Investigating the regulatory role of Anti-Müllerian Hormone in the growing follicles of a Monovulatory Species

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

May 2016

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Declaration

I declare that the studies undertaken for this thesis were devised and conducted by me. Where other sources have been used, they are acknowledged by reference. No part of this work has been previously submitted or accepted for any other award.

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May 2016

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Abstract

Evidence from recent research suggests that folliculogenesis is regulated by stage specific locally produced growth factors and hormones including mediators of proliferation, differentiation and angiogenesis. AMH has been identified as one of these potentially important local factors and in polyovulatory species has been implicated in the regulation of the follicles growth, throughout its developmental stages. However in monovulatory species, despite its circulating levels being use as a marker of ovarian reserve and its suggested link to the aetiology of PCOS, little is known about its role and the means of its control in the growing follicle. Using the sheep model, the over-riding objective of this research project was the further elucidation of the roles of AMH during the antral stages of follicular development in monovulatory species. It was hoped the work would also provide valuable insights into the aetiology of PCOS. The study has focussed on four main research goals: characterisation of GC and TCs in terms of AMH and AMHR2 expression; confirmation of the utilisation of a SMAD signal transduction pathway in GC and TCs; an androgen involvement in AMH production; and the elucidation of two putative mechanisms of AMH regulation.

Both AMH and AMHR2 were found to be expressed in ovine GC and TCs, although levels of AMH mRNA in TCs were very low and may not be biologically relevant. The outcomes also confirmed that in both ovine GC and TCs, AMH binds to a receptor complex containing the type 2 AMH receptor and that this mediates transduction of an intra-cellular signal via the SMAD 1/5/8 pathway. This finding in TCs indicates that GCs derived AMH may act in a paracrine way on TCs, disputing the

long held belief of AMH only functioning in an autocrine manner.

The effect of androgens on AMH production in FSH-stimulated GCs was previously not clarified but in this present study a significant increase in steroidogenesis in the presence of a non-aromatisable androgen now suggests that androgens may have a direct role in the regulation of AMH production, and thus at least in part, be involved in the modulation of folliculogenesis.

The final aspect of this project was to study novel inter-ovarian interactions. This focussed on the hypothesis that VEGF, a potent ovarian angiogenic factor, and SOX8, a member of the SOX family of transcription factors, could be involved in AMH regulation in GCs. Results indicate that the two VEGF variants associated with growing follicles ($VEGF_{120}$ and $VEGF_{164}$) may have direct roles in AMH regulation. While on the other hand, SOX8 may have a role in regulating folliculogenesis and altering oestradiol production, however, it was not shown that this effect was mediated via AMH regulation.

In conclusion this present study, not only supports earlier work showing TCs responsiveness to AMH, but for the first time in a monovulatory species, provides compelling evidence that AMH signal transduction in TCs is via receptor complexes containing AMHR2. Collectively these studies indicate a probable regulatory loop between GC produced AMH and TC produced androgens. From these outcomes it seems likely that the AMH regulatory loop may involve fine-tuning by external stimuli. A further novel finding has been that the VEGF variants particularly associated with growing follicles, and the

transcription factor SOX8 appear to have some involvement in a complex system of AMH regulation. These findings should help in the design of future studies to elucidate how perturbations in this complex configuration may be involved in the aetiology of common forms of anovulatory infertility.

Acknowledgements

The past four years have been enjoyable but testing at times, and there are certain people who have helped me through the tough times and kept me going.

I would like to thank Dr Peter Marsters and Professor Bruce Campbell for giving me the opportunity to undertake this PhD, and for offering guidance and support throughout the entirety, while allowing me to find my own way along the path. I would also like to thank all the staff in the department especially the technical team for their endless help and support. A special thank you goes to Catherine Pincott-Allen and Rana Alhamdan for teaching and guiding me through most of the laboratory techniques. Without their help I would have felt lost.

I would also like to thank the Libyan Embassy and the Libyan government for financially sponsoring this research project and making it possible.

Last but not least, I would like to thank my family especially my wife for believing in me and being by my side through this life changing experience.

Abdullatif Elfituri

February 2016

Abbreviations

A4	Androstenedione
ALK	Activin Like Kinase
AMH	Anti Mullerian Hormone
AR	Androgen Receptor
bFGF	basic Fibroblast Growth Factor
BMP	Bone Morphogenic Protein
BRE	BMP Responsive Element
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMH1	Dorsomorphin Homologue 1
E2	Oestradiol
E4	Inhibin A
EGF	Epidermal Growth Factor
ELISA	Enzyme linked immunosorbent Assay
ERK	Extracellular Signal Regulated Kinases
FSH	Follicle Stimulating Hormone
GC	Granulosa Cell
GDF	Growth Differentiation Factor
GnRH	Gonadotrophin Releasing Hormone
HMG	High Mobility Group
ICC	Immunocytochemistry

IGF	Insulin-Like Growth Factor
IHC	Immunohistochemistry
IVF	*In Vitro* Fertilisation
KGF	Keratinocyte Growth Factor
LH	Luteinising Hormone
LIF	Leukaemia Inhibitory Factor
MAPK	Mitogen Activated Protein Kinase
OCP	Oral Contraceptive Pills
OHSS	Ovarian Hyper-Stimulation Syndrome
PCOS	Polycystic Ovary Syndrome
PCR	Polymerase Chain Reaction
PGC	Primordial Germ Cells
PIGF	Placental Growth Factor
SOX	SRY Related Box
SRE	Serum Response Element
SRY	Sex Determining Region Y
T4	Testosterone
TC	Theca Cell
TGF	Transforming Growth Factor
TMB	Tetramethylbenzidine
TRE	Testosterone Response Element
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor

Chapter 1 Literature Review

1.1 Introduction

Female fertility in mammals depends on the ability of the ovary to produce a Graafian follicle that can potentially release a mature competent and fertilisable, oocyte. This coordinated process of folliculogenesis is controlled by peripheral hormones and local factors which either facilitate or attenuate the development and selection of an ovulatory follicle (Webb & Campbell 2007).

It is estimated that 80-85% of women will become pregnant within the first year of unprotected intercourse, 90% will succeed in 18 months, and 95% will conceive within two years (Salhan 2011). Therefore, the NICE guidelines have defined infertility as the inability to achieve pregnancy after two years of regular unprotected sexual intercourse without contraception (NICE Clinical Guideline 2004). This health problem is rising throughout the world, not only in the developed countries where one out of six couples undergoes assisted reproductive techniques but also, according to the latest reports, in developing countries too (Skakkebæk et al. 2006). The cause of infertility could be due to either male or female factors. The female factors can contribute up to 40% of all infertility causes which may be due to uterine abnormalities, tubal defects, and of this large percentage, around 15-25% can be due to ovarian causes (Salhan 2011). Significantly, over 75% of anovulation cases involve a common metabolic dysfunction called polycystic ovary syndrome (PCOS).

PCOS is one of the most common endocrine defects which affects around 6.6-8% of women in their reproductive life (Azziz *et al.* 2004) and is considered the main cause of anovulatory infertility (Franks 1995). The cause of this complex syndrome remains largely unknown. Of the many local factors, which have been shown to be involved in the pathophysiology of PCOS (Giovanni Artini et al. 2007), anti mullerian hormone (AMH) may be of particular importance. It has been shown to be involved in normal follicular development (Durlinger *et al.* 2002a), and a key feature of PCOS is markedly elevated levels of this TGFβ/BMP family member (Fallat *et al.* 1997). However, as to date relatively little is known about its roles and mechanisms of action in the developing follicles of monovulatory species, it remains a promising candidate for further study. Recent reports suggest that AMH may be linked in some way to the disruption of folliculogenesis in PCOS (Pigny et al. 2003, Laven et al. 2004). This present study proposes to explore this possibility further and to elucidate the functionality of AMH in normally developing follicles. In order to clarify the signalling mediators and pathways of AMH it is intended to utilise a novel immuno-cytochemical approach specifically selected for its ability to detect very small amounts of target. The work will also utilise a number of other techniques to validate the immunocytochemistry work and determine signal transduction activation. It is felt that these approaches while helping to elucidate the underlying mechanisms of action of AMH, may also provide important insights into the aetiology of PCOS. It is of note that several studies have reported an inverse relationship between AMH and aromatase (Campbell *et al.* 2012), which may involve availability of androgen. As a result an important aim of this work is to investigate the existence of

an intra-ovarian regulatory loop between granulosa cell (GC)-derived AMH and androgens produced in the theca cells (TC). Another area which is to be further investigated is local factor regulation of AMH. Prime candidates are VEGF (vascular endothelial growth factor), which like AMH is characteristically produced at elevated levels in PCOS, and SOX8, a close family member of which acts as a transcription factor for up-regulation of AMH in the testes (Salmon *et al.* 2005). $VEGF_{120}$ and $VEGF_{164}$, the two prominent VEGF-A variants found in sheep follicles, as well as SOX8 will be evaluated in respect of their links to AMH regulation.

1.2 Ovarian Anatomy

As described by (Standring 2008), the ovaries of a non-pregnant female lie in the pelvis, close to the lateral pelvic wall and on each side of the uterus suspended by a double layer of peritoneum called the mesovarium which connects it to the upper posterior aspect of the broad uterine ligament. The ovaries are dull white in colour and are composed of dense fibrous tissue in which the ova are embedded. Before ovulation commences the ovarian surface is smooth, but soon becomes distorted with scarring due to degeneration of successive corpora lutea once ovulation begins. The dimensions of the ovary change throughout the female life span. An average mature woman's ovary dimensions are 4 x 2 x 3 cm. During neonatal life the ovary's size is considerable smaller reaching about a third of the normal adult size just prior to menarche. The size gradually increases with body growth. In pregnancy, the ovaries more than double in size. But as menopause approaches, the average size of the ovary

starts to diminish and in late menopause the ovarian dimensions can reach 1.5 x 0.75 x 0.5 cm.

Laterally, the ovary lies in the ovarian fossa which is a parietal peritoneal layer that covers retroperitoneal structures including the ureter, internal iliac vessels, obturator vessels and nerves, and the origin of the uterine artery. Medially, the ovary faces the uterus and the uterine blood vessels in the broad ligament. The distal part of the uterine tube and the fimbria lie superior to the ovary while inferiorly it points towards the pelvic floor. The anterior aspect of the ovary faces the posterior fold of the broad ligament where it is attached to the mesovarium and posteriorly it is free (Standring 2008).

The ovary is suspended in the pelvis with three peritoneal and ligamentous supports consisting of the infundibulopelvic, ovarian, and mesovarian ligaments. The Infundibulopelvic ligament, also known as the suspensory ligament, is a peritoneal fold that connects the lateral aspect of the ovary to the pelvic wall. The peritoneal attachment is higher on the left side than on the right. It conveys the ovarian vessels and nerves. The ovarian ligament attaches the inferiomedial part of the ovary to the lateral angle of the uterus. It lies inferior and posterior to the uterine tube. It runs in the posterior leaf of the broad ligament and is composed of some smooth muscular cells. The ovarian ligament is a continuation of the medial border of the round ligament, which are both remnants of the gubernaculum. The mesovarium, is a short ligament composed of a peritoneal fold that attaches the ovary to the posterior aspect of the broad ligament. It transmits the blood vessels and nerves to the ovarian hilum (Figure 1.1).

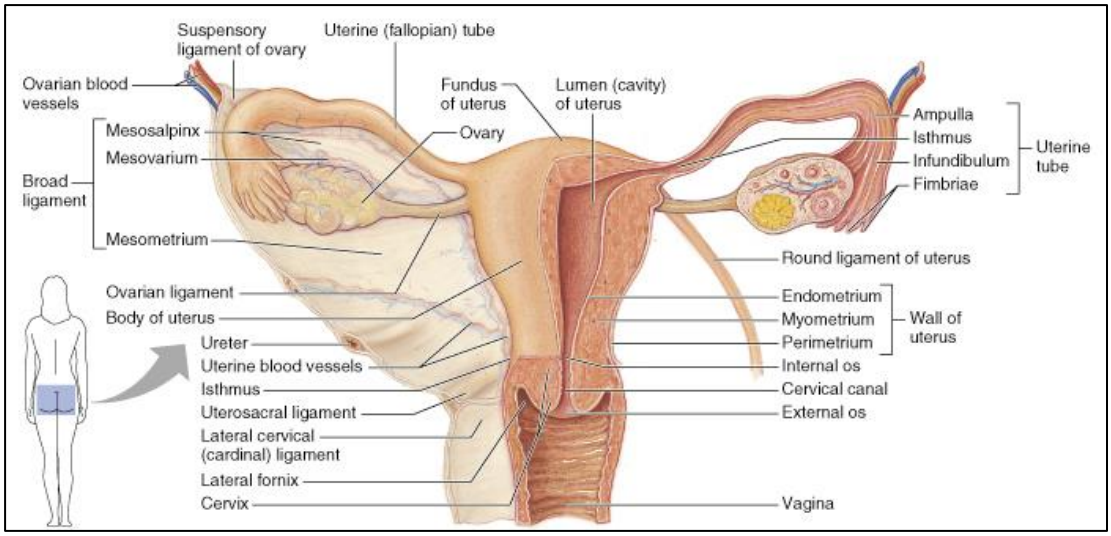


Figure 1.1 The female reproductive anatomy
Adapted from (Cummings 2006).

The arterial blood supply to the ovary originates from the ovarian arteries which are branches of the abdominal aorta below the renal arteries. As it enters the pelvis it runs medially in the suspensory ligament where it then splits into two branches. One enters the mesovarium to supply the ovary and the other continues into the broad ligament to supply the uterus and tube. The ovarian venous drainage of the ovary originates as a plexus called the pampiniform plexus in the mesovarium and infundibulopelvic ligament. From this plexus two veins arise that follow the artery. They usually merge into a single vein before connecting to the inferior vena cava on the right side and the renal vein on the left. The ovaries main lymphatic drainage is into the para-aortic nodes, but can also drain into the pelvic nodes and rarely follow the round ligament to the inguinal nodes. The ovary is innervated through autonomic plexuses derived from branches of the renal, aortic, superior and inferior hypogastric plexuses.

1.3 Ovarian Histology

As described by (Standring 2008), in brief, the surface of the ovary is covered by a dull white appearing single layer of cuboidal epithelium. This layer is continuous with the smooth shiny peritoneal mesothelial covering of the mesovarium. The transitional border between the peritoneum and the ovarian epithelium is marked by a white line around the anterior mesovarian border. This surface epithelial layer is also called the germinal epithelium, which is a misnomer, since this layer does not contain any germ cells. The next layer beneath the epithelium is collagenous coat called the tunica albuginea. This coat surrounds the ovarian tissue which is composed of an outer cortical layer that contains the ovarian follicles, and an inner medulla that conveys the ovarian vessels and nerves.

The sheep ovary is considered an adequate candidate for research due to its close resemblance in structure to that seen in humans with very minor differences. Its size is about 80% of the premenopausal human ovary and contains a similar cortical layer that accommodates the pool of primordial follicles (Fransolet *et al.* 2014). Minor differences also exist between humans and the sheep model during folliculogenesis and this will be discussed in more detail in section 1.4.

1.3.1 Ovarian Cortex

Before puberty, the ovarian cortex only occupies about a third of the overall volume of the ovary (35%). While the largest percentage of the ovarian volume is composed of interstitial cells (45%) and the medulla forms only 20% of the total volume (Standring 2008). These figures change after puberty, as the cortex becomes the major part of the ovary. The cortex contains the ovarian follicles at different stages of

development, the corpora lutea, and the degenerative remnants, depending on the cycle stage and age. A meshwork of collagen fibers and fusiform fibroblast like cells form the dense stromal tissue that surrounds the follicles and the structures derived from them. These stromal cells are different to the general connective tissue fibroblasts in that contain lipid droplets. The maturing ovarian follicle thecal layer arises from these stromal cells.

1.3.1.1 Ovarian Follicles

The development of ovarian follicles can be divided into different stages:

1.3.1.1.1 Primordial Follicles

At birth, a superficial zone of primordial follicles is embedded in the ovarian cortex. These follicles contain a primary oocyte with a diameter of 25 micrometre, which is surrounded by a single layer of flat follicular cells. Histologically, the oocyte nuclei are eccentric and have a prominent nucleolus. Since before birth, they contain the diploid number of chromosomes, which are arrested in the diplotene stage of meiotic prophase.

Many of these follicles will degenerate either prenatally or throughout the female's life until reaching puberty, or undergo atresia at some stage following the beginning of the cyclical maturation process throughout their reproductive age. The remnants of these follicles accumulate throughout the reproductive life and are visible as atretic follicles. The development of primordial follicles to become an ovulatory follicle is estimated to take over 175 (Gougeon 1986, Baerwald *et al.* 2012), whereby a cohort of primordial follicles are activated and usually only one follicle from one or the

other ovaries reaches maturity and becomes dominant and releases its oocyte at the time of ovulation.

1.3.1.1.2 Primary follicles

The first sign of change from primordial follicles to primary follicles is that the flattened follicle cells become cuboidal. These cells then proliferate to form a multi-layered follicle composed of GCs enclosed by a thick basal lamina. The cells immediately surrounding the follicle, also known as stromal cells, differentiate to become spindle shaped cells which will form the theca interna. These are then surrounded by a more fibrous layer known as the theca externa. During this period the oocyte also undergoes changes. It increases in size and secretes a thickened layer of extracellular material that is proteoglycan rich that is called the zona pellucida. This layer is formed between the plasma membrane and the GCs surrounding the follicle. As the follicle progresses to the late primary follicle stage the GCs which are in functional contact with each other through gap junctions continue to proliferate resulting in thickening of the follicular wall.

An important step in follicular development is the establishment of blood vascularity in the TC layer starting from the preantral follicle stage. Endothelial cells derived from the ovarian stroma are employed to form the thecal vascular sheet which is comprised of two networks of blood vessels that run between the theca interna and externa (Findlay 1986). GCs are separated from the TCs by the basement membrane and stay devoid of blood vessels until after ovulation (Stouffer et al. 2001). Follicles that have not reached the preantral stage do not possess a vascular network, while antral follicles are rich in vascularity. This uneven distribution points to an

important role for angiogenesis in determining the fate of the follicle to either be selected for ovulation or undergo atresia. An important local factor has been found to be associated with ovarian angiogenesis which is called vascular endothelial growth factor (Fraser 2006). This factor will be discussed in more detail in this review.

1.3.1.1.3 Secondary follicles

As the GC numbers continue to increase, cavities begin to form between the cells that are filled with a clear fluid that contains hyaluronate, steroid hormones and growth factors produced by the GCs. The follicle at this stage lies deep into the cortex. The cavities formed start to coalesce to form a single fluid filled space, called the antrum, which is walled by a single layer of GCs except at one end where the follicle contains a thickened GC layer that encircles the eccentrically placed oocyte, the cumulus oophorus. The inner and outer theca layers at this stage are clearly distinguished. As the follicle continues to mature, the theca interna layer becomes more noticeable and its cells become more round and resemble typical steroid producing endocrine cells. These cells secrete androgens which are used by GCs to produce oestrogens (primarily oestradiol) (Hillier *et al.* 1994).

1.3.1.1.4 Tertiary (Graafian) follicles

Although many follicles may progress to reach the secondary stage, usually only one follicle continues to become a tertiary follicle. The rest become atretic. This follicle increases considerably in size as the antrum expands. The oocyte and its surrounding tightly adherent cells, the corona radiate, project on a stalk into the antral cavity. At the time of the LH surge and ovulation, the primary oocyte that has remained dormant

in the first meiotic prophase since foetal life completes the first meiotic division resulting in a large secondary oocyte and a small first polar body that contains very little cytoplasm. The oocyte then begins its second meiotic division immediately, but gets arrested when it reaches metaphase. The oocyte remains arrested at this stage until fertilisation has occurred. The ovary surface bulges as the follicle moves to the superficial cortex. The tissue where the tunica albuginea and ovarian surface epithelium become in contact becomes eroded resulting in the rupture of the follicle and the release of its contents into the peritoneal cavity. The oocyte at ovulation remains surrounded by the zona pellucida and corona radiata of GCs. The oocyte is captured by the fimbria of the uterine tubes where fertilisation usually occurs. If fertilisation does not happen, degeneration of the oocyte starts to begin after 24 to 48 hours.

1.3.1.1.5 Corpus luteum

Following ovulation the remains of the follicle becomes the corpus luteum. As the antum is no longer filled with fluid, the walls collapse and form folds. The GCs that have now increased in size start to secrete a cytoplasmic carotenoid pigment called lutein that gives the follicle the distinctive yellowish colour. The basal lamina encircling the GCs breaks down resulting in infiltration of numerous small theca lutein cells to the folds of cellular mass accompanied by capillaries and extravasated blood, forming a clot in the centre of the mass. As time progresses this clot is resolved and replaced by connective tissue. All lutein cells have characteristics of steroid producing cells. Granulosa lutein cells produce oestradiol by aromatising androgens produced by theca lutein cells, and progesterone. If fertilisation does not occur the corpus luteum

continues to function for 12 to 14 days following ovulation, and then undergoes atrophy by fatty degeneration, autolysis, macrophage removal and is finally replaced by fibrous tissue (Standring 2008).

If fertilization does occur, the chorionic gonadotropin produced by the embryonic trophoblast, stimulates the corpus lutetium to grow and secrete progesterone, oestrogen and relaxin. Its function continues throughout pregnancy although it regresses as the placenta replaces its function at about 8 week's gestation.

1.3.2 Ovarian Medulla

Numerous veins and spiral arteries enter the hilum of the ovary from the mesovarium, making the medulla highly vascular. The vessels all lie within a loose connective tissue stroma.

1.4 Folliculogenesis

Ovarian folliculogenesis is an intricately regulated process marked by a lengthy dramatic proliferation and accurately orchestrated differentiation of both germ and somatic cells (Webb & Campbell 2007). As a gametogenic and endocrine organ the function of the ovary is to produce developmental competent oocytes at the appropriate time that will result in fertilisation, implantation and pregnancy, leading at the end to the birth of offspring in optimal environmental conditions. In order to achieve this, complex homeostatic control mechanisms have developed that rely on physiological and environmental information to control the reproductive cycle either directly or through the hypothalamic pituitary axis.

Over many year researchers have been trying to advance our understanding of the somatic and germ cell biology of the ovarian follicle. Follicles have been classified in many different ways based on both structure and size. However, a classification based on the physiological function of the follicle was introduced by (Scaramuzzi *et al.* 1993) and later revised in 2011 (Scaramuzzi *et al.* 2011). For the purpose of this review the classification by Scaramuzzi *et al*. (2011) will be used as a base for describing the stages of folliculogenesis (Figure 1.2). It must be noted that folliculogenesis is a continuous process, and only for descriptive purposes follicles have been divided into five different classes. Starting with the primordial follicles, this is followed by committed follicles, gonadotrophic responsive follicles, gonadotrophic dependant follicles and ending with the ovulatory follicles.

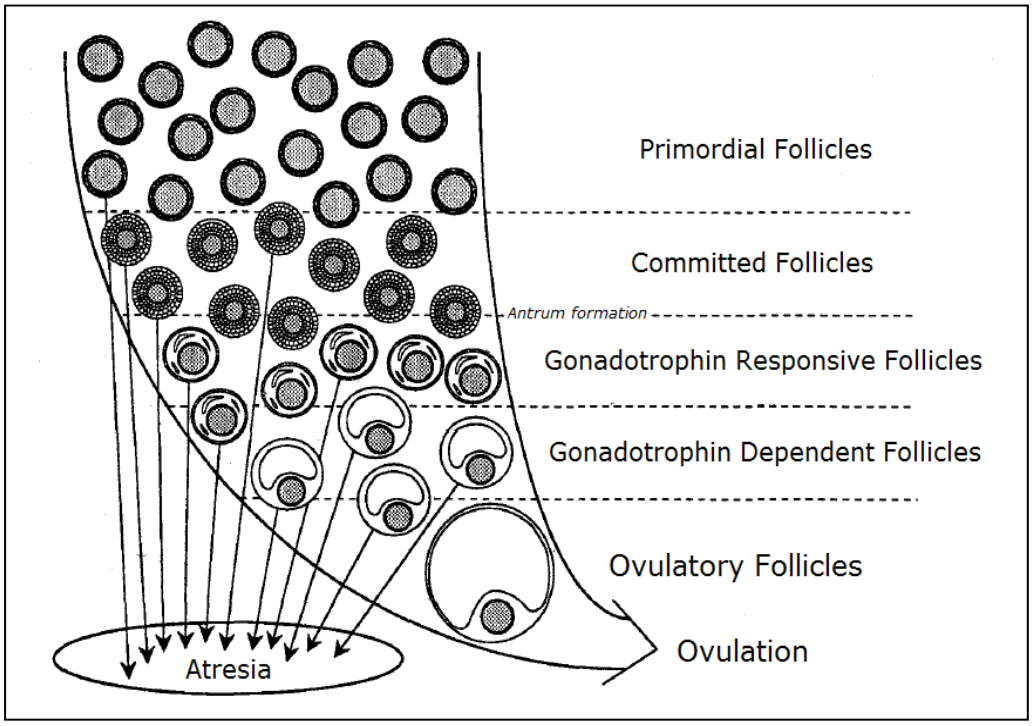


Figure 1.2 Illustrates a model of follicular growth showing the stages of development.
Adapted from (Scaramuzzi et al. 1993, Scaramuzzi et al. 2011).

1.4.1 Primordial Germ Cells and Primordial Follicle Formation

Primordial germ cells (PGC) are undifferentiated large cells that originate from epiblast cells which later form the extra embryonic mesoderm (Lawson *et al.* 1999). PGCs are first seen in the yolk sac at around 8 days of embryonic life in mice (Grudzinskas 1995) and 21-22 days gestation in humans (De Felici *et al.* 2004). (Hirshfield 1991) has shown that PGCs are motile. They move into the developing hindgut, pass through the dorsal mesentery and at about 10 days of embryonic life in mice reach the genital ridge (Grudzinskas 1995). In sheep, although it is not clear when they start to migrate but at about 23 days following conception evidence of these cells can be found in the genital ridge and by 40 days most of these cells have completed this migration (McNatty *et al.* 2000).

Between 23 and 120 days of embryonic life in the sheep the PGCs go through a stage of prolific mitosis producing an increase in the gonadal germ cell population (McNatty *et al.* 1995). Following several divisions these cells differentiate to form oogonia (Grudzinskas 1995). The oogonia further perform a number of species specific mitotic divisions and at about 55 day following conception in the sheep they enter into meiosis to form primary oocytes which become arrested in the diplotene stage of meiosis 1 (Grudzinskas 1995, McNatty *et al.* 1995). In humans this process starts to occur at about 2 months gestation, while in rats it starts at around 16.5 days of embryonic life (Hirshfield 1991).

Many factors have been shown to be necessary for PGC formation and migration. These include bone morphogenic protein (BMP) 4 (Fujiwara *et al.* 2001), and BMP 8b (Ying *et*

al. 2000) and the gap junction protein connexion 43 (Francis & Lo 2006). Transforming growth factor (TGF) β-1 may also play a role in PGC migration as a chemotropic agent (Godin & Wylie 1991) and interleukin 4 may be important for PGCs survival (Cooke *et al.* 1996). *In vitro* studies by (Silva *et al.* 2004) also showed that Activin A may play a role in the survival and proliferation of human PGCs. However, these results were contrary to results by (Richards *et al.* 1999) on mice. Kit ligand (KL also known as stem cell factor) and its receptor, c-kit; have also been shown to support migration, survival and proliferation of the PGCs (Godin *et al.* 1991, Matsui *et al.* 1991, Driancourt *et al.* 2000, Runyan *et al.* 2006). However, (Godin *et al.* 1991) did not find KL to influence migration of the PGCs. Downregulation of KL has also been shown to be important in apoptosis of the PGCs that have not migrated successfully to the genital ridge which may persist and latter cause tumour formation (Runyan *et al.* 2006).

The maximum number of germ cells is seen at around 75 days following conception in sheep. At this point the first primordial follicles can be seen in the inner cortex (McNatty *et al.* 1995). These are formed from the interaction between primary oocytes with somatic pre-GCs. These pre-GCs are thought to originate from mesonephric tissue which then forms cell streams seen at about 30 days of foetal life in sheep. They then extend to the ovarian cortex, progressively surrounding the oogonia to form individual follicles (McNatty *et al.* 1995, McNatty *et al.* 2000). Immense oocyte apoptosis also occurs at around this time resulting in an 80% reduction in number (McNatty *et al.* 1995). The primary oocyte association with the precursor GCs is thought to protect them from cell death (Silva *et al.* 2004).

In sheep, primordial follicles are about 30-40 μm in diameter (Scaramuzzi *et al.* 1993). They consist of an oocyte surrounded by a single pre GC layer contained by a basal lamina (Scaramuzzi *et al.* 1993). Once the primordial follicles start to form, the rapid proliferation of the pre-GCs halts and become quiescent (Hirshfield 1991). In many species such as sheep, cows and humans, primordial follicle formation only occurs in foetal life (McNatty *et al.* 1992, Faddy & Gosden 1995, Braw-Tal 2002). While in other species such as rodents, it is thought that primordial follicle formation occurs after birth (Hirshfield 1991, Johnson *et al.* 2004).

The formation of primordial follicles is thought to be regulated by many factors (Skinner 2005). Some may enhance primordial follicle formation such as a transcription factor encoding gene known as Fig alpha, studied in mice (Soyal *et al.* 2000), growth differentiation factor (GDF)9, Kit Ligand (KL), oestradiol and follicle stimulating hormone (FSH) studied in hamsters (Wang & Roy 2004). While research on rats, found that progesterone inhibited primordial follicle formation (Kezele & Skinner 2003).

1.4.2 Primordial Follicle Activation

The primordial follicles may stay dormant for months or years until activated (Fortune *et al.* 2000). In sheep, at around 100 days of foetal life, continuous activation of primordial follicles begins, which then join the growing pool of irreversibly committed follicles to growth (Scaramuzzi *et al.* 1993). The pre-GCs change to cuboidal GCs and begin to proliferate, and the oocyte increases in size (Scaramuzzi *et al.* 1993, McNatty *et al.* 2007). The follicle that contains a primary oocyte surrounded by a single layer of GCs is called a primary follicle

and in the earliest form of a committed follicle (Kezele & Skinner 2003). In humans and rodents, the zona pellucida forms around the oocyte at this early stage. However, in ruminants, this does not occur until the small antral stage (Lundy *et al.* 1999).

Primordial follicle activation is independent of gonadotrophic action and it is suggested that local factors play the dominant role in this action (McNatty *et al.* 2007). Many factors have been shown to effect primordial follicle activation. In rodents, KL and basic fibroblast growth factor (bFGF) have been shown to promote primordial follicle activation and development (Nilsson & Skinner 2004) but they are not essential (Ortega *et al.* 1998). In other species their role is still unclear (McGee & Hsueh 2000, McNatty *et al.* 2007). Recently, it has been suggested that the PI3K pathway may have a role in follicle activation (Kim 2012). Primordial follicles in mouse ovaries deficient in forkhead box O3 (Foxo3a), a downstream effector of the PI3K pathway, were found to be prematurely activated (Castrillon *et al.* 2003). Other factors such as members of the BMP family seem to play a significant role in rodents. These include BMP7 (Lee *et al.* 2001), and BMP4 (Nilsson & Skinner 2003). However, these two factors are not expressed in sheep follicles (Juengel *et al.* 2006b). Another factor that may be involved is BMP15. In sheep which are heterozygous for a BMP15 gene mutation had a greater than normal ovulation rate and a larger than normal littler size, while animals that were homozygotes for this mutation were sterile (Davis *et al.* 2001). In the latter, the ovarian follicles did not exceed beyond the primary stage of development (Braw-Tal *et al.* 1993). Similar abnormal pathological finding have been shown in animals which were actively immunised for BMP15 and the

closely related GDF9 (Juengel *et al.* 2004b), which suggests that these factors perform similar actions in sheep. Other promoting factors of primordial follicle activation include leukaemia inhibitory factor (LIF) (Nilsson *et al.* 2002), and keratinocyte growth factor (KGF) (Skinner 2005) both studied in rodents.

Serum free culture systems development for thin strips of ovarian cortex and isolated preantral follicles have provided experimental methods to study both primordial follicle initiation and follicular growth into the antral stages (Gutierrez *et al.* 2000, Picton *et al.* 2003, Walters *et al.* 2006). Using these techniques has provided evidence that primordial follicle activation may be under inhibitory control since higher than expected primordial follicles were activated in these *in vitro* studies than *in vivo* estimates (Wandji *et al.* 1996). The identity of this inhibitory factor is still to be precisely identified. But studies on knockout mice (Durlinger *et al.* 2002b) suggests that anti mullerian hormone is a possible candidate. AMH role will be reviewed thoroughly below.

