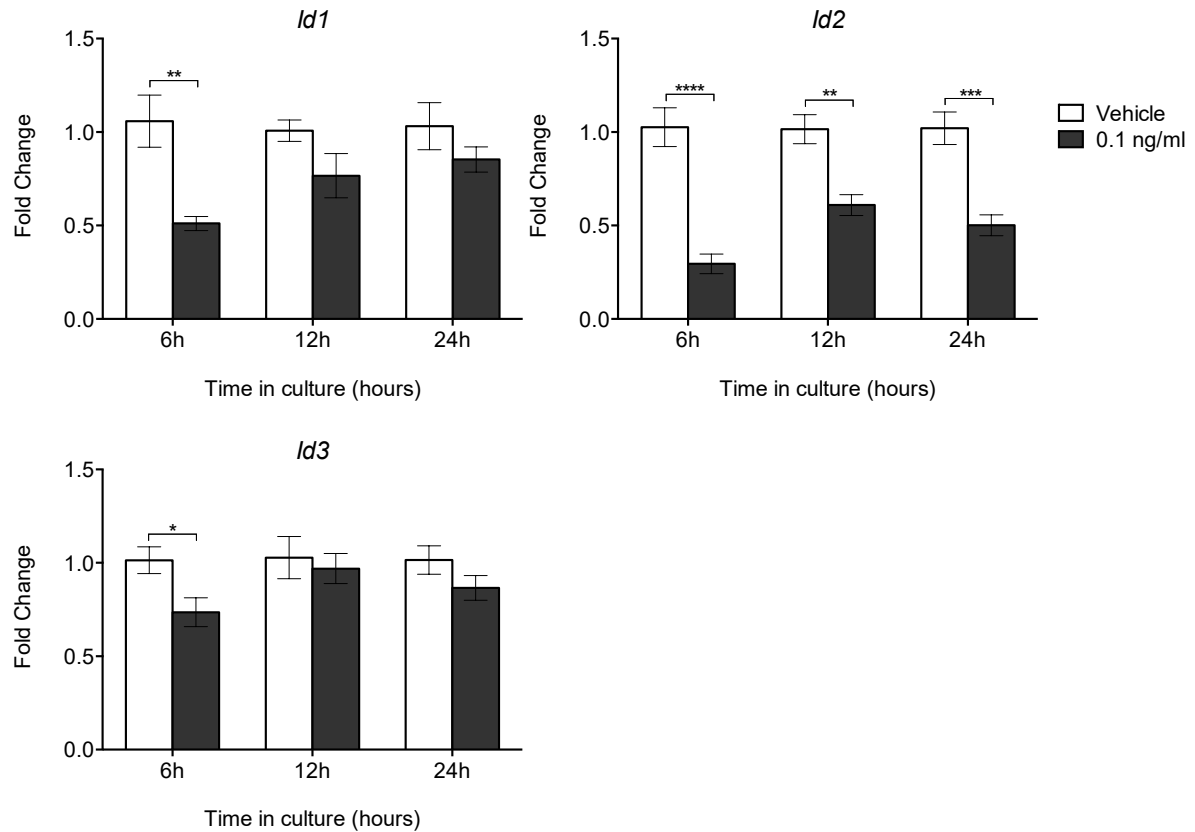


Figure 6.11. The effect of TGFB2 on the expression of regulators of cell proliferation and differentiation in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or 0.1 ng/ml TGFB2 for 6, 12 or 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression within each time point. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment and vehicle were made by unpaired t test within each time point where * p < 0.05 ** p < 0.01, *** p < 0.001, **** p < 0.0001.

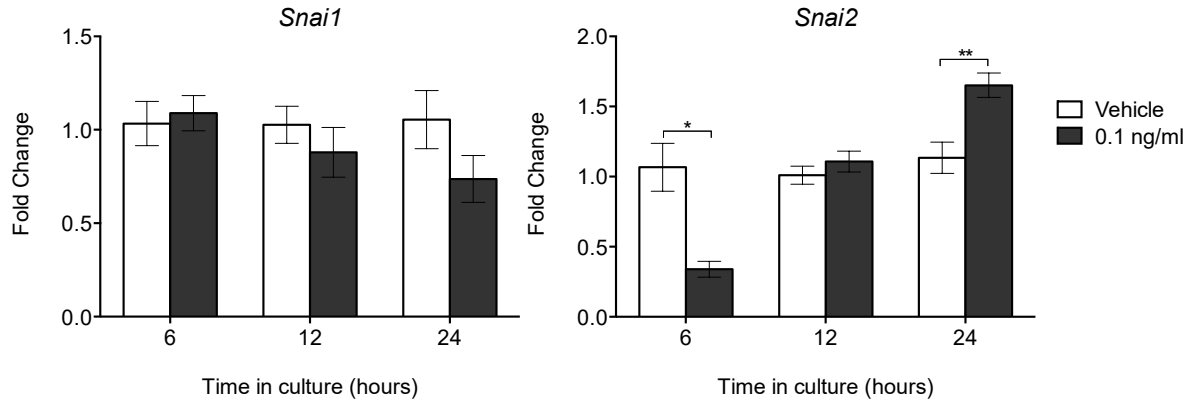


Figure 6.12. The effect of TGFB2 on the expression of regulators of EMT in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or 0.1 ng/ml TGFB2 for 6, 12 or 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression within each time point. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment and vehicle were made by unpaired t test within each time point where * $p < 0.05$, ** $p < 0.01$.

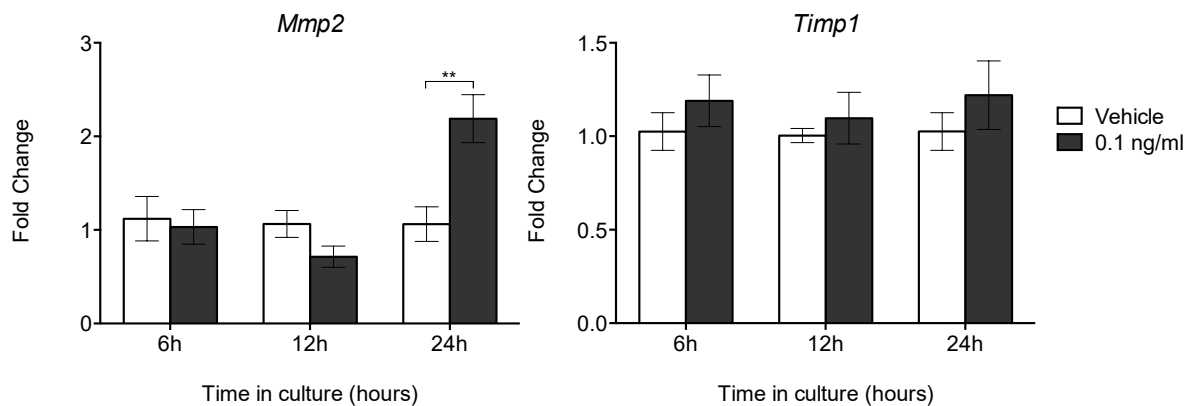


Figure 6.13. The effect of TGFB2 on the expression of regulators of tissue remodelling in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or 0.1 ng/ml TGFB2 for 6, 12 or 24 hours. Transcript expression was quantified by RT-PCR. Each transcript was normalised to ATP5B and fold changes calculated relative to vehicle expression within each time point. Data presented as mean $\pm$ SEM (6 follicles from a single 16 dpp ovary for each treatment (n = 6 ovaries)). Comparisons between treatment and vehicle were made by unpaired t test within each time point where ** $p < 0.01$.

6.2.2.3 Increased follicle growth is accompanied by an increase in GC proliferation

Subsequent to the onset of follicle activation, preantral follicle development is characterised by proliferation and multilayering of GCs accompanied by extensive ECM remodelling to accommodate the increase in follicle size (Curry & Osteen, 2003; Lintern-Moore & Moore, 1979). Given the TGFB2 induced perturbation of transcripts involved in regulation of the cell cycle and tissue remodelling it was proposed that these transcriptional effects induce functional cellular changes which underlie the observed increase in follicle growth. The mitotic activity of GCs was therefore assessed by expression of Ki67, a cellular marker of proliferation, to investigate whether the observed increase in follicle growth in response to TGFB2 was accompanied by increased levels of GC proliferation. Apoptotic activity was additionally examined by double labelling for cleaved caspase-3 and TUNEL. Evidence of tissue remodelling was investigated by the qualitative examination of the ECM component laminin. In line with the observed growth and survival of follicles in culture, even without TGFB2 stimulation, expression of Ki67 was observed. The amount of Ki67 positive nuclei however were increased at 24 hours in TGFB2 (0.1 ng/ml) treated follicles ($p < 0.0001$) compared with vehicle (**Figure 6.14 A, A', C**). A few apoptotic cells were present with or without TGFB2 stimulation, as evidenced by expression of cleaved caspase-3 and TUNEL labelling. Follicles exposed to TGFB2 however had a significantly higher proportion of cleaved caspase-3 cells compared with controls ($p = 0.02$), whereas the proportion of TUNEL positive cells was similar between the two groups (**Figure 6.14 B, B', D, E**). Laminin expression did not appear to differ qualitatively between vehicle and TGFB2 (0.1 ng/ml) treated follicles (**Figure 6.15**). This suggests that the rapid growth inducing effect of TGFB2 is due to increased GC proliferation.

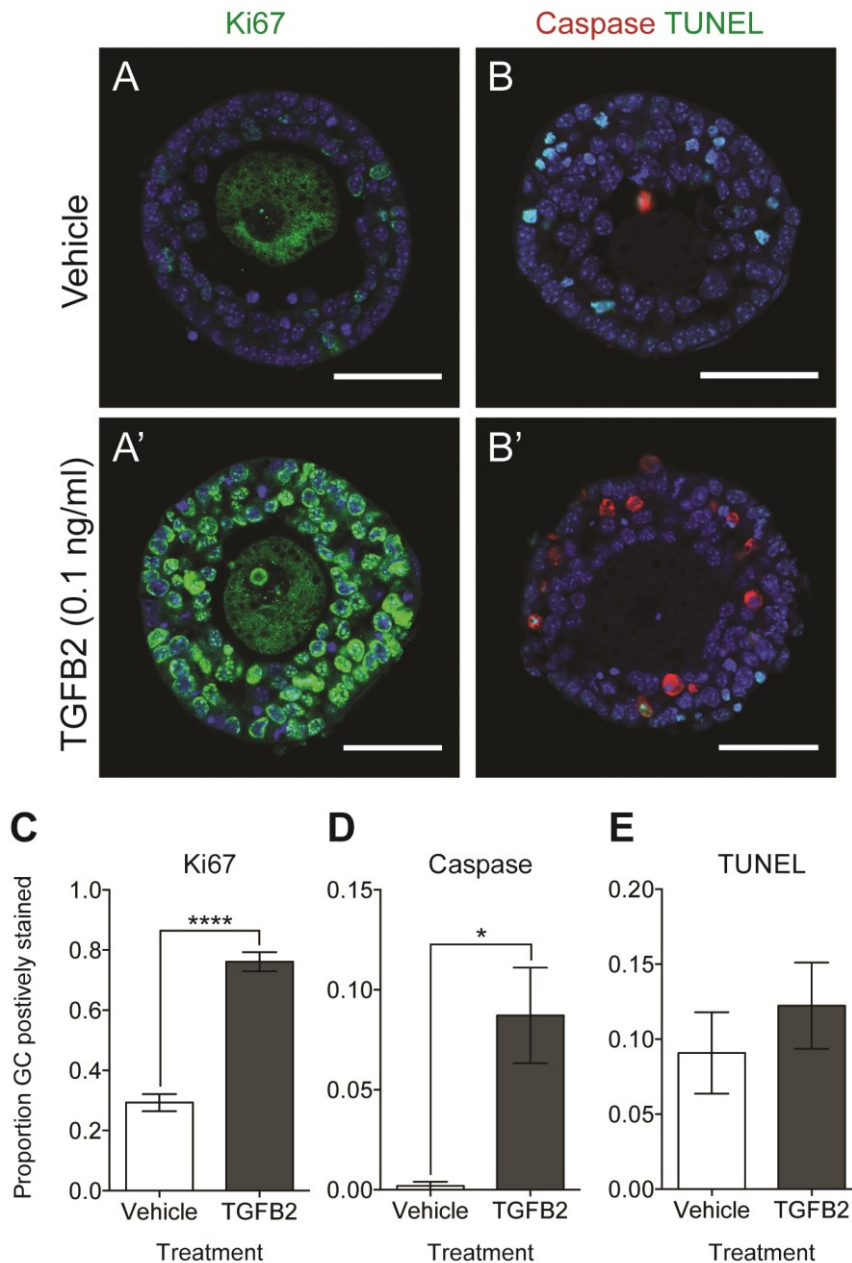


Figure 6.14. TGFB2 promotes GC proliferation in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or TGFB2 (0.1 ng/ml) for 24 hours. Cultured follicles were fixed and sectioned for immunohistochemical localisation of proliferation marker Ki67 (green) (A, A'), or apoptotic markers cleaved caspase-3 (red) and TUNEL (green) (B, B'), and nuclear DAPI (blue). Representative images of vehicle control (A, B) and 0.1 ng/ml TGFB2 treated (A', B') follicles. The proportion of positive nuclei in each treatment was quantified for each marker, Ki67 (C), cleaved caspase-3 (D) and TUNEL (E). Data presented as mean $\pm$ SEM ($n = 5 - 8$ follicles analysed for each treatment). Comparisons between vehicle and treatment were performed using an unpaired t test where * $p < 0.05$, $p^{****} < 0.0001$. Scale bar, 40 μ m.

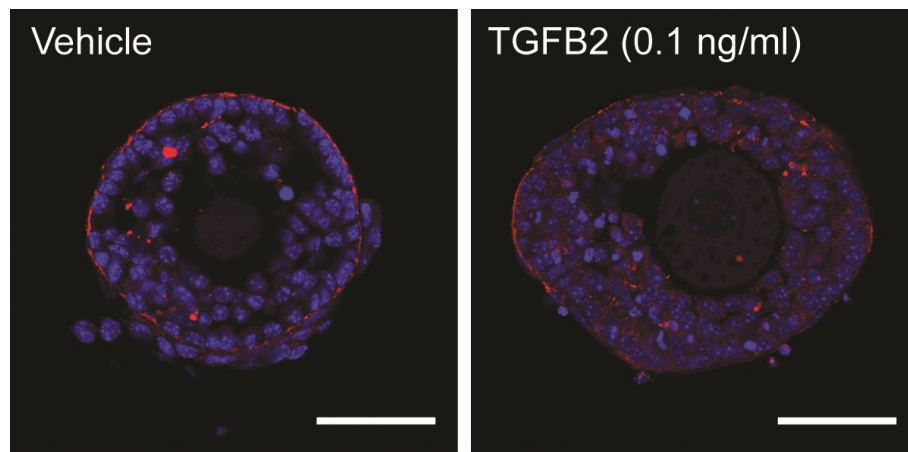


Figure 6.15. TGFB2 does not induce qualitative changes in laminin expression in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control or TGFB2 (0.1 ng/ml) for 24 hours. Cultured follicles were fixed and sectioned for immunohistochemical localisation of ECM component laminin (red) and nuclear DAPI (blue). Representative images of vehicle control and 0.1 ng/ml TGFB2 treated follicle. Scale bar, 40 μ m.

6.3 Discussion

The current study demonstrates, for the first time, that TGFB2 promotes preantral follicle growth.

Previous reports investigating the role of TGFB in the preantral follicle are limited and have focussed primarily on the TGFB1 isoform. The current results however are comparable with a study by Liu et al. (1999) who reported TGFB1 induced growth of preantral follicles in an in vitro culture system. Subsequent to the onset of follicle activation, preantral follicle development is characterised by extensive proliferation and multilayering of GCs (Lintern-Moore & Moore, 1979). In the current study, TGFB2 induced follicle growth was accompanied by a prolific increase in GC proliferation. This corroborates previous work describing TGFB1 as a potent stimulator of GC proliferation (Dorrington et al., 1988; Saragueta et al., 2002). Moreover, Roy (1993) reported a specific GC targeted mitogenic effect of the TGFB2 isoform in the rodent preantral follicle as demonstrated by increased follicular [^{3} H]thymidine incorporation. Cell cycle progression and proliferation are controlled by a balance of positive and negative regulators converging on cell cycle kinase cascades (Sherr & Roberts, 2004). GC proliferation during follicle development is regulated by cyclin

D2 (CCND2), the action of which permits progression through G1 phase of the cell cycle. Indeed, mice null for CCND2 are sterile due to impaired GC proliferation and preantral follicle growth arrest (Sicinski et al., 1996). In correlation with the observed increase in GC proliferation in the current study, increased transcript levels of cell cycle regulator *Ccnd2* were observed while levels of opposing cell-cycle inhibitor *Cdkn1b* decreased. It is therefore plausible that TGFB2 plays an integral role in the extensive proliferation and multilayering of GCs in the preantral follicle by promoting GC progression through the G1 phase of the cell cycle via upregulation of CCND2 and downregulation of CDKN1B. The oocyte secretes potent TGFB superfamily growth factors including BMP15 and GDF9 to stimulate GC proliferation in the preantral follicle. This oocyte-paracrine communication is a long recognised signalling paradigm, a process fundamental to ovarian follicle development, disruption of which has profound effects on fertility (Eppig, 2001). Given the abundant expression of TGFB2 in the oocytes of small multilayered preantral follicles, as demonstrated in Chapter 5, it is plausible that TGFB2 acts in a paracrine manner to stimulate follicle growth. TGFB2 treatment additionally resulted in an increased proportion of caspase-positive GCs. Similar stimulation of both proliferation and apoptosis has previously been observed in follicles cultured in the presence of both BMP15 and GDF9, and FSH, both treatments of which resulted in an increase in follicle growth, suggesting an increase in cell turnover may be induced as a self-regulatory mechanism to prevent excessive follicle growth (Fenwick et al., 2013; Hardy et al., 2016). Finally, although not directly quantified, no apparent change in oocyte size was observed in response to TGFB2. Oocyte growth and GC proliferation do not take place simultaneously, indeed the extensive phase of oocyte growth is accompanied by relatively limited GC proliferation from one layer of flattened pre-GCs to three layers of cuboidal GCs (Wasserman & Albertini, 1994). Given that oocyte growth is largely complete at the follicle stage selected for culture (2-3 layers GCs), the absence of a TGFB2 effect is not entirely unexpected.

Extensive remodelling of the ECM is a fundamental element of follicle development. There is considerable evidence to suggest that ECM and connective tissue remodelling during follicular growth is facilitated by the matrix metalloproteinase (MMP) system (Curry & Osteen, 2003). Furthermore, abundant expression of MMP2 has been demonstrated in the juvenile rodent ovary indicative of a role in early preantral follicle development (Bagavandoss, 1998). Consistent with this the current study demonstrated increased transcript

levels of *Mmp2* transcript in parallel to TGFB2 induced preantral follicle growth while the concurrent levels of MMP inhibitor *Timp1* remained unchanged, suggestive of an environment conducive for remodelling. The role of TGFB in stimulation of the MMP system is well documented and upregulation in response to TGFB1 has specifically been demonstrated in the GCs of the chicken ovary (Zhu et al., 2014). Evidence of tissue remodelling was investigated by the qualitative examination of the ECM component laminin. Characterisation of laminin in the murine follicle demonstrates a defined ring at the exterior of the follicular GCs marking the basal laminin component of the basement membrane with low levels apparent in the stroma and GC compartment (Berkholtz et al., 2006). Despite the observed increase in follicle growth, laminin expression did not appear qualitatively different in response to TGFB2 exposure. Expansion of the basement membrane in growing follicles however must occur in a manner that maintains integrity throughout the expansion process therefore the absence of a qualitative difference was not unexpected. In this regard, future investigations concerning the action of the MMP system on ECM remodelling in response to TGFB2 would be best modelled by employing zymography to examine enzymatic activity.

TGFB typically mediates its effects by regulation of target gene expression (Massague, 2012). Activated SMAD proteins translocate to the nucleus where they recruit additional transcription regulators, including DNA binding transcription factors, co-activators and co-repressors, that control the expression of multiple target genes. The inhibitors of differentiation (*Id*) proteins (also known as inhibitors of DNA binding) are a subfamily of transcription factors that regulate multiple genes including those required for growth and differentiation. The *Id* proteins act to promote proliferation and inhibit differentiation and are a gene target of TGFB (Kang et al., 2003). In this regard the influence of TGFB2 on *Id* gene expression in the current study is particularly intriguing. The TGFB2 induced repression of *Id* transcript levels is suggestive of suppressed cellular proliferation with a commitment to differentiation, in contrast with the observed increase in GC proliferation and follicle development. TGFB mediated repression of cellular *Id* expression has previously been reported and is believed to underlie the typical cytostatic programme of TGFB action (Kang et al., 2003). In vivo the *Id* proteins act to coordinate the balance of proliferation and differentiation and a functional role has been demonstrated in the postnatal ovary (Hogg et al., 2010). Given this it may simply be that decreased *Id* expression, as observed in the

current study, is a repressive response to the rapid onset of GC proliferation induced by TGFB2.

It has been reported that activated GCs express morphological characteristics that are akin to mesenchymal rather than epithelial cells, suggesting that a partial EMT takes place as they activate and develop (Childs & McNeilly, 2012; Mora et al., 2012). The initiation and maintenance of EMT are regulated by members of the Snail family of zinc finger proteins which directly repress the expression of E-cadherin among other epithelial markers (Cano et al., 2000; Nieto, 2002). Transcripts encoding the Snail superfamily member *Snai2* are highly expressed in the mesenchymal theca within the ovary (Parent et al., 2004). TGFB is a potent initiator of epithelial mesenchymal transition (EMT), responsible for the induction of a number of EMT inducing transcription factors, including *Snai1* and *Snai2* (Ahmed & Nawshad, 2007). Consistent with this the current study demonstrates increased transcript levels of *Snai2* in response to TGFB2. TGFB2 may therefore regulate the putative EMT events accompanying follicle development.

The previous chapter demonstrated an inhibitory role for TGFB2 in the maintenance of the primordial follicle pool whereas the current chapter revealed a stimulatory role for TGFB2 in preantral follicle growth. When interpreted collectively it appears that the functional role of TGFB2 in preantral follicle development is dictated by the developmental stage of the follicle. The bifunctional role of TGFB is well-documented, indeed the TGFB isoforms characteristically elicit both context-dependent and cell-specific effects (Massague, 2012). This however is the first time a context dependent role for TGFB2 has been demonstrated in preantral follicle development. The TGFB signalling cascade is complex and promiscuous in nature whereby multiple ligands converge on various combinations of heteromeric receptor complexes and transduce their signals via both canonical and non-canonical signalling cascades to modify the expression of specific sets of target genes (Moustakas & Heldin, 2009). The given response of a cell is therefore determined by the extracellular and intracellular composition of the TGFB signal transduction system. Given this it is likely that the observed context-dependent role of TGFB2 is a result of differential signal transduction within the preantral follicle. Indeed, differing cellular signalling machinery, the influence of non-canonical signalling pathways, or differentially expressed transcription factors within the different staged follicles most likely underlie the bifunctional role of TGFB2 (Massague, 2012; Mullen et al., 2011).

In conclusion, *in vitro* studies demonstrate increased growth of the preantral follicle in response to TGFB2. When interpreted collectively with the previous chapter, the results clearly demonstrate a differential role for TGFB2 during preantral follicle development. The mechanism of signal transduction underlying the differential role of TGFB2 in preantral follicle development warrants further investigation. The following chapter will therefore examine the signalling mechanism underlying the role of TGFB2 in the preantral follicle.