1.4.3 Committed Follicles

Once the primordial follicle is activated it becomes a committed follicle. From the committed follicle stage to the preantral stage the GCs proliferate to produce 2-3 layer and the oocyte rapidly enlarges. This is followed by TC differentiation from the surrounding stromal cells (Scaramuzzi *et al.* 1993). The growth of the committed follicles is known to be independent of gonadotrophins (Webb *et al.* 1999a) despite the evidence of FSH receptor mRNA expression in sheep at this follicular stage (Tisdall *et al.* 1995, Fortune *et al.* 1998). The two closely related members of the TGF-β family (BMP15

and GDF9) have been shown to be vital for the development of the follicle beyond the primary stage (Braw-Tal *et al.* 1993, Juengel *et al.* 2002). KL and c-kit may also play a role at this stage of follicular development in rodents (Packer *et al.* 1994, Yoshida *et al.* 1997) but their role in humans is still not clear (Carlsson *et al.* 2006a). Other promoters of committed follicle development include the Epidermal growth factor (EGF) (Hemamalini *et al.* 2003), and androgens (Yang & Fortune 2006, Gleicher *et al.* 2011). Other local factors have also been shown to be expressed during the early stages of gonadotrophin independent follicle development including activins and inhibins and follistatin which may play a role in regulating this stage of development (McNatty *et al.* 1999).

Thecal cell formation is also an important event that occurs around the primary follicular stage (Young & McNeilly 2010). There is a species specific difference, where in sheep, theca interna can be seen in primary follicles with 1-2 GC layers and most preantral follicles with 2-4 GC layers possess a theca interna layer (Lundy *et al.* 1999). In mice, TCs start to be seen in follicles with around 2-4 GC layers while in hamsters it is not until follicles have 7-8 GC layers that TCs become evident (Hirshfield 1991).

1.4.4 Gonadotrophin Responsive Follicles

Follicles continue to grow independent of gonadotrophins into the early antral stage, where further continual growth becomes dependant on circulating luteinising hormone (LH) and FSH. However, from preantral stage follicles have been found to respond to gonadotrophin challenges (Scaramuzzi *et al.* 1993). In sheep, follicles at this stage measure around >100μm in diameter and follow a hierarchical pattern of

response to gonadotrophins (McNatty *et al.* 1992). The effect of FSH on the follicles growth at this stage is not permissive but due to its proliferative effect on GCs can cause growth in the preantral follicles (Campbell *et al.* 1996, Campbell *et al.* 2000, Campbell *et al.* 2004). As the sheep preantral follicles growth continues, GCs start to express FSH receptors and just before the formation of the antrum, theca intera cells starts to develop LH receptors (Logan *et al.* 2002). In gonadotrophin responsive follicles that are approximately 220μm, the antrum starts to form in response to FSH (Webb *et al.* 1999b), whereby follicles begin to form cavities and accumulate follicular fluid between GCs. These latter coalesce to produce a single cavity or antrum (Rodgers & Irving-Rodgers 2010). As the antrum starts to form the GC and TCs begin to differentiate, becoming less proliferative and more steroidogenic (Webb & Campbell 2007). With the expression of LH receptors, follicles begin to produce androgens in reaction to LH binding (McNatty *et al.* 1992) which are then converted in the GCs to oestradiol (Scaramuzzi *et al.* 1993).

1.4.5 Gonadotrophin Dependant Follicles

For a follicle to develop from a gonadotrophin responsive to a gonadotrophin dependant follicle it critically depends on the action of FSH on the acquired FSH receptors (Scaramuzzi *et al.* 1993). This transition which involves many local factors, non the less appears to be largely depends on a transient increase in FSH levels seen following luteal regression or as a result of a preovulatory follicle collapse (le Nestour *et al.* 1993). This produces a fall in oestradiol levels, decreasing the negative feedback effect on FSH (le Nestour *et al.* 1993). It has been noted that follicles are more likely to leave the follicular pool and enter a process called cyclic recruitment if

they have a higher level of FSH responsiveness (McGee & Hsueh 2000). From here on, any further follicle growth is dependent on gonadotrophic support (Scaramuzzi *et al.* 1993). LH has also been found to be important for follicle steroidogenesis (Campbell *et al.* 2007), however, high amplitude LH pulses affects follicle development and cause the follicle to undergo atresia (Picton *et al.* 1990). From the gonadotrophin dependant follicle pool a breed specific number of follicles are chosen to continue their growth (Campbell *et al.* 1995). This selection is associated with a decline in the level of FSH resulting in the loss of the majority of the gonadotrophin dependant follicles while only a few continue to become ovulatory follicles (Baker & Spears 1999). The mechanism of this selection is not fully understood, but is thought to not only depend on endocrine factors but also local factors as well (Baker & Spears 1999). The theory is that local factors are expressed differently in the recruited follicles resulting in a difference in sensitivity to the decrease in FSH levels at critical points of development (Campbell *et al.* 1995). Many locally produced hormone and growth factors have been shown to effect follicle selection and the acquisition of the dominant follicle (Knight & Glister 2003, Webb & Campbell 2007). These systems include the insulin/Insulin-Like Growth Factor (IGF) system (Webb *et al.* 1999a), the inhibin/activin system (Campbell & Baird 2001, Campbell *et al.* 2003) and the bone morphogenic system (Souza *et al.* 2001a, Souza *et al.* 2002, Campbell *et al.* 2006). The oocyte may also produce factors that may affect late follicular development and ovarian function including GDF9 (Juengel *et al.* 2004a), BMP6 (Knight & Glister 2003, Campbell *et al.* 2006) and BMP15 (Galloway *et al.* 2000). Studies from AMH null mice show that in addition to its role on follicle initiation, AMH may also have an effect on

the dominant follicle selection (Campbell *et al.* 2012). AMHs role in follicle selection will be examined in more detail later in this review.

1.4.6 Ovulatory Follicles

In sheep, usually no more than 4 follicles reach the ovulatory follicle stage (Campbell *et al.* 1995). These follicles are large in size, have extensive vascularity, and express LH receptors on both GCs and Theca intera cells (Campbell *et al.* 1995). A low but critical level of FSH is an important requirement for the progression of a gonadotrophin dependant follicle to an ovulatory follicle (Scaramuzzi *et al.* 1993). This occurs when selected follicles grow and start to produce increasing amounts of oestradiol which inhibits the production of FSH from the anterior pituitary (Baird 1983). Subordinate gonadotrophin dependant follicles undergo atresia in response to the sub threshold levels of FSH. While follicles that become dominant, produce LH receptors on the GCs, in response to FSH and oestradiol (Webb *et al.* 1999a, Mihm *et al.* 2006). With this upgrade and ideal LH pulse frequency and amplitude, follicles are able to transfer dependence from FSH to LH just in time to survive the self-induced decrease in FSH, and in the end result to the ovulation of 1-2 large antral follicles in most sheep (Campbell *et al.* 1999).

1.5 The Ovarian Reproductive Cycle

In order to fully understand the process of folliculogenesis, it is important to comprehend the basis physiology of the reproductive cycle. Gonadal function in the female is primarily under the control of a complex but necessary interplay between the hypothalamus, the pituitary and the ovaries to

form what is known as the hypothalamo-pituitary-ovarian axis.

1.5.1 The Hypothalamo-Pituitary-Ovarian Axis

The hypothalamus is located at the middle of the base of the brain, just above the pituitary gland that rests in the sella turcica cleft of the skull (Amar & Weiss 2003). The pituitary is composed of the anterior and posterior lobes. Hypothalamic neurons contact the hypophyseal portal system with the pituitary, facilitating the transport of hormones and neurotransmitters produced by the hypothalamus to interact with receptors on the cell membrane of various cell types in the anterior pituitary (Amar & Weiss 2003).

Unlike in the male, the feedback regulation by both the pituitary and hypothalamus is more complicated due to the fact that gamete development and release is not continuous. Further, the unavailability of gamete replenishing stem cells in the ovary, dictates the release of the ovum under tight regulation by the brain at a specific time during the menstrual or oestrous cycles. Ovarian produced oestradiol and inhibin in response to FSH and LH, feedback to the pituitary and hypothalamus. At around the time of ovulation, oestradiol increases to a threshold that causes a feed forward effect, resulting in a GnRH (gonadotrophin releasing hormone) surge from the hypothalamus which then induces the LH surge and causing ovulation. Feedback systems which regulate ovulation and corpus luteum formation differ between species, and therefore resulting in the differences in length of menstrual and oestrous cycles. Oestradiol is not produced by the corpus luteum in ruminants, releasing the pituitary from its inhibitory

affect which leads to a rise in FSH. Therefore, follicle development restarts, resulting in a shorter oestrous cycle than human menstrual cycle ranging in length between 17 to 21 days (Baird *et al.* 1975).

1.5.2 The Oestrous Cycle

In ovine and bovine species which are considered the core research models for human reproductive physiology, the reproductive cycles are called oestrous cycles. During these cycles, follicles develop in a wave like manner, under hormonal control (Figure 1.3). Once ovulation occurs, a new batch of follicles commences. This starts with the recruitment of follicles from the primordial follicle pool within the ovary, in a mechanism that is still poorly understood. Once follicular growth is initiated, the follicle continues until reaching one of two fates either ovulation or atresia. In monovulatory animals like the cow, only a minute fraction of the growing follicles will ovulate, less than 0.1% (Webb *et al.* 2003). In cattle, the oestrus cycle is characterised by 2 or 3 follicular waves with each having 3 to 5 developing follicles reaching more than 4 mm in diameter. Once they reach about 6-8 mm a mechanism of selection and dominance is employed resulting in one follicle dominating and inhibiting the rest of the group (Webb *et al.* 2003).

1.5.3 The Difference between the Menstrual & Oestrus Cycles

Higher primates do not undergo oestrus. The periodic shedding of the endometrial lining distinguishes these species from others in a process known as the menstrual cycle (Figure 1.4). Although, in both types, cyclical changes occur in the ovary and the reproductive organs, however, the days at

which these cycles start differs. In the oestrous cycle, day 1 is considered to be the first day of oestrus, while in the menstrual cycle the first day corresponds with the initiation of flow.

Another difference between the two cycles is the length of the follicular phase. In the menstrual cycle the length of the follicular phase usually equals that of the luteal phase, whereby luteolysis is followed by menstruation and the start of a new cycle. However, in the oestrus cycle, the follicular phase is shorter, due to the fact that follicular growth takes place in the luteal phase of the preceding cycle. Menstruation also does not occur in oestrus cycles however, there are usually changes seen in the reproductive behaviour around that time.

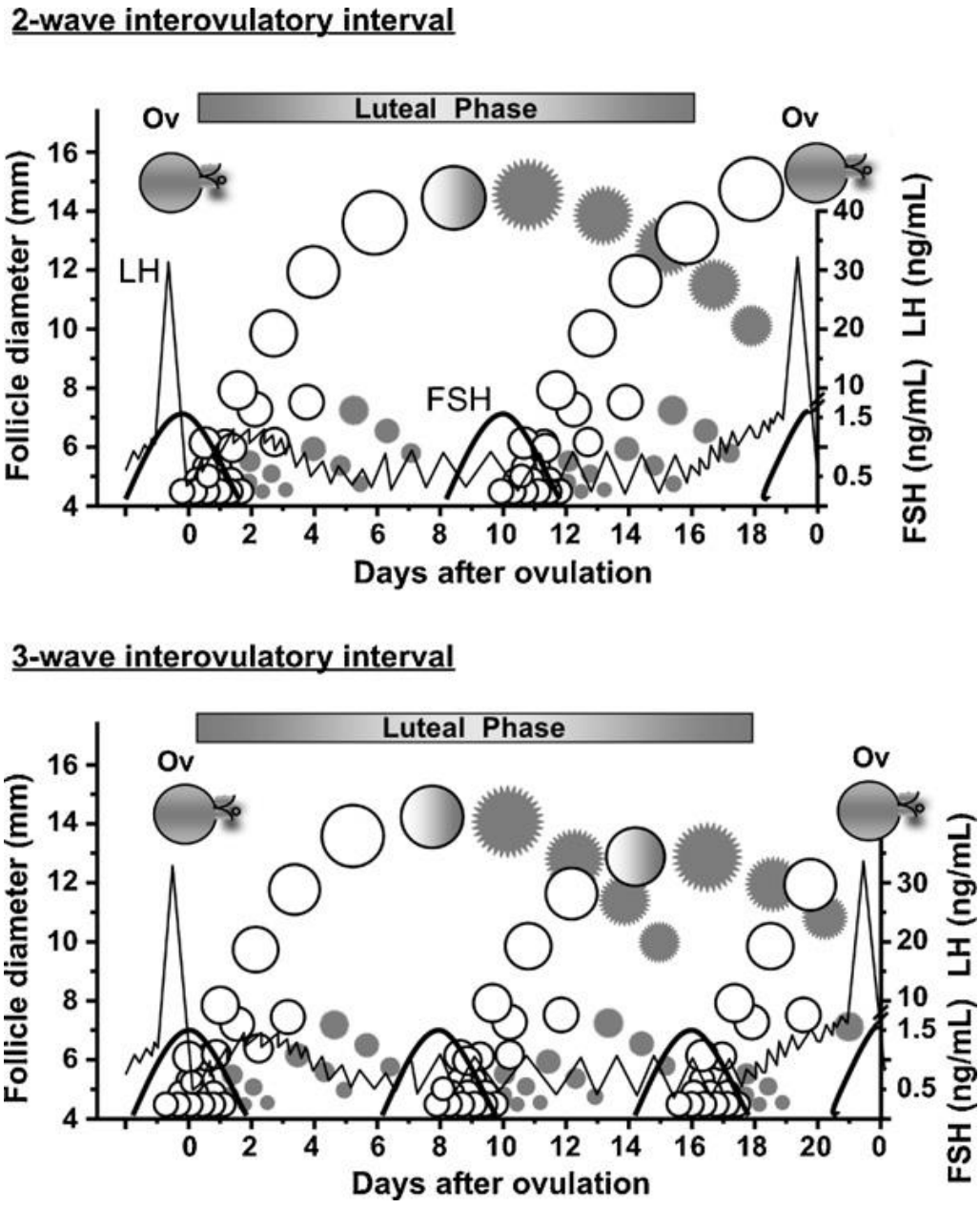


Figure 1.3 Bovine two and three wave oestrous cycles.
The image illustrates the follicular development and gonadotropin secretion in a two and three wave oestrous cycle in cattle. An FSH surge precedes the emergence of each follicle wave. An LH surge precedes ovulation (Adams *et al.* 2008).

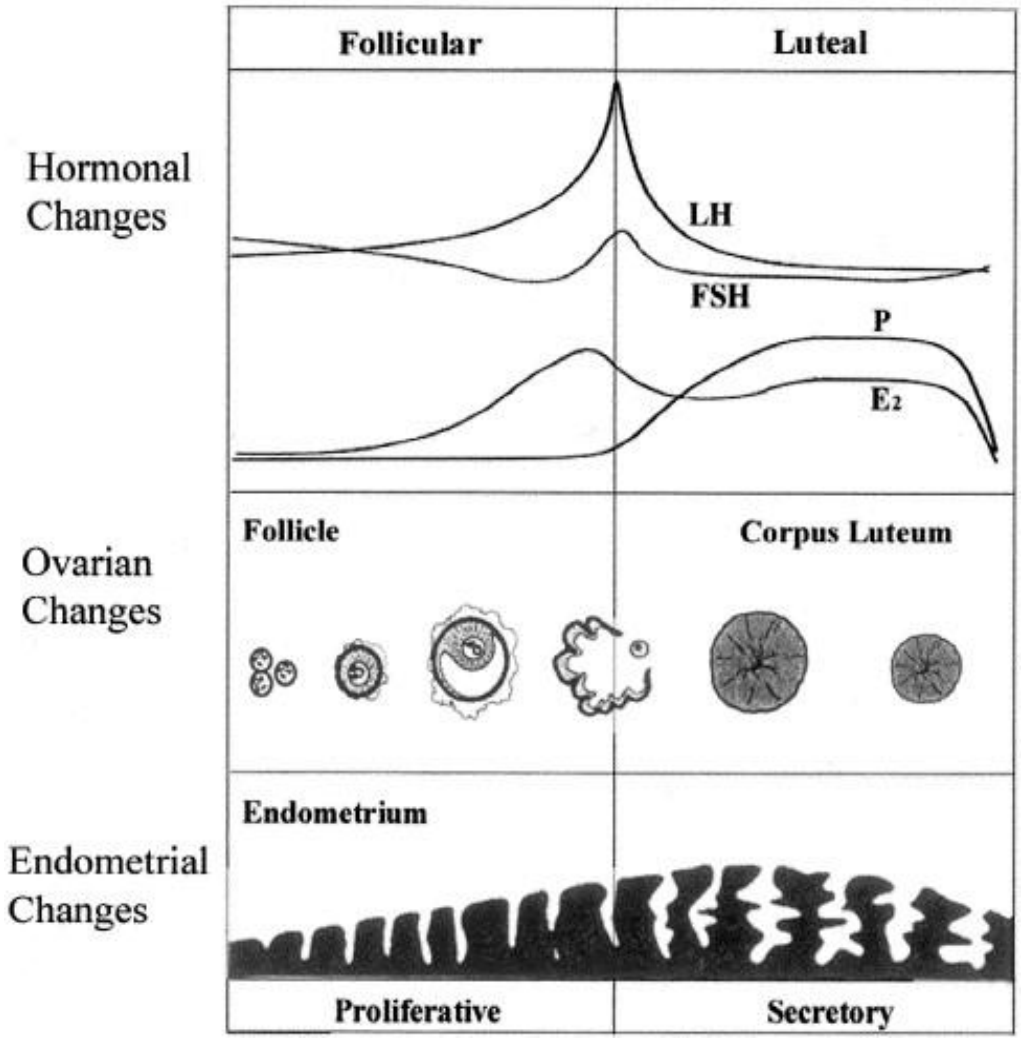


Figure 1.4 The menstrual cycle.
(Petroff *et al.* 2001)

1.6 Anti-Mullerian Hormone

1.6.1 Ovarian Anti-Mullerian Hormone

Anti-Mullerian hormone, also known as Mullerian inhibiting substance has been classified as a member of the (TGF-β) family (Cate *et al.* 1986), despite the fact that it only shares 20-30% homology with members of the Decapentaplegic/bone morphogenetic proteins (Dpp/BMPs) family and TGF-β family itself (Josso & Clemente 2003).

The AMH gene has been mapped to chromosome 19 p133, which is spread over 2.8 kbp and divided into five exons. The fifth exon encodes the C terminal bioactive fragment of the protein. The full length protein monomer that is 70 kDa is composed of 535 amino acids long divided into two unequal domains. A long N terminal that is 426 amino acid long and a short C terminal that is 109 amino acid long that carries the bioactivity. Two monomers associate to form disulphide linked dimers (140 kDa). The full length AMH dimeric glycoprotein can be cleaved by a proteolytic agent into a 110 kDa N terminal and a 25 kDa C terminal (Josso & Clemente 2003) (Figure 1.5).

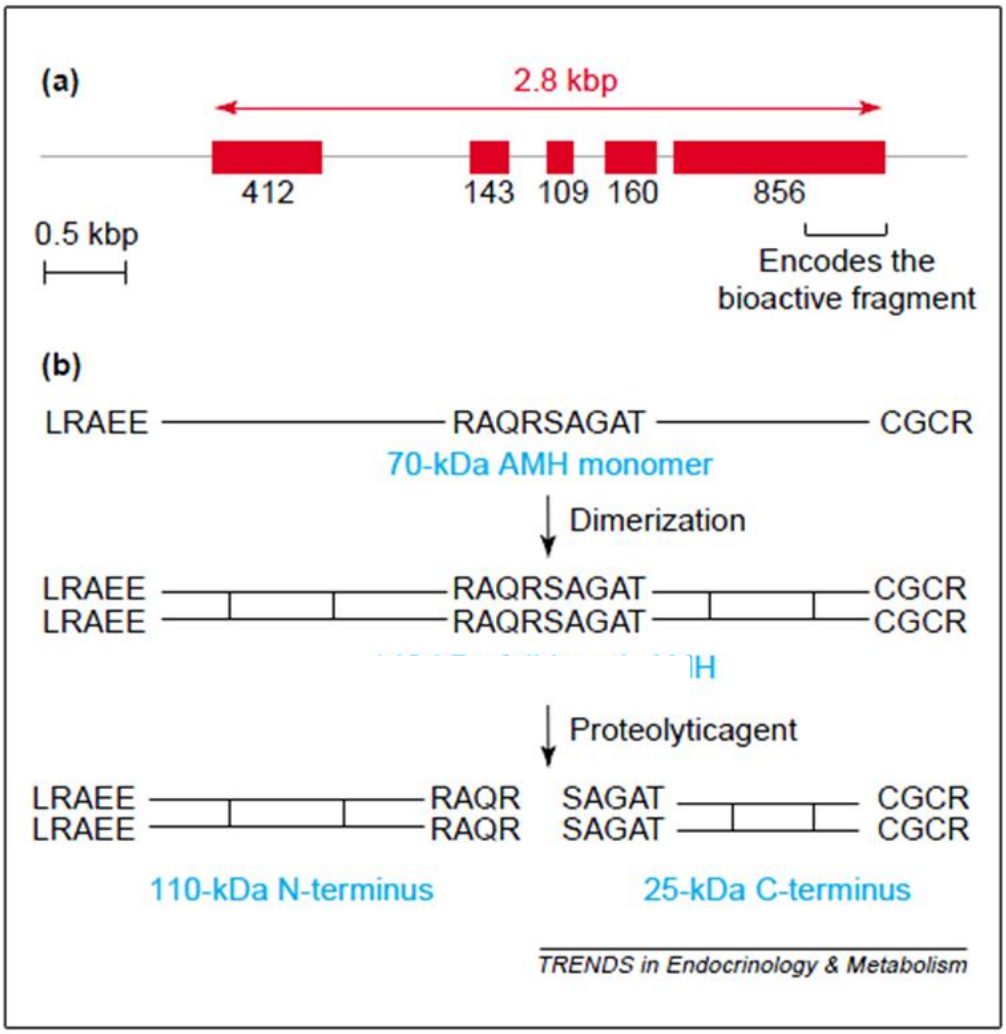


Figure 1.5 AMH and its gene product.
Human AMH divided into five exons (a). Processing of the AMH protein (b) (Josso & Clemente 2003).

While other members of the family are expressed broadly and have a wide range of biological activities depending on the cellular environment and context, AMH is predominantly produced in the gonads and has an inhibitory effect on function and development in the reproductive organs. However, recent publications indicate to a possible extra gonadal function of AMH in the central nerves system, which needs further investigation (Wang *et al.* 2005, Lebeurrier *et al.* 2008, Wang *et al.* 2009, Cimino *et al.* 2016).

Due to the striking effects of AMH came the origins of the name Mullerian inhibiting substance which had the capacity to induce regression of the Mullerian ducts, the rudimentary basis of the reproductive internal organs (Behringer 1994). In the male, a process regulated by the SRY gene located on the Y chromosome warrants the development of the testis through three critical hormones. These include testosterone and Insulin-like factor 3 produced by the Leydig cells and AMH produced by the Sertoli cells. The consequence of a mutation in either the AMH gene or the AMH receptor 2 gene result in a rare autosomal recessive disease known as persistent mullerian duct syndrome. It is characterised by having persistent mullerian duct derivatives whereby a normally virilised genetic male has a uterus, fallopian tubes and upper vagina (Belville *et al.* 2004).

However, as research progressed, other functions were identified for AMH including its inhibitory role on differentiation and steroidogenesis of immature ovaries, the follicles of adult female ovaries and prenatal and postnatal male leydig cells. The functions in the female ovaries will be discussed in length in this review. Other suggested targets for AMH action include breast, prostate, ovarian and uterine cancer cells (Teixeira *et al.* 2001).

In the early 1980s, AMH was detected in GCs and was found to be produced from 36 weeks gestation and continues to be expressed until menopause (Vigier *et al.* 1984, Rajpert-De Meyts *et al.* 1999). Many follow-up studies using *in situ* hybridisation or immune-staining have shown the expression of AMH in the ovaries of rats (Ueno *et al.* 1989, Baarends *et al.* 1995b), mice (Munsterberg & Lovell-Badge 1991), sheep (Sweeney *et al.* 1997), and humans (Stubbs *et al.* 2005, Modi

et al. 2006). It has been difficult to determine precisely what stage during folliculogenesis AMH starts to be expressed since studies in the literature on primordial follicles have produced equivocal results (Stubbs *et al.* 2005, Modi *et al.* 2006). In a recent paper by (Campbell *et al.* 2012), found that the expression of AMH was first distinguished at the secondary follicle stage. However, it has been found that AMH is at its highest expression during the preantral and small antral follicle stages, where the latter are involved in FSH dependant cyclic recruitment (Weenen *et al.* 2004). Following selection, AMH expression gradually decreases in the mural GCs and AMH positive staining becomes mainly localised to cumulus GCs (Munsterberg & Lovell-Badge 1991). This was confirmed by a study by (Pellatt *et al.* 2007), which showed that the highest protein production of AMH in human GCs and follicular fluid was in small antral follicles which subsequently decreasing to very low or undetectable levels in 10mm and over-sized follicles. These results suggest that the cessation of production of AMH is an imperative requirement for the dominant follicle selection. AMH mRNA expression and staining disappeared in follicles that became atretic. It was also not observed in oocytes, corpus luteum or TCs in mice, rates or humans ovaries (Ueno *et al.* 1989, Baarends *et al.* 1995b, Weenen *et al.* 2004, Modi *et al.* 2006). Therefore, this confirms that to date GCs are the only know source of AMH in the ovary.

1.6.2 The Role of Anti-mullerian Hormone

As previously discussed, there is now strong evidence that in order to achieve an ovulatory follicle that is capable of fertilisation, the follicle is submitted to a complex biological process that is regulated by stage specific expression of local

hormones and growth factors, secreted by both the oocyte and the somatic cells, and by peripheral hormones, which not only control oocyte maturation but also the proliferation, metabolism, angiogenesis and differentiation of somatic cells (Knight & Glister 2003, Webb & Campbell 2007). AMH has been identified as one of the local factors that regulate follicle development (Durlinger *et al.* 2002a). While extensively researched, our knowledge of the exact biological role of AMH in ovarian function of monovular species remains relatively little, despite the use of AMH circulating levels as an index of ovarian reserve (Themmen 2005) and its suggested link with the aetiology of PCOS (Stubbs *et al.* 2005, Themmen 2005).

In order to study the action of AMH, many experiments were conducted on knockout mice. AMH null mice were found to be fertile (Durlinger *et al.* 1999, Durlinger *et al.* 2001, Durlinger *et al.* 2002a) but primordial follicle recruitment was increased resulting in the depletion of the primordial pool and early ovulation cessation leading to premature ovarian failure (Durlinger *et al.* 1999). (Durlinger *et al.* 2002a) showed that this effect could be reversed by culturing the mice ovaries with AMH. A confirmatory experiment by (Gigli *et al.* 2005), whom cultured AMH receptor type 2 null mice and wild type mice ovaries beneath chorioallantoic membrane of chick embryos to prevent normal follicular loss seen in normal cultures. They found that there was an increase in follicular growth in the AMH receptor type 2 null mice ovaries when compared to the wild type. This was further consolidated by work conducted by (Nilsson *et al.* 2007) on rats, whereby microarray analysis of mRNA transcripts showed a down regulation in expression of the stimulatory growth factors, kit ligand and KGF in the presence of AMH. This may present a negative regulatory

mechanism of AMH on the transition of primordial to primary follicles by interacting with the factors that enhance recruitment.

Although the available evidence from the literature on polyovular species supports the functional role of AMH in the suppression of primordial follicle initiation, in humans and other monovulatory species this picture is less straightforward. One study by (Carlsson *et al.* 2006b) on human tissue, found that cultured ovarian cortical samples treated with AMH showed a reduced growth in primordial follicles when compared to untreated tissue. (Gigli *et al.* 2005) also showed that AMH may play a role in suppressing primordial follicle initiation in bovine tissue. While in another study, AMH had a positive effect and caused an increase in primordial follicle growth (Schmidt *et al.* 2005). However in the latter study, the samples had been previously cryopreserved and a higher dose of AMH was used which may explain the difference in results. A study by (Campbell *et al.* 2012) on sheep also produced interesting results. They found that active immunisation against AMH to knock down its bioactivity did not affect primordial follicle recruitment. In this study the bioactivity of AMH was supressed for a period of 6 months. This short period may not have been sufficient to cause a remarkable change in the large pool of primordial follicles. Therefore, all the evidence to support the functional role of AMH in the early stages of the follicle development is still equivocal and demands further research.

In preantral follicles, studies on cultured mice follicles have shown that AMH may cause a decrease in gonadotropin dependant growth (Durlinger *et al.* 2002a, Themmen 2005), and inhibit the progress of follicles from the preantral follicle

to the antral follicle stage by causing a decrease in follicle FSH sensitivity (Durlinger *et al.* 2001, Durlinger *et al.* 2002a).

Similarly, AMH has been shown to play an inhibitory role on antral follicle growth. A number of studies on different species have been conducted in this area. All have demonstrated similar findings that the general effect of AMH on antral follicles is to decrease the sensitivity of the follicle to FSH (Diclemente *et al.* 1994, Durlinger *et al.* 2001, Grossman *et al.* 2008, Pellatt *et al.* 2011, Campbell *et al.* 2012). (Diclemente *et al.* 1994) studied rat GCs, and found that following treatment with AMH, there was a significant decrease in FSH stimulated aromatase activity. In the same paper, AMH also caused a decrease in the expression of aromatase mRNA and LH receptor mRNA in FSH stimulated porcine granulosa cells. In another study on AMH null mice, a low FSH environment still caused an increase in the number of follicles growing when compared to the wild type mice (Durlinger *et al.* 2001). In sheep active immunisation against AMH caused a decrease in the preantral and small antral follicle pool and an increase in the gonadotropic dependant antral follicles and in the rate of ovulation (Campbell *et al.* 2012). In human GCs, the production of oestradiol and aromatase mRNA expression has also been shown to be inhibited by AMH (Grossman *et al.* 2008). furthermore, (Pellatt *et al.* 2011) showed that AMH caused a decrease in human granulosa cell sensitivity to FSH through a reduction in expression of aromatase mRNA by an effect on PII promoter activity. They also found a reduction in the FSH receptor mRNA expression.

Collectively, all this research demonstrates the inhibitory role of AMH in the ovary. As small antral follicles possess the

highest concentration of AMH, this may cause steroidogenesis, FSH responsiveness and LH receptor acquisition to be held back until the point of follicle selection. As the FSH increases during the cycle, the AMH production decreases dramatically, resulting in a decline in its concentration. This allows the release of the inhibitory effect of AMH and permits the production of oestrogen (Pellatt *et al.* 2010). Therefore, this removal seems to play a crucial role in the selection of the dominant follicle and progression to ovulation (Pellatt *et al.* 2010). The factor that causes the decrease in AMH is still unclear.

1.6.3 AMH Signal Transduction Pathway

Members of the TGF-β family usually signal through a set of membrane bound receptors that have serine/threonine kinase activity. The type2 receptor, which comprises an extracellular domain, binds the ligand resulting in the formation of a tetrameric complex composed of 2 molecules of the type 2 receptor and two molecules of the type 1 receptor. The type2 receptor causes phosphorylation of transmembrane serine and threonine residues of the type 1 receptor in a region that is glycine/serine rich. This phosphorylation causes release of the kinase domain, allowing the type 1 receptor to phosphorylate cytoplasmic proteins known as SMADs which will then act as receptor substrates. A choice of several type1 and/or type 2 receptors are activated depending on the binding ligand, which then funnels their activity through one of the two groups of Smad proteins. In general, activins, nodals and TGF-βs employ receptors that phosphorylate SMADs 2 and 3. While BMPs and GDFs, engage receptors that signal through SMADs 1, 5 and 8. Phosphorylated SMADs translocate into the nucleus, binding on their way a common SMAD 4 protein that

acts as a transcriptional activator (Josso & Clemente 2003) (Figure 1.6).

Once the SMAD complex enters the nucleus, they bind to the promoter of the target genes. This interaction is not selective whereby both activin/TGF-β/nodal pathways and the BMP pathways all recognize the same sequence, CAGAC (Shi *et al.* 1998). This interaction is also of low affinity and therefore necessitates that DNA binding cofactors be involved to provide a tight and highly specific recognition of regulatory elements in target genes (Massague & Wotton 2000, Chen *et al.* 2002).