Chapter 7 Investigating the signalling mechanism underlying the role of TGFB2 in preantral follicle growth

7.1 Introduction

TGFB classically regulates gene expression by receptor-mediated activation of the SMAD signalling cascade (Moustakas & Heldin, 2009). The TGFB isoforms exert their cellular effects by binding transmembrane type I and type II serine/threonine kinase receptors on the cell surface leading to phosphorylation-dependent activation of the receptor regulated SMAD2/3. Activated SMAD2/3 then forms a heteromeric complex with the common SMAD4, which translocates into the nucleus to regulate target gene expression. Further to the canonical SMAD signalling cascade however, the cellular response to TGFB can be additionally regulated by an array of non-SMAD pathways including the ERK branch of the MAPK pathway and the PI3K/Akt pathway (Derynck & Zhang, 2003). This regulation can occur in response to direct activation of non-SMAD proteins by the TGFB receptor complex initiating a parallel signalling cascade or be elicited by cross talk with parallel pathways (Moustakas & Heldin, 2005). In this manner, cellular responses to TGFB can be reinforced, attenuated or modulated, which permits context dependent interpretation of TGFB signals. A summary of the potential cellular responses to TGFB are summarised in **Figure 7.1**.

In vitro studies in the preceding chapters have demonstrated a differential role for TGFB2 in preantral follicle development, whereby the functional role appears to be dictated by the developmental stage of the follicle. Given the well-documented ability of TGFB to utilise both SMAD and non-SMAD intracellular signalling pathways to regulate cellular function it is plausible that the observed context-dependent role of TGFB2 is a result of non-classical signal transduction within the preantral follicle. Based on this a detailed examination of the signal transduction mechanism underlying TGFB2 induced follicle growth was undertaken in the preantral follicle. It was hypothesised that non-classical signal transduction may be facilitating and/or modulating the TGFB2 response in the multilayered preantral follicle. More specifically, non-SMAD pathways may be independently facilitating TGFB2 signal transduction or cross talk between collateral signalling pathways may be modulating the canonical SMAD2/3 signalling cascade.

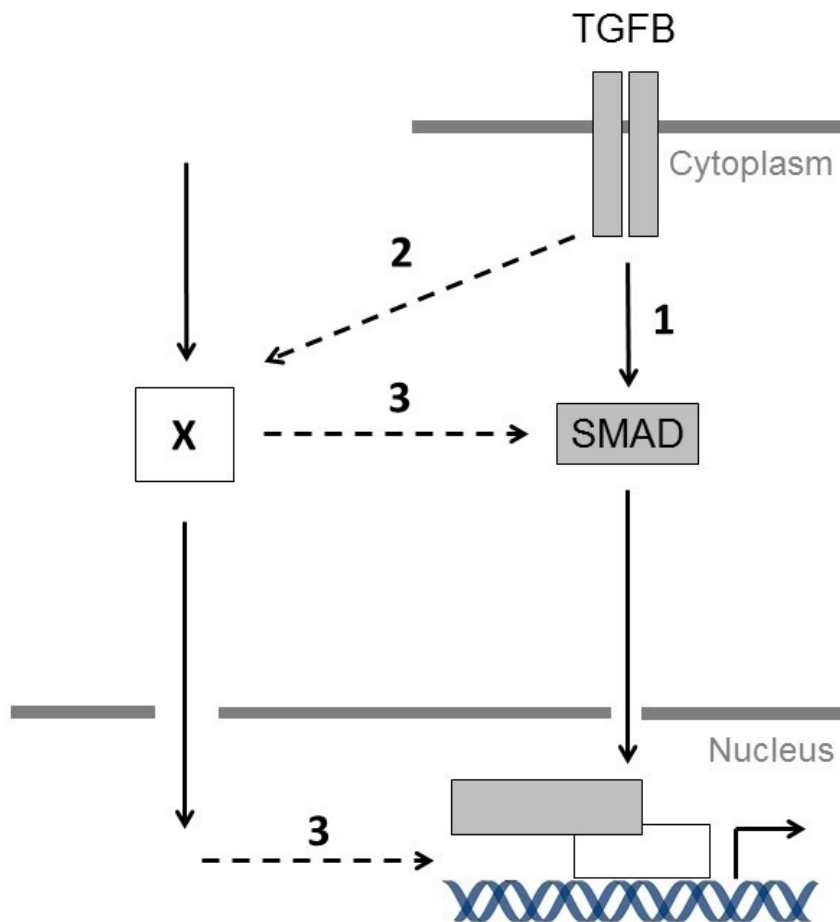


Figure 7.1. The cellular response to TGFβ. Receptor-mediated activation of the canonical SMAD pathway (1). Receptor-mediated activation of a non-SMAD pathway (2). Modulation of the canonical SMAD pathway by cross-talk from parallel signalling pathways (3).

7.1.1 Specific aims

- Establish the role of SMAD and non-SMAD signalling in TGFβ2 induced preantral follicle growth
- Examine whether the action of TGFβ2 is being directly facilitated by SMAD and non-SMAD signalling cascades

7.2 Results

7.2.1 The role of SMAD and non-SMAD signalling in TGFB2 induced preantral follicle growth

In order to investigate the signal transduction mechanism underlying TGFB2 induced follicle growth, small molecule inhibitors were employed to disrupt both SMAD (SMAD2/3 and SMAD1/5/8) and non-SMAD (MAPK/ERK and PI3K/Akt) signalling pathways in combination with TGFB2 in culture (detailed in **Figure 7.2**). As in the previous chapter, an isolated preantral follicle culture system was employed.

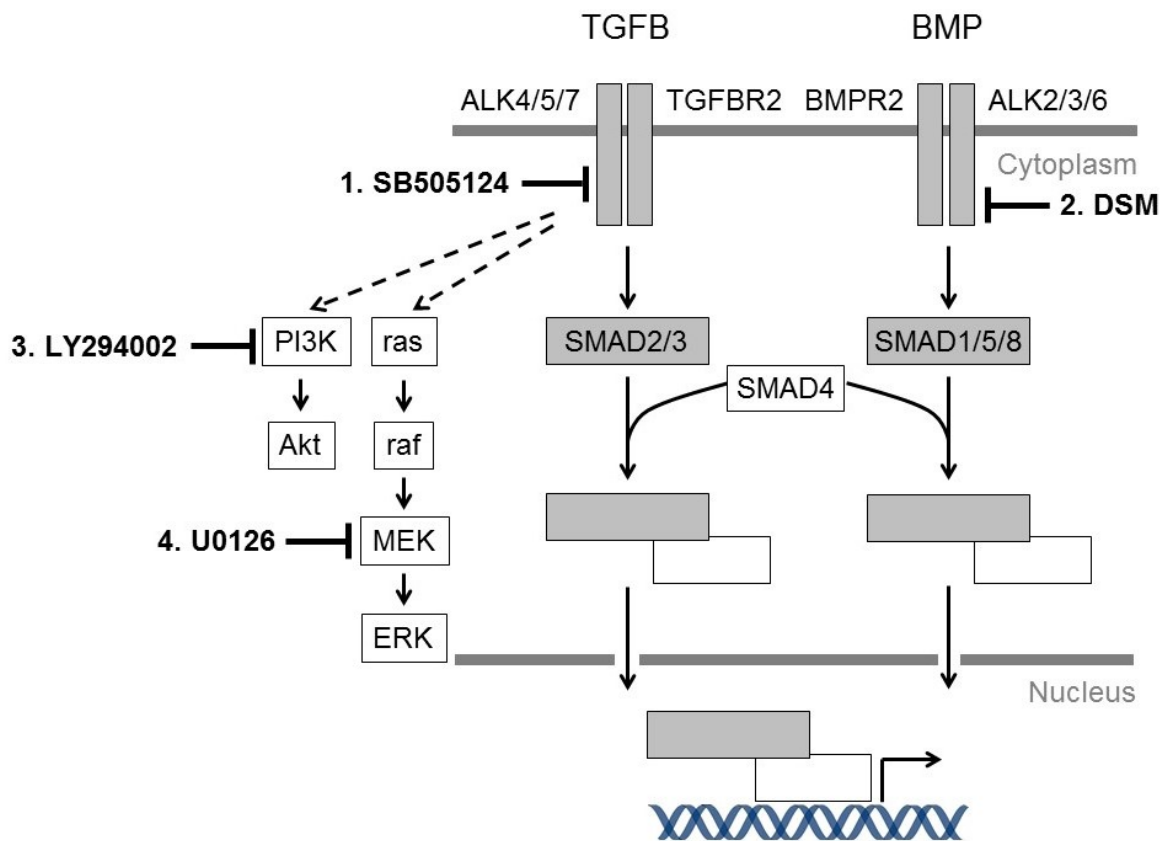


Figure 7.2. Small molecule kinase inhibitors – mechanism of action. 1. SB505124 is a selective inhibitor of the TGFB type I receptors ALK4, ALK5 and ALK7 which act upstream to phosphorylate SMAD2/3. 2. DSM is an inhibitor of the BMP type I receptors ALK2, ALK3 and ALK6 which act upstream to phosphorylate SMAD1/5/8. 3. LY294002 is an inhibitor of PI3K 4. U0126 is a specific inhibitor of MEK1 and MEK2.

7.2.1.1 Inhibition of ALK4/5/7 signalling prevents TGFB2 induced preantral follicle growth

To establish whether TGFB2 induced follicle growth is dependent on the SMAD2/3 signalling pathway, isolated follicles were cultured with TGFB2 in the presence or absence of SB505124 (1 μ M). SB505124 is a competitive inhibitor of the TGFB type I receptors ALK4, ALK5 and ALK7, which act upstream to phosphorylate SMAD2/3 (**Figure 7.2**). Follicle area was quantified every 24 hours (**Figure 7.3 A**). As observed in the previous chapter TGFB2 promoted a rapid increase in follicle growth relative to vehicle after 24 and 48 hours ($p = 0.0003, 0.02$). SB505124 treatment completely inhibited the growth promoting effects of TGFB2 at both 24 and 48 hours reducing follicle growth to vehicle control levels. In addition, SB505124 alone significantly reduced follicle growth relative to vehicle control at 48 hours ($p = 0.0001$). Despite the observed inhibition of follicle growth to levels below that of the vehicle control, no morphological evidence of atresia was noted in response to inhibitor treatment (**Figure 7.3 B**). Collectively, this suggests that activation of the SMAD2/3 pathway is necessary for TGFB2 induced follicle growth. Furthermore, signalling via the SMAD2/3 pathway may be essential for multilayered preantral follicle growth.

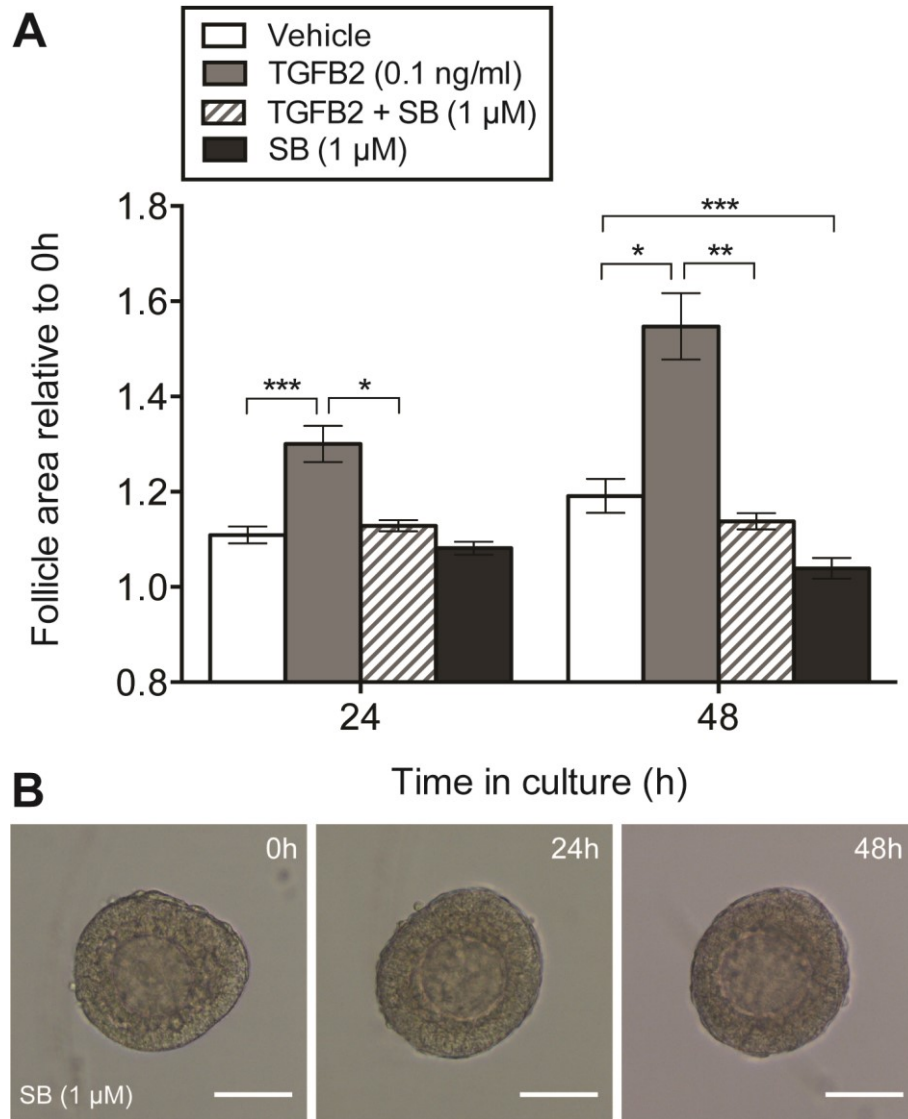


Figure 7.3. SB505124 inhibits TGFB2 induced preantral follicle growth. Follicles (2 - 3 layers of GCs (100 – 130 μm diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control (n = 34), TGFB2 (0.1 ng/ml) (n = 20), TGFB2 (0.1 ng/ml) + SB505124 (1 μM) (n = 24) or SB505124 (1 μM) alone (n = 36) for 48 hours. Follicle area was quantified every 24 hours and calculated relative to the area at time 0 (0h) (A). Data presented as mean ± SEM. Comparisons between treatment groups were performed within each time point by Kruskal-Wallis with Dunn's post hoc test where *p < 0.05 ** p < 0.01, *** p < 0.001. Representative images of SB505124 treated follicle at 0h, 24 and 48 h (B). Scale bar, 60 μm.

7.2.1.2 ALK2/3/6 signalling does not regulate TGFB2 induced preantral follicle growth

Unopposed SMAD2/3 signalling is speculated to underlie the increased rate of GC proliferation and tumour development observed in mice with targeted deletions of the BMP receptors ALK3 and ALK6, and downstream signalling intermediates SMAD1 and SMAD5 (Edson et al., 2010; Pangas et al., 2008). The inhibitory influence of SMAD1/5/8 signalling on the SMAD2/3 cascade may therefore be necessary for normal regulation of GC proliferation and follicle growth. To establish whether the SMAD1/5/8 pathway regulates TGFB2 induced follicle growth, follicles were cultured with TGFB2 ligand in the presence or absence of DSM (1 μ M). DSM is an inhibitor of the BMP type I receptors ALK2, ALK3 and ALK6, which act upstream to phosphorylate SMAD1/5/8 (**Figure 7.2**). TGFB2 promoted a rapid increase in follicle growth relative to vehicle after 24 and 48 hours ($p = 0.0001, 0.0001$) (**Figure 7.4 A**). DSM treatment did not influence the growth promoting effects of TGFB2 at either time point. Finally, no morphological evidence of atresia was noted in response to inhibitor treatment (**Figure 7.4 B**). This suggests that SMAD1/5/8 signalling does not regulate TGFB2 induced follicle growth.

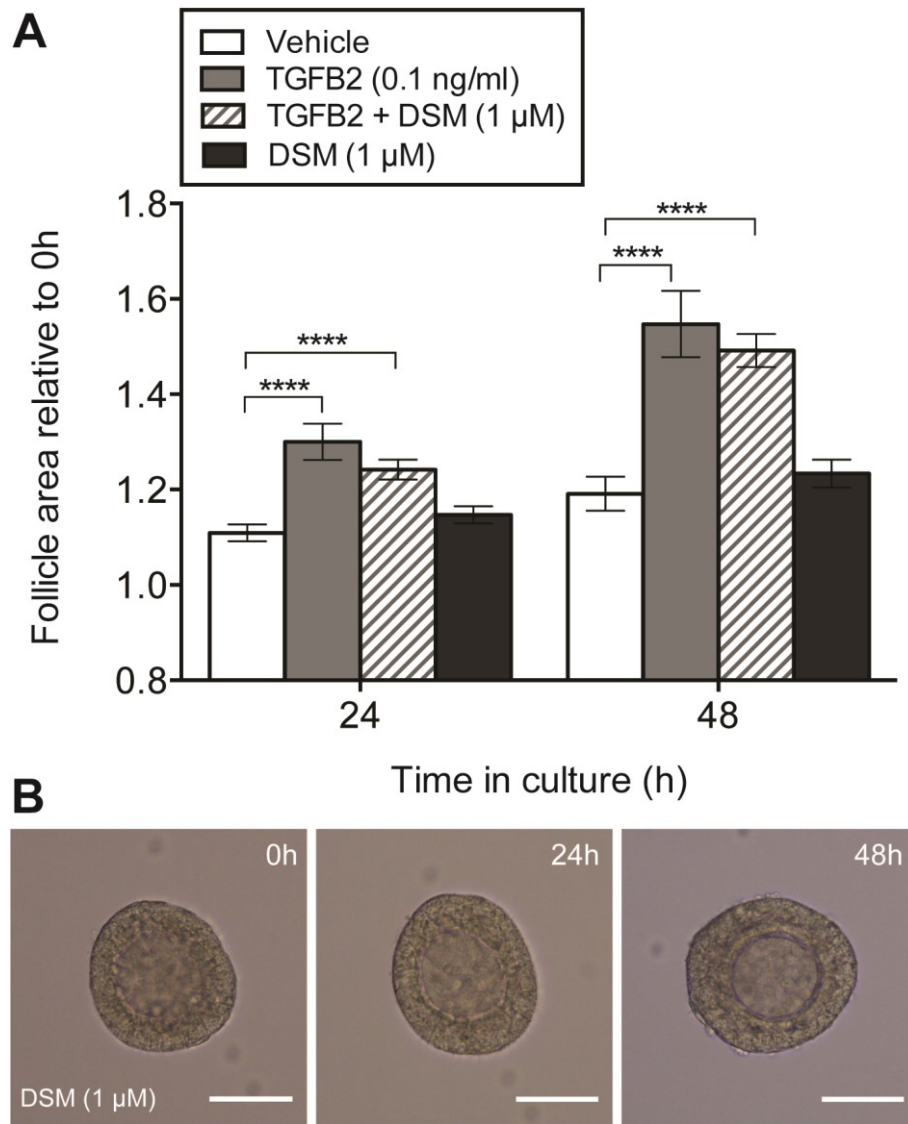


Figure 7.4. DSM has no effect on TGFB2 induced preantral follicle growth. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control (n = 34), TGFB2 (0.1 ng/ml) (n = 20), TGFB2 (0.1 ng/ml) + DSM (1 μ M) (n = 31) or DSM (1 μ M) alone (n = 32) for 48 hours. Follicle area was quantified every 24 hours and calculated relative to the area at time 0 (0h) (A). Data presented as mean $\pm$ SEM. Comparisons between treatment groups were performed within each time point by one-way ANOVA with Sidak's post hoc test where **** $p < 0.0001$. In the case of not normally distributed data comparisons were confirmed by Kruskal-Wallis with Dunn's post hoc test. Representative images of DSM treated follicle at 0h, 24 and 48 h (B). Scale bar, 60 μ m.

7.2.1.3 Inhibition of MAPK/ERK signalling prevents TGFB2 induced preantral follicle growth

TGFB stimulated activation of the MAPK/ERK pathway is a well-documented mechanism demonstrated in a multitude of cell types (Zhang, 2009). Furthermore, ERK MAP kinases can modulate SMAD activity via pathway cross talk, regulating nuclear translocation and transcriptional output (Kretzschmar, Doody, et al., 1997). To investigate whether the MAPK/ERK signalling pathway contributes to the mitogenic response of GCs to TGFB2, follicles were cultured with TGFB2 in the presence or absence of U0126 (10 μ M), a MAPK/ERK pathway inhibitor. U0126 is a selective inhibitor of both MEK1 and MEK2, which act upstream to phosphorylate MAPK (**Figure 7.2**). TGFB2 promoted a rapid increase in follicle growth relative to vehicle after 24 and 48 hours ($p = 0.0001$, 0.0001) (**Figure 7.5 A**). U0126 treatment partially inhibited TGFB2 induced follicle growth at 24 hours, whereby follicle growth was reduced in comparison to TGFB2 treated follicles ($p = 0.0009$) however was still greater than that of the vehicle control follicles ($p = 0.002$). By 48 hours U0126 reduced the growth of TGFB2 treated follicles to vehicle control levels. In addition, U0126 alone significantly reduced follicle growth relative to vehicle control at 48 hours ($p = 0.04$). Despite the observed inhibition of follicle growth to levels below that of the vehicle control, no morphological evidence of atresia was noted in response to inhibitor treatment (**Figure 7.5 B**). Collectively, this suggests that activation of the MAPK/ERK pathway is necessary for TGFB2 induced follicle growth. Furthermore, the MAPK/ERK signalling pathway appears to be essential for multilayered preantral follicle growth.