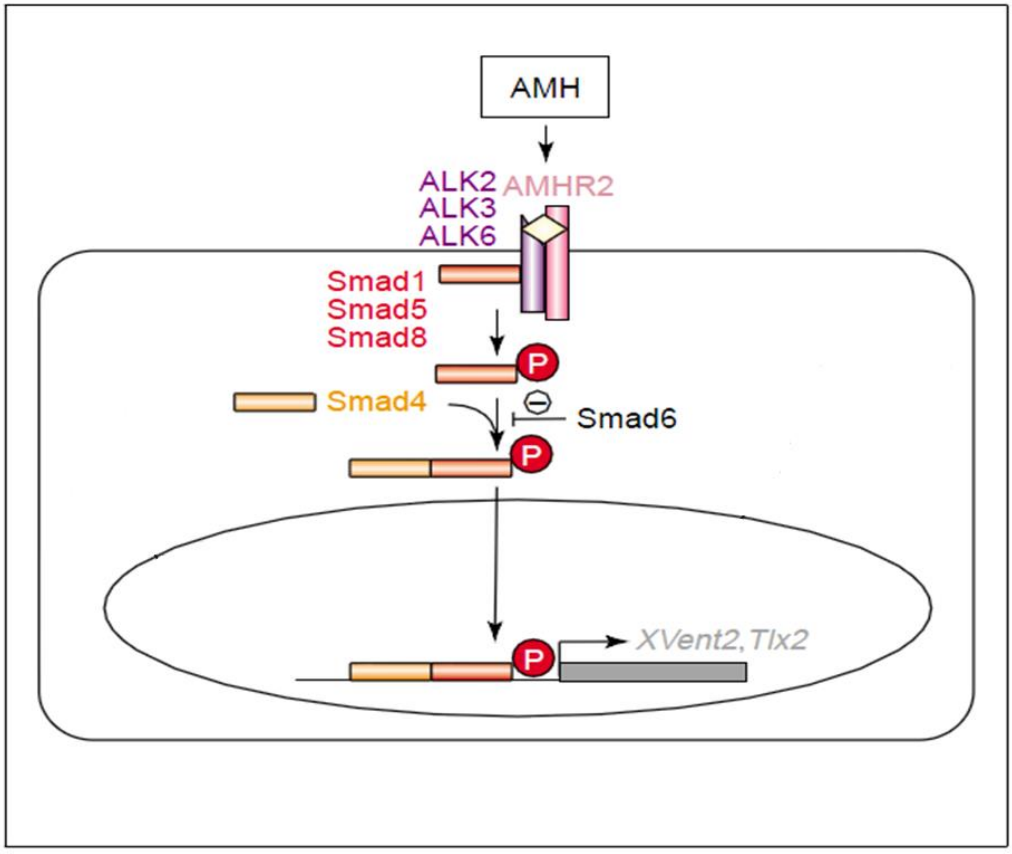


Figure 1.6 AMH signal transduction pathway.
Adapted from (Josso & Clemente 2003).

1.6.4 AMH Receptor Type 2

The AMH receptor type 2 (AMHR2) gene was first cloned in 1994 by two independent groups. The first was a Dutch groups (Baarends *et al.* 1994) that isolated a cDNA clone (C14), from a subtracted rat sertoli cell library. The fact that this clone was structurally similar to other serine/threonine receptors of the TGF-β family, and its expression pattern that correlated with the expected sites of AMH action, drew the attention of the researchers to the possibility of this clone encodes the AMH receptor. In the same year, (di Clemente *et al.* 1994) also cloned the AMHR2 using a degenerate probe that encoded serine/threonine kinase consensus sequence of the transforming growth factor-beta receptor superfamily to screen a fetal rabbit ovarian library. In 1995, the human AMH receptor type 2 was cloned and mapped (Imbeaud *et al.* 1995). This group also provided genetic evidence indicating that AMHR2 gene is required for Mullerian regression (AMH signalling) in human males by identifying a mutation in the AMH receptor of a patient with persistent mullerian duct syndrome.

The AMHR2 has been mapped on chromosome 12q13 and is 8kbp long and is distributed over 11 exons, each encoding a specific part of the receptor (Imbeaud *et al.* 1995). Exons 1 to 3 encode the signal sequence and the extracellular domain, exon 4 resembles the transmembrane domain, and exons 5 to 11 encode the intracellular domain (Imbeaud *et al.* 1995) (Figure 1.7).

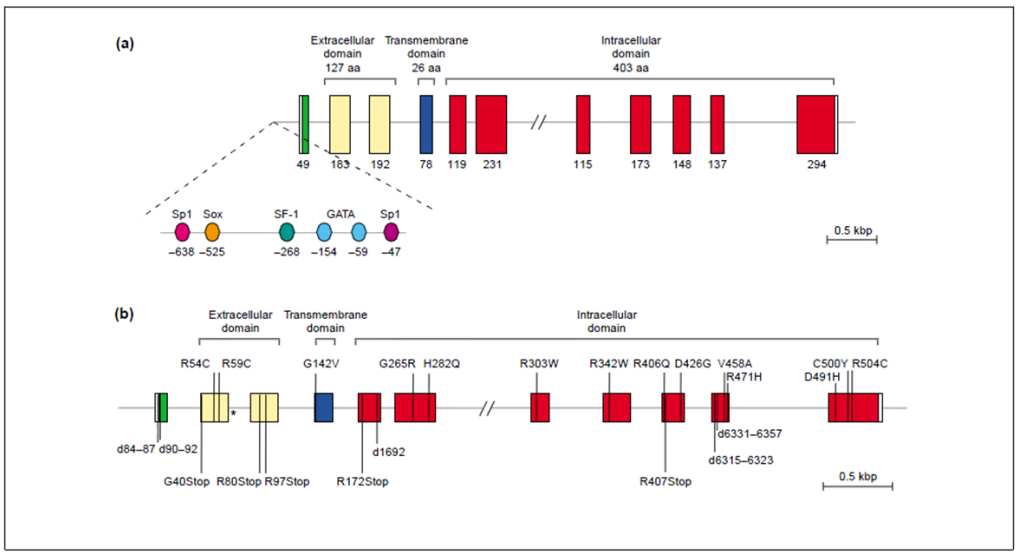


Figure 1.7 The AMH receptor type 2.
The human AMHR2 contains 11 exons spread over 8kbp (a). Mutations in AMHR2 lead to the persistent Mullerian duct syndrome (b) (Josso & Clemente 2003).

The human AMHR2 protein is 573 amino long and in its mature wild type form has a mass of 82kDa (Faure *et al.* 1996). It is divided into an extracellular domain that binds the ligand and has two potential glycosylated sites, a single short transmembrane domain and an intracellular domain that has serine/threonine kinase enzymatic activity (Faure *et al.* 1996).

Unlike other members of the TGF-β family that have numerous functions and hence multiple target organs, the AMHR2 is only expressed in the reproductive tract where AMH causes its biological effects. The receptor is expressed in the mullerian ducts of both male and female embryos (Baarends *et al.* 1994). This expression is seen in the mesenchyme but not in the epithelium (Baarends *et al.* 1994, di Clemente *et al.* 1994), as it has been shown that it is through the mesenchyme that AMH acts to kill or dedifferentiate the mullerian epithelial cells (Tsuji *et al.* 1992). Unexpectedly, the

mullerian duct continues to express the receptor even after it becomes resistant to AMH (Baarends *et al.* 1994), and it has been shown that this receptor is expressed in the normal (Arango *et al.* 1999) and the gravid uterus (Teixeira *et al.* 1996a). In the GCs, the sertoli cells, the cell line AT29C derived from GCs (Dutertre *et al.* 2001), and the SMAT-1 cell line derived from sertoli cells (Dutertre *et al.* 1997), the AMH and the AMHR2 are coexpressed (Josso & Clemente 2003). In the adult rat testis, the seminiferous tubes in the VII stage of their cycle showed the highest level of expression of the AMHR2, which corresponds with the time of greatest ligand expression (Baarends *et al.* 1995a). While, in the leydig cells, AMHR2 alone was found to be expressed (Racine *et al.* 1998). In the rat ovary, AMH and AMHR2 were both expressed in small growing follicles mainly in GCs (Baarends *et al.* 1995b). However, in monovular species this is less clear and will be discussed in greater detail in further chapters. AMHR2 was also shown to be expressed in granulosa like tumour cells also known as KGNs (Pellatt *et al.* 2011) but its expression was also reported in other cell lines derived from breast (Segev *et al.* 2000, Segev *et al.* 2001), prostate (Segev *et al.* 2002) and human ovarian cancer (Masiakos *et al.* 1999, Ha *et al.* 2000). However in these later group, AMHR2 expression was very low and its size (63-65kDa) differed from the mature form (82kDa) (Faure *et al.* 1996) previously described.

In order to elucidate the follicular stage at which AMH starts to perform its action, studies have looked at the existence of the AMHR2 in different stages of follicular development. (Rice *et al.* 2007) measured the mRNA expression of the receptor in human preantral follicles and found that it was rarely expressed. These findings may challenge the hypothesis of a

direct inhibitory effect of AMH on the early stages of folliculogenesis. However, in this study only mRNA was measured which does not prove the absence of the protein. On the other hand in rodents, using in situ hyberdisation AMHR2 expression was seen in preantral follicles but not in primordial follicles (Baarends *et al.* 1995b). This plus the effect AMH has on preantral follicular growth seen by (McGee *et al.* 2001) in rats, shows that AMH exerts its action through its receptor in these small follicles. Therefore, all these results may indicate to a species difference in the pattern of expression of the AMHR2.

AMHR2 is expressed in the rat GCs at the preantral and small antral follicular stages but this expression diminishes in the GCs of large antral follicles and preovulatory follicles (Baarends *et al.* 1995b, Teixeira *et al.* 1996b). The corpus luteum was found to produce very little of the receptor while no expression was seen from the oocyte and primordial follicles (Baarends *et al.* 1995b). AMHR2 is expressed in very low levels in atretic follicles. This is lost eventually but seems to persist longer than AMH mRNA expression (Baarends *et al*. 1995). In rodents, it has also been found that this receptor is expressed in the TCs mainly from preantral and small antral follicles but unlike in the GCs it continues to be expressed in antral and early atretic follicles (Baarends *et al.* 1995b, Ingraham *et al.* 2000).

1.6.5 AMH Receptor Type 1

Since AMH is a member of the TGF-β family it has been assumed that it signals through two serine/threonine kinase receptors. However, numerous fruitless attempts to clone an AMH specific type 1 receptor have failed. Which then lead

researchers to suggest that AMH may share one of the known type 1 receptors of the same family. Because, AMH activates the SMAD 1-5-8 pathway (Gouédard *et al.* 2000, Clarke *et al.* 2001, Visser *et al.* 2001), this lead scientists to point towards the type 1 receptor of the BMPs and GDFs which use the same pathway, unlike the other possibilities, the TGF-β and activin, that signal through the SMAD2 and 3 (Clarke *et al.* 2001). Another important finding was that AMH upregulated SMAD6, the BMP specific inhibitory SMAD (Clarke *et al.* 2001). Three BMP type 1 receptors have been considered, activin like kinase (ALK)2 also known as ActR1, ALK3 also known as BMPR1-A and ALK6 also known as BMPR1-B. Several criteria were studied to determine which of these type 1 receptors AMH uses. These included studying the ability of the type 1 receptor to interact with the AMHR2, its expression in the AMH target organs, its ability to repress reporter genes by dominant negative isoforms, and the induced defects caused by its disruption or mutation *in vivo* (di Clemente *et al.* 2003). However, all the research conducted in the area remains inconclusive and the identity of the AMHR1 is still unclear, especially in the gonads. The possibility proposed is that AMH may have access to different type 1 receptors and depending on the condition and availability of accessory molecules will determine which given type 1 receptor is used in a particular cell type (di Clemente *et al.* 2003).

1.6.6 AMH SMAD Pathway

The AMH SMAD pathway was first identified in two testicular cell lines, the SMAT-1 cells that originate from sertoli cells and the MA-10 cells that originate from leydig cells. Both cell lines were sensitive to AMH and expressed the AMHR2. AMH caused the suppression of the AMHR2 and P450 side chain cleavage

mRNAs (Messika-Zeitoun *et al.* 2001). Experiments on cell lysates indicated that like BMP2, AMH induced specific phosphorylation of SMAD1, resulted in SMAD1/SMAD4 co-precipitation and the nuclear translocation of SMAD1 (Gouédard *et al.* 2000). Further, using P19 cells, AMH treatment caused the activation of a specific reporter gene for the SMAD1 pathway, while it had little activity on reporter genes for the SMAD2 pathway indicating that it does not use TGF-β or activin pathways for signalling (Gouédard *et al.* 2000). Using a cell line cloned form a mouse ovarian tumour, the AMH activation of the SMAD1 pathway was confirmed in GCs (Dutertre *et al.* 1997, Dutertre *et al.* 2001). The drawback of these studies is that the antibodies used in these experiments were unable to differentiate between SMAD1, 5 or 8 and therefore unable to determine which of these was activated by AMH in testicular and ovarian cell lines used. However, a recent publication has indicated that SMAD1 and SMAD5 are the main SMAD proteins used by AMH in mice GCs (Sedes *et al.* 2013). They also found that the most important type 1 receptor used by AMH in GCs is the BMPR-1A (Sedes *et al.* 2013).

1.7 Androgens

Researchers in recent years have had an increased interest in the role of androgens in follicular development regulation and female fertility. This has resulted in a considerable change in the overall understanding of the effect of androgens in the reproductive field over the last few years. Originally, androgens were considered to be detrimental to ovarian function and were frequently associated with infertility. However, with advancements in research techniques including

in vitro and *in vivo* studies, the development of different androgen receptor (AR) knockout models, and increasing clinical trials, has resulted in better understanding of the physiology of androgen actions and its necessity for normal follicular development and function (Prizant *et al.* 2014).

Excess androgens result in enhanced follicular development and the formation of dysfunctional antral follicles is associated with the development of PCOS. While, diminished androgen levels are associated with abnormal follicle growth, low ovarian reserve and primary ovarian insufficiency. Therefore, researchers now believe that a critical balance between the necessity of androgens in normal ovarian function and the harmful effects of androgens in excess androgenic conditions is vital for regulation of female fertility (Prizant *et al.* 2014).

1.7.1 Androgen Synthesis in the Ovary

In the ovary, androgens are primarily produced from cholesterol in a sequential manner along with other sex steroids, progestogens and oestrogens, whereby each steroid is used as a substrate for a subsequent one in a cascade of events known as steroidogenesis (Hillier *et al.* 1994, Palermo 2007, Miller & Auchus 2011). In the classical two cell two hormone model which explains the role of the follicular cells (granulosa and theca) and the two gonadotropins (FSH and LH) in the synthesis and secretion of steroids in the ovary. Briefly, androstenedione is synthesised from TCs progestogens, which is then converted to testosterone through the action of the enzyme 17β-hydroxysteroid dehydrogenase under the influence of LH. The produced androgen is then transported through the basement membrane to the GCs to be converted to oestrogen under FSH stimulation through the

action of the aromatase enzyme (Hillier *et al.* 1994) (Figure 1.8).

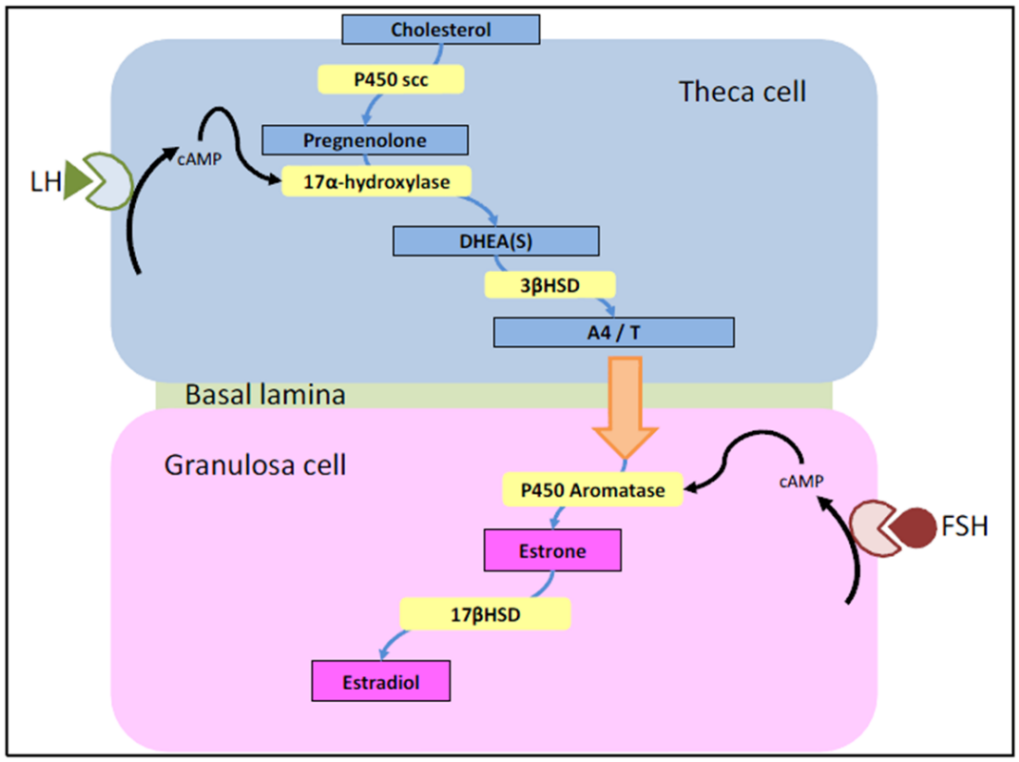


Figure 1.8 The two cells (granulosa and theca) and the two gonadotropins (FSH and LH) model.
Adapted from (Young & McNeilly 2010).

There are four major endogenous androgens, testosterone (T4), androstenedione (A4), dihydrotestosterone (DHT), and dehydroepiandrosterone (DHEA). DHT is considered the most potent against the androgen receptor but is non aromatisable. T and A4 are second and third in potency and are both aromatisable. While DHEA is the weakest and is also non aromatisable (Rang *et al.* 2003).

Since androgens are converted to oestradiol in the GCs, many of their actions on follicular growth and differentiation can be mediated indirectly by their action as precursors in the oestrogens biosynthesis pathway. However, little is known

about the involvement of androgens in the regulation and maintenance of folliculogenesis when they interact with their specific receptor (Gervasio *et al.* 2014).

1.7.2 Expression of the Androgen Receptor in the Ovary

The AR is considered a member of the nuclear and steroid receptor superfamily which also includes the mineralocorticoids, glucocorticoids, progesterone, and oestrogen receptors (Montgomery *et al.* 2001, Heinlein & Chang 2002a). They are also classified as class I receptors as part of the larger nuclear receptor superfamily in which thyroid hormone receptors, retinoic acid receptors, peroxisome proliferator-activated receptors and vitamin D receptors are part of (Detera-Wadleigh & Fanning 1994).

The AR protein and mRNA have been found in the ovary of various species, such as the rodent (Tan *et al.* 1988, Hirai *et al.* 1994), sheep (Juengel *et al.* 2006a), cattle (Hampton *et al.* 2004), swine (Cárdenas & Pope 2002), non-human primates (Hild-Petito *et al.* 1991), and humans (Chadha *et al.* 1994) (Horie *et al.* 1992). Androgen receptor expression is mostly found in GCs when compared to other ovarian cell types (theca and ovarian stromal cells) (Lenie & Smitz 2009). While in oocytes, there was a species difference in expression, were amphibians expression is high, rodents moderate, ruminants little and incipient or absent in primates and humans (Gervasio *et al.* 2014).

1.7.3 Mechanism of Action of Androgens

Similar to other steroid hormones, androgens exert their primary function through specific nuclear receptor binding and activation which results in the initiation of intracellular events

that are responsible for transcription of target genes (Mangelsdorf *et al.* 1995, Rosenfeld *et al.* 2006). Without ligand the receptor is located in the cytoplasm (He *et al.* 2000, Loy *et al.* 2003). Upon binding the ligand, the AR undergoes changes that affects AR protein and AR DNA interactions, and nuclear shuttling (Bennett *et al.* 2010). More recently, androgens have been shown to have another mode of action. They have been found to exert their effects through interactions with receptors on the cell membrane resulting in rapid non genomic functions that are involved in the activation of different transcription factors (Kousteni *et al.* 2001, Lösel *et al.* 2003, Lutz *et al.* 2003, White *et al.* 2005, Li *et al.* 2009). Therefore, once the AR is activated it may regulate the expression of a certain group of genes through direct or indirect interactions with the regions of upstream elements (Quigley *et al.* 1995, Gelmann 2002, Heinlein & Chang 2002b).

While many autocrine and/or paracrine factors associated with the regulation of the ovarian follicle development have been defined (Eppig 2001, Matzuk *et al.* 2002, Thomas & Vanderhyden 2006), only little is known about the AR targeted genes linked with the transcription of these factors. Of the particularly important genes that have been tested as AR targets include the ones related to FSH receptors, IGF 1, and the aromatase enzyme (Vendola *et al.* 1999a, Vendola *et al.* 1999c, Weil *et al.* 1999b, Wu *et al.* 2011).

1.7.4 The Role of Androgens in the Ovary

Androgens have been found to have a fundamental role in follicle maturation and ovulation by either enhancing or

inhibiting steroidogenesis in the GCs depending on the stage of follicular development (Cheng *et al.* 2002).

In primary and preantral follicles, aromatase activity is low while AR expression is high (Cheng *et al.* 2002). It is proposed that androgens that have diffused from TCs to GCs activate the AR, resulting in increased expression of IGF-1R, enhanced GCs proliferation, and increasing the effect of FSH on aromatase activity (Vendola *et al.* 1999c, Cheng *et al.* 2002). Therefore, suggesting an essential role for androgens in these early stages of follicular maturation. In rodents and primates, androgen receptor mRNA and protein were found in high concentrations in ovaries which mostly contained preantral and early antral follicles (Tetsuka *et al.* 1995, Weil *et al.* 1998). It was also found that in bovine follicles, testosterone treatment stimulated the transition from primary to secondary follicles (Yang & Fortune 2006), further extending this idea. *In vitro* studies on cultured whole mouse follicles with androgens lead to increased immature follicle diameter and preantral follicle development (Murray *et al.* 1998a, Sen *et al.* 2014).

Moreover, the importance of androgens during the early stages of follicle maturation was further observed by treating mouse follicles *in vitro* with anti-androgenic compounds. This resulted in a reduction in follicular growth during the preantral phase and altered steroidogenesis (Lenie & Smitz 2009). Studies on androgen receptor knockout mouse models provided further clarity to the importance of androgens and androgen receptors in normal follicular maturation (Sen & Hammes 2010). They found that GC specific androgen receptor knockout mice were sub-fertile, had premature ovarian failure, and had less ovulated oocytes and longer oestrous cycles. Ovaries from these mice also had more

preantral and atretic follicles and less antral follicles and corpus lutea (Sen & Hammes 2010).

On the contrary, as the follicle progresses, AR expression decreases (Lenie & Smitz 2009) leading to the conversion of the follicle from an androgenic environment to an oestrogenic environment which is important for the development of the follicle from preantral to antral and preovulatory stages (Cheng *et al.* 2002). This increase in circulating oestradiol will exert a positive feedback regulation resulting in the induction of the LH surge and subsequently ovulation. Therefore, the reduction in AR expression is crucial for follicular survival as continued AR expression will result in atresia due to follicles not being able to convert to oestrogen dominance (Cheng *et al.* 2002).

Recently, evidence has arisen that point towards the involvement of androgens in the activation of primordial follicles. However, this theory has only been suggested by a few studies (Abbott *et al.* 2005, Smith *et al.* 2009, Magamage *et al.* 2011) which were criticised due to the finding of a normal number of primordial follicles in granulosa specific ARKO mouse models. It has therefore been speculated that androgens may not be crucial for the activation of primordial follicles but may promote it (Prizant *et al.* 2014). Many questions are yet to be answered on whether or how androgens may affect primordial follicle recruitment necessitating further research in this area.

Despite the vast number of conducted studies, the question still remains whether the reported actions of androgens are the consequence of a direct androgen action through its receptor or as a result of aromatization of androgens to

oestrogen, which makes this an attractive area for further research.

1.8 The involvement of AMH and Androgens in PCOS

1.8.1 Overview

PCOS is one of the most common endocrine disorders among females in their reproductive age. The prevalence of PCOS has been reported to be anywhere from 4% to 12%, with mild racial variations. PCOS is a complex, heterogeneous disorder, both clinically and biochemically (Chang 2004).

This complex disorder which was originally described by Stein and Leventhal's case reports in 1935 as a reproductive disorder of a collection of clinical symptoms which included amenorrhea, infertility, obesity, hirsutism and polycystic appearing ovaries (Stein & Leventhal 1935), has since evolved into a syndrome that we now understand to affect multiple organs including the cardiovascular, metabolic and endocrine systems (Teede *et al.* 2010).

1.8.2 AMH and PCOS

Patients with PCOS have a two to three fold increase in the number of follicles. This increase is mainly in the early stages of follicular growth, starting from the time they start growing to when they reach the antral stage at a size of around 2-5mm (Hughesdon 1982, Webber *et al.* 2003). In PCOS, follicles that reach 4-7mm also stop growing and developing (Dewailly *et al.* 2003) which may point to a defect in the maturation and selection of the dominant follicle. As previously discussed AMH may play a role in altering early

follicle growth making it a possible attributing candidate for the change seen in PCOS. Further, the ability of AMH to alter early follicle growth was investigated by determining the expression levels of AMH in stained sections of polycystic ovaries from anovulatory and ovulatory women using immunostaining (Stubbs *et al.* 2005). They found that anovulatory ovaries contained significantly lower positively stained primordial and transitional follicles when compared to normal and ovulatory polycystic ovaries. However, in preantral and antral follicles, the intensity of staining was found to be similar in all three groups (Stubbs *et al.* 2005). The suggestion was that in the early stages of development, the attenuated AMH expression in anovulatory polycystic ovaries may allow for an increasing number of follicles to start growth and it is not until theca cells appear that the increasing level of AMH expression is seen. However, until the factors that control AMH production are identified and studied this can only remain speculations and demands further research. Although the outcomes of Stubbs *et al.* (2005) study may need further investigating to confirm the immune expression levels due to signal quantification issues. However these results may provide important findings that are related to work that will be presented in this project, as the universal belief that AMH only functions in an autocrine nature may be inaccurate and potentially have a more extended paracrine function which in this case is to affect theca cells, forming a local regulatory loop between GC and TC.

According to (Fallat *et al.* 1997), AMH in women with PCOS increases two to three fold when compared to women with normal ovaries. At first this expression was speculated to be caused by the vast increase in small antral follicle number but

studies by (Pellatt *et al.* 2007), showed that AMH production per GC was 75 times higher in anovulatory polycystic ovaries when compared to normal ovaries. AMH concentration in follicular fluid from follicles of anovulatory PCOS women was also found to be 5 times higher than in follicles from ovulatory women (Das *et al.* 2008). Another study also showed that the serum concentration of AMH corresponded to the severity of PCOS symptoms and again ovulatory women had lower AMH levels when compared to anovulatory women (Piouka *et al.* 2009). In this study, the increase in the number of follicles only contributed 5.3% to the variance in AMH concentration. Another study that found a higher level of AMH mRNA in GCs from polycystic ovaries even following stimulation may also strengthen the evidence that the increased production of AMH seen in PCOS is an intrinsic property of GCs (Catteau-Jonard *et al.* 2008). Collectively, these studies have shown that the increase in the concentration of AMH may largely be due to the increase in the production of individual follicles and not only due to the raise in follicle number. The cause of increased AMH production in PCOS is still poorly understood and is therefore an area of interest for future work.

As formerly illustrated AMH has an inhibitory function in the ovary and results show that its down regulation seems to be crucial for follicle maturation and dominant follicle selection. It is therefore possible that in order for anovulatory polycystic ovaries to start ovulating, AMH production in GCs would have to be greatly reduced (Pellatt *et al.* 2010). The factor that causes the decrease in AMH is still unclear. Therefore, understanding the mechanism that regulate and control AMH in the dominant follicle is vital, making it a research priority.

1.8.3 Androgens and PCOS

Hyperandrogenemia has been described as one of the intraovarian factors characterising PCOS. It is mainly due to excessive production of androgens by the ovaries, with contribution from the adrenals and to a lesser extent from the adipose tissue (Qiao & Feng 2011). PCOS follicles are hyperandrogenic due to the raised biosynthesis of androgens by TCs (Nelson *et al.* 2001, Dumesic *et al.* 2008). Since PCOS is a heterogeneous ovarian syndrome and not all PCOS patients are hyperandrogenic. Therefore, hyperandrogenemia cannot be the only aetiology of PCOS (Qiao & Feng 2011). Animal model studies in mice have shown that dehydroepiandrosteron (DHEA) treatment induces PCOS phenotypes in ovaries that were previously normal (Luchetti *et al.* 2004, Sander *et al.* 2006). Recently, this has also been demonstrated in humans, where long term treatment with DHEA resulted in an increase in antral follicle count even in decreased ovarian reserve patients. (Barad & Gleicher 2005).

In order to further understand the relationship between PCOS and hyperandrogenimia to elucidate the molecular mechanisms behind it, (Yang *et al.* 2010) treated cultured neonatal mouse ovaries that contained mainly primordial follicles with testosterone to induce a mouse PCOS model. They found an increase in the primary follicles, and they showed that androgens induced factors which are likely to be a key step in the activation of primordial follicles. However, in this study testosterone also caused a down regulation in the expression of GDF9, which in this model led to follicular arrest. Therefore, androgens again appear to have a positive and a negative effect on the follicle development and maturation. The heterogeneity seen in human PCOS phenotypes is

potentially the principal reason why until now it has been difficult to isolate these two opposing effect in these patients. Understanding when and how androgens effect follicular development and their inter-ovarian signalling pathways may help customise therapeutic interventions in clinical practice to target these isolated effects.

PCOS women with increased levels of testosterone and/or LH have been associated with high levels of AMH and are suggested to be the cause of impaired follicle/oocyte development and embryo quality (Dumesic *et al.* 2008, Franks *et al.* 2008, Patel & Carr 2008). The extent of whether intra-follicular AMH levels reflects the quality of the follicle maturation is still highly debated (Qiao & Feng 2011) and demands further investigation.

1.9 Vascular endothelial growth factor

1.9.1 Overview

As previously discussed normal folliculogenesis involves an integrated harmony of a myriad of systemic and local ovarian factors (Webb *et al.* 1999a). While, pituitary gonadotrophins are considered the primary regulators of folliculogenesis, local factor are essential in modulating the actions of these pituitary hormone starting from follicular recruitment, through selection, dominance and ending with ovulation (Webb *et al.* 1999a).

Ovarian angiogenesis has been extensively studied throughout the literature and collectively researches have shown that angiogenesis is of vital importance in folliculogenesis and follicle selection (Stouffer *et al.* 2001, Wulff *et al.* 2001, Zimmermann *et al.* 2001, Danforth *et al.* 2003, Fraser 2006,

Taylor *et al.* 2007). VEGF, a potent ovarian angiogenic factor has been suggested to have a vital role in folliculogenesis by increasing delivery of hormones, fluids, growth factors and nutrition to the dominant follicle to support its development (Zimmermann *et al.* 2001). Angiogenesis is considered essential since dominant follicles were found to have increased vascularisation when compared to other smaller antral follicles (Zelezni *et al.* 1981). Similarly, following ovulation the luteinised granulosa cells showed increased vascularisation to accommodate the increasing demand for cholesterol that is carried by low density lipoproteins which is needed in large quantities for the production of progesterone in the Corpus luteum (Carr *et al.* 1983).

VEGF-A and its receptors have been studied in numerous species including cattle, pigs, and humans (Ravindranath *et al.* 1992, Kamat *et al.* 1995, Barboni *et al.* 2000, Greenaway *et al.* 2004, Yang & Fortune 2007) and were found to be expressed in the GCs of developing follicles , varying in the degree of expression depending on the stage of follicular development and increasing in the dominant follicle. Further, following the LH surge GCs VEGF expression and follicular fluid VEGF protein were both found to be increased (Anasti *et al.* 1998). Studies on rats (Danforth *et al.* 2003) and cattle (Yang & Fortune 2007) have also shown that treatment with VEGF may promote follicular development. On the other hand, restricting follicular angiogenesis using VEGF trap significantly compromised follicular development (Wulff *et al.* 2001). Similarly, treatment with a VEGF antagonist (Hazzard *et al.* 2002) or an antibody to VEGF (Fraser *et al.* 2000) resulted in impaired ovulation and the subsequent development and functional capacity of the corpus luteum.

1.9.2 VEGF Protein

VEGF, also known as vascular permeability factor (VPF), is a glycoprotein dimer (34-42kd) that can enhance vascular permeability and has potent mitogenic activity on endothelial cells (Gospodarowicz *et al.* 1989, Keck *et al.* 1989, Leung *et al.* 1989). Initially VEGF was identified as a product secreted by tumour cell that could induce vascular leakage (Senger *et al.* 1983). It was not until the late 80s that VEGFs molecular structure was identified and subsequently showing that it had the ability to induce proliferation migration and survival of endothelial cells (Ferrara & Henzel 1989).

After extensive research extending over many years, researchers have found that VEGF signalling is considered a crucial step in a series of extremely complex coordinated process that necessitates sequential activation of multiple receptors that can result in mitogenesis, endothelial cell survival, mobilisation of bone marrow endothelial progenitors into the circulation, cell differentiation and migration, and increased vascular permeability (Ferrara *et al.* 2003).

VEGF has been found to be overexpressed in many physiological process necessitating angiogenesis and stromal generation, including ovarian folliculogenesis (Kamat *et al.* 1995), corpus luteum formation (Ravindranath *et al.* 1992), placentation and implantation (Stouffer *et al.* 2001), and wound healing (Brown *et al.* 1992). It is also amply expressed in many pathological conditions including tumours (Senger *et al.* 1986, Brown *et al.* 1995), rheumatoid arthritis (Koch *et al.* 1994) and retinopathy (Aiello *et al.* 1994). Moreover, VEGF mRNA has been found to be expressed in normal tissue throughout the body including adrenal glands, heart, kidney,

lung, and activated macrophage (Berse *et al.* 1992, Kamat *et al.* 1995).

Members of the VEGF family include, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PIGF) (Ferrara *et al.* 2003, Tammela *et al.* 2005). The VEGF-A gene is composed of 8 exons and through alternative splicing of the gene, various isoforms are formed which are named based of the number of amino acids in the VEGF protein chain (Tischer *et al.* 1991). Many isoforms of the VEGF–A have been identified in humans (Cui *et al.* 2004). Initially, four were identified $VEGF_{121,}$ $VEGF_{165}$ $VEGF_{189}$ $VEGF_{206}$, however, less frequent splice variants have also been described such as $VEGF_{145}$ and $VEGF_{183}$ (Ferrara *et al.* 2003). $VEGF_{121}$ is a freely diffusible isoform, while $VEGF_{189}$ and $VEGF_{206}$ are bound to the extracellular matrix. On the other hand, $VEGF_{165}$ has intermediate properties, it can be found in both the soluble and sequestered forms (Ferrara *et al.* 2003). $VEGF_{121}$ and $VEGF_{165}$ are considered the most abundant isoform found in the ovary (Greenaway *et al.* 2004, Marsters *et al.* 2014). In other species, VEGF isoforms have been found to have one amino acid less, due to a single amino acid deletion in the N-terminal region, when compared to humans. In sheep, $VEGF_{120}$ and $VEGF_{164}$ are equivalent to human $VEGF_{121}$ and $VEGF_{165}$ respectively (Redmer *et al.* 1996).

The expression of VEGF-A isoforms are suggested to be different depending on the type of tissue (Ferrara 2000). Little is known about the mechanisms that control the expression of any particular isoform (Rosales-Torres *et al.* 2010). In the ovary, progesterone and oestradiol have been found to regulate differential splicing of VEGF. Progesterone inhibited $VEGF_{164}$ expression but increased $VEGF_{120}$ expression (Shimizu

& Miyamoto 2007). While oestrogen, increased $VEGF_{120}$ expression without affecting $VEGF_{164}$ expression (Shimizu *et al.* 2007b). This suggests that not only are the various isoforms secreted differently depending on the expressing organ but may, at least in the ovary, be related to the stage of follicular development (Rosales-Torres *et al.* 2010).

VEGF-B and VEGF-C have also been found to be expressed in the ovary, however, they are thought to function differently to VEGF-A (Laitinen *et al.* 1997, Stouffer *et al.* 2001).

1.9.3 VEGF Receptors

VEGF can bind to two related tyrosine kinase receptors to mediate its biological effect, VEGFR-1 (also known as Flt1) and VEGFR-2 (also known as kinase domain region (KDR) or Flk-1), which have very different signalling properties (Ferrara *et al.* 2003). VEGFR-3 is also a member of the RTK family but does not bind VEGFR-A, instead it binds to VEGF-C and VEGF-D (Karkkainen *et al.* 2002). VEGF-A can also interact with another family of receptors, the neuropilins (Nrp-1 and Nrp-2) (Tammela *et al.* 2005).

The VEGF family members bind VEGF receptors with varying specificities (Hicklin & Ellis 2005). While all VEGF-A isoforms utilise both VEGFR-1 and VEGFR-2, the main signalling pathway of VEGF-A in endothelial cells and other tissue types is through the VEGFR-2 (Bates 2010). VEGF-A has minor signalling activity when bound to VEGFR-1 (Carmeliet *et al.* 2001). Therefore in normal circumstances, VEGFR-2 activity may be regulated by competitive binding of VEGF to the VEGFR-1 to prevent any unwanted activation. This fine-tuned regulation may become disrupted resulting in pathological conditions such as PCOS (Carmeliet *et al.* 2001).

1.9.4 Regulation of Ovarian VEGF

Many factors have been shown to modulate VEGF expression levels including oxygen tension, and systemic and local endocrine factors and ageing (Hazzard & Stouffer 2000).

Angiogenesis has been found to be induced by hypoxia in both normal and pathological tissue. Exposure of GCs *in vitro* to hypoxia resulted in an increase in VEGF production two to four folds (Hazzard & Stouffer 2000). Recently, Marsters and colleagues also showed that GCs grown under hypoxic conditions either by culturing cells in high density or by using hypoxia inducing agent resulted in enhancing VEGF expression (Marsters *et al.* 2014). However it is still unclear whether oxygen tension is considered a primary regulator of angiogenesis and VEGF expression (Hazzard & Stouffer 2000).

Gonadotrophins are considered major regulators of ovarian angiogenesis and VEGF expression, especially LH (Hazzard & Stouffer 2000). Around the time of ovulation, at the time of the LH surge or following hCG treatment, GCs undergo neovascularisation and increased VEGF production (Hazzard *et al.* 1999). This is consistent with other publications which found similar results (Anasti *et al.* 1998). VEGF expression was also shown to be increased in bovine GCs following FSH treatment (Shimizu *et al.* 2007b). The effect of gonadotrophins extends beyond the time of ovulation, since studies on monkeys treated with GnRH antagonists resulted in a reduction in VEGF expression in the corpus luteum (Ravindranath *et al.* 1992). Already illustrated, oestradiol and progesterone have also been shown to influence VEGF expression and production (Shimizu *et al.* 2007b, Shimizu & Miyamoto 2007).

As discussed earlier, AMH has been shown to be an important local intra ovarian factor that has an inhibitory role in folliculogenesis. A single study has addressed the effect of VEGF on AMH expression (Thomas *et al.* 2007). While this will be discussed below in more detail, it was suggested that a direct link may exists between VEGF and AMH. Further research is necessary to consolidate this finding. Therefore in this study we aim to address this point and hopefully reach a better understanding of the effect of VEGF on AMH and folliculogenesis.

1.10 The SOX Gene Family

1.10.1 Overview

The first member of the SOX gene family identified was the male sex determination gene SRY (Sex determining region Y). After many years of research, scientists classified SRY as part of a larger gene family named SOX (SRY-related box) (Gubbay *et al.* 1990). The SOX proteins belong to the high mobility group (HMG) superfamily. This superfamily is divided into two subfamilies that have different characteristics. The TCF/SOX/MATA family members bind specific sequences of DNA, while the HMG/UBF proteins non-specifically bind DNA (Soullier *et al.* 1999). The three groups of the TCF/SOX/MATA family are separated based on the analysis of sequences of the HMG domains (Soullier *et al.* 1999). Members of the Sox family are considered transcriptional facts that bind the DNA sequence (A/T)(A/T)CAA(A/T)G (Kiefer 2007). Some are thought to be activators or repressors of transcription, while others have been found to lack transactivation domains. Based on this phylogenetic analysis they are subdivided into groups

from A to H (Bowles *et al.* 2000). The SOX proteins also depend on mandatory partners for target specificity (Kamachi *et al.* 2000, Koopman 2005). Like other members of the HMG subfamily, SOX attaches to the minor groove of DNA, causing conformational changes that bring gene promoters and enhancers closer to each other and thereby permitting their functional interaction(Kiefer 2007). These genes are expressed in many tissues and regulate a vast number of developmental events throughout the body (Bowles *et al.* 2000), and are therefore implemented in the aetiology of many diseases and cancers (Dong *et al.* 2004).

One of the many functions of the SOX gene is to regulate testicular development. The mammalian embryo possesses gonadal precursors that have equal ability to differentiate into either testis or ovary (Koopman 2005). From the bipotential precursors in the normal XY gonad primordium, the first male cells to appear are the sertoli cells. These coordinate many testis cells development, making them vital in the determination of the male sex (Kiefer 2007).

1.10.2 SRY (Sex Determining Region Y)

SRY, the testis determination factor, is essential in the development of the testis. In its absence female genitalia develop. Further, its ectopic expression was found to be sufficient to prompt testis development in XX transgenic mice (Berta *et al.* 1990, Koopman 2005). Sertoli cell precursors transiently express the SRY gene, which initiates the male determination process by inducing their differentiation into sertoli cells (Polanco & Koopman 2007). Very little is known about how SRY is regulated. No genes have been identified that target the SRY protein and the mechanism which

regulates its developmental functions are still vague (Polanco & Koopman 2007). The difficulty in identifying targets and SRY protein partners is due to the fact that the protein is expressed for short periods of time and from a small number of cells (Wilhelm *et al.* 2005). SRY's main and maybe only function is to activate SOX9, which starts the male differentiation pathway (Vidal *et al.* 2001, Jiang *et al.* 2013).

1.10.3 SOX9

SOX9 has a similar function to SRY, making it another important factor in testis determination. Shortly after SRY, it too is expressed from sertoli precursor cells (Kiefer 2007). Transgenic mice studies, also showed that SOX9 is capable of inducing testis formation (Chaboissier *et al.* 2004). Fgf9 has been implemented in the regulation of SOX9 (Kim *et al.* 2006). During the early stages of embryo development, before obvious sexual differentiation, SOX9 is expressed faintly on the genital ridges in both sexes (Chaboissier *et al.* 2004). As the embryo's development progresses, SOX9 expression becomes significantly different with a strong staining seen in the male genital ridges (Sekido *et al.* 2004). At 12.5 days post coitum, the expression of SOX9 was found on both the Mullerian duct in the female and the mesonephric duct in the male, but by 13.5 days post coitum, the staining was only found in the male reaching the mesenchymal cells around the Mullerian ducts (Sinclair *et al.* 1990). From this time until their regression, SOX9 continues to be expressed in the cells surrounding the mullerian ducts, an area where AMH is found to be expressed, indicating to the likely possibility that SOX9 is essential for the expression of AMH (Kent *et al.* 1996, Vidal *et al.* 2001, Chaboissier *et al.* 2004, Jiang *et al.* 2013). Further, studies on the proximal sequence of the AMH

promoter, have found undisputed evidence that SOX9 binding to a specific response element located at -141 is necessary for the activation of AMH expression in the early developmental stages of the embryo (da Silva *et al.* 1996, De Santa Barbara *et al.* 1998, Arango *et al.* 1999).

1.10.4 SOX8

Like SOX9, another member of the subgroup E, SOX8, has also been found to have a role in testis development, but to a lesser extent (Koopman 2005). Experiments on SOX8 $^{-/-}$ mice, which were SOX9 depleted, at late stages of gonadal development, showed that both genes have overlapping functions in testis differentiation (Chaboissier *et al.* 2004). However, mice deficient of only SOX8, showed no testicular defects indicating to the minor role of SOX8 in testis development (Sock *et al.* 2001).

1.10.5 The Role of SOX9 and SOX8 in the Regulation of Ovarian AMH

In sertoli cells, several transcription factors are involved in the regulation of AMH through binding specific representative site in the AMH promoter including SOX9, steroidogenic factor 1 (SF1), and GATA4 (Shen *et al.* 1994, Giuili *et al.* 1997, De Santa Barbara *et al.* 1998, Viger *et al.* 1998, Arango *et al.* 1999). These factors with the exception of SOX9 are all expressed in the GCs (Ikeda *et al.* 1996, Kent *et al.* 1996, Anttonen *et al.* 2003, Salmon *et al.* 2005). As previously discussed, in the male sertoli cells, the binding of SOX9 is essential for AMH expression (Arango *et al.* 1999), raising the question of whether SOX proteins are also involved in the regulation of AMH in the GCs.

SOX8, which can substitute SOX9 in the transactivation of AMH in sertoli cells, has been found to be expressed in the oocyte of preantral follicles and in the oocyte and cumulus cells of pre-ovulatory follicles (Salmon *et al.* 2005). And a recent paper has shown that the cumulus cell layer in sheep continues to express AMH even in pre-ovulatory follicles, while the membrana GC layer showed no AMH expression and high aromatase levels (Campbell *et al.* 2012). This raises the question of whether the SOX proteins have a direct or indirect role in regulating AMH in the growing follicle through oocyte somatic cell interaction. This research question will be discussed in greater detail in this review.

1.11 Hypothesis, Aims and Objectives

It was hypothesised that in a monovular species, AMH signalling in TC would be similar to signalling in GC where its cellular responses are mediated via receptor complexes containing AMHR2. Docking of AMH onto this receptor complex results in the stimulation of a BMP-like signal transduction pathway involving SMAD-SMAD interactions. It was further hypothesised that androgens have a direct effect on folliculogenesis and AMH regulation in sheep (a monovulatory species), resulting in a regulatory cycle between GC-derived AMH and androgens produced in the TC. Finally, it was hypothesised that intra-ovarian factors such as, alternatively spliced variants of VEGF, and SOX8, a member of the SOX family of transcriptional factors, locally produced intra-ovarian factors have a role in modulating folliculogenesis and AMH production in the ovary.

In order to test the above hypothesis the following objectives were projected:

- To characterise sheep GC and TC in terms of AMH and AMHR2 expression.
- To elucidate the signal transduction pathway of AMH in ovine TCs to determine its similarity to that seen in ovine GCs in this monovular species. Further, to determine if AMH signals through the MAP kinase pathway as an alternative signalling pathway in both GC and TC.
- To clarify the stimulatory effect of androgens on GC and AMH production by treating cultured cells with a mix of aromatisable and non aromatisable androgens and measuring the effect on oestradiol, inhibin A, and AMH

production, as well as the effect on aromatase expression, and activation of the AR pathway in ovine granulosa.

- To illustrate the role of VEGF isoforms on the modulation of folliculogenesis by assessing their effect on oestradiol and AMH production, and their effect on VEGF receptor type 1 and 2 expression levels in cultured ovine GCs.
- Lastly, to determine the role of SOX8 on folliculogenesis and AMH by evaluating the effect of SOX8 knock out, and exogenous SOX8 protein substitution, on oestradiol and AMH production in Human KGN cells (a secondary human GC line).

Chapter 2 Materials and Methods

This chapter will illustrate the general methodologies used in this study. However, each set of experiments will be described in greater detail in the relevant chapter. All materials were purchased from Sigma Aldrich, UK unless otherwise specified.

2.1 Cell Culture

2.1.1 Follicle Dissection and GC & TC Isolation

The isolation and separation of GC and TC was performed following the process described by (Campbell *et al.* 1996, Campbell *et al.* 1998). Somatic follicular cell culture conditions attempt to mimic physiological conditions in the follicular phase of ovarian development from the inclusion of physiological amounts of FSH, IGF, insulin and LH.

Ovine ovaries were collected from a local abattoir and placed immediately into dissection media (Medium 199 containing 20mM Hepes, 1% penicillin (5000U/ml)/streptomycin (5mg/ml), and 1% fungizone (amphotericin B-250µg/ml)) at 37^{o}C for transfer to the laboratory. They were then washed in 70% ethanol, and transferred to fresh warmed dissection medium. Under sterile conditions, small follicles (approximately 2-3mm in diameter) were dissected from the ovaries and morphologically atretic follicles were discarded. Once the desired number of follicles was obtained, the extraneous stromal tissue was removed using watchmakers' forceps. The follicles were then transferred to approximately 10ml of Dulbecco's PBS (phosphate buffered saline) and hemisected using fine bow scissors and forceps under a stereo

microscope, to release the granulosa cells from the thecal shells (Figure 2.1).

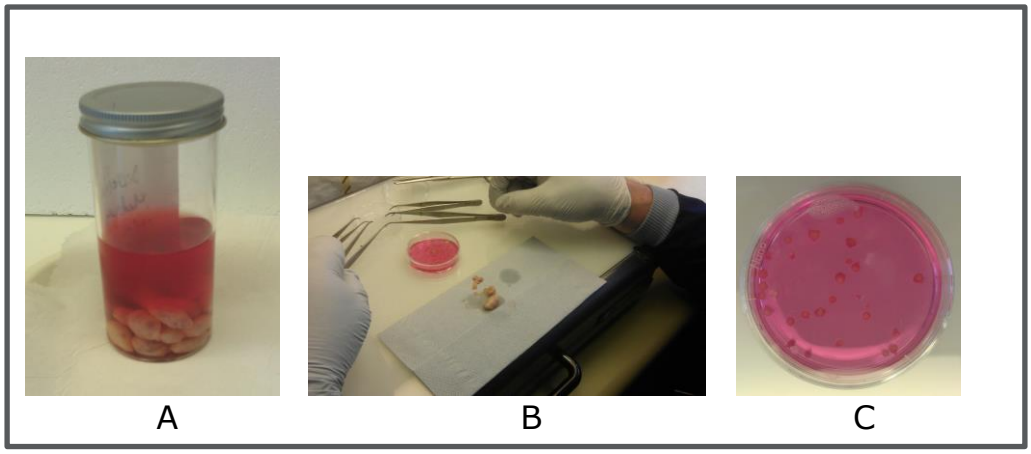


Figure 2.1 Follicle hemisection and GC andTC isolation.
Ovine ovaries collected and placed in dissection media (A). Ovary dissection process (B). Dissected and cleaned follicles (C).

The hemisected follicles were further flushed up and down a 1ml syringe repeatedly in a 25 ml Universal container to further facilitate the release of the GCs from the TCs. The thecal shells were left to settle and the supernatant containing the GCs was collected and divided equally into two 15ml centrifuge tube. The supernatant was centrifuged at 800g for 10 min, the supernatant discarded, and the resulting cell pellet was resuspended in 10 ml complete GC culture media (McCoy's 5A with sodium bicarbonate plus, 1mg/ml bovine serum albumin (BSA), penicillin/streptomycin cocktail solution (500U/ml Penicillin, 5mg/ml streptomycin), 1.5% L-glutamine, 20mM HEPES, 10µg/ml transferrin, 4ng/ml selenium, testosterone (100ng/ml), 10ng/ml IGF-I LR3, 10ng/ml insulin and 1ng/ml NIAMDD-FSH S20). Following another wash, the

number of cells and viability was estimated using Trypan blue. 50µl of the Trypan blue were mixed with 50µl of the cells suspension. 10µl of this mix was used to fill a haemocytometers chambers. The transparent cells that did not stain blue were considered to be viable and were therefore included in the count. All cells in the 25 small squares of the haemocytometer were counted. The cell count was multiplied by 2 (dilution factor) and then multiplied by 10^4 to calculate the number of cells per ml.

Meanwhile, the thecal shells were flushed repeatedly and then dispersed with an enzyme preparation previously described by (Marcus *et al.* 1984) containing 5mg/ml collagenase, 1mg/ml hyaluronidase, 1mg/ml protease, and 1% foetal calf serum (FCS) (v/v) in 10ml DPBS at 37^{o}C for 20 minutes. This enzyme mix not only dismantled the theca shells, releasing the TCs but also had the capability of destroying any remaining GCs contaminating the mix. The reaction was then stopped by adding 1ml of FCS. The theca cells solution was then divided equally into two separate conical tubes, avoiding any remaining sells. 5ml of warmed complete TCs media (Duldecco's Modified Eagle Medium: Ham's F12 mixture with 15mM HEPES, NaHCO3, L-Glutamine and pyridoxine, with 1mg/ml BSA, 1% penicillin/streptomycin solution, Transferrin (10µg/ml), Selenium (4ng/ml), IGF-1LR3 (10ng/ml), insulin (10ng/ml) and LH-526 (0.1ng/ml)) was added to each tube and centrifuged for 10 minutes at 800g. Carefully avoiding both the compacted and more disperse pellet at the bottom of the tubes, the supernatant was removed and discarded. The TCs from both tubes were resuspended and combined in complete TC media and centrifuged again at 800g for 10 min. Again the supernatant was removed and the pellet was

resuspended in a specific volume of TC media. The cells were then counted in the same way as for GCs using Trypan blue.

2.1.2 GC and TC Culture

GC and TCs were added to specific volumes of fresh GC or TC media. The media was either treated with different stimulants at this stage or during the culture period depending on the experiment performed (each one will be discussed in more detail in later chapters). The cells were seeded in 24, 48 or 96-well Nuclon™ microtitre plates (Nalge Nunc International) or in 8-well labtek culture slides. Cells were seeded at 500,000 cells per well of the 24 well plate in 1ml media, 250,000 cells were seeded in each well of the 48 well plate in 500µl media, and 75,000 cells were seeded per well of the 96 well plate and 8-well labtek culture slides in 250µl media. The cells were cultured for up to 192 hours in humidified 5% CO_2 incubators at 37^{o}C depending on the experiment setup. Every 48 hours, 80% of the media from each well was carefully removed and stored for later analysis and replaced with fresh media. In all experiments, at least 2 replicates of each treatment were included and each experiment was repeated at least 3 times.

A small volume of suspended GC and TCs were centrifuged separately in 1.7ml tubes at 800g for 1 min. The resulting pellet was then lysed in RNeasy™ RLT buffer containing 1% β-mercaptoethanol and stored at -20^{o}C to be later used as a zero time point calibrator for real time PCR. In some experiments, alongside the cultured plates, 1-2 million cells were cultured in a flask containing 5 ml of media that were later used to assess the cells viability and construct standard curves.

At the end of the culture period, the media from each well was removed and stored for later immunoassays. The cells were then either harvested and lysed in RLT buffer containing 1% β-mercaptoethanol and stored at (-20°C) for further qualitative and quantitative detection of genes of interest or fixed for future immunocytochemistry studies.

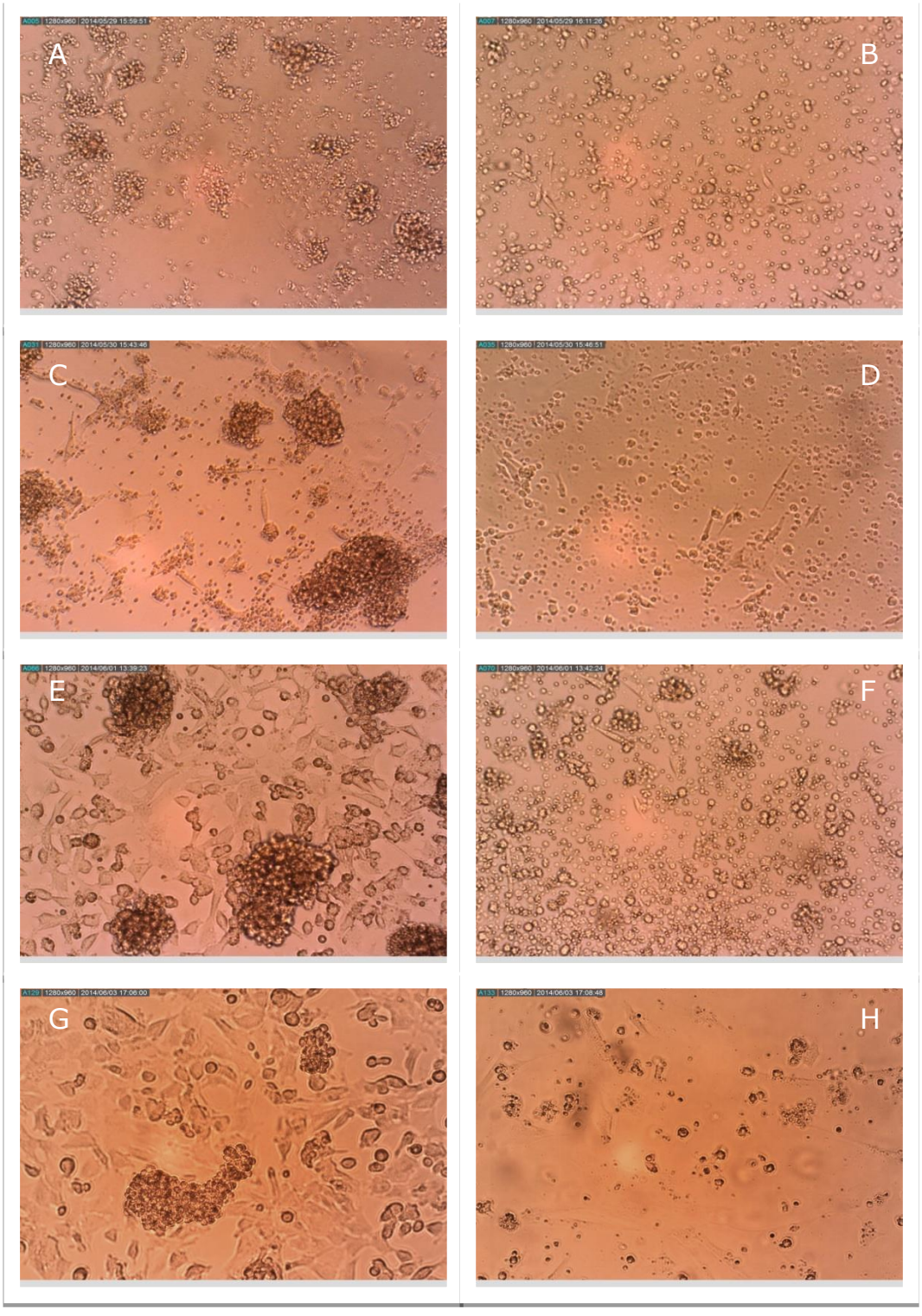


Figure 2.2 Untreated granulosa and theca cell cultured cells at different time points.
Granulosa cells at 24 hours (A), 48 hours (C), 96 hours (E) and 144 hours (G). Theca cells at 24 hours (B), 48 hours (D), 96 hours (F) and 144 hours (H). The cells visualised under the microscope at x200 magnification.

2.1.3 Estimation of Viable Cell Number

The neutral red method (Borenfreund & Puerner 1985) was used in some experiments to estimate the number of viable cells per well. Briefly, at the end of the culture period, spent media was removed and replaced with 200µl of fresh media containing 10µg neutral red (5mg/100ml). The plate was then incubated for 2-3h at 37^{o}C, to allow uptake of the dye. Following incubation, the neutral red solution in the 96 well plates was removed and replaced with 200µl of formal calcium (4% w/v formaldehyde and 1% w/v calcium chloride) and left for 2-3 minutes to fix the cells to the bottom of the wells. This was then removed and replaced with 200µl of acetic acid ethanol (50% v/v ethanol and 1% v/v glacial acetic acid), and left for 1 hour until colour developed from lysed cells. The absorbance was read using a plate reader at 540nm and compared against a standard curve prepared from the flask cells (described below).

At the same time, cells were cultured under similar conditions to the main culture in a flask. At the end of the culture period, media in the flask was also replaced with 5ml of neutral red media and incubated for 2 hours. The neutral red solution in the flask was then removed and replaced with 5ml trypsin-EDTA solution (0.2% (w/v) of trypsin and 0.08% w/v EDTA in DPBS), and incubated for 20 min, to detach the cells from the flask surface. The suspension was then transferred to a 15ml centrifuge tube and 1ml foetal calf serum was added to inactivate the enzyme. The cell suspension was then centrifuged at 800rpm for 10 minutes. The pellet was resuspended in McCoy's 5A medium and the pink viable cells were counted with a haemocytometer. The cells were re-centrifuged at 800rpm for 10 min and the pellet was

resuspended in acetic acid ethanol and then serially diluted. 200µl aliquots in quadruplicate were placed in a new 96 well plate and the absorbance was read at 540nm using a multi-plate reader. The relationship between the absorbance and the number of cells was linear ($r>0.99$) and from the resulting linear regression equation, the number of cells in the culture plate was estimated.

2.2 Tissue Processing and Staining

Whole ovaries collected in the abattoir were washed with 70% ethanol and immediately submerged in 4% paraformaldehyde for an appropriate length of time until they were completely fixed. The ovaries were then subjected to a series of processes in order to obtain slide sections for histological staining.

Initially, an automated tissue processor (Shandon Excelsior, Thermo Scientific, Electron Corporation) was used, whereby the ovaries were dehydrated with a series of increasing ethanol gradients (70%, 90%, 95%) each for 60 minutes, then 100% ethanol 3 times for 60 minutes each. They were then treated with xylene 4 times each time for 60 minutes. Finally the machine embedded the tissue in molten paraffin wax 3 times for a period of 1 hour 20 minutes each time. The ovaries were then removed from the machine and fixed into paraffin blocks using the Tissue Embedding centre (Tissue-TEK III). Serial 5µm sections were cut from the tissue blocks using a rotary microtome, and two sections were mounted onto each slide. The slides were then dried at room temperature before incubation overnight at 37^{o}C to allow adequate adhesion. The slides were then reserved for later immunohistochemistry

(IHC) studies using an automated staining machine (Leica BOND-MAX™, Milton Keynes, UK).

Epitope retrieval was performed on the machine, before the Bond staining protocol was initiated. The Bond Polymer Refine Detection Kit (DS9800, Leica Microsystems, Milton Keynes, UK) which visualizes the complex via a brown precipitate and a haematoxylin counterstain which allows the visualization of cell nuclei, was used in the machine. One hour incubation with an unsupplied target antibody was performed during the bond staining protocol. Once the staining was complete, the slides were soaked in decreasing concentrations of alcohol to ensure better staining and then, mounted with a coverslip using DPX mountant. The specificity of the staining was confirmed by inclusion of negative control slides processed without the primary antibody to control against nonspecific binding. However, to control against false positive signalling a negative control using tissue that was known not to produce AMHR2 was also processed. The follicles were examined using a Nikon Eclipse 90i microscope.

2.3 Sample Analysis

2.3.1 Reverse Transcriptase Polymerase chain reaction (PCR)

In order to perform reverse transcriptase PCR, mRNA was extracted from somatic cell lysates, followed by construction of cDNA libraries.

2.3.1.1 Total RNA Extraction

Using QIAshredder columns (Qiagen 79654), cell lysates that were stored in RLT buffer with β- mercaptoethanol were first

homogenised. The cell lysate was loaded onto the columns that were fitted into 2ml collection tubes and centrifuged at 13,000rpm for 2 minutes. The collected homogenized lysate was then used for mRNA extraction.

A commercially available Qiagen RNeasy mini kit (Qiagen 74104) was used for mRNA preparation from the cell lysate, which contains spin columns that have an RNA binding membrane. A volume of 350µl (70% ethanol) was added to each of the collected lysate, making sure they were mixed well before transfer to the RNeasy spin columns. The columns were then centrifuged for 15 seconds at 10,000rpm and the flow through was discarded. RW1 buffer (700µl) was added to each of the columns and they were then centrifuged at 10,000rpm for 15 seconds. The flow through was again discarded. The columns were then washed twice using 500µl of RPE buffer and spun for 15 seconds at 10,000rpm and the flow through was again discarded. The empty columns were then subjected to another centrifugation step at full speed for 2 minutes to eliminate any carry over ethanol that may interfere with the downstream reactions. The columns were removed from the 2 ml collection tubes and placed in new 1.7ml collection tubes. RNase-free water (33µl) was added to each column directly on to the membrane and spun for 1 minute at a speed of 10,000fpm to elute the target RNA. The columns were then discarded and the tubes were placed directly onto ice to minimise loss. Total RNA concentration and purity was measured using a spectrophotometer linked to Nanodrop computer software. The eluted RNA samples were then divided into three tubes each containing 10µl and stored at -80^{o}C for future use.

2.3.1.2 cDNA synthesis

Each RNA sample was reverse transcribed in a 20µl reaction volume (10µl sample+10µl 2X RT master mix) using a high capacity cDNA reverse transcriptase kit (Applied biosystem cDNA kit) to produce cDNA libraries. The 2X RT master mix (per 20µl reaction) was composed of 0.5µl MultiScribeTM Reverse Transcriptase, 0.8µl of the 25X dNTP Mix (100mM), 1.0µl of RNase inhibitor, 2µl of the 10X RT buffer, 2.0µl of the 10X RT Random Primers, and 3.7µl of RNase free water. The master mix and samples were all kept on ice until the next step. The master mix (10µl) was added to each of the tubes containing the 10µl total RNA samples. The tubes were then loaded into the RT-PCR thermal cycler machine. The thermo-cycling condition steps were as follow: 25°C for 10 min, 37°C for two hours, 85°C for 5min, and a final hold steps at 4°C. The samples were then stored at -20°C for later use in RT-PCR.

2.3.1.3 Real Time PCR (RT-PCR)

Semi-quantitative real-time PCR was performed using the ABI PRISM 7700 Sequence Detection System (Applied Bio-systems) to study the relative levels of specific target gene expression by utilising the cDNA libraries as templates.