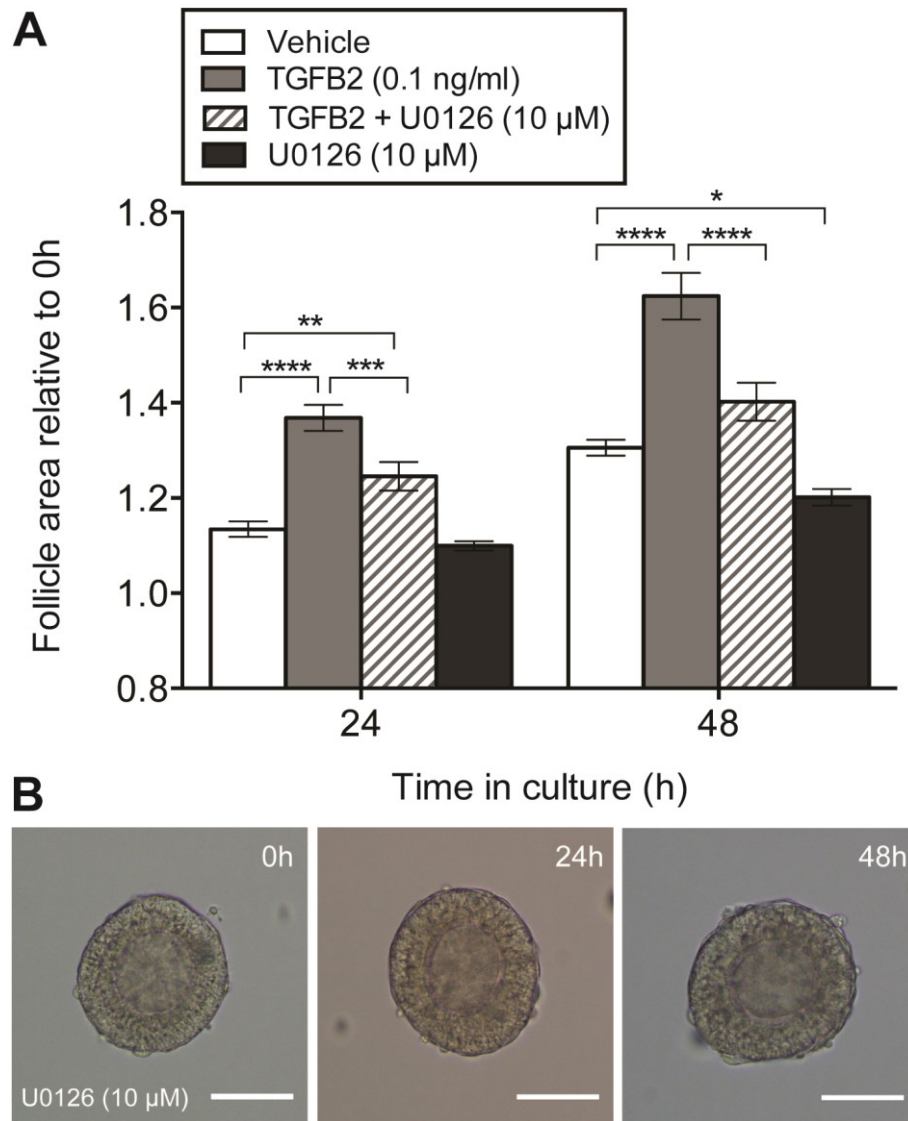


Figure 7.5. U0126 inhibits TGFB2 induced preantral follicle growth. Follicles (2 - 3 layers of GCs (100 – 130 μm diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control (n = 33), TGFB2 (0.1 ng/ml) (n = 25), TGFB2 (0.1 ng/ml) + U0126 (10 μM) (n = 18) or U0126 (10 μM) alone (n = 29) for 48 hours. Follicle area was quantified every 24 hours and calculated relative to the area at time 0 (0h) (A). Data presented as mean ± SEM. Comparisons between treatment groups were performed within each time point by one-way ANOVA with Sidak's post hoc test where **** p < 0.0001. In the case of not normally distributed data comparisons were confirmed by Kruskal-Wallis with Dunn's post hoc test. Representative images of U0126 treated follicle at 0h, 24 and 48 h (B). Scale bar, 60 μm.

7.2.1.4 Inhibition of PI3K/Akt signalling prevents preantral follicle growth

TGFB stimulated activation of the PI3K/Akt pathway has been documented in an array of cell types (Reviewed in Zhang et al, 2009). To investigate whether the PI3K/Akt signalling pathway plays a role in TGFB2 induced preantral follicle growth, follicles were cultured with TGFB2 in the presence or absence of LY294002, a PI3K/Akt pathway inhibitor. LY294002 is an inhibitor of PI3K which acts upstream to phosphorylate Akt (**Figure 7.2**). TGFB2 promoted a rapid increase in follicle growth relative to vehicle after 24 and 48 hours ($p = 0.0001$, 0.0004) (**Figure 7.6 A**). LY294002 treatment completely inhibited the growth promoting effects of TGFB2 at both 24 and 48 hours to levels below that of the vehicle control ($p = 0.0001$, 0.0001). In addition, LY294002 alone significantly reduced follicle growth relative to vehicle control at both 24 and 48 hours ($p = 0.0001$, 0.0001). Despite the observed inhibition of follicle growth to levels below that of the vehicle control, no morphological evidence of atresia was noted in response to inhibitor treatment (**Figure 7.6 B**). Collectively, this demonstrates that the PI3K/Akt pathway is essential for multilayered preantral follicle growth. Given the complete inhibition of follicle growth in response to LY294002 it is unclear whether activation of the PI3K/Akt pathway is necessary for TGFB2 induced follicle growth.

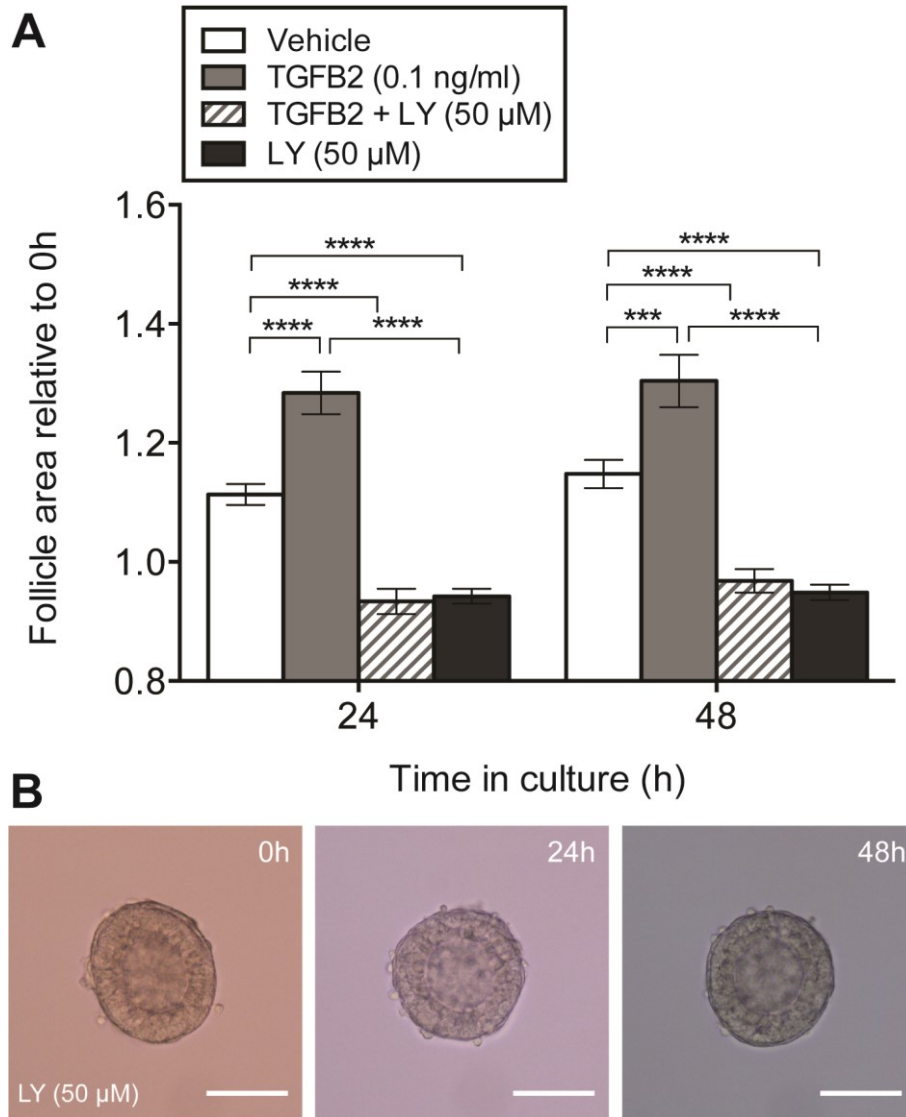


Figure 7.6. LY294002 inhibits preantral follicle growth. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control (n = 14), TGFB2 (0.1 ng/ml) (n = 10), TGFB2 (10 ng/ml) + LY294002 (50 μ M) (n = 14) or LY294002 (50 μ M) alone (n = 15) for 48 hours. Follicle area was quantified every 24 hours and calculated relative to the area at time 0 (0h) (A). Data presented as mean $\pm$ SEM. Comparisons between treatment groups were performed within each time point by one-way ANOVA with Sidak's post hoc test where ***p < 0.001, **** p < 0.0001. Representative images of LY294002 treated follicle at 0h, 24 and 48 h (B). Scale bar, 60 μ m.

7.2.2 Investigating the molecular mechanism underlying TGFB2 induced preantral follicle growth

The current results suggest that the canonical SMAD2/3 and non-SMAD MAPK/ERK signalling cascades are fundamental to TGFB2 induced preantral follicle growth. Given this it was hypothesised that non-classical signal transduction may be facilitating and/or modulating the TGFB2 response in the multilayered preantral follicle. In order to investigate whether TGFB2 was inducing direct activation of both SMAD and non-SMAD pathways phosphorylation studies were undertaken using the isolated preantral follicle model to examine whether TGFB2 treatment led to phosphorylation of SMAD3 and ERK1/2.

7.2.2.1 TGFB2 does not induce phosphorylation events at 8 hours

In order to investigate which pathways are directly activated in response to TGFB2 treatment, a preliminary study was undertaken whereby isolated follicles were cultured with vehicle or TGFB2 in the presence or absence of SB505124 (1 μ M) for 8 hours and western immunoblotting was performed to examine the phosphorylation levels of SMAD3 and ERK1/2. Following 8 hour treatment with TGFB2, the phosphorylation levels of SMAD3 did not differ from vehicle control (**Figure 7.7 A, B**). Similarly, levels of phospho-ERK1/2 were unchanged at 8 hours (**Figure 7.7 A, C**). Correspondingly, SB505124 did not influence levels of phosphorylation of any pathway in response to TGFB2.

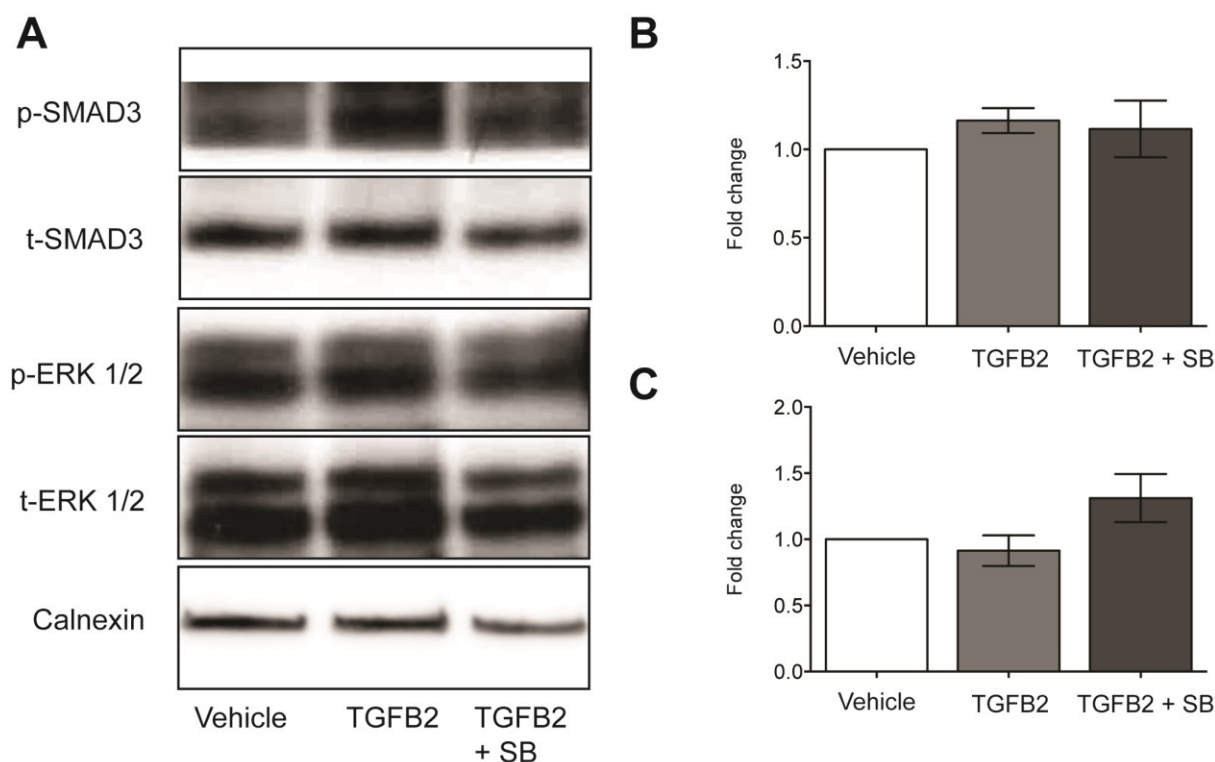


Figure 7.7. TGFB2 treatment does not promote SMAD3 or ERK1/2 phosphorylation in cultured preantral follicles. Follicles (2 - 3 layers of GCs (100 – 130 μ m diameter)) were mechanically isolated from 16 dpp mouse ovaries and cultured individually with vehicle control, TGFB2 (0.1 ng/ml) or TGFB2 (0.1 ng/ml) + SB505124 (1 μ M) for 8 hours. Phosphorylation of SMAD3 and ERK1/2 was determined by Western immunoblotting. Calnexin was used as a loading control. Representative blots are shown (A). Densitometric analysis of phospho (p)-proteins was normalised to the total (t) protein/calnexin ratio and fold change calculated relative to vehicle control; p-SMAD3 (B) and p-ERK1/2 (C). Data presented as mean $\pm$ SEM (Data represents 3 experimental replicates, ~ 30 follicles from three pooled 16 dpp ovaries for each treatment in each experimental repeat; 9 ovaries. Comparisons between treatment groups were performed by one-way ANOVA with Tukey's post hoc test.