PCR Master Mix (Biosystems) was freshly made up before every cycle of RT-PCR. Unless otherwise stated a typical PCR reaction, Master Mix contained the following ingredients: 4.5 µl RNase free water, 7.5µl 2x SYBR Green, 1µl sense primer and 1µl antisense primer. Together these produced a final volume of 14µl per reaction. Stock concentrations of the forward and reverse primers used were 25mM for the target gene and 12.5mM for the 18S endogenous control. Target

genes are fully described in each relevant chapter. The rRNA 18S was used as a universal endogenous control, to be compared with the gene of interest.

Unless otherwise stated a typical PCR reaction mix contained 14µl of the master mix and 1µl of cDNA samples that had been pre-diluted 1 in 4. These were added to each well sequentially with each sample, standard and control being loaded in triplicate on to a 96 well reaction plate. The standards and calibrator were prepared using cDNA samples obtained from previously collected GCs at zero time point. Optical adhesive film was used to seal the plate and placed into the 7500 Fast RT-PCR machine. The Applied Biosystems™ 'V2.0.6' software was used to run the RT-PCR. A total of 40 RT-PCR cycles were performed with the following step-cycle profile for 1 cycle: Holding stage at 95 °C for 10 minutes and Cycling stage denaturation at 95 °C for 15 seconds; annealing at 58 °C for 15 seconds; extension at 72 °C for 30 seconds. The results were then analysed using the same software. The plate was laid out in a way that half of it would target the gene of interest and the other half the endogenous control (18S) (Table 2.1). All products were verified by sequencing (in house automated sequencing).

In order to maximise efficiency of PCR, primer sets were optimised to similar PCR parameters. All the utilised primers were designed from previously published target gene sequences. Melt-curve analysis was performed to confirm the absence of non-specific spurious products for each primer set and product identity was confirmed by sequencing.

Table 2.1 Layout of PCR plates where U is the sample used S is the standard curve.

	Gene of interest						Endogenous control (18S)					
	1	2	3	4	5	6	7	8	9	10	11	12
A	U	U	U	S1	S1	S1	U	U	U	S1	S1	S1
B	U	U	U	S2	S2	S2	U	U	U	S2	S2	S2
C	U	U	U	S3	S3	S3	U	U	U	S3	S3	S3
D	U	U	U	S4	S4	S4	U	U	U	S4	S4	S4
E	U	U	U	S5	S5	S5	U	U	U	S5	S5	S5
F	U	U	U	CAL	CAL	CAL	U	U	U	CAL	CAL	CAL
G	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
H												

2.3.2 ELISA

2.3.2.1 Oestradiol (E2) ELISA

An in-house validated and optimised method (Marsters *et al.* 2014) was used for oestradiol ELISAs. In all experiments the intra-assay and inter-assay coefficients of variation was less than 10%.

E2 antibody (ABCAM ab1024) was diluted at 1:32000 in coating buffer. This was then used to coat an appropriate number of plates, depending on the experiment conducted, by adding 100µl of the dilute into each of the 96 wells. The plates were then covered with parafilm and incubated overnight at 4^{o}C.

The following morning, plates were washed with a wash buffer (PBS containing 0.005% Tween-20) 4 times on an automated plate washer, to remove any excess unattached antibodies from the plate. A volume of 100µl blocking solution (3% BSA

in PBS) was added to each well and incubated for 2 hours at room temperature to block all other potential binging sites and prevent non-specific binding by the sample antigen or labelled competitor conjugate (HRP). Following this incubation, the plates were washed a further 4 times using the plate washer.

Standards were prepared using E2 which was serially diluted to produce concentrations (0, 3.9, 7.8, 15.6, 31.25, 62.5, 125, 500, 1000pg/100µl). A volume of 100µl from the prepared standards and controls were pipetted into each well in triplicates. Samples were diluted 1 in 2 using assay buffer and loaded at a volume of 100µl per well. E2-HRP conjugate (ABIOX ED-006) was diluted at 1:64,000 in assay buffer and 100µl was added to each well. The plates were then incubated for 2 hours at room temperature.

The plates were then washed again 4 times and 50µl of tetramethylbenzidine (TMB) was added to each of the wells. They were incubated at room temperature for approximately 12 minutes or until colour development was sufficient and then 50µl of stop solution (H_2SO_4) was added to each well. Plates were read within 30 minutes at 450nm absorbance referencing at 620nm on plate reader and sample concentrations estimated compared to the standard curve.

2.3.2.2 Androstenedione (A4) ELISA

A4 ELISA was used to measure androstenedione levels synthesised from cultured theca cells. Depending on the number of samples per experiment, the number of Nunc-Immuno 96 well plates need was calculated. Donkey Anti-Rabbit Serum (SAPU), that was diluted 1:800 in coating buffer, was pipetted into each well of the plates (100µl). And

as per E2 ELISA, the plates were sealed and incubated overnight at 4^{o}C.

The following day, plates were washed 4 times using the appropriate programme on the automated plate washer. Plates were blotted dry to eliminate any residual fluid. 100µl of Blocking Buffer (3% BSA (w/v) in PBS) was added to each well and incubated for 2 hours at room temperature. After incubation, plates were washed again 4 times and inverted as previously. A4 stock (10ng/ml in Ethanol Sigma A-9630), was double diluted in assay buffer (0.1% BSA (w/v) in PBS), and used to prepare the standards. Controls (QCs) were prepared using A4 stock (1µg/ml in Ethanol Sigma A-9630) diluted in Assay Buffer. Standards, controls and samples were pipetted 25µl per well in triplicates. Following this, 50µl of A4-HRP conjugate (Position 11 ABIOX HRP-AD) diluted 1:64000 in Assay Buffer was added to each well, this was followed by an addition of 50µl A4 Antibody (Fitzgerald Industries Position 11 20R-AR040) diluted 1:20000 in Assay Buffer into each well. Plates were covered with parafilm and lid, mixed for 10 seconds on a multi-vortex mixer, and incubated overnight at 4°C.

The next day, the plates were washed 4 times on the plate washer, and then 100µl of TMB was added to each well for approximately 20 minutes at room temperature, or until the colour change had sufficiently developed. Stop Solution (1N H2SO4) (100µl) was then added to each well and immediately, plates were read at 450nm, referencing at 620nm using a Benchmark Plate Reader. The intra-assay and inter-assay coefficient of variation were less than 10%.

2.3.2.3 Inhibin A (E4) ELISA

A sandwich ELISA involving two antibodies (E4 Anti-Inhibin βA (Groome & Lawrence 1991) and Biotinylated PPG 14/6 (Bleach *et al.* 2001)), was used to measure Inhibin A levels in GC culture media following the protocol described by (McNeilly *et al.* 2002).

On the first day of the assay protocol, E4 antibody was diluted 1:65 in coating buffer. 100µl of the antibody solution was pipetted into each of the 96 wells in the Maxisorp microtiter plate and incubated overnight at room temperature.

The following day, standards (through double dilution of the top standard) and controls were prepared. 150µl of the standards and the controls were pipetted into corresponding micro-centrifuge tubes. A set amount of 100µl of samples diluted 1 in 5 in assay buffer (0.1M Tris Buffer, 10% BSA and 5% Triton X-100), was also pipetted into corresponding micro-centrifuge tubes. A volume of 150µl of 3% oxygen peroxide solution was added to the standards and controls tubes and 100µl to the samples tubes, mixed well and incubated for 30 minutes at room temperature. Assay buffer (150µl) was added to standards and controls tubes and 100µl of the same assay buffer was added to samples tubes and mixed well. The plate was washed 5 times with wash buffer on an automated plate washer using the appropriate program. A 100µl from each of the standards, controls, and the samples tubes was added to the corresponding wells in the E4 coated plate and incubated overnight in a moist box at room temperature.

On the following day, the plate was washed 5 times with wash buffer on the plate washer. A volume of 50µl of the PPG 14/6 antibody that was diluted 1:500 in assay buffer, was added to

each well. The plate was then incubated for 3 hours in a moist box at room temperature. The plate was washed again 5 times on the plate washer. A 100µl of freshly prepared streptavidin poly HRP diluted at 1:80 in 1% casein in PBS was added to each well and incubated for 1 hour at room temperature.

To visualise the reaction, the plate was first washed 5 times with wash buffer and 3 times with dH_2O. A 100µl of TMB solution was added to each well and incubated for 30 minutes at room temperature, until colour developed. The reaction was stopped by adding 100µl of stop solution to each well. Immediately, plates were read at 450nm, referencing at 620nm using a Benchmark Plate Reader. The intra-assay and inter-assay coefficient of variation of this assay were less than 10%.

2.3.2.4 AMH ELISA

AMH ELISA was carried out to estimate the level of AMH produced in GC culture media. A simple competitive AMH ELISA kit (CSB-E12756) was used, which had a detection range of 0.375ng/ml to 150ng/ml. All the reagents were supplied with the kit. Undiluted samples or standards (50µl) were loaded in duplicates to the wells. Then, 50µl of HRP conjugate was added to each well and the plate incubated for 1 hour at 37^{o}C.

Following this incubation, the plate was washed on the micro-plate washer using the appropriate program. Then, 50µl of both substrate A and substrate B was added to each well and the plate incubated for 15 minutes at 37^{o}C. a volume of 50µl of stop solution was added to each well and the plate was then read on a micro-plate reader at 450nm with a correction wavelength of 620nm. All the samples fell within the

detectable range, and the intra-assay and inter-assay coefficient of variation were less than 10%.

2.3.3 Western Blot

Western blot analysis was performed to detect and quantify AMHR2 protein in both GC and TCs. Cells were cultured following the same protocol as above. At the end of the culture period, cells were lysed using Radioimmunoprecipitation (RIPA) Lysis Buffer (SC-24948). Immediately prior to use 10µl of protease inhibitor, 10µl of sodium orthovanadate, and 10µl of PMSF solution was added per ml of 1X RIPA Lysis buffer to prepare complete RIPA. After removing the culture media, 100µl of this buffer was added to each well of cells and were stored at -80^{o}C for later use in western blot.

On the day of the procedure, samples were thawed out on ice and 24µl of each sample mixed with 8µl of electrophoresis sample buffer in separate Eppendorf tubes. Together with a separate tube containing 12µl of a biotinylated protein ladder, the sample tubes were boiled at 95^{o}C for 7 minutes on a thermocycler, and placed immediately on ice.

The NuPage Novex 4-12% Bis-Tris Gel was set in the electrophoresis chamber and electric transfer apparatus (Invtrogen). Then, 12µl of the biotinylated protein ladder and 25µl of the samples were loaded into the designated wells of the polyacrylamide gel. Electrophoresis was run at 100mA for 1.5 hours. The gel was then transferred onto the nitrocellulose membrane of the gel transfer stack and placed in the electric transfer apparatus and run for 7 minutes. The nitrocellulose membrane was placed in 1X TBS for 5 minutes and then transferred into blocking buffer (15ml 1X TBS, 7.5g non-fat

milk and 0.15ml Tween-20) for 1 hour at room temperature in the orbital incubator at 100rpm. The membrane was then washed for 5 minutes, three times in TBS/T (1X TBS and 0.1%Tween 20), and then incubated with the primary antibody solution (AMHR2 rabbit polyclonal 1gG (Santa Cruz D2909) diluted 1:200 in primary antibody diluent, and β-actin rabbit monoclonal antibody diluted 1:2000 in the same diluent), with gentle agitation at room temperature for 1 hour before further incubation overnight at 4^{o}C.

On the following day, the membrane was washed three times in TBS/T wash buffer and then incubated with secondary antibody anti rabbit IgG HRP-linked antibody diluted 1:1000 and anti-biotin HRP-linked antibody diluted 1:1000 in blocking buffer. The membrane was then washed again with wash buffer.

Next, the membrane was incubated in 10ml LumiGlo solution, composed of 0.5ml 20X LumiGlo , 0.5ml 20X peroxide and 9ml distilled water, for 1 minute at room temperature. The membrane was then immediately wrapped in plastic film and chemiluminescence visualised for 5 minute using Fujifilm LAS-3000 image reader.

2.4 Bacterial Cultures and Plasmid DNA Extraction

Amplification of plasmid constructs was performed by cloning reserved stab cultures of transformed bacteria. Plasmids included the BMP-responsive element (BRE) fused to the firefly luciferase reporter gene (BRE-Luc) and the Renilla luciferase (pRL) endogenous control plasmid.

In brief, 2.5g of Luria-Bertani (LB) broth were dissolved per 100ml distilled water and boiled for 10 minutes and allowed to cool at room temperature. Ampicillin (100mg/ml) was then added. A volume of 50µl of BRE-Luc or PRL stab cultures (stored 1:1 in glycerol) was added to 5ml of LB broth, and incubated at 37^{o}C in an orbital shaking incubator at a speed of 225rpm.

The following day, plasmid DNA was cultivated and harvested from bacterial cells using the Nucleospin® Plasmid/Plasmid (NoLid) Kit (MN1308/001). Briefly, the bacterial cultures were divided into 1.5ml microcentrifuge tubes and centrifuged for 30 seconds at 11,000g. The formed pellet was resuspended in 250µl of buffer A1. Then, 250µl of buffer A2 was added, followed by 300µl of buffer A3 and then the whole solution was centrifuged for 5 minutes at 11,000g. The NucleoSpin® Plasmid/Plasmid (No Lid) column was placed in a 2ml collection tube, and then the supernatant was transferred into the column and centrifuged for 1 minute at 11,000g. The flow-through was removed, and 500µl of AW buffer was added to the column and centrifuged for 1 minute at 11,000g. The flow-through was again discarded and 600µl of Buffer A4 was then added to the column and centrifuged for 1 minute at 11,000g. Finally, the flow-through was discarded and the column was centrifuged for 2 minutes at 11,000g, and then transferred from the collection tube to a new 1.5ml microcentrifuge tube. Buffer AE (50µl) was carefully added to the column to elute the plasmid DNA, and after 1 minute the column was centrifuged for 1 minute at 11,000g. The flow through was stored at -20^{o}C for later use.

2.5 Cell Cultures and Transfection

GC and TCs were cultured in 24 well plates for a specific period of time at 38C^{o} in a humidified atmosphere of 5% CO_2 and 95% air. A transfection mix was prepared on the day of transfection that was composed of a 3 to 1 ratio of a transfection reagent FuGENE6 (Roche, 1815091) and the plasmid DNA, all mixed in a Bovine Serum Albumin and antibiotic free culture media. Existing media was removed from the 24 well plates and replaced with the transfection mix media and returned to the incubator for another specific period of time. The cells were then stimulated with different treatment for specific periods of time depending on the experiment protocol. After the spent treatment period, all the media was removed and 100µl of diluted Passive lysis buffer was loaded into each well, followed by manual scraping of the wells to release the cells from the plate surface and allow proper mixing with the buffer. All the cell lysates were then frozen at -20C^{o} prior to assay.

2.6 Dual Luciferase Reporter Assay System

The Firefly and Renilla luciferase activity were measured sequentially from each sample using the Dual-Luciferase® Reporter Assay System (Promega, Cat. No. E1910). The firefly luciferase reporter was measured first by adding 50µl of Luciferase Assay Reagent II (LAR II) to 20µl of cell lysate sample and the generated luminescent signal was measured using a Modulus II Microplate Multimode, automated Reader. After measuring the firefly luminescence, this reaction was quenched, and the Renilla luciferase reaction was initiated simultaneously by adding 50µl of Stop & Glo® Reagent to the

same sample. The luminescence generated by this reaction was then measured using the Modulus II Microplate Multimode Reader. The amount of luciferase produced and subsequent activity determined by the light emissions is assumed to have a direct relationship to the activation of the BMP responsive element.

2.7 Fluorescent Immunocytochemistry (ICC)

GC and TC were cultured in labtek slides for a predetermined period of time. Following the culture period, the cells were then treated for a specific period of time. The culture media was then removed and the cells washed with PBS. The cells were then fixed with 4% Paraformaldehyde (PFA) for 7 minutes followed by another wash with PBS. Two drops of Duolink II blocking buffer were added per well and incubated in a pre-heated humidity chamber for 30 minutes at 37°C. A primary antibody was added in the appropriate dilution per well and incubated for 1 hour at room temperature and then overnight at 4°C.

The following day, the cells were washed twice with TBS and 0.05% tween for 5 minutes each time. A fluorescent secondary antibody that is specific for the primary antibody species was diluted at 1:1000 in dilution buffer. A volume of 100µl of this solution was added to each well for 1 hour at room temperature. The cells were then washed twice with TBS and 0.05% tween. Then, 40µl of Duolink II mounting medium with DAPI was added to the middle of each well and gently covered with a coverslip. After, 15 min the slides were visualised using a fluorescent microscope.

2.8 Proximity Ligation Assay (PLA)

This novel technology extends the traditional immunoassays capabilities for protein identification, to include the detection of protein interactions with high sensitivity and specificity. Even with only few cells, subcellular events, which are transient or weak, can be shown *in situ.* The principle behind this technique, involves two primary antibodies that were raised in different species which target the antigen or antigens of interest. Secondary antibodies that are species specific, known as probes, target the primary antibody. These probes have a short DNA strand attached to it. When the PLA probes are in close proximity the DNA stands interact, and are amplified using a polymerase resulting in a signal that can be detected and visualised as an individual fluorescent spot under the microscope.

After a period of culture (specified in the appropriate 'Results' section) in the eight well glass, Lab-Tek slides, GC and TC were treated for different time periods. The culture media was then removed and the cells washed with PBS. The cells were then fixed with 4% PFA for 7 minutes followed by another wash with PBS. Two drops of Duolink II blocking buffer were added per well and incubated in a pre-heated humidity chamber for 30 minutes at 37°C. A primary antibody was added in the appropriate dilution per well and incubated for 1 hour at room temperature and then overnight at 4°C.

The next day, the PLA probes were diluted 1:5 using the provided diluent. The primary antibody was discarded (tapped off), and the slides were washed twice for 5 minutes using the provided wash buffer A. PLA probes solution at a volume of 80µl was added to each well and incubated in a pre-heated

humidity chamber for 1 hour at 37°C. Next, the Ligation-Ligase solution was prepared from the provided kit. The PLA probe solution was removed and the slides were washed twice with wash buffer A for 5 minutes. Then, 40µl of the Ligation-Ligase solution was added to each well and incubated for 30 minutes in a pre-humidity chamber at 37°C. Near the end of the incubation, the Amplification-Polymerase solution was prepared. After the incubation period, the slides were washed again with wash buffer A twice for 2 minutes and then incubated with 40µl of the prepared Amplification-Polymerase solution per well for 100 minutes in a pre-humidity chamber at 37°C in a dark environment (reagents light sensitive). The slides were then washed twice with wash buffer B for 10 minutes and with 0.01×wash buffer B for 1 minute. The slide wells were removed and the slides kept in the dark to dry. A minimal volume of Duolink II mounting medium with DAPI was added to the middle of each well and gentle covered with a coverslip. After, 15 min the slides were visualised using a fluorescent microscope.

Chapter 3 AMH Receptor Type 2 detection in Granulosa and Theca cells

3.1 Introduction

In contrast to other members of the TGF-β family that are widely expressed and have different biological activities depending on the cellular content, AMH is only expressed in the gonads causing inhibitory effects on the function and development of the reproductive organs. It is well established that it is only produced in sertoli cells in male testis and GCs in female ovarian follicles (Rey *et al.* 2000). Evidence to-date is consistent with AMH's cellular effects being transduced only via heterodimeric receptor complexes which specifically contain AMHR2 (Josso & Clemente 2003).

The AMHR2 has also been found to be expressed in the GCs. In rats it was detected in GC but not TCs at the preantral stage of follicular development, reaching its highest expression in small antral follicles (Baarends *et al.* 1995b). In the Baarends et al., (1995b) study the expression of AMHR2 diminished in the GCs of large antral follicles and preovulatory follicles. From the limited amount of published data on the AMH receptor in monovulatory species, there is an indication of a species differences in the pattern of its expression (Baarends *et al.* 1995b, McGee *et al.* 2001, Rice *et al.* 2007).

The initial aim of this series of experiments was to characterise ovine GC and TCs in terms of AMH and AMHR2 mRNA expression in cells taken from small antral follicles and grown under conditions which support normal cellular development and differentiation. The effect of time and AMH

treatments on levels of AMHR2 mRNA was also to be analysed. A secondary aim was to characterise the same cells (taken from small antral sheep follicles) in terms of AMHR2 protein and to determine the effect of time in culture and treatments of AMH on AMHR2 amount and spread. The ovine model was used in these sets of experiments because of the close resemblance of its reproductive physiology to humans.

3.2 Experimental Design

3.2.1 AMHR2 Expression in Freshly Collected and Cultured GC and TC using real-time PCR

GC and TCs taken from small (3-4mm in diameter) ovine antral follicles either underwent a process of total RNA extraction, as described in the 'Methods' chapter (Section 2.3.1.1.), or were cultured over time under the specialised conditions optimised in an earlier study (Campbell *et al.* 1996, Campbell *et al.* 1998), and fully described in the 'Methods' chapter (Section 2.1.2.). After 24, 48, 96 and 144 hours of culture the media was removed. The cells were then harvested at each time point separately and isolated total RNA from each, along with the total RNA set aside from the cells that were not cultured, were used to generate first strand cDNA libraries. Specific fragments of AMH and AMHR2 genes were amplified from each of the cDNA libraries, utilising quantitative real-time PCR, as fully described in the 'Methods chapter (Section 2.3.1.). Outcomes relative to an endogenous control (18S) were compared. The primers used for the genes of interest are illustrated in (Table 3.1).

Table 3.1 Forward and reverse primer sequences for internal standard (18S), AMH and AMHR2.

Target Gene	Forward Primer Sequence (Sense)	Reverse Primer Sequence (Antisense)
18 S	5'-TTGACTCAACACGGGAAACCT-3'	5'-AGAAAGAGCTATCAATCTGTCAATCCT-3'
AMH	5'-GTGACCCTGCATGGGAGT-3'	5'-CGGTGACAGTGACCTCCAG-'3
AMHR2	5'-CTGGGCACTGCTTCCTACAGCT-'3	5'-CATCCAGCAGCTTCCCCAGGTT-'3

3.2.2 AMHR2 Expression in GC and TC using Western Blot

In a further round of experiments GC and TCs were again cultured under the conditions described in the 'Methods' chapter (Section 2.1.2.), for 24, 48, 96 and 144 hours. Over the first 24 hours cells were either treated with AMH (50 ng/mL) or were left untreated. The cells were left without AMH treatment for the remainder of the culture period. At each time point the media was removed and the cells lysed for analysis by Western Blot as described in the Methods chapter (Section 2.3.3.), utilising AMHR2-specific antibodies.

3.2.3 AMHR2 Expression using Fluorescent ICC

It was felt that the Western Blot approach lacked sufficient sensitivity for detection of AMHR2 in TCs so fluorescently labelled antibodies were utilised in a further round of experiments. ICC was performed on cells maintained in culture as before. After either 24 or 144 hours cells were either treated for 1 hour (as optimised) with AMH (50 ng/mL), or left untreated for the same period. The ICC process was performed as described in the 'Methods' chapter (Section 2.7.). A primary AMHR2 rabbit polyclonal antibody (Santa Cruz D2909) and a fluorescent anti rabbit secondary antibody (CF 555, biotium) were used. To further consolidate results this experiment was repeated again using an AMHR2 primary mouse monoclonal antibody (AbCam, 64762) and a fluorescent anti mouse secondary antibody (CF 555, biotium). Negative controls were included in each slide for background control.

3.2.4 AMHR2 Expression using PLA

PLA is a powerful tool that uses an amplification process that can detect single targets or identify interactions between two proteins. In an attempt to optimise the PLA procedures, the first set of experiments conducted using this technique were to illustrate the expression of AMHR2 in GC and TCs.

The cells were cultured for 144 hours and either non-treated or treated with AMH for different time points (1 hour, 30 minutes, 20 minutes), to determine the optimal AMH treatment time in detecting the highest AMHR2 expression signal. The procedure (as described in detail in the methods chapter section 2.8.) involved the use of a primary AMHR2 rabbit polyclonal antibody (Santa Cruz D2909) and again negative controls were included in each slide for background control.

3.2.5 AMHR2 Expression In Vivo

In order to compare the ICC outcomes with the *in vivo* situation IHC was performed on sectioned whole ovaries using two different primary antibodies, the rabbit polyclonal primary antibody and the mouse monoclonal antibody in accordance to the process described in the 'Methods' chapter (Section 2.2.). Human testes section slides were ran at the same time, to be used as positive controls and sheep lymph node section slides were used as negative controls. The slides were then examined using the same microscope. Finally, IHC florescent studies were conducted on previously prepared ovine ovarian sections. Dewaxing, rehydration and epitope retrieval were performed on the automated Bondmax machine (Leica BOND-MAX™, Milton Keynes, UK). The slides were then subjected to a rabbit polyclonal primary antibody (Santa Cruz D2909) and

then to a fluorescent anti rabbit secondary antibody (CF 555, biotium) and visualised under a fluorescent microscope.

3.3 Statistical Analysis

In all cases, data from this chapter are presented as mean +/- SEM of at least three different experiments conducted in triplicate. Statistical significance was set as $p<0.05$. Results of the qPCR were normalised against the cell number by utilising an endogenous control (18S mRNA) and analysed relative to a standard curve according to the software provided by Applied Biosystem, version 2. Data were tested for normality and statistical significance was compared using a two way ANOVA analysed on the statistical package for the social sciences (SPSS).

3.4 Results

3.4.1 AMHR2 Expression in Freshly Collected and Cultured GC and TC

AMHR2 mRNA was expressed in both freshly collected GC and TCs. However, the expression in GCs was more than 3 times the expression levels seen in TCs. AMH mRNA expression was also measured in both GCs and TCS. It was found that its expression in GCs was considerably high when compared to the minimal expression seen in TCs ($p<0.001$) (Figure 3.1).

AMHR2 mRNA expression was measured by PCR in GC and TCs that were cultured for different time periods (24, 48, 96, and 144 hours). Results show that the expression of AMHR2 in GCs decreased with time ($p<0.001$), while receptors expression was stable in TCs throughout the culture period. A significantly higher expression ($p<0.001$) was seen in GCs when compared to TCs that were cultured for 24 hours. Minimal difference in expression between GC and TCs was measured thereafter (Figure 3.2).

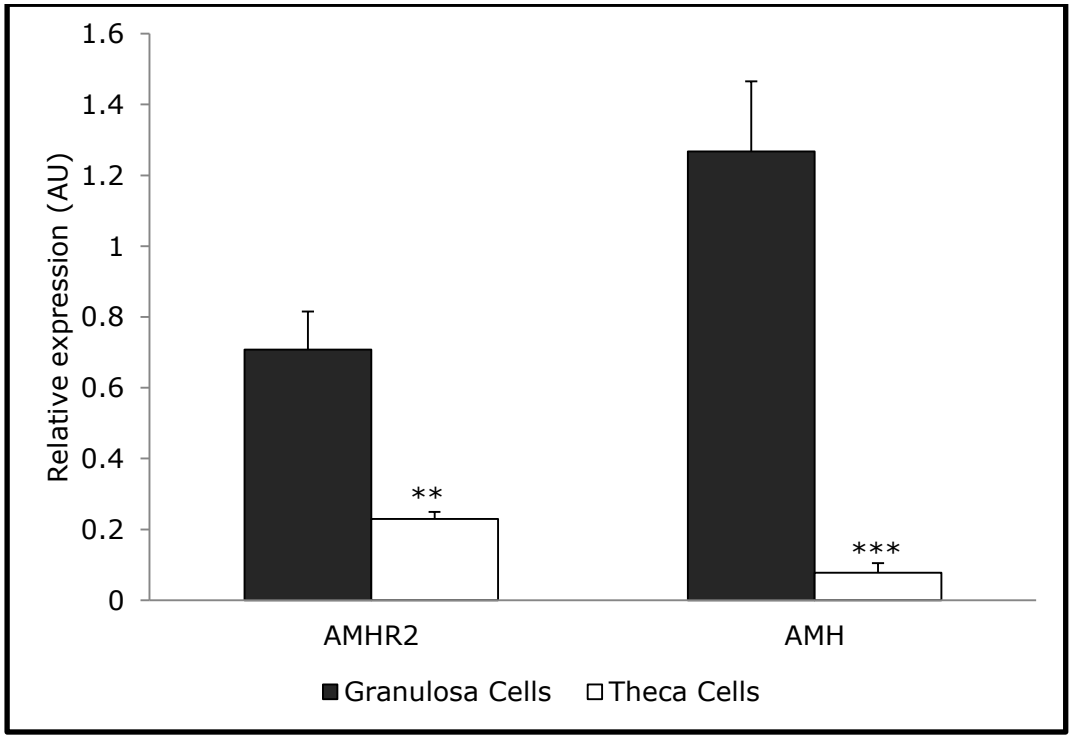


Figure 3.1 mRNA expression of AMH receptor type 2 and AMH in granulosa and theca cells at zero time point.
Values represent mean ± SEM. **, P< 0.01, ***, P<0.001 for comparisons between GC and TCs.

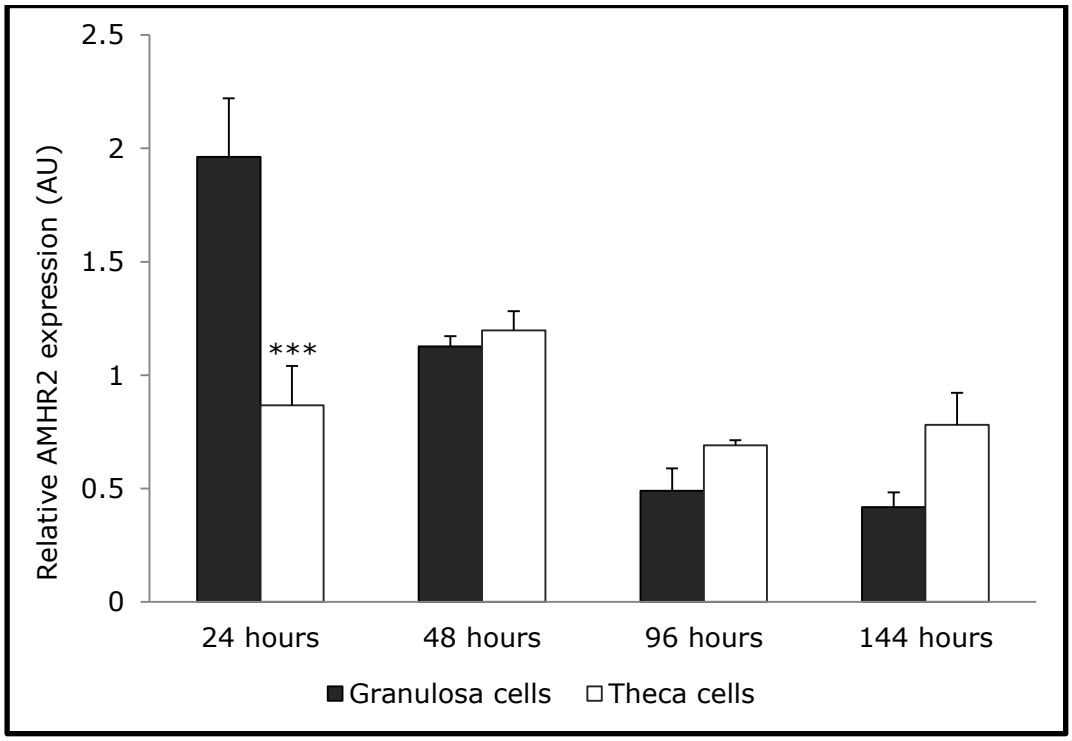


Figure 3.2: mRNA expression of AMH receptor type 2 in cultured granulosa and theca cells at different time points.
Values represent mean ± SEM. ***, P<0.001 for comparison between GC and TCs at each time point.

3.4.2 Elucidation of AMHR2 Expression in GC and TC using Western Blot

This analysis showed that AMHR2 was expressed predominately in GCs that were cultured for 24 hours and to a lesser extent in the cells that were cultured for 48 hours while very minimal expression was seen in cells cultured for 96 and 144 hours. AMH receptor expression was undetected in TCs throughout the culture period. To note however, that in this experiment, the number of GCs was higher than the number of TCs used evident by the beta actin band (Figure 3.3).

Therefore, another experiment was conducted to exclude the possibility of cell number causing the difference in expression. GC and TCs were cultured for 24 hours and one group was treated with 50ng/ml of AMH while the other group was devoid of treatment. Results showed that AMHR2 was expressed in both the treated and non-treated GCs, while AMH receptor expression was undetected in TCs at the same time point (Figure 3.4).

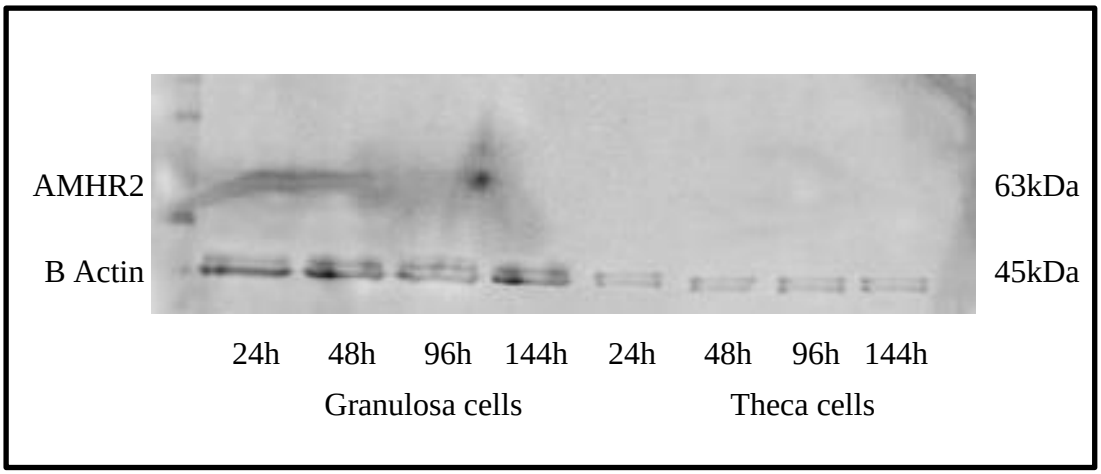


Figure 3.3 Western blot analysis showing the expression of AMHR2 in cultured ovine GC and TC at different time points.

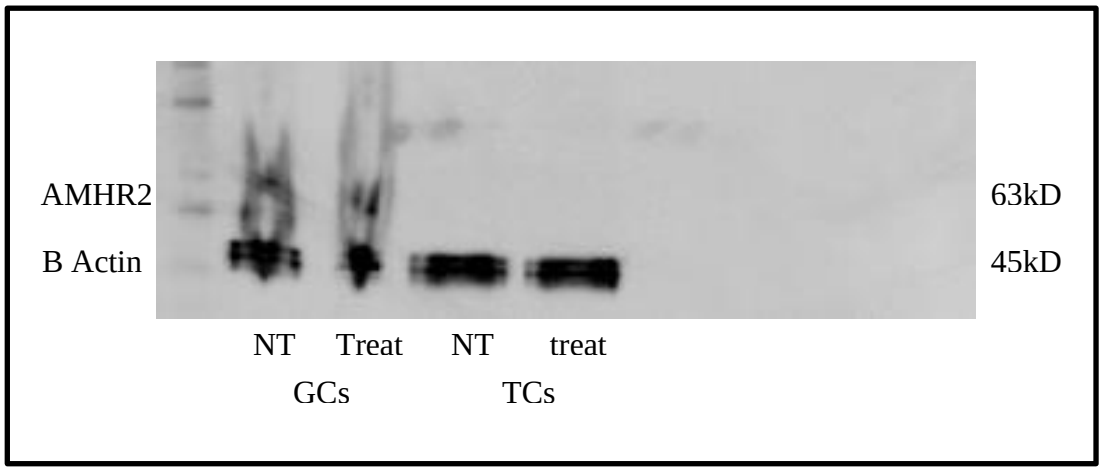


Figure 3.4 Western blot analysis showing the expression of AMHR2 in 24 hour cultured GC and TC, treated and non-treated with 50ng/ml of AMH.

3.4.3 AMHR2 Expression using Fluorescent ICC

ICC studies using two different primary antibodies revealed that AMHR2 was abundantly expressed in GCs that were cultured for 24 hours both in the treated and non-treated group (Figure 3.5). In the parallel experiment on TCs cultured for 24 hours, AMHR2 was expressed, but appeared to be less than that seen in GCs both in the treated and non-treated cells (Figure 3.6).

The same experiment layout was repeated as above on GC and TCs that were cultured for 144 hours. This showed that in GCs, AMHR2 was highly expressed in both the treated and non-treated group with the fluorescent signal completely covering the cells cytoplasm leaving a clear nucleus in the middle (Figure 3.7). Immunolocalisation of AMHR2 occurred in AMH treated and non-treated TCs that were tested using the monoclonal antibody (Figure 3.8 A, C). While, the expression level appeared to be markedly less evident in AMH treated TCs that were tested against the Polyclonal antibody (Figure 3.8 D) and nearly undetectable in the non-treated TCs that were exposed to the Polyclonal antibody (Figure 3.8 B).

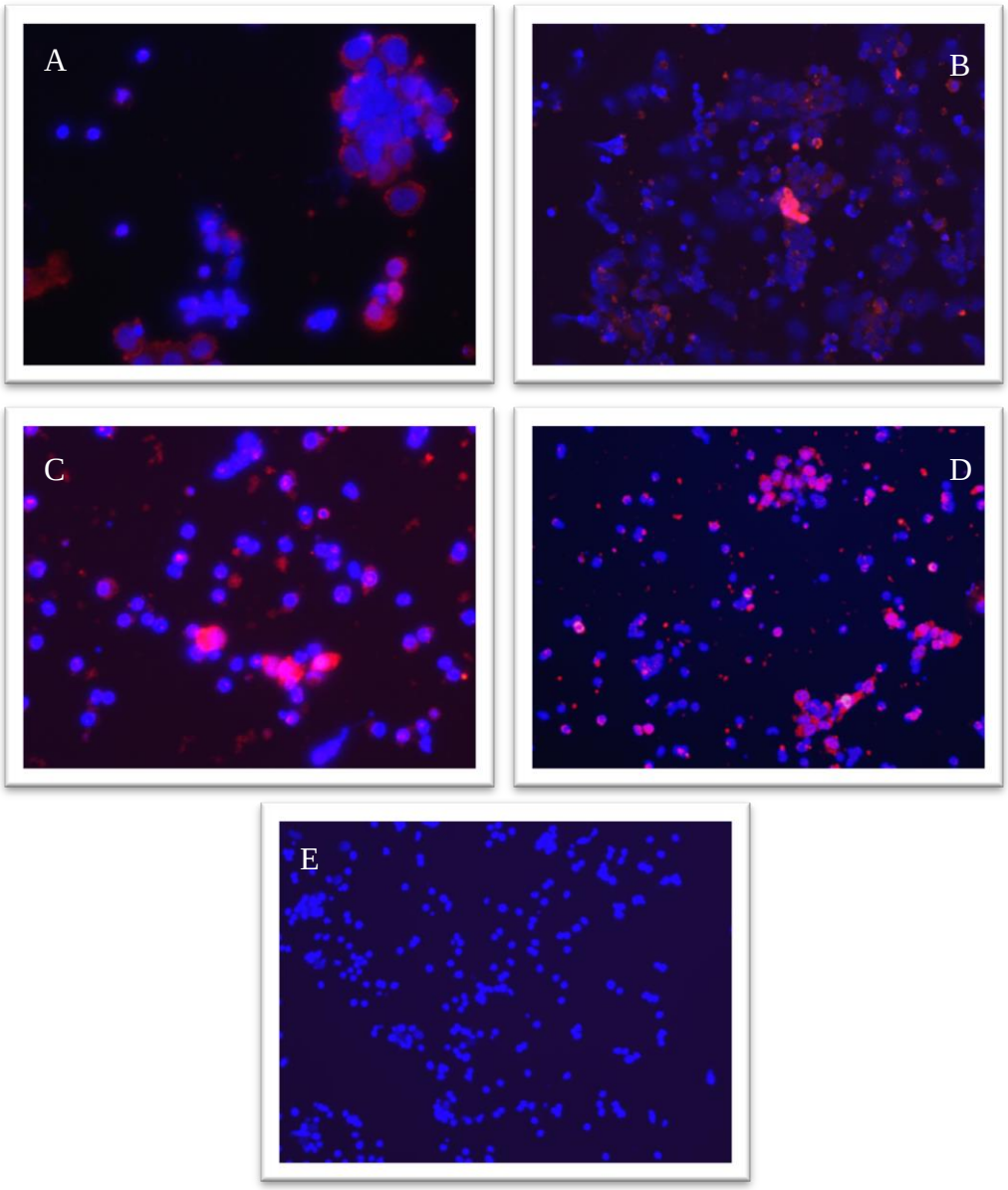


Figure 3.5 The expression of AMHR2 in 24h cultured, ovine GC using fluorescent ICC studies.

Two different primary antibodies were used and in each case cells were either non-treated (NT) or treated with 50ng/ml of AMH for 1 hour. Monoclonal antibody, cells non-treated (A). Polyclonal antibody, cells non-treated (B). Monoclonal antibody, cells treated with AMH for 1 h (C). Polyclonal antibody, cells treated with AMH for 1 h (D). Negative control (E). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

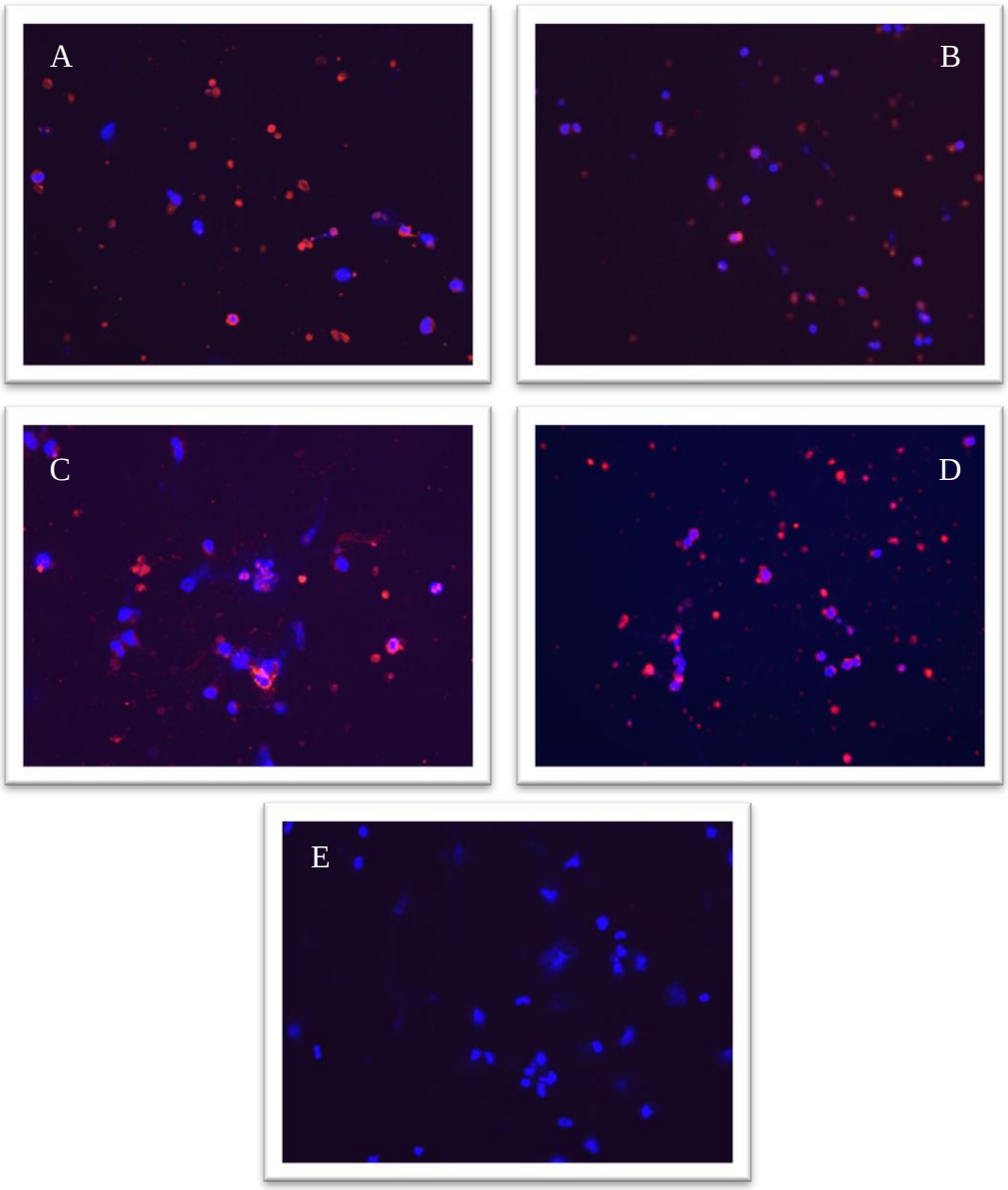


Figure 3.6 The expression of AMHR2 in 24h cultured, ovine TC using fluorescent ICC studies.

Two different primary antibodies were used and in each case cells were either non-treated (NT) or treated with 50ng/ml of AMH for 1 hour. Monoclonal antibody, cells non-treated (A). Polyclonal antibody, cells non-treated (B). Monoclonal antibody, cells treated with AMH for 1 h (C). Polyclonal antibody, cells treated with AMH for 1 h (D). Negative control (E). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

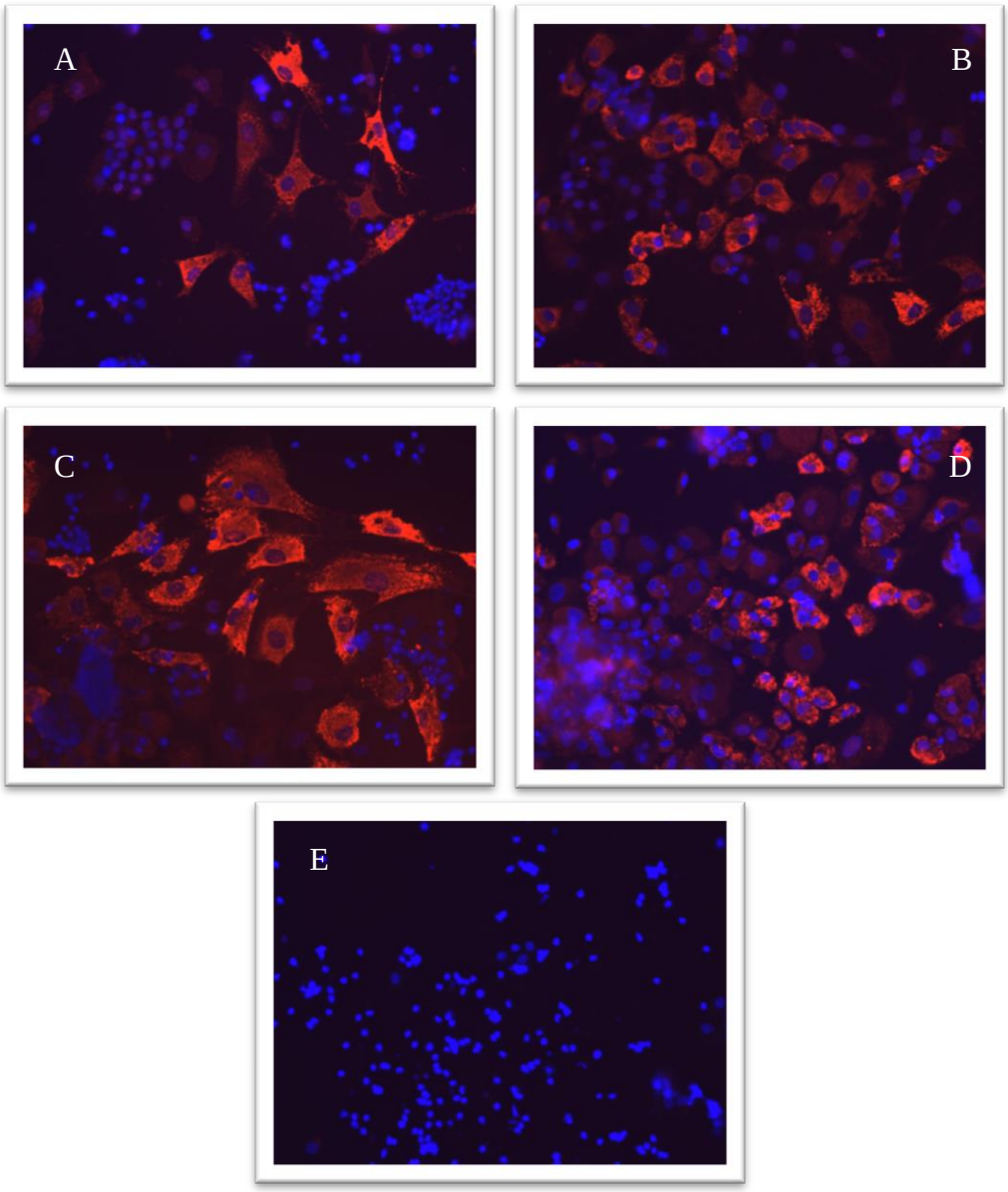


Figure 3.7 The expression of AMHR2 in 144h cultured, ovine GC using fluorescent ICC studies.

Two different primary antibodies were used and in each case cells were either non-treated (NT) or treated with 50ng/ml of AMH for 1 hour. Monoclonal antibody, cells non-treated (A). Polyclonal antibody, cells non-treated (B). Monoclonal antibody, cells treated with AMH for 1 h (C). Polyclonal antibody, cells treated with AMH for 1 h (D). Negative control (E). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

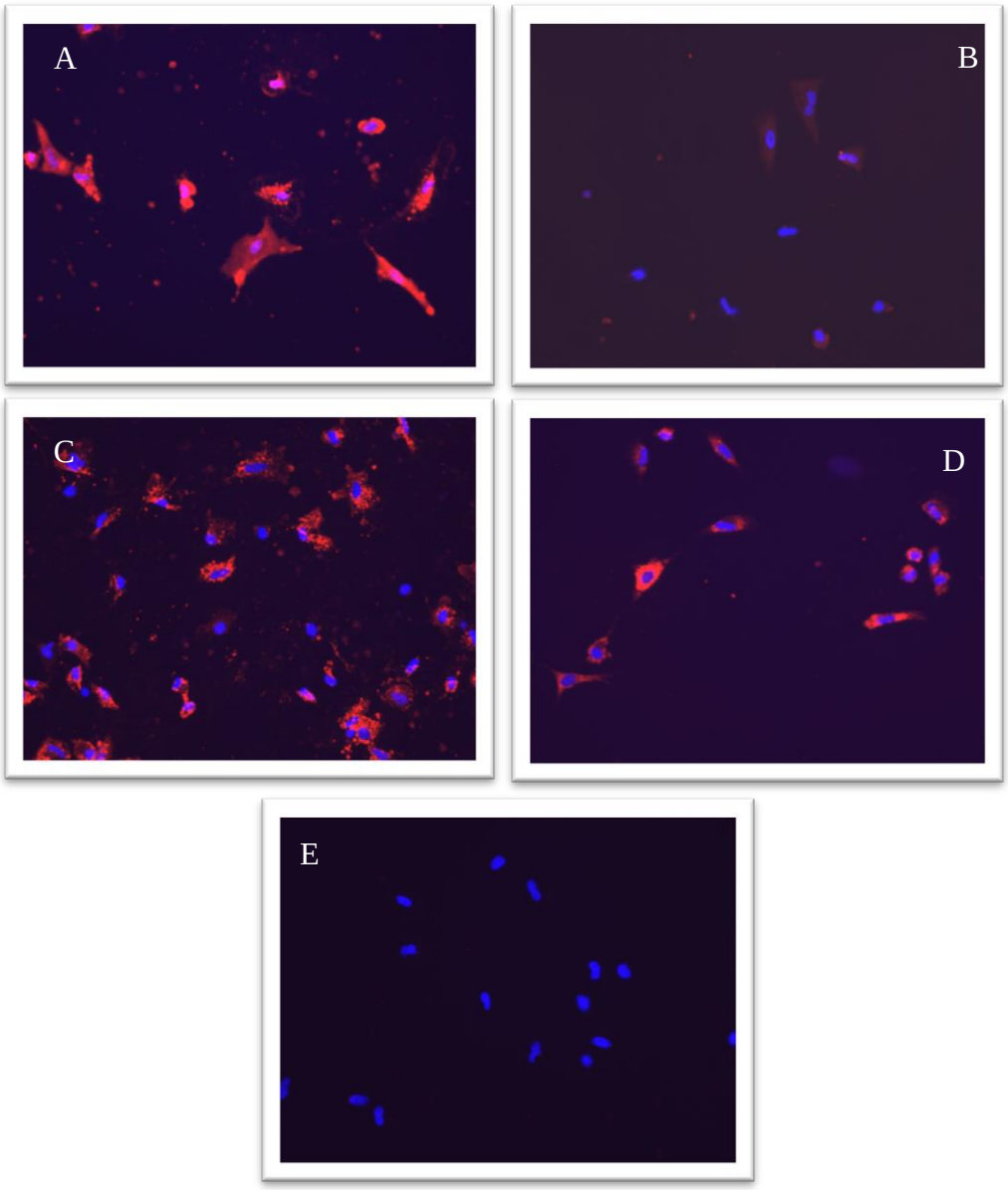


Figure 3.8 The expression of AMHR2 in 144h cultured, ovine TC using fluorescent ICC studies.

Two different primary antibodies were used and in each case cells were either non-treated (NT) or treated with 50ng/ml of AMH for 1 hour. Monoclonal antibody, cells non-treated (A). Polyclonal antibody, cells non-treated (B). Monoclonal antibody, cells treated with AMH for 1 h (C). Polyclonal antibody, cells treated with AMH for 1 h (D). Negative control (E). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

3.4.4 AMHR2 Expression using PLA

AMHR2 expression was studied in GC and TCs that were cultured for 144 hours using single target detection PLA. Results show that in GCs, AMHR2 was expressed in the cells that were AMH treated at different time points and in the non-treated cells (Figure 3.9).

In TCs, there was no clearly observed difference in the AMHR2 expression between the cells that were treated with AMH at different time points (1h, 30min, 20min). However, the receptor expression level was notably less in the TCs that were not AMH treated (Figure 3.10).

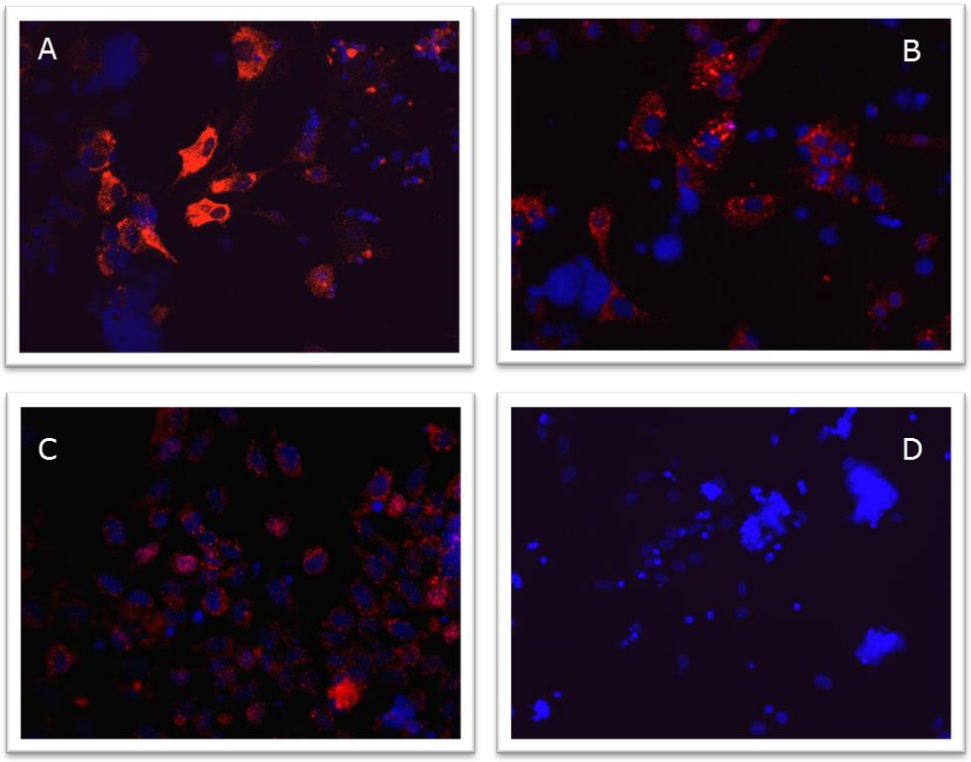


Figure 3.9 The expression of AMHR2 using PLA in 144h cultured, ovine GC that were treated with AMH for different periods of time.
Cells were treated for 1 hour (A), 30 minutes (B), no treatment (C). Negative control (D). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

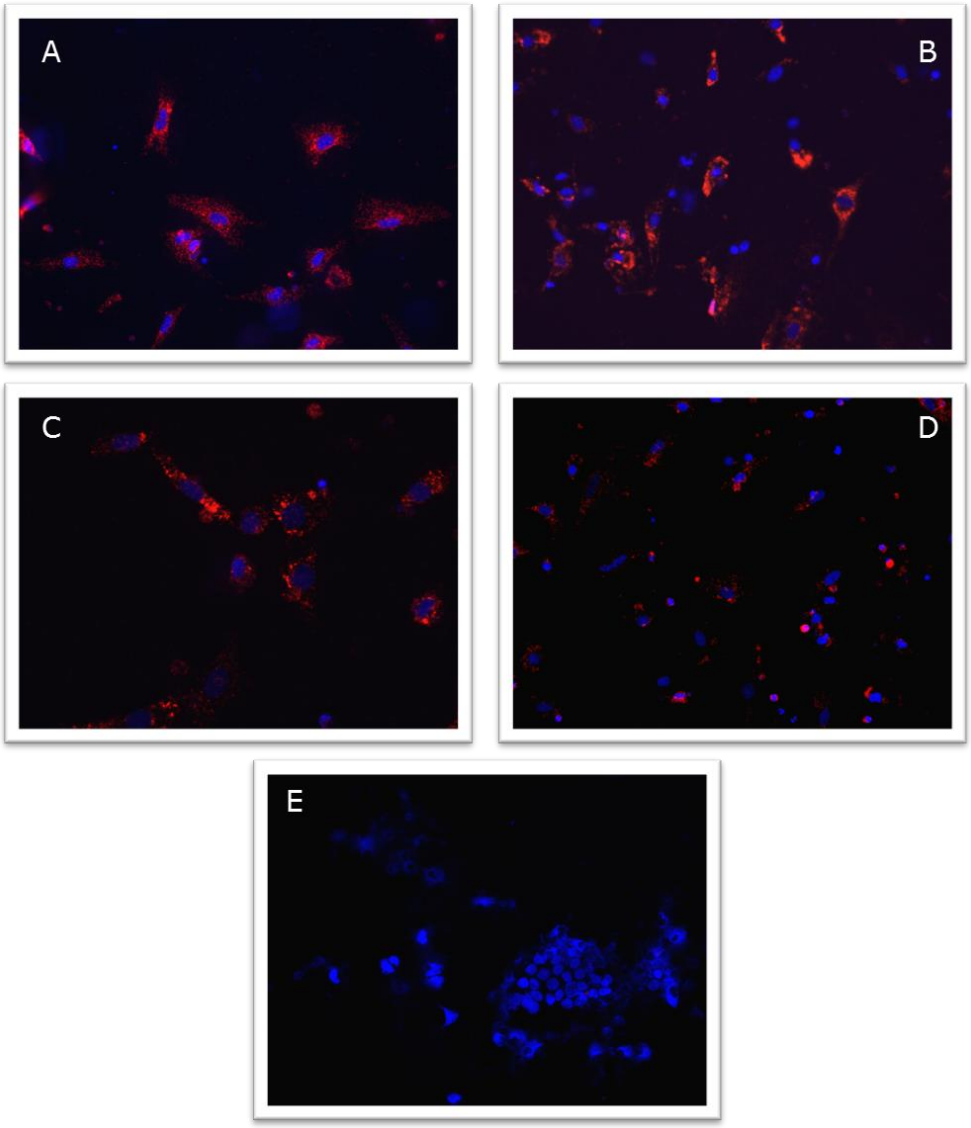


Figure 3.10 The expression of AMHR2 using PLA in 144h cultured, ovine TC that were treated AMH for different periods of time.
Cells were treated for 1 hour (A), 30 minutes (B), 20 minutes (C), no treatment (D). Negative control (E). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

3.4.5 AMHR2 Expression In Vivo

IHC studies show that in GCs the expression level of AMHR2 was first detected in the preantral follicles, while the primary and secondary follicles did not show any expression of the receptor. The highest level of expression of the receptor in GCs was seen in the small antral follicles (Figure 3.11, Figure 3.12). In TCs, the expression of the receptor was undetected in the early follicular stages. Low levels were first detected in the TCs of the preantral follicles and the highest expression was seen in the small antral follicles, but the expression in TCs was lower than that seen in GCs (Figure 3.11,Figure 3.12). Fluorescent studies further confirm the high expression of AMHR2 in the GCs and to a lesser extent in the TCs of small antral follicles (Figure 3.13).

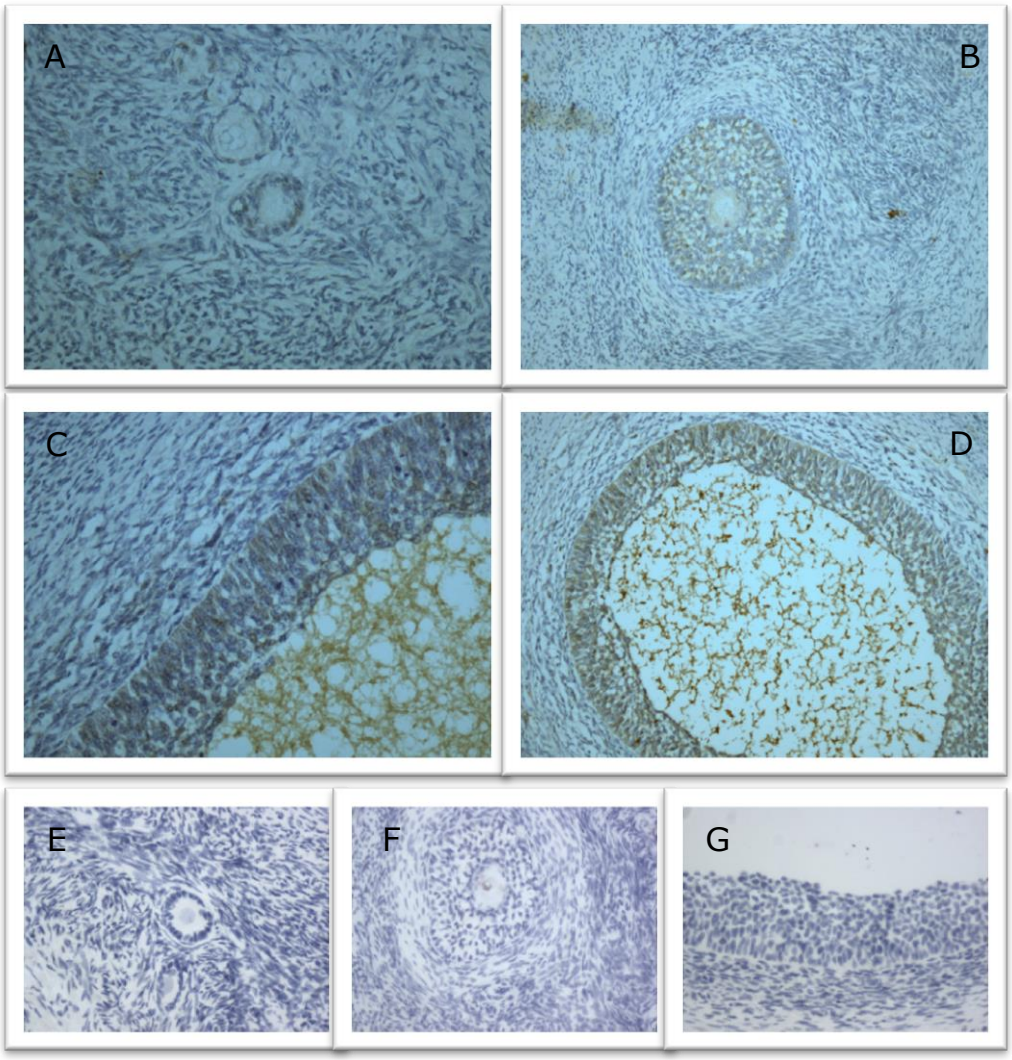


Figure 3.11 IHC studies showing AMHR2 expression in ovine ovarian follicles of different sizes.
Specific AMHR2 expression in primary and secondary follicles (A), preantral follicles (B), and antral follicles (C, D). A primary AMHR2 polyclonal antibody was used. Negative controls with no primary antibodies in primary (E), preantral (F), and antral (G). All were at X200 magnification except (D) at X100.