7.3 Discussion

The current study reveals that TGFB2 induced preantral follicle growth is ablated by pharmacological inhibition of both the type I TGFB receptors ALK4/5/7 and the MAPK/ERK signalling cascade suggesting that both SMAD and non-SMAD signalling is required for the action of TGFB2 in the preantral follicle.

TGFB2 induced preantral follicle growth was ablated by inhibition of the type I ALK4/5/7 receptors. Based on the well-defined serine/threonine activity of the type I receptor it is likely that ablation of TGFB2 action stems from inhibition of the canonical SMAD2/3 pathway. However, given the pleiotropic nature of the TGFB receptors the relative contribution of the SMAD2/3 and non-SMAD cascades cannot be definitively proven without direct evidence of stimulation. It was further observed that SB505124 treatment alone resulted in the reduction of baseline follicle growth, indicative of a fundamental role for ALK4/5/7 signalling in multilayered preantral follicle growth. The SMAD2/3 signalling cascade is promiscuous in nature, impinged on by numerous ligands of the TGFB superfamily. The observed follicle phenotype associated with inhibition of ALK4/5/7 signalling is therefore a converged manifestation of the loss of numerous ligands that signal through SMAD2/3. In this regard it is likely that the reduction in follicle growth is due to the concurrent inhibition of oocyte specific GDF9, a mitogenic factor critical for normal GC proliferation beyond the primary one-layer follicle stage (Dong et al., 1996).

As described in Chapter 4, inhibition of ALK4/5/7 signalling in the neonatal ovary led to accelerated activation of the primordial pool. In a further observation however it was noted that ALK4/5/7 signal ablation resulted in the absence of multilayered preantral follicles (**Figure 4.4**) an observation which complements the current data. Collectively the results suggest that ALK4/5/7 signalling is required for maintenance of the primordial follicle pool, however in a paradoxical scenario the active cascade is also required for growing follicles to undergo normal GC proliferation and multilayering. This in itself is an interesting paradigm which will be considered further in the final discussion.

Unopposed SMAD2/3 signalling is proposed to underlie the increased rate of GC proliferation and tumour development observed in mice with targeted deletions of the BMP receptors ALK3 and ALK6, and downstream signalling intermediates SMAD1 and SMAD5 (Edson et al., 2010; Pangas et al., 2008). The inhibitory influence of SMAD1/5/8 signalling on the SMAD2/3 cascade may therefore be necessary for normal regulation of GC proliferation and follicle growth. In this regard it would be expected that inhibition of SMAD1/5/8 would lead to an increase in follicle growth in response to TGFB2. Contrary to this however inhibition of the SMAD1/5/8 cascade in the current study did not influence the proliferative response to TGFB2 suggesting SMAD pathway cross talk does not regulate preantral follicle growth in this context. Direct counteraction of TGFB activity via the BMP-

SMAD1/5/8 pathway is reported to occur via opposing regulation of a common gene promotor (Zeisberg et al., 2003). Indeed, the two pathways have been shown to coregulate approximately 25 genes in an epithelial cell line (Kowanetz et al., 2004). In this context it may be that the negligible effect of SMAD1/5/8 inhibition on TGFB2 activity is due to an absence of common gene promoters in the GC of the early preantral follicle.

The cellular response to TGFB can be regulated by an array of non-SMAD pathways (Derynck & Zhang, 2003). This regulation can occur in response to direct activation of non-SMAD proteins by the TGFB receptor complex initiating a parallel signalling cascade or be elicited by cross talk with parallel pathways. Pharmacological inhibition of the MAPK/ERK pathway in the current study ablated TGFB2 induced preantral follicle growth. TGFB stimulated activation of the MAPK/ERK pathway is a well-documented mechanism demonstrated in a plethora of cell types (Reviewed in Zhang, 2009). The TGFB receptors have both serine/threonine kinase activity and weaker tyrosine/kinase activity (Heldin & Moustakas, 2016; Lawler et al., 1997). TGFB induced phosphorylation of tyrosine residues on both the TGFB type I and type II receptor enables recruitment of signal transduction adaptor protein complex SchA/Grb2/Sos (Lee et al., 2007). Subsequent Ras activation leads to sequential activation of Raf, MEK and ERK which culminates in a variety of cellular events including proliferation and apoptosis via ERK1/2 phosphorylation of an array of transcription factors (Qi & Elion, 2005). In addition to facilitating SMAD-independent signalling, non-SMAD signalling proteins can modulate SMAD activity via pathway cross talk. ERK MAP kinases phosphorylate serine residues in the linker domain of receptor-activated SMADs, regulating nuclear translocation and transcriptional output (Kretzschmar, Doody, et al., 1997). In this manner, Sasseville et al. (2010) demonstrated that maintained phosphorylation of the SMAD3 linker domain by ERK was necessary for GDF9 induction of GC proliferation. In the same study it was also reported that the mitogenic role of TGFB1 required cooperative ERK1/2 and SMAD2/3 signalling. Based on this it could be postulated that the proliferative response of GCs to TGFB2 in the current study requires simultaneous stimulation of the ERK1/2 pathway. A further level of pathway modulation comprises interaction of downstream MAPK/ERK components with nuclear SMADs to regulate gene expression. In this manner MAPK/ERK substrate AP-1 functions in conjunction with SMADs to regulate gene expression mediating EMT (Davies et al., 2005). Furthermore, MAPK/ERK signalling has been reported to assist the SMAD nuclear complex in inducing

Snail expression, a key regulator of EMT (Horiguchi et al., 2009). Both studies complement the TGFB2 induced upregulation of EMT regulating genes observed in Chapter 6, perhaps further supporting a role for MAPK/ERK pathway regulation of TGFB2 action in the preantral follicle. In an alternative signalling paradigm, the requisite for MAPK/ERK signalling in TGFB2 induced follicle growth may be the consequence of an indirect action of TGFB2. In this context, Kay et al. (1998) report that autocrine production of FGF is necessary for TGFB stimulated fibroblast proliferation. Furthermore, in a more recent study TGFB induced TGF- α expression led to stimulated endothelial cell angiogenesis via EGF receptor (EGFR)-MAPK/ERK signal transduction (Vinals & Pouyssegur, 2001). The current data therefore demonstrate MAPK/ERK signalling is a requisite for TGFB2 induced preantral follicle growth. The precise mechanism with which this pathway contributes to the mitogenic response of GCs to TGFB2 however remains to be determined.

The current results demonstrate that the PI3K/Akt pathway is crucial for normal follicle development. Indeed, baseline follicle growth was completely ablated by the PI3K inhibitor LY294002 at the dose used. In this regard the pathway cannot be directly implicated in TGFB2 induced follicle growth. Numerous growth factors that are speculated to play a role in early preantral follicle development, including KL, platelet-derived growth factor (PDGF) and keratinocyte growth factor (KGF), converge on the PI3K/Akt cascade (Adhikari & Liu, 2009; Kezele et al., 2005; Nilsson et al., 2006; Yoshida et al., 1997). The observed follicle morphology is therefore a manifestation of the loss of numerous ligands that signal via the PI3K/Akt pathway. The results do not however exclude the possibility that the PI3K/Akt cascade contributes to the mitogenic effects of TGFB2. Given the complete inhibition of baseline follicle growth in response to LY294002 in the current study, a lower dose should be used in future studies to elucidate whether the PI3K/Akt signalling pathway plays a role in TGFB2 induced preantral follicle growth.

In an attempt to reconcile the diverse cellular mechanism underlying the mitogenic role of TGFB2 in the preantral follicle, phosphorylation studies were undertaken. A preliminary study was performed to initially investigate whether TGFB2 was inducing direct activation of SMAD and non-SMAD pathways in the multilayered preantral follicle. Following 8 hour treatment with TGFB2, the phosphorylation levels of SMAD3 and ERK1/2 were unchanged. Correspondingly, SB505124 did not influence levels of phosphorylation of any pathway in response to TGFB2. The results however do not exclude the role of SMAD and non-SMAD

cascades in TGFB2 induced follicle growth. Indeed, based on the functional studies in the isolated follicles it is highly unlikely that none of these pathways are activated by TGFB2. It is most probable that the time point selected missed key phosphorylation events. The array of possible mechanisms underlying the TGFB2 elicited effect in the follicle gives rise to a multitude of potential phosphorylation kinetics. The TGFB-SMAD signalling pathway is characteristically laboured following ligand stimulation when compared to cascades such as the MAPK/ERK pathway. Indeed, Inman et al. (2002) describe peak levels of pERK1/2 at 5 minutes compared to maximal levels of pSMAD at 45 minutes in response to TGFB stimulation. In contrast a delayed response of ERK phosphorylation has also been demonstrated, with peak levels reported hours after ligand stimulation suggestive of an indirect response requiring protein translation (Vinals & Pouyssegur, 2001). In retrospect, given the diverse manner in which non-SMAD MAPK/ERK signalling could be regulating the cellular response to TGFB future work should encompass multiple time points to decipher whether a direct or indirect mechanism is being employed.

In conclusion, in vitro studies suggest a role for both canonical-SMAD2/3 and non-SMAD signalling cascades in TGFB2 induced preantral follicle growth. The exact mechanism of signal transduction remains to be determined. Whether this is due to direct signal transduction via non-SMAD pathways or modulation of the canonical-SMAD2/3 pathway, it is likely that this integrated signalling network allows contextual interpretation of the TGFB2 signal in the preantral follicle. This study, for the first time, sheds some light on the complex regulatory networks underlying the differential effects of TGFB in the ovary.

Chapter 8 Final Discussion

Investigations into the spatiotemporal expression pattern of SMAD2/3 in the postnatal ovary reveal a stage specific expression in the GCs of the preantral follicle (Fenwick et al., 2013; J. Xu et al., 2002). Furthermore targeted disruption of SMAD2/3 results in perturbation of follicle development and subsequent ovarian function (Li et al., 2008). Despite this the functional role of the SMAD2/3 cascade in early preantral follicle development remains unexamined. The basis of this thesis was to examine the role of TGF β -SMAD2/3 signalling in primordial follicle activation and subsequent early preantral follicle development.