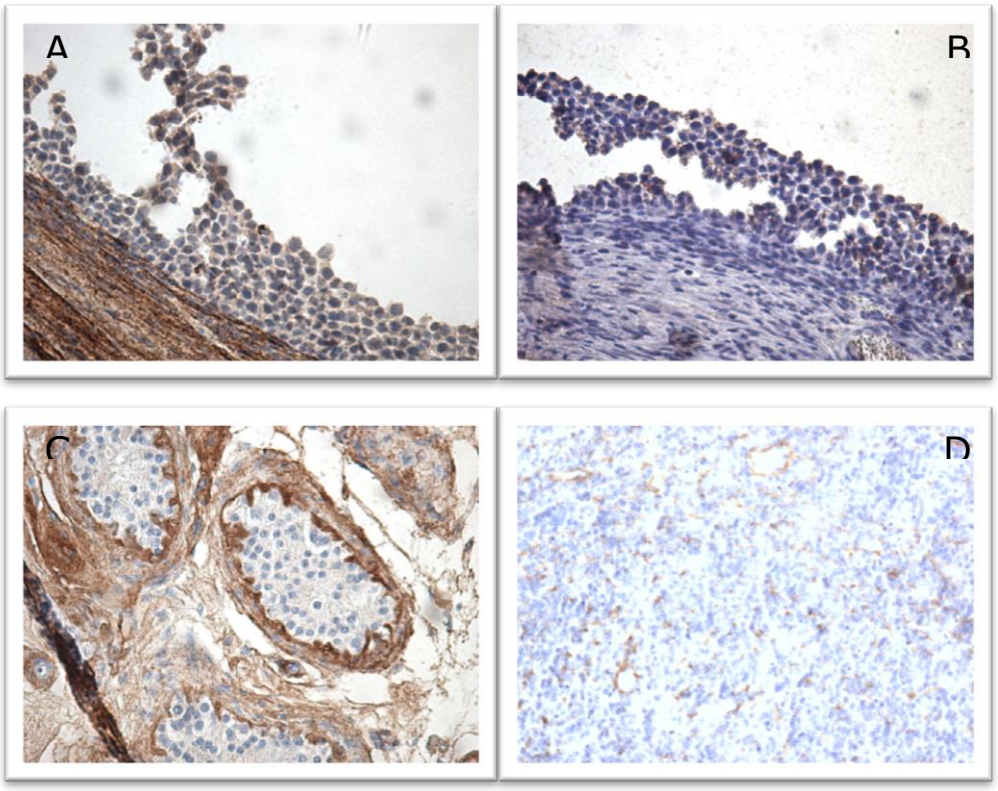


Figure 3.12 IHC studies showing AMHR2 expression in an ovine antral follicle

Expression in ovine antral follicle (A), Human testes section as positive control (C), ovine lymph node section as negative control (D), and ovine antral follicle with no primary as negative control (B). AMHR2 monoclonal antibody was used. All were at X200 magnification.

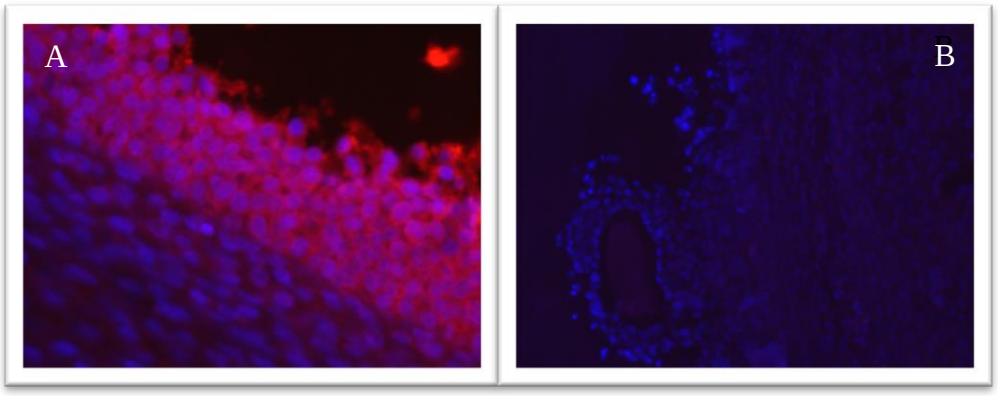


Figure 3.13 IHC florescent studies showing the expression of AMHR2 in ovine granulosa and theca cells of an antral follicle

Expression in ovine GC and TC of an antral follicle (A). A primary AMHR2 rabbit polyclonal antibody was used. Antral follicle devoid of primary antibody used as negative control (B). Both slides are at X200 magnification.

3.5 Discussion

Using both *in vitro* and *in vivo* studies, this chapter provides evidence that both ovine GC and TCs may possess an AMHR2 by which AMH can interact. Three different *In vitro* studies were conducted and showed that AMHR2 mRNA was expressed in both GCs and TCs. This expression was considerably higher in GCs when compared to TCs. However, as time in culture progressed, the levels in GCs dramatically decreased after 48 hours, reaching theca cell levels that were somewhat steady throughout the culture. This is in agreement with western plot studies, where AMHR2 protein was measurable in cultured GC but not in TC. The expression of the receptor was clearly detected at 24 hours and to a lesser extent at 48 hours but was not shown at 96 or 144 hours. As expression levels were considered low throughout the culture period in TCs, the protein was therefore undetectable. This novel finding may explain why to date researchers have been unsuccessful in elucidating the receptor in TCs while many have shown that it exists in GCs (Baarends *et al.* 1995b). This finding may have major implications as this may provide evidence of an important paracrine pathway for AMH in the regulation of steroids in the ovary and perturbation of this pathway could in fact cause an imbalance in the steroid production resulting in different pathologies. In order to consolidate these results, specialised ICC techniques were adopted that had the ability to detect minute levels of proteins produced by cells. As these studies were very expensive to conduct, cultured GCs and TCs were analysed at only 2 different time points 24 and 144 hours , which corresponded with findings of previous researchers (Campbell *et al.* 2012), using two different primary antibodies to further reinforce the

results. Interestingly, AMHR2 was abundantly expressed in AMH treated and non-treated GCs that were cultured for 24 hours, while the receptor was expressed in AMH treated and non-treated TCs, but to a lesser extent than what was evident in GCs. However, at 144 hours of culture, similar expression levels of the receptor were seen in both GC and TCs. Therefore, these findings confirm previous results but extend them by showing that the AMHR2 seems to be expressed in both cell types but to a lesser extent in TCs, throughout the culture period.

In vivo IHC studies further confirm the above findings. The receptor was found to be expressed in both GC and TCs, with the highest level of expression being at the small antral follicle stage. Again the level of its expression was considerably higher in GCs when compared to TCs. In this study little or no expression of the receptor was observed in the early follicles before the preantral follicular stage. This contradicts previous studies that have shown that AMHR2 knock out affected the primordial follicle pool in polyovular rodents (Durlinger *et al.* 2002a). Another study on rats also showed that AMH exerts its action through its receptor in these small follicles (McGee *et al.* 2001). However, in a study on sheep where active immunization was used to regulate AMH bioactivity showed no marked effect on the primordial pool (Campbell *et al.* 2012). Further, AMHR2 mRNA expression was measured in the human early follicular stages and was found to be rarely expressed (Rice *et al.* 2007). Collectively these studies point to a species difference in the expression of the receptor, which can be explained due to the differences seen in the reproductive physiology (Turnbull *et al.* 1977, Franks 1995, de

Souza *et al.* 1998), between monovular species and polyovular rodents.

In conclusion, this chapter provides evidence that in this monovular species, the AMHR2 is expressed in TCs. Although not quantified the level of expression in TCs seems to be at a lower level than that seen in GCs and therefore laying the groundwork for a possible paracrine role for AMH which will be discussed in more detail in the next chapter.

Chapter 4 The Signal Transduction Pathway of AMH in GC and TC

4.1 Introduction

Members of the TGF-β family, of which AMH belongs to, have been shown to signal through the type2 receptor resulting in the formation of a tetrameric complex composed of 2 molecules of the type 2 receptor and 2 molecules of the type 1 receptor. This causes release of the kinase domain, allowing the type 1 receptor to phosphorylate cytoplasmic proteins known as SMADs which will then act as receptor substrates. BMPs and GDFs, engage receptors that signal through SMADs 1, 5 and 8. Phosphorylated SMADs translocate into the nucleus, binding on their way a common SMAD 4 protein that acts as a transcriptional activator (Josso & Clemente 2003).

It has been reported that sertoli cells within the seminiferous tubules of adult rat testis express both AMH and AMHR2 (Baarends *et al.* 1995a). A further study (Racine et al. 1998) found that although AMH was not expressed by leydig cells, AMHR2 was. Thus it was suggested that, in the testis, as well as having autocrine roles, sertoli-derived AMH may also act in a paracrine way to mediate responses in leydig cells. This present study has hypothesised that a similar pattern of expression may exist in the ovary with AMH produced by the GC targeting specific receptors on TC. Further support for this hypothesis was produced by Baarends et al., (1995b) who showed that, in rodents, low levels of this receptor are expressed in the TCs mainly from preantral and small antral follicles. In addition to this, ovine TC have also been reported to express at least two of the type 1 receptors thought to be

used by AMH (Souza *et al.* 2001b). A recent publication (Campbell et al. 2012) has also shown that cultured sheep TC are AMH-responsive. The Campbell et al., (2012) study suggested a direct role for AMH in modulating thecal androgen production by demonstrating that AMH caused a dose dependent inhibition of LH-induced A4 production in TC taken from small (>3 mm in diameter) antral sheep follicles.

Taken together this evidence does suggest a possible paracrine effect on TC by granulosa-derived AMH. However, to-date it has remained unclear whether the reported cellular responses elicited in sheep TC, are mediated by AMHR2 with the intra-cellular signal being transduced as in GC (Josso & Clemente 2003) or via another unknown pathway.

The MAPK signalling pathway is a cascade of protein Ser/Thr kinases that can convey extracellular stimuli into a range of cellular responses and are known to have cellular roles in growth, differentiation and proliferation (Cargnello & Roux 2011), which are very important during folliculogenesis. The MAPK pathway has previously been shown to be involved in AMH mediation in the early stages of rodents follicular growth at the time of primordial to primary follicle transition (Nilsson *et al.* 2007). However, it is still unclear whether AMH, due to its myriad of functions in the ovary, utilises the MAPK pathway in the latter stages of follicular development as an alternative to the SMAD pathway.

The aim of this series of experiments was to address these shortfalls in the current understanding by further characterising the signal transduction pathway of AMH in ovine GC and TC. It was expected that this process would also provide further insights into how AMH exerts its ovarian

inhibitory function. Because of the similarity of sheep reproductive physiology to humans it was again felt to be an ideal model for this part of the study. The essential aim of this set of experiments was to characterise and compare the AMH signalling pathway in sheep TC and GCs. The final aspect studied in this chapter was to determine if AMH uses the MAP kinase pathway as an alternative signalling pathway in GC and TCs.

4.2 Experimental Design

The central aim of this study, to extend the present understanding of how AMH signals to the nucleus in follicular somatic cells and in TC in particular, was undertaken utilising highly sensitive immunochemical technology. Associations between AMH signal transduction pathway entities in GC and TC were determined utilising PLA which has been fully described in the 'Methods' chapter (Section 2.8).

4.2.1 The AMH/AMHR2 Interaction using PLA

In the first part of this work the PLA process was used to determine AMH associations with AMHR2 on GC and TC taken from small (>3 mm in diameter) antral follicles. The cells were cultured on eight-well glass microscope slides, under the conditions described in the 'Methods' chapter (Section 2.1.2.), for periods of time where steroid production would be either low (24 hours) or high (144 hours). At these time points the cells were treated for 1 hour with AMH (50 ng/mL), after which time the media was removed and the cells fixed and prepared for PLA analysis (Section 2.8.), utilising AMH, and AMHR2-specific primary antibodies and primary antibody-specific PLA probes (secondary antibodies). Negative controls were included in all slides to control against secondary antibody cross-reactivity. To consolidate outcomes the PLA process was performed using both a polyclonal and a monoclonal primary antibody for the AMH and AMHR2.

4.2.2 The Interaction of SMAD1/5/8 Complex with SMAD4 using PLA

The next phase of experiments was to determine whether AMH stimulation induced the activation of the canonical BMP

signalling pathway in ovine GC and TC. To do this PLA technology was utilised to determine whether either SMAD 1, 5 or 8 became closely associated with SMAD 4, in these cells, after an AMH (50 ng/mL) challenge. Ovine GC and TC were cultured for 144 hours as described in detail in the methods chapter (Section 2.1.2.). Following this culture period, cells were either left untreated (NT) or were treated with either AMH (50ng/ml), or a positive control (BMP6, 50ng/ml) for 5, 10, 20, 45, 90, and 135 minutes before being fixed (Section 2.8.) in preparation for immunolocalisation of either SMAD 1, 5 or 8 while in complex with SMAD 4. This process utilised SMAD1/5/8 rabbit polyclonal antibody (Santa Cruz Biotechnology) and SMAD4 mouse monoclonal antibody (Santa Cruz Biotechnology) and antibody-specific, PLA probe-conjugated secondary antibodies (Section 2.8.).

4.2.3 The Interaction of AMH Receptor Complex with SMAD 1/5/8 complex using PLA

In parallel experiments in which GC and TC were again cultured for 144 hours and then, either treated with AMH (50ng/ml) or left untreated for 5, 10, 20, 45, and 90 minutes (135 minutes was not included due to a signal not being seen in the previous experiment). The PLA procedure was, again performed as described in the methods chapter (Section 2.8.), but in this experiment AMHR2 mouse monoclonal antibody and a SMAD1/5/8 rabbit polyclonal antibody were the primary antibodies used. Background signalling was controlled for by including negative control wells processed in the absence of primary antibodies.

4.2.4 AMH Induced BMP Responsive Element Activation

Activation of SMAD-responsive genes was determined by measuring expression of a SMAD-responsive luciferase reporter gene. Ovine GC and TC were transfected with the luciferase reporter plasmid construct (BRE-Luc) carrying a SMAD (BMP)-responsive element and an endogenous control (pRL) after 48 hours of culture. The timing of the transfection was set at 48 hours of culture, to ensure that Luciferase was assayed from cells producing measurable steroids. The full method can be found in the Methods chapter (Section 2.5.). After 24 hours of culture in the transfection media it was removed from both the GC and TC and replaced with fresh media alone or containing either AMH (50ng/ml), or BMP6 (50ng/ml) and cultured for a further 45, 90, 135 or 180 minutes. Cells were then lysed and luciferase production determined by a dual luciferase reporter assay as described in the Methods chapter (Section 2.6.).

4.2.5 The Effect of SMAD Signal Transduction Pathway Inhibition on AMH Ovarian Function in GC and TC

To provide further insights into AMH signalling cells were subjected to dorsomorphin homologue 1 (DMH1) a novel second generation inhibitor of BMP signalling that specifically targets the SMAD pathway (Hao *et al.* 2010). This section of the work sought to characterise the effect of inhibiting the SMAD pathway in ovine GC and TC, on E2 and A4 respectively.

4.2.5.1 DMH1 Induced SMAD Signalling Inhibition in GC and TC

The first stage was to verify the effect of DMH1 on the formation of SMAD activation complexes. Ovine GC and TC

were seeded on to 8-well culture slides as previously described (Section 2.1.2.) and allowed to settle for a period. At the end of either a 24 hour or 144 hour culture period, cells were treated with either DMH1 (0.5μM) resuspended in DMSO (to a final concentration of 0.5%) or left untreated. A negative control was also set up with DMSO alone (0.5% final working concentration) as a control against DMSO effects. After a further 30 minutes the cells were treated with either AMH (50ng/ml), BMP6 (50ng/ml), or left untreated and then incubated for a further 30 minutes. In the cultures left for 144 hours before treatments, DMH1 was administered at both 0.5μM (low) or a 20μM (high) concentration. In all other respects the same protocol as above described was utilised. After the 30 minute treatment period (chosen as the optimal time for SMAD pathway activation), ICC was performed using the PLA process to determine SMAD1, 5 or 8 interactions with SMAD 4.Again, as earlier described) this process involved the use of a rabbit polyclonal SMAD 1/5/8 antibody and a SMAD 4 mouse monoclonal antibody.

4.2.5.2 The Effect of SMAD Signalling Inhibition on Ovarian Steroidogenesis

A further set of cell cultures were performed in order to determine the effect of AMH on steroidogenesis, oestradiol (E2, produced by GC cells) and androstenedione (A4, produced by TC) levels were measured. GC and TC were cultured and assayed after 24, 48, 96, 144, and 192 hours of culture. At each time point the cells were either left untreated in DMSO alone (as negative controls), treated with DMH1 (at 0.5μM in DMSO) for 1 hour, treated with DMH1 (at 0.5μM in DMSO) for 30 minutes and then stimulated with AMH (50 ng/mL) for a further 30 minutes or stimulated with AMH (50

ng/mL) alone in only DMSO for 30 minutes. This culture layout was repeated with a higher concentration of DMH1 (at 20 μM in DMSO) and challenged for a longer period of time. The cells were either left untreated in DMSO alone, treated with DMH1 (at 20μM in DMSO) for 24 hour prior to media removal at each time point, treated with DMH1 (at 20μM in DMSO) and stimulated with AMH (50 ng/mL) for 24 hours or stimulated with AMH (50 ng/mL) alone in only DMSO for the same period of time, before media removal. Following the planned treatment, at each specific time point, all the media from the wells was stored, for later analysis by ELISA to measure either E2 (GC) or A4 (TC) production as described in chapter 2 (Section 2.3.2.). Cell lysates were also collected and stored and used to measure total RNA as a means to normalise against differences in the original cell number again, as described in chapter 2 (Section 2.3.1.1).

4.2.5.3 The Effect of AMH with or without DMH1 treatment on the MAPK signalling pathway

The final aim in this stage of the study was to test a putative alternative signalling pathway for AMH in ovine GC and TC. This utilised another luciferase reporter plasmid construct assay, although in this case the construct was designed to determine the activation of the serum response element (SRE) pathway in which mitogen activated protein kinase (MAPK) is an intermediary. The reporter construct incorporates an SRE-responsive element controlling expression of a luciferase reporter gene. Ovine GC and TC were transfected (Section 2.5.) with the SRE-Luciferase construct (Cignal) after 48 hours of culture. Again the transfection time was chosen to ensure that cells would be maximally producing steroids by the time of Luciferase (SRE pathway-activation) analysis.

The cells were then left in culture for a further 24 hours, after which time they were either left untreated or treated with 20μM DMH1 (resuspended in DMSO at a working concentration of 0.5%). The DMH1-treated cells also had additions of AMH (50ng/mL). After a further 23 hours of culture, the untreated cells were treated with 0.5μM DMH1 (resuspended in DMSO at a working concentration of 0.5%). All cells were then cultured for a further 30 minutes when the 0.5μM DMH1 cultures were additionally treated with AMH (50ng/mL). All cells were then maintained for a further final 30 minutes (96 hours of culture in total). After lysis the cell lysates were assayed for luciferase production using the dual luciferase reporter assay as described in the Methods chapter (Section 2.6.).

4.3 Statistical Analysis

In all cases, data from this chapter are presented as mean +/- SEM of at least three different experiments conducted in triplicate. Data were tested for normality before using a two way ANOVA on SPSS to determine sample group differences for E2, and A4 production; and BMP induced activation of the BMP signalling pathway; and AMH induced activation of the MAP signalling pathway. Statistical significance was set as $p<0.05$. For all ELISAs the inter-and intra-assay coefficients of variation were all within 10%, and cell-number differences were normalised against total RNA amounts for the same sample and relative quantities measured in arbitrary units (AU).

4.4 Results

4.4.1 The Signal Transduction Pathway of AMH in GC and TC

4.4.1.1 The AMH/AMHR2 Interaction using PLA

Using PLA, the interaction between AMH and the AMH2 was clearly noted in both the GC and TC that were cultured for 24 and 144 hours and treated with AMH for 1 hour (Figure 4.1,Figure 4.2 and Figure 4.3).

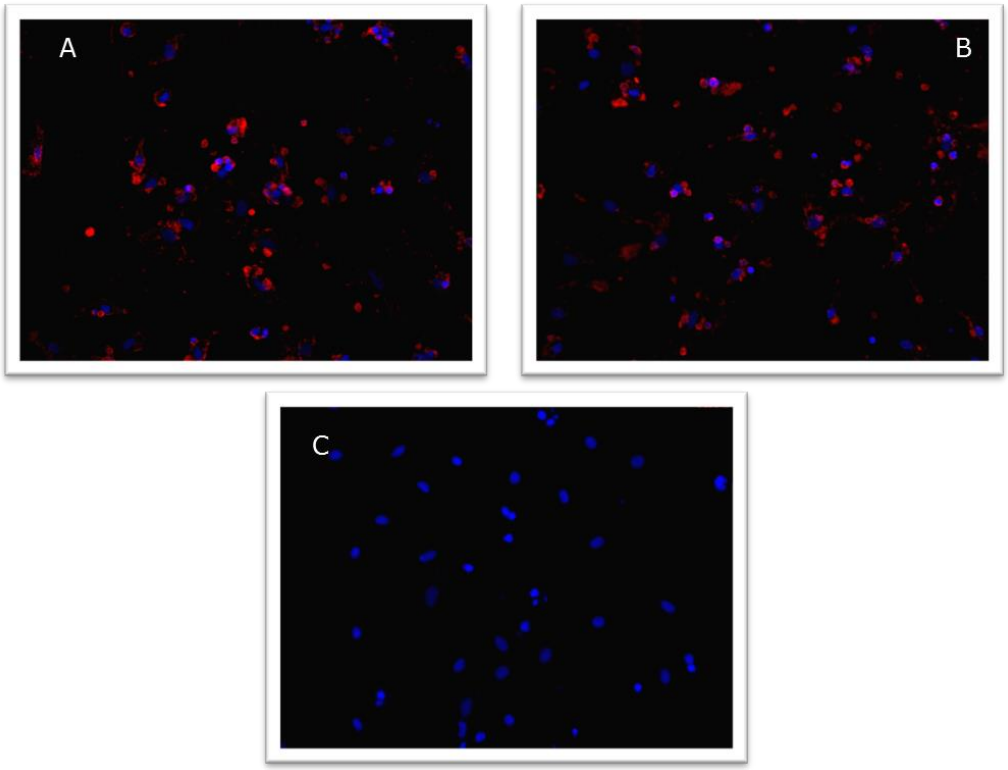


Figure 4.1 The AMHR2/AMH interaction using PLA in 144h cultured, ovine TC that were treated with AMH for different periods of time.
A primary AMHR2 polyclonal antibody and an AMH Monoclonal antibody were used. Cells were treated for 1 hour (A), 30 minutes (B). Negative control (C). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x200 magnification.

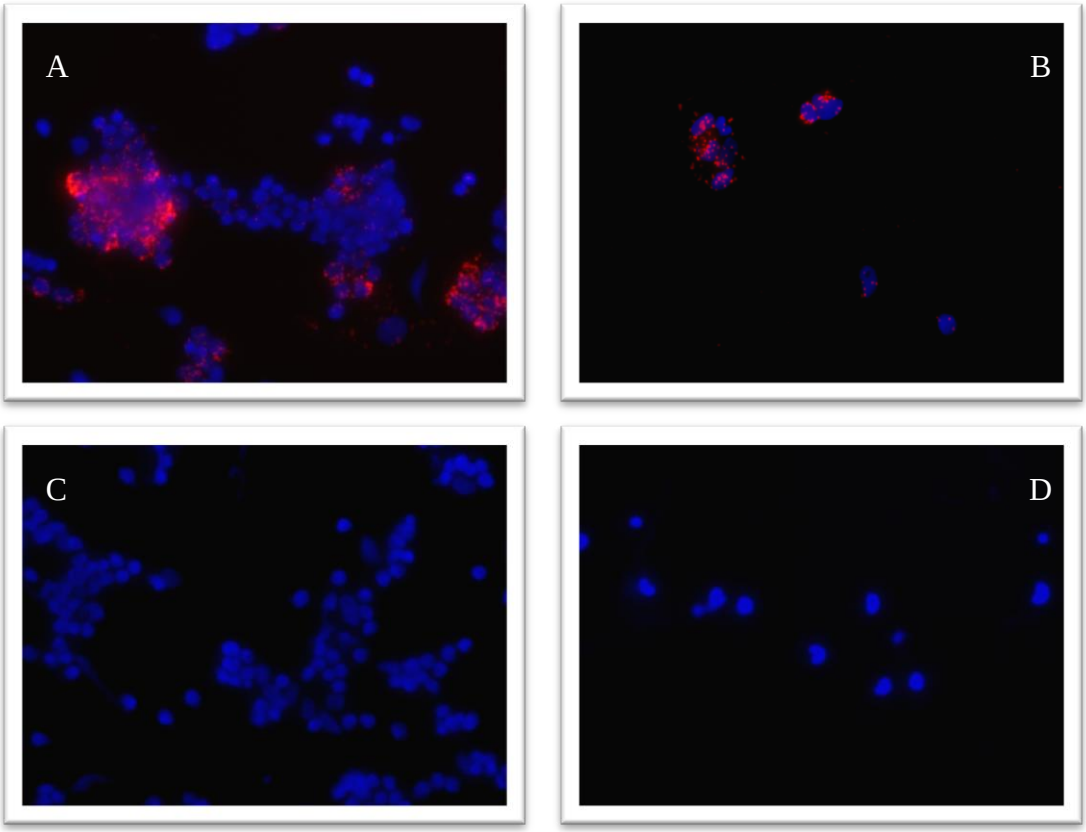


Figure 4.2 The AMHR2/AMH interaction using PLA in 24h cultured, ovine GC and TC that were treated with AMH for 1 hour.
A primary AMH polyclonal antibody and an AMHR2 Monoclonal antibody were used. GC treated for 1 hour (A), TC treated for 1 hour (B), GC negative control (C), and TC negative control (D). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

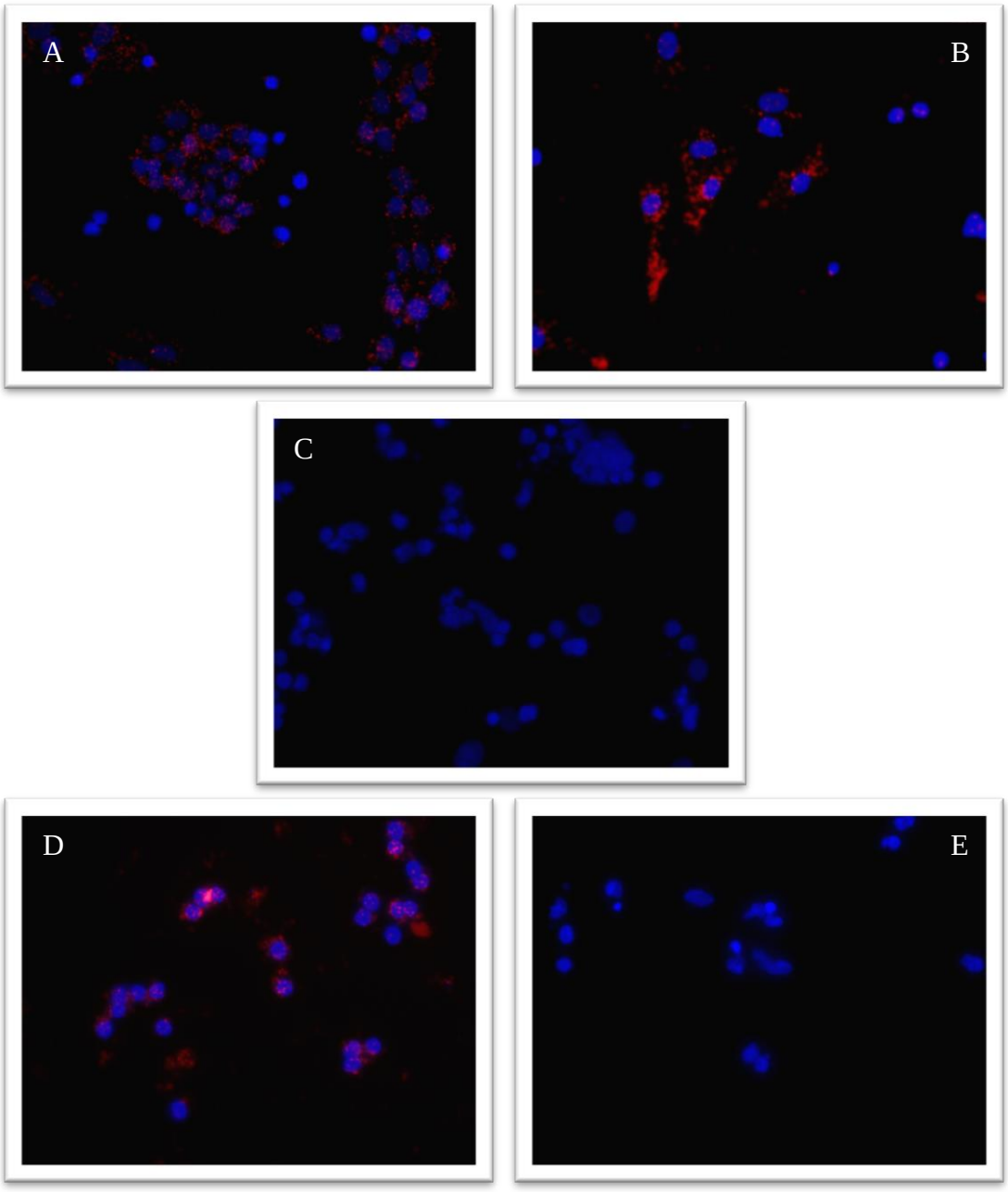


Figure 4.3 The AMHR2/AMH interaction using PLA in 144h cultured, ovine GC and TC.

Elucidation of the AMHR2/AMH interaction using PLA in 144h cultured, ovine GC (A and B) and TC (D) that were treated with 50ng/ml of AMH for 1 hour. A primary AMHR2 polyclonal antibody and an AMH Monoclonal antibody were used in (A). While, a primary AMH polyclonal antibody and an AMHR2 Monoclonal antibody were used in (B and D). GC negative control (C), and TC negative control (E). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

4.4.1.2 The Interaction of SMAD1/5/8 Complex with SMAD4 using PLA

Untreated GC showed some evidence of SMAD 1/5/8 and SMAD 4 interaction. However, treatment with 50mg/ml AMH or BMP6 induced the formation of the SMAD 1/5/8 and SMAD 4 complex, with the amount of complex formation reaching its highest at around 10-20 minutes. Thereafter, the amount of complex formation declined steadily reaching levels similar to the non-treated cells by 135 minutes (Figure 4.4 and Figure 4.5).

In untreated TCs, virtually no immunoflorescent signal was detected, which indicates little or no interaction occurring between SMAD 1/5/8 and SMAD 4. However, treatment with 50mg/ml AMH induced the formation of the SMAD 1/5/8 and SMAD 4 complex which appeared to increase over time, reaching a peak at about 10-20 minutes. Thereafter, the signal appeared to decline noticeably, reaching no-treatment levels by 90 minutes (Figure 4.6).

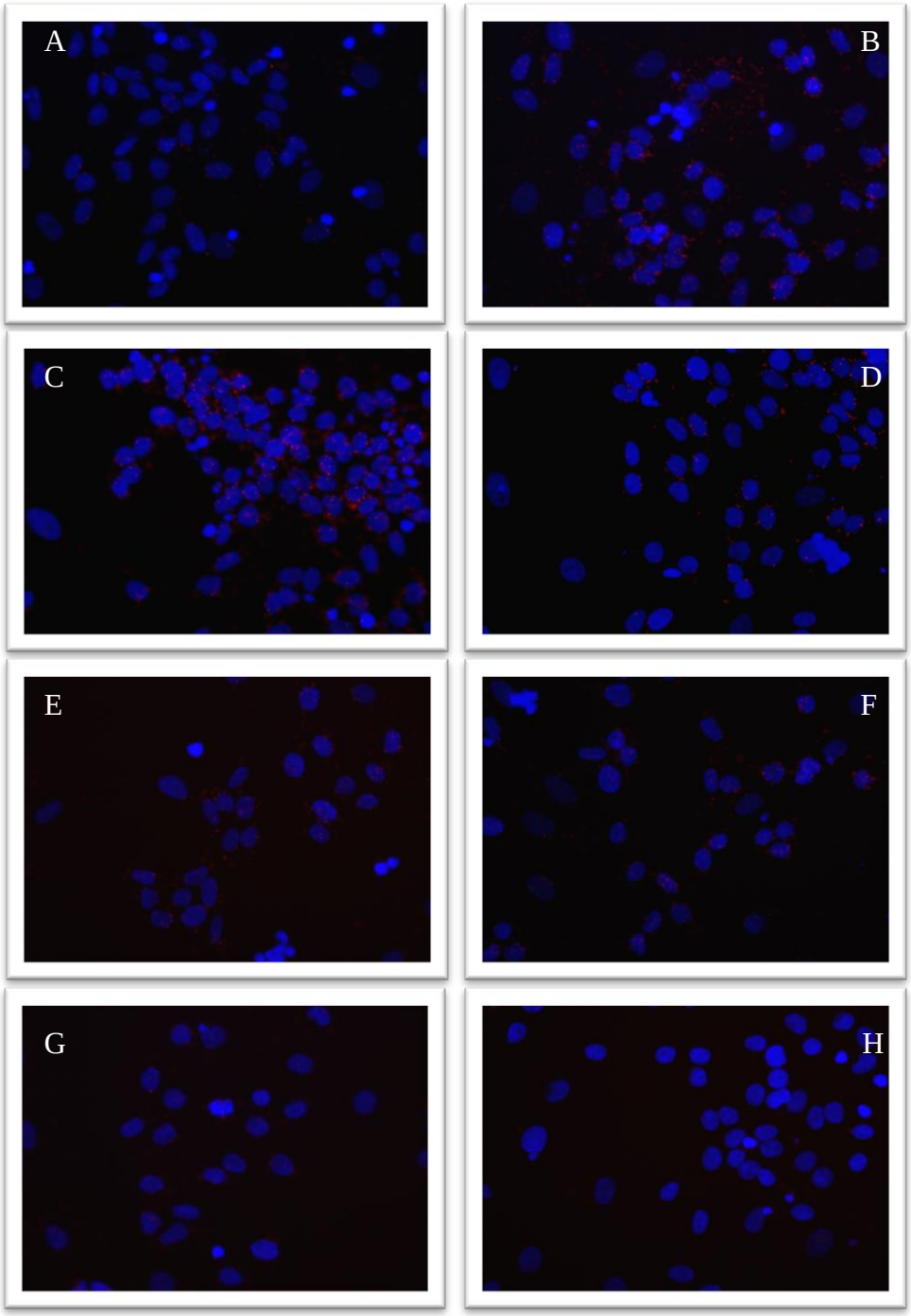


Figure 4.4 The AMH induced SMAD 1/5/8/-SMAD 4 interactions using PLA in 144h cultured ovine GC following different treatment durations.