8.1 A dual role for ALK4/5/7 signalling in early follicle development

In accordance with observations by Fenwick et al. (2013), a clear stage-specific expression of SMAD2/3 was demonstrated in the juvenile ovary. Indeed, abundant SMAD2/3 expression in the GCs of single-layered (primordial, transitional and primary) and small multilayered follicles (1-3 layers of GCs) with considerably less prevalent staining observed in large multilayered follicles supports a role in both maintenance of the primordial follicle pool and early preantral follicle development. To attenuate activation of the SMAD2/3 cascade, pharmacological inhibition of the type I ALK4/5/7 receptors using the small molecule inhibitor SB505124 was undertaken to ablate the action of all ligands that converge preferentially to SMAD2/3. In concordance with the stage specific expression of SMAD2/3, it was demonstrated that pharmacological attenuation of the type I receptors ALK4/5/7 in a neonatal ovary culture led to perturbation of early follicle development. Inhibition of ALK4/5/7 signalling led to accelerated activation of the primordial follicle pool and, in a paradoxical manner, resulted in failure of growing follicles to undergo normal GC proliferation and multilayering. This was an observation further corroborated in Chapter 6, whereby inhibition of ALK4/5/7 signalling in an isolated preantral follicle culture led to the inhibition of multilayered preantral follicle growth. Collectively the results suggest that ALK4/5/7 signalling is required for maintenance of the primordial follicle pool and in a paradoxical scenario the active cascade is also required for growing follicles to undergo normal GC proliferation and multilayering. When considered in concert with a study by Wang et al. (2014), who report accelerated activation of the primordial pool in response to treatment with SD208, a specific inhibitor of the ALK5 receptor, it appears that the common

target ALK5 is an essential step of the signalling cascade that regulates primordial follicle activation. Whether ALK5 inhibition is also the crucial step underlying the inhibition of subsequent preantral follicle development requires further study. When considering the context dependent action of ALK4/5/7 signalling it is imperative to acknowledge the pleiotropic nature of the TGFB receptors. Further to the canonical SMAD signalling cascade the TGFB receptors can directly interact with an array of non-SMAD signalling pathways of which the MAPK cascades, primarily composed of the ERK, p38 MAPK and the c-Jun N-terminal kinases (JNK), represent a major TGFB signalling pathway independent of SMAD activation (Hartsough & Mulder, 1995; Hocevar et al., 1999; Sano et al., 1999). Indeed DaCosta Byfield et al. (2004) directly demonstrate that 1 μ M SB505124 potently inhibits TGFB-induced activation of ERK1/2, JNK and p38 in addition to SMAD2. Whether the dual role of the ALK4/5/7 signalling cascade is reflective of non-SMAD signalling pathways therefore requires further investigation, in particular work should focus on direct demonstration of the specific downstream pathway being stimulated. Finally given the promiscuous properties of the TGFB type I and type II receptors it would be informative to examine inhibition of the SMAD2/3 cascade below the receptor level. The use of SMAD3 inhibitor, SIS3, which specifically targets SMAD3 phosphorylation, could be used to confirm results in both the neonatal ovary culture and the isolated preantral follicle model (Jinnin et al., 2006). The clear expression of SMAD2/3 in the juvenile ovary in addition to the observed perturbation of follicle development in response to ALK4/5/7 signalling inhibition is therefore suggestive of a role for the SMAD2/3 pathway in early follicle development. In this regard a screen of upstream SMAD2/3 signalling components in the preantral follicle was performed to determine which ligand would most likely contribute to the regulation of early follicle development. Of the ligand transcripts examined, *Tgfb2* was the most abundantly expressed, in concert with *Alk5*, the TGFB type I receptor. Subsequent investigations therefore concerned the examination of the role of the TGFB2 ligand in early preantral follicle development.

8.2 A role for TGFB2 in primordial follicle activation

Given the accelerated activation of the primordial follicle pool in response to attenuation of ALK4/5/7 signalling, it was postulated that the action of a factor through the SMAD2/3 pathway maintains the primordial follicle in an inactive state. A study was then undertaken to

investigate whether a concomitant inhibitory action of TGFB2 was observed in primordial follicle development. Indeed, in a manner consistent with the inhibitory action of the ALK4/5/7 cascade, and in a completely novel finding, TGFB2 was demonstrated to inhibit activation of the primordial follicle in a neonatal ovary culture. Given the simultaneous expression of SMAD2/3 and TGFB2 in the flattened pre-GC of the primordial follicle and the common maintenance role in primordial follicle activation, it is plausible that TGFB2 acts in an autocrine manner to maintain the flattened pre-GC in an inactive state. This is a postulation further reinforced by the inherent antimitogenic role of TGFB, inducing cell cycle arrest in the late G1 phase via upregulation of cyclin-dependent kinases inhibitors, a mechanism facilitated by SMAD regulated transcription (Derynck et al., 2001; Pardali et al., 2000). However direct confirmation of GC growth inhibition was not revealed in the current study. The onset of mammalian development is a protracted process whereby primordial follicles leave the resting pool and initiate a phase of slow growth in which the granulosa cells enter the cell cycle but proliferation is exceedingly slow (Hirshfield, 1991). This is reflected by the very low proportion (<10%) of GCs that are positively labelled for proliferation marker Ki67 in primordial and early growing transitional follicles (Da Silva-Buttkus et al., 2008). In retrospect the low level of proliferative activity in the early preantral follicle warrants the use of a proliferation marker with a longer half-life which, given the longer persistence of the label, would allow for more accurate detection of GCs that are in the process of, or have recently, undergone cell division. In this regard, PCNA with a biological half-life of 20 hours, would likely prove a more suitable marker than Ki67 as employed in the current study, which has an estimated half-life of one hour (Bravo & Macdonald-Bravo, 1987; Scholzen & Gerdes, 2000). Whether an antimitogenic action of TGFB2 underlies the observed maintenance of the follicle pool therefore remains to be determined. The localisation of the ALK5 receptor to the oocyte membrane was an intriguing finding, and led to ambiguity regarding the manner of signal transduction underlying the inhibitory effect of TGFB2. Indeed, localisation on the oocyte membrane is suggestive of an alternative signalling paradigm whereby TGFB is signalling solely via the oocyte in a SMAD independent mechanism. In this context Wang et al. (2014) report inhibition of primordial follicle activation by TGFB1 in a neonatal ovary culture through tuberous sclerosis complex (TSC)/mammalian target of rapamycin complex 1 (mTORC1) signalling in oocytes. Whether this occurred in concurrence with SMAD signalling however was not clarified. Certainly, TGFB stimulated activation of the PI3K/Akt pathway has been documented in an array of

cell types (Reviewed in Zhang, 2009). Furthermore, the PI3K/PTEN and TSC/mTORC1 signalling cascades are fundamental to maintenance of the primordial follicle pool (Adhikari et al., 2009; Adhikari et al., 2010; Reddy et al., 2008). In a further scenario it could be that ALK5 is not the sole physiological type I receptor for TGFB2 in the preantral follicle. In this manner ligand promiscuity has been demonstrated in the endothelial cell whereby Goumans et al. (2002) report TGFB activation of two distinct type I receptor/SMAD signalling pathways; ALK5/SMAD2/3 and ALK1/SMAD1/5. However, this is a cell specific mechanism unlikely to be implicated in the current context given the absence of SMAD1/5/8 and ALK1 in the primordial follicle (Fenwick et al., 2013; Pangas et al., 2008). Nevertheless, it could be postulated that TGFB2 signals via ALK4 or ALK7 to activate the SMAD2/3 pathway. Whether the ALK5 receptor is truly absent in the GCs of the preantral follicle however remains to be clarified. The unexpected expression pattern observed in the current study is corroborated by previous immunolocalisation studies demonstrating considerable variation in the spatiotemporal expression pattern of ALK5 in the murine preantral follicle. Li et al. (2011) describe minimal if any expression in the GCs of preantral follicles, while Gueripel et al. (2004) and Juneja et al. (1996) both describe strong ALK5 immunostaining in oocytes from primordial and growing follicles and, to a lesser extent, in GCs at all stages of follicle development. A more recent examination of ALK5 localisation in the murine ovary describes expression in the oocytes of primordial and primary follicles in addition to the cuboidal cells of growing follicles (Wang et al., 2014). It is unclear why there is such disparity in the reported staining patterns of ALK5. Perhaps such considerations simply reflect a very low abundance of ALK5 in the GCs of the preantral follicle. Clearly the signal transduction pathway and indeed the underlying cellular mechanism employed by TGFB2 to inhibit primordial follicle activation are both aspects that require further study. In particular, whether TGFB2 and SMAD2/3 act in concert to regulate activation of the primordial pool needs to be elucidated. Regardless, the revelation of an inhibitory role for both ALK4/5/7 and TGFB2 in maintaining the primordial pool is indeed an exciting discovery and one which warrants further investigation. To summarise, the proposed mechanisms underlying TGFB2 inhibition of primordial follicle activation are illustrated in **Figure 8.1**.

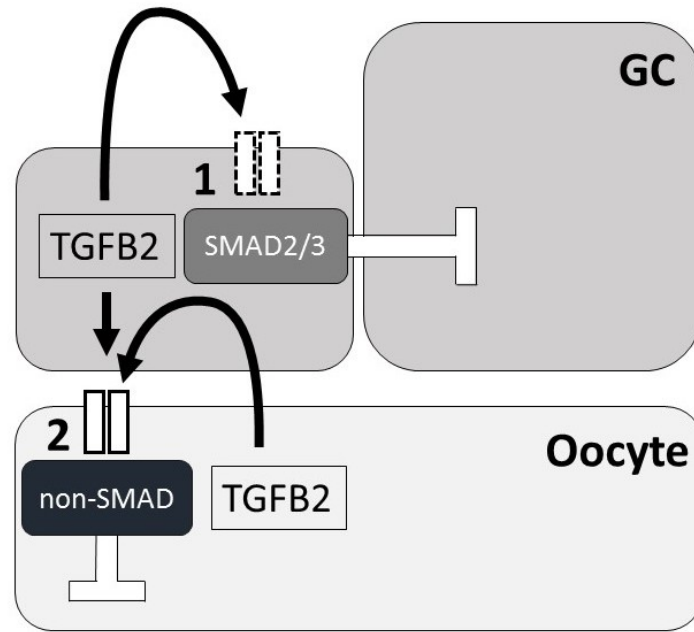


Figure 8.1. The proposed mechanisms underlying TGFB2 inhibition of primordial follicle activation. TGFB2 from the GC acts in an autocrine manner on the flattened GCs through a putative ALK5-SMAD2/3 mechanism to maintain cell cycle arrest (1). Alternatively, TGFB2 acts in a paracrine or autocrine manner on the oocyte through an ALK5-SMAD independent mechanism to inhibit oocyte growth (2). Dashed receptor represents putative type I ALK5 receptor on GC membrane.

8.3 A role for TGFB2 in preantral follicle growth

Given that disruption of multilayered preantral follicle growth was observed in response to attenuation of ALK4/5/7 signalling, it was proposed that activation of the SMAD2/3 cascade is necessary for normal preantral follicle development. Based on the abundant expression of TGFB2 in the oocyte of the preantral follicle, it was further postulated that the action of TGFB2 through the SMAD2/3 pathway may be important for preantral follicle growth. Indeed, TGFB2 was demonstrated to potently stimulate preantral follicle growth in an in vitro preantral follicle culture. Given the localisation of SMAD2/3 in the GCs and TGFB2 in the oocyte of the multilayered preantral follicle it is plausible that TGFB2 acts in a paracrine manner to stimulate follicle growth. Oocyte-paracrine communication is a long recognised signalling paradigm, a process fundamental to ovarian follicle development, disruption of which has profound effects on fertility (Eppig, 2001). Once more however, the absence of ALK5 receptor on the GC membrane leads to ambiguity regarding the manner of signal

transduction underlying the mitogenic action of TGFB2. It is therefore possible that a non-SMAD signalling paradigm encompassing the autocrine action of TGFB2 on the oocyte is the mechanism underlying the regulation of preantral follicle growth. Nevertheless, a speculative role for ALK5-SMAD2/3 facilitated signal transduction in the GC can be drawn from a study by Gilchrist et al. (2006), who report induction of a SMAD2/3 reporter in transfected GC in response to both TGFB1 and GDF9, both of which classically utilise the ALK5 receptor. TGFB2 induced follicle growth was accompanied by an increase in GC proliferation, an observation that was corroborated by increased transcript levels of cell cycle regulator *Cdnd2* and decreased levels of opposing cell-cycle inhibitor *Cdkn1b*. It is therefore plausible that TGFB2 plays an integral role in the extensive proliferation and multilayering of GCs in the preantral follicle by promoting GC progression through the G1 phase of the cell cycle via upregulation of CCND2 and downregulation of CDKN1B. In accordance with the morphological increase in follicle size, as induced by GC proliferation, an environment conducive for tissue remodelling was demonstrated whereby expression levels of protease enzyme *Mmp2* were increased while concurrent levels of MMP inhibitor *Timp1* remained unchanged. Indeed, the role of TGFB in stimulation of the MMP system is well documented and upregulation in response to TGFB1 has specifically been demonstrated in the GC (Zhu et al., 2014). Collectively the results suggest that TGFB2 plays an integral role in preantral follicle growth and development by promoting prolific GC proliferation while concurrently regulating the extensive ECM remodelling process that is fundamental to follicle development. An intriguing revelation was the upregulation of EMT regulator *Snai2* in response to TGFB2. In this context, it has been reported that activated GCs express morphological characteristics that are akin to mesenchymal rather than epithelial cells, suggesting that a partial EMT takes place as they activate and develop (Childs & McNeilly, 2012; Mora et al., 2012). The upregulation of an EMT transcriptional regulator in concurrence with TGFB2 induced follicle growth follicle development is therefore an interesting parallel. Whether TGFB2 is a regulator of the putative EMT events accompanying follicle development is an intriguing concept which warrants further study. Future work should explore the expression of further mesenchymal markers in response to TGFB2. To summarise, the proposed mechanisms underlying TGFB2 induced preantral follicle growth are illustrated in **Figure 8.2**.

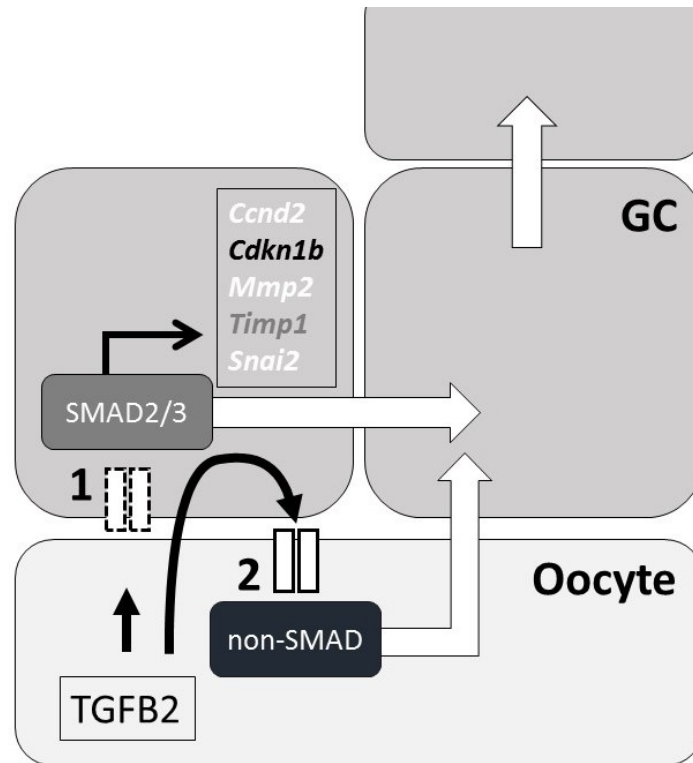


Figure 8.2. The proposed mechanisms underlying TGFB2 induced preantral follicle growth. TGFB2 from the oocyte acts in a paracrine manner on the GC through a putative ALK5-SMAD2/3 mechanism to induce follicle growth (1). TGFB2 acts in an autocrine manner on the oocyte in an ALK5-SMAD independent mechanism to induce follicle growth (2). Follicle growth is accompanied by altered gene expression. Dashed receptor represents putative type I ALK5 receptor on GC membrane. Upregulated transcripts white, downregulated black, unchanged grey.

8.4 The context dependent effect of TGFB2: a role for non-SMAD signalling

The data presented from in vitro studies in this thesis have demonstrated a context-dependent role for TGFB2 in preantral follicle development, whereby the functional role appears to be dictated by the developmental stage of the follicle. Given the well-documented ability of TGFB to utilise both SMAD and non-SMAD intracellular signalling pathways to regulate cellular function it was proposed that the observed context-dependent role of TGFB2 is a result of non-classical signal transduction within the preantral follicle. Indeed, in a completely novel finding, the mitogenic action of TGFB2 in the preantral follicle was ablated by pharmacological inhibition of both the type I TGFB receptors ALK4/5/7 and the MAPK/ERK signalling cascade. The influence of non-SMAD signalling is an intriguing revelation, however the exact manner in which the MAPK/ERK pathway is regulating the

mitogenic action of TGFB2 requires further investigation. One mechanism may be the direct facilitation of TGFB2 signalling via the MAPK/ERK pathway. Although the type I and type II TGFB receptors are well-defined serine/threonine kinases they possess weak tyrosine/kinase activity enabling TGFB induced stimulation of the MAPK/ERK pathway (Heldin & Moustakas, 2016; Lawler et al., 1997). Indeed, rapid activation of ERK in response to TGFB stimulation is a well-documented mechanism, demonstrated in a multitude of cell types (Reviewed in Zhang, 2009). Furthermore, inhibition of the type I ALK4/5/7 receptors additionally ablated the mitogenic action of TGFB2 in the preantral follicle. However, given the pleiotropic nature of the TGFB receptors the relative contribution of the SMAD2/3 and non-SMAD cascades cannot be definitively proven without direct evidence of stimulation. In addition to facilitating SMAD-independent signalling, non-SMAD signalling proteins can modulate SMAD activity via pathway cross talk. In this regard it is known that ERK MAP kinases have the capacity to phosphorylate serine residues in the linker domain of receptor-activated SMADs in response to growth factors (for example fibroblast growth factor (FGF) and epidermal growth factor (EGF)), regulating nuclear translocation and transcriptional output (Kretzschmar, Doody, et al., 1997). In this manner, Sasseville et al. (2010) demonstrate that maintained phosphorylation of the SMAD3 linker domain by EGFR-ERK activity is necessary for GDF9 induction of GC proliferation. Whether the balance of SMAD and non-SMAD pathways plays a key role in the coordinated cellular response to TGFB2 remains to be determined. Regardless it is tempting to speculate that the plethora of growth factors differentially expressed in the intrafollicular milieu during follicle development may provide an integrated signalling network that allows contextual interpretation of TGFB signals, and moreover enables the paradoxical action of the ALK4/5/7 cascade observed in the current study. Finally, in an alternative signalling paradigm the requisite for MAPK/ERK signalling may be the consequence of an indirect action of TGFB2. In this context, Kay et al. (1998) report that autocrine production of FGF is necessary for TGFB stimulated fibroblast proliferation. Furthermore, in a more recent study TGFB induced TGF- α expression led to stimulated endothelial cell angiogenesis via EGF receptor (EGFR)-MAPK/ERK signal transduction (Vinals & Pouyssegur, 2001). The concept of an indirect signalling paradigm in the current context is further reinforced by the documented role of growth factors such as FGF2 and TGF α (amongst others), in ovarian follicle development (Nilsson & Skinner, 2001). Given the diverse manner in which the MAPK/ERK cascade could be facilitating the cellular response to TGFB2 future work should define whether the

cascade is being directly or indirectly activated by TGFB2 or alternatively whether the MAPK/ERK cascade is modulating the SMAD2/3 pathway.

Given the extensive use of pharmacological inhibition in the current thesis it is important to recognise the limitations of small molecule inhibitors when considering the interpretation of results. Both the specificity and effective doses of such inhibitors must be evaluated with great caution due to proven cross-reactivity against structurally similar kinases. When examining the in vitro kinase action of small molecule inhibitors against a panel of protein kinases, Vogt et al. (2011) demonstrated 1 μ M Dorsomorphin to inhibit the activities of 29 out of 119 kinases more potently than ALK3 proving Dorsomorphin a poor candidate for the selective inhibition of BMP-activated type I receptor kinases ALK2/3/6. In contrast when the selectivity of SB505124 was assayed p38 MAPK was the single kinase inhibited more than 50% by 10 μ M SB505124 (DaCosta Byfield et al., 2004). Furthermore downstream responses to MAPK α were proven to be unaffected at the concentrations used in the current study (DaCosta Byfield et al., 2004).

Collectively, in vitro studies suggest a role for both canonical-SMAD2/3 and non-SMAD signalling cascades in TGFB2 induced preantral follicle growth. Whether this integrated signalling network allows contextual determination of the TGFB2 signal in the preantral follicle however remains to be determined.

8.5 Implications and future perspectives

A significant revelation in the current study was the identification of a novel inhibitory role for TGFB signalling in maintaining the primordial follicle pool. The rate at which the primordial follicle pool is depleted has a profound influence on fertility and defines the reproductive lifespan. POI, defined as the onset of menopause before the age of 40, affects approximately 1% of women (Coulam et al., 1986). The majority of cases of POI are idiopathic however the proposed pathogenesis is failure to establish a normal supply of primordial follicles during development or a consequence of accelerated depletion of the cohort during life (Goswami & Conway, 2005). The latter of which underscores the potential clinical importance of the novel data described here. In patients for whom POI can be predicted, such as those requiring radio- or chemotherapy, cryopreservation of a primordial follicle-rich section of ovarian cortex represents a potential strategy for overcoming infertility

(Lobo, 2005). By advancing our knowledge of the mechanisms regulating primordial follicle activation and subsequent early preantral follicle development, there is the potential to perform in vitro maturation of follicles preserved in this way; the application of which would bypass the need to reintroduce the cryopreserved tissue to the patient, which currently represents the greatest obstacle in this technique (Lobo, 2005).

A further result in the current study was the context dependent role for TGFB signalling in early preantral follicle development, a finding speculated to be regulated by the role of both classical SMAD and SMAD independent signalling. The context-dependent action of TGFB is a well-documented phenomenon; indeed the switch from the classic cystostatic, pro-apoptotic action of TGFB to a pro-oncogenic role, as observed by resistance to growth inhibition, induction of EMT and increased levels of MMPs, is considered the hallmark of cancer (Jakowlew, 2006). In this context it has been suggested that the pro-tumorigenic effects of TGFB involve a pathological switch from SMAD-mediated tumour suppression to non-canonical malignant pathways (Massague, 2012). Therefore, when paralleled to preantral follicle development it could be postulated that inhibitory maintenance of the primordial pool by TGFB2 is facilitated by canonical SMAD signalling whereas the subsequent mitogenic role in multilayered follicle development is a consequence of non-classical signal transduction. Differing cellular signalling machinery, the influence of non-canonical signalling pathways, or differentially expressed transcription factors within the different staged follicles may therefore be the switch which governs the context dependent interpretation of the TGFB2 signal. A further understanding of the differential action of TGFB in the preantral follicle would therefore have the potential to inform new therapeutic strategies regarding cancer therapy.

8.6 Conclusions

The work in this thesis has identified a dual role for ALK4/5/7 signalling in early follicle development. Furthermore, it has highlighted a novel and context dependent role for TGFB2 in both primordial follicle activation and subsequent preantral follicle development. Finally, it has demonstrated a role for non-SMAD MAPK/ERK signalling in TGFB2 induced preantral follicle growth, a finding speculated to underlie the context dependent action of TGFB2 in the preantral follicle. Collectively these novel data have provided a further understanding of the signalling events underlying the early stages of preantral follicle

development and an insight into context dependent action of TGFB signalling in the ovary. Further exploration of the signal transduction and underlying cellular mechanisms employed by TGFB2 to differentially regulate follicle development will have the potential to inform therapeutic interventions in both oncology and fertility preservation.

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