The cells were either non treated (A), or treated for 5 min (B), 10 min (C), 20 min (D), 45 min (E), 90 min (F), 135 min (G), -ve control with no primary antibody (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

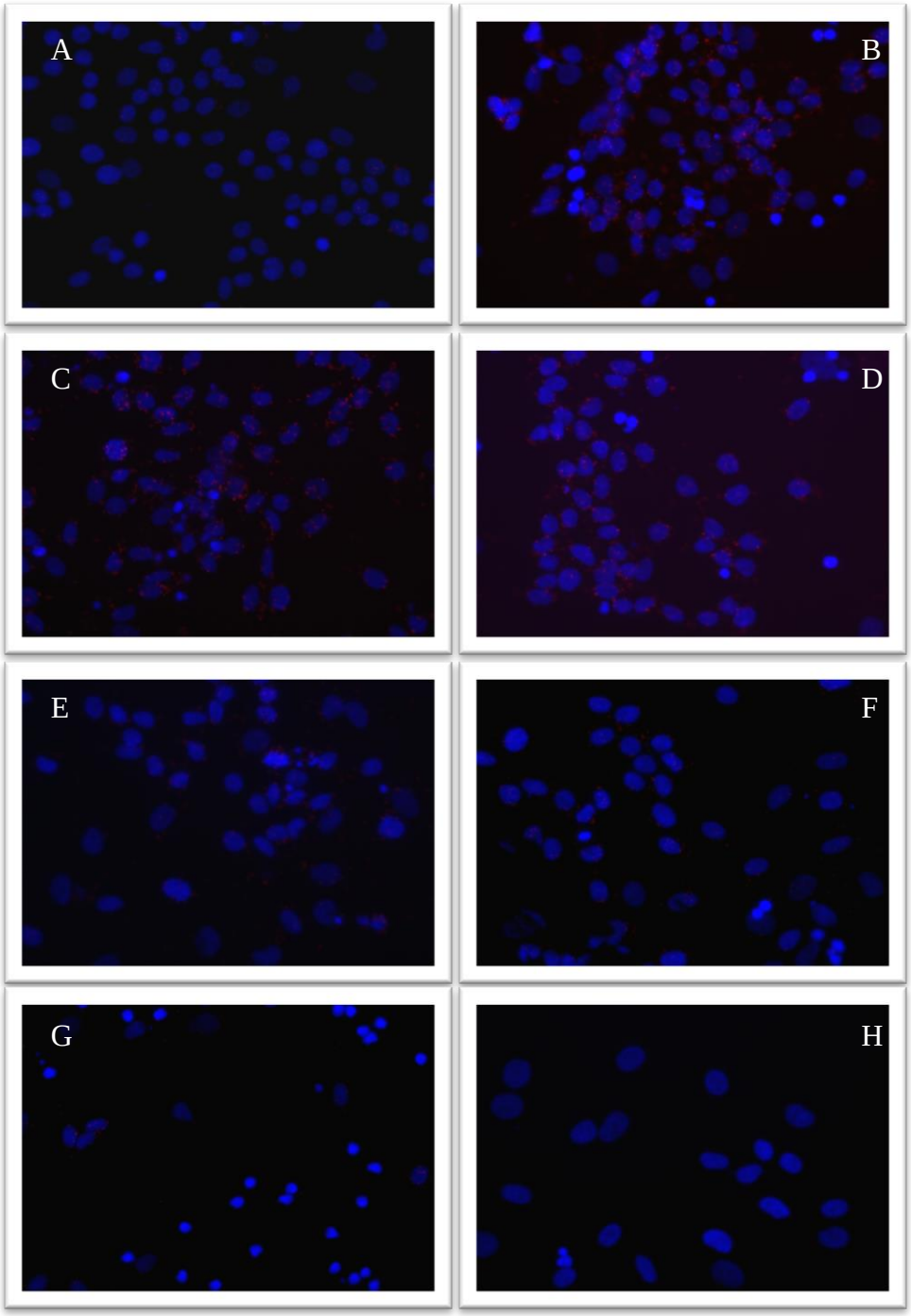


Figure 4.5 The BMP6 induced SMAD 1/5/8/-SMAD 4 interactions using PLA in 144h cultured ovine GC following different treatment durations.

The cells were either non treated (A), or treated for 5 min (B), 10 min (C), 20 min (D), 45 min (E), 90 min (F), 135 min (G), -ve control with no primary antibody (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

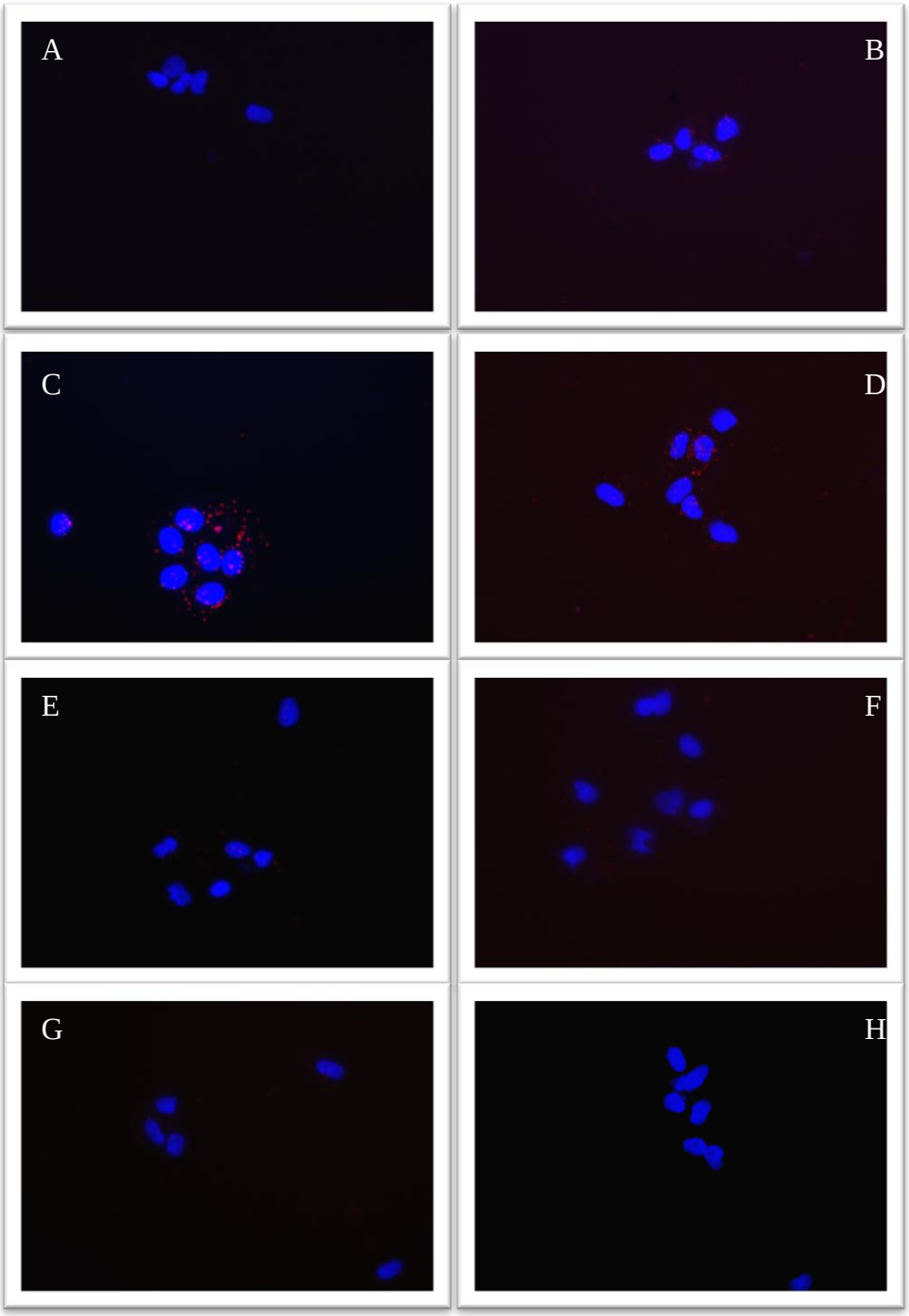


Figure 4.6 The AMH induced SMAD 1/5/8/-SMAD 4 interactions using PLA in 144h cultured ovine TC following different treatment durations.

The cells were either non treated (A), or treated for 5 min (B), 10 min (C), 20 min (D), 45 min (E), 90 min (F), 135 min (G), -ve control with no primary antibody (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

4.4.1.3 The Interaction of AMH Receptor Complex with SMAD 1/5/8 complex using PLA

The AMH induced receptor/SMAD 1/5/8 interaction in both GC and TCs showed a similar pattern to what was observed in the experiment involving SMAD 1/5/8 and SMAD 4 interaction.

No interaction was observed between AMH receptor complex and SMAD 1/5/8 in untreated TC. However, treatment with AMH induced the formation of the complex starting from 5 minutes reaching a peak at about 10-20 minutes and declining thereafter to non-treatment levels by 90 minutes (Figure 4.8).

Non treated GC showed minimal complex formation. However, treatment with AMH resulted in the AMH receptor and SMAD 1/5/8 complex formation starting from 5 minutes of treatment, then peaking at about 10-20 minutes and finally declining to non-treatment levels by 90 minutes (Figure 4.7).

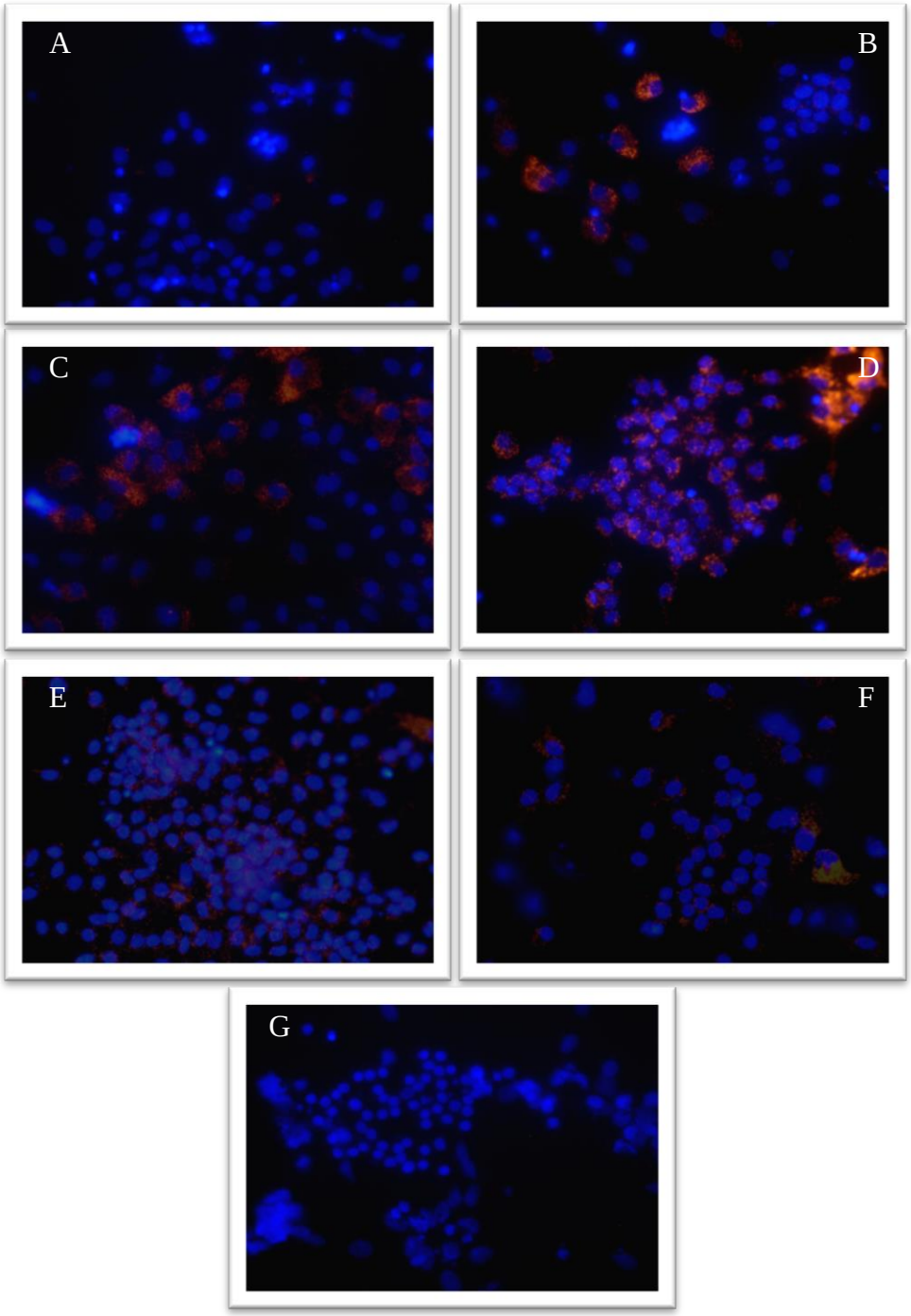


Figure 4.7 The AMH induced AMHR2-Smad 1/5/8 interactions using PLA in 144h cultured ovine GC following different treatment durations.

The cells were either non treated (A), or threated for 5 min (B), 10 min (C), 20 min (D), 45 min (E), 90 min (F), -ve control with no primary antibody (G). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

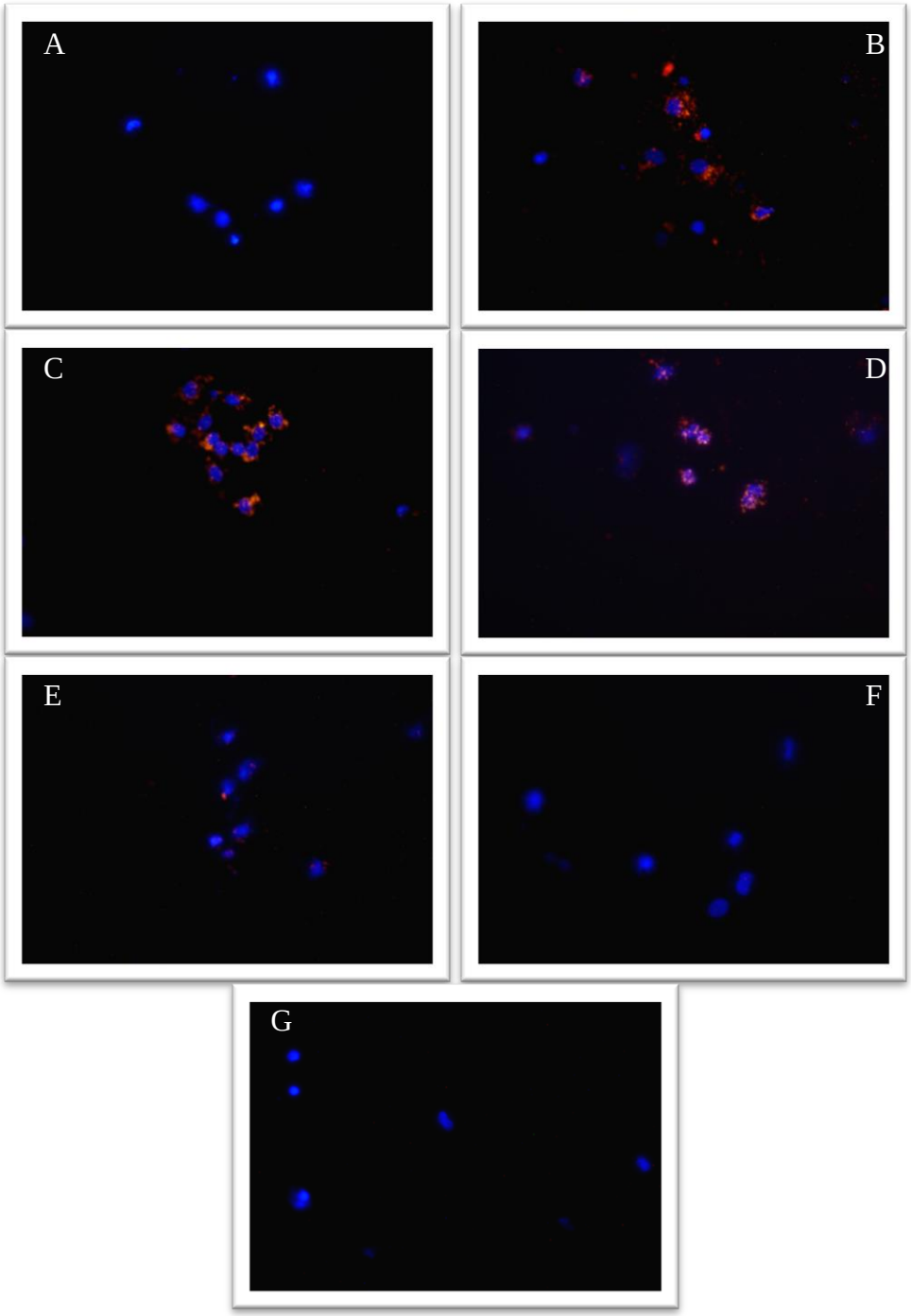


Figure 4.8 The AMH induced AMHR2- SMAD 1/5/8 interactions using PLA in 144h cultured ovine TC following different treatment durations.

The cells were either non treated (A), or treated for 5 min (B), 10 min (C), 20 min (D), 45 min (E), 90 min (F), -ve control with no primary antibody (G). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

4.4.1.4 AMH Induced BMP Responsive Element Activation

Untreated GCs showed a steady low level of luciferase activity throughout. Treatment with BMP6 showed a significantly higher ($p<0.01$) luciferase production than non-treated cells following 45 minutes of treatment. The level of luciferase production decreased steadily thereafter but still continued to be statistically higher ($p<0.05$) at 90 minute, reaching non-treated luciferase production levels at 135 minutes of treatment. AMH induced significantly higher levels of luciferase activity ($p<0.01$) compared to non-treated cells throughout the treatment period until 135 minutes and then declined to non-treatment levels by 180 minutes (Figure 4.9).

In TC, untreated cells also showed a steady low level of luciferase activity throughout. AMH induced a similar pattern of luciferase activity to that seen in GCs. On the other hand, BMP6 treatment induced a significantly higher level of luciferase production ($p<0.01$) at 45 minutes of stimulation but thereafter showed an early drop in luciferase activity reaching non-treatment levels by 90 minutes of treatment (Figure 4.10).

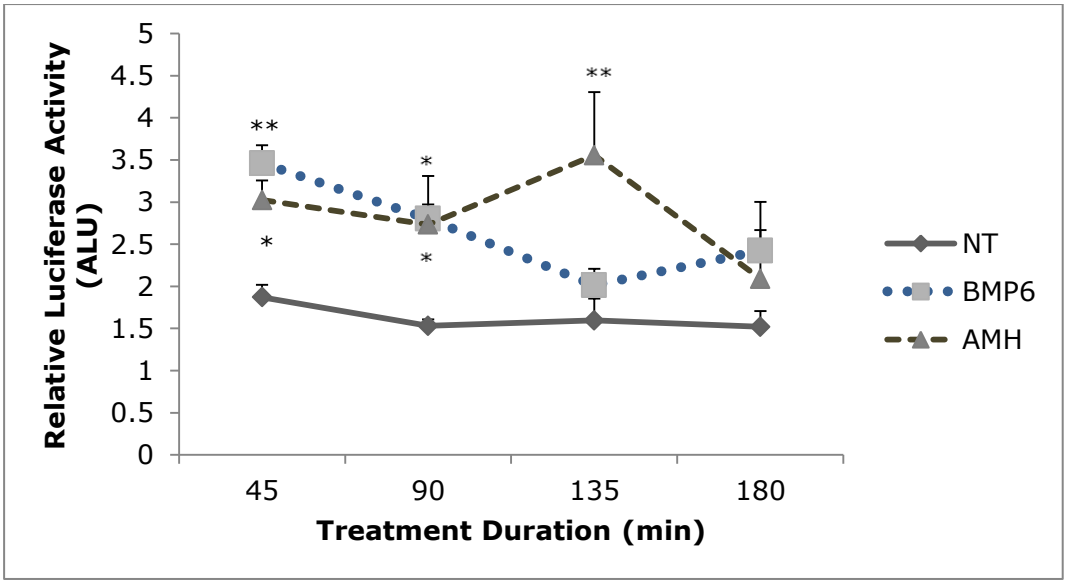


Figure 4.9 The effect of NT, AMH, and BMP6 on the activation of the BRE, after different periods of time, in cultured ovine GC.
Values represent mean ± SEM. *, P<0.05, and **, P< 0.01 indicates a significant difference from NT at the same treatment duration.

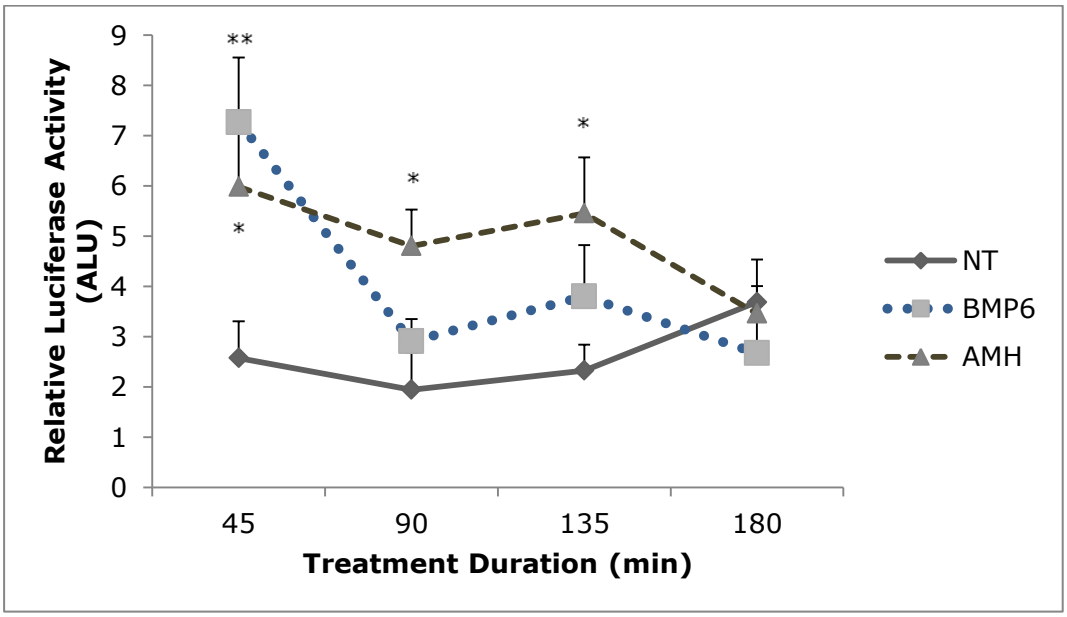


Figure 4.10 The effect of NT, AMH, and BMP6 on the activation of the BRE, after different periods of time, in cultured ovine TC.
Values represent mean ± SEM. *, P<0.05, and **, P< 0.01 indicates a significant difference from NT at the same treatment duration.

4.4.2 The Effect of SMAD Signal Transduction Pathway Inhibition on AMH Ovarian Function in GC and TC

4.4.2.1 DMH1 Induced SMAD Signalling Inhibition in GC and TC

GC and TCs stimulated with BMP6 were used as positive controls, while cells that were treated with DMSO alone and not stimulated with either AMH or BMP6 were used as negative controls, with DMSO acting as a non-inhibitory carrier for DMH1.

In GC that were cultured for 24 hours, an immuno-fluorescent signal was detected in AMH treated cells (50ng/ml for 30 minutes) that were not subjected to either DMH1 suspended in DMSO or DMSO alone, indicating that an interaction occurring between SMAD1, 5 or 8 and SMAD 4 (Figure 4.11 A). A similarly intense immuno-fluorescent signal was also observed in cells that were treated with AMH (50ng/ml for 30 minutes) in DMSO (0.5μM) alone, for 1 hour (Figure 4.11 C). However, this was highly attenuated when the GC were treated for 1 hour with DMH1 (0.5μM) suspended in DMSO (Figure 4.11 D). A diminished level of signalling was observed in cells that were treated with either DMSO or DMH1 but were not exposed to a stimulant (Figure 4.11 G, H).

Similar results were seen in TCs that were incubated for the same period of time. Although, signalling was generally not as widespread as that seen in GCs (Figure 4.12 A, C). Signalling in the DMH1 treated cells appeared to be curtailed (Figure 4.12 D, F, H).

In GC that were cultured for 144 hours, an intense immuno-fluorescent signal was detected in AMH stimulated cells

(50ng/ml for 30 minutes) that were not treated with either DMSO or DMH1 (Figure 4.13 A) and in AMH stimulated cells that were exposed to DMSO (0.5μM for 1 hour) alone (Figure 4.13 C), Whilst non quantified, the immune-fluorescent signal appeared slightly lower in the wells that were treated with DMH1 (Figure 4.13 D, F, H). However, cells that were not stimulated by AMH but treated with DMSO still showed a high level of signalling perhaps indicating to peak SMAD mediated endogenous AMH signalling within GC at this time point (Figure 4.13 G).

Similar findings were seen in TC that were subjected to the same culture conditions as that of GC. An immune-fluorescent signal was detected in AMH stimulated cells (50ng/ml for 30 minutes) that were not treated with either DMSO or DMH1 (Figure 4.14 A). A signal was also detected in cells that were stimulated with AMH in DMSO only (Figure 4.14 C). However, the signal in these wells appeared not as intense as that seen in GC. Further, very minimal signalling was seen in the cells that were treated with DMSO but not stimulated by AMH (Figure 4.14 G), perhaps explained by the lack or minimal endogenous AMH production in TC. The immune-fluorescent signal was attenuated in the wells that were treated with DMH1 but the signal was still evident (Figure 4.14 D, F, H).

The previous experiment was repeated but the GC and TCs that were cultured for 144 hours were exposed to a higher treatment dose of DMH1 (20μM for 1 hour).

In GC, a high immune-fluorescent signal was detected in AMH stimulated cells (50ng/ml for 30 minutes) that were not subjected to either DMSO or DMH1 (Figure 4.15 A) and in AMH stimulated cells in DMSO only (Figure 4.15 C). However, the

immune-fluorescent signal appeared nearly completely shut down in the wells that were treated with DMH1 (20μM for 1 hour) (Figure 4.15 D, F, H). Likewise, cells that were not stimulated by AMH but treated with DMSO showed minimal signalling (Figure 4.15 G).

Although not quantified, signalling in TC appeared markedly higher in AMH stimulated TC that were devoid of DMSO and DMH1 Treatment (Figure 4.16 A), and in cells that were stimulated with AMH in DMSO only (Figure 4.16 C), when compared to DMH1 treated TC (Figure 4.16 D, F, H).

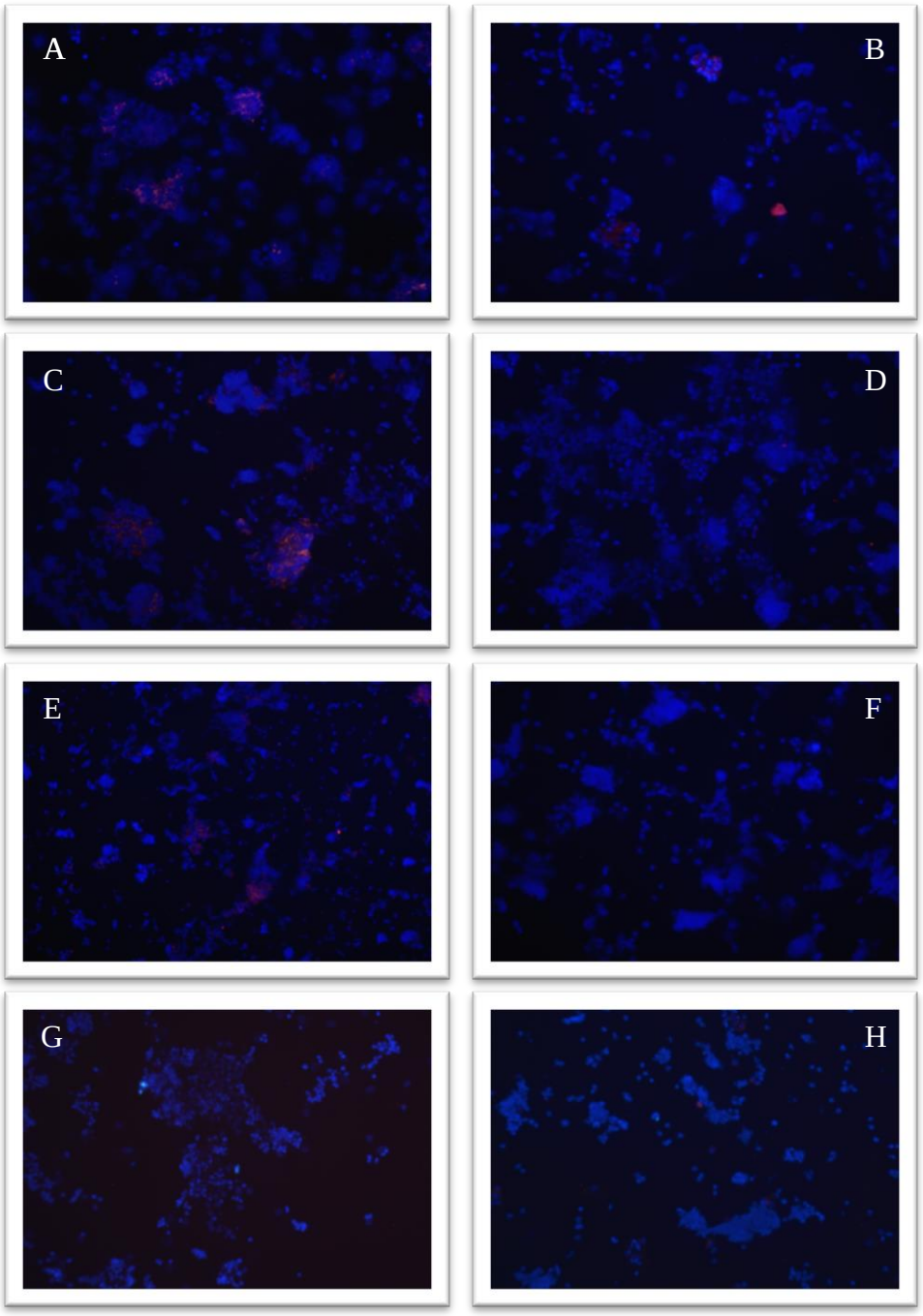


Figure 4.11 The effect of AMH or BMP6 on SMAD 1/5/8 and SMAD 4 interaction using PLA in 24h cultured GC with or without DMH1.
The cells were NT, DMSO only, or 0.5μM DMH1 for 1 h. AMH (A), BMP6 (B), AMH in DMSO (C), AMH and DMH1 (D), BMP6 in DMSO (E), BMP6 and DMH1 (F), DMSO with no stimulant (G), DMH1 with no stimulant (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x200 magnification.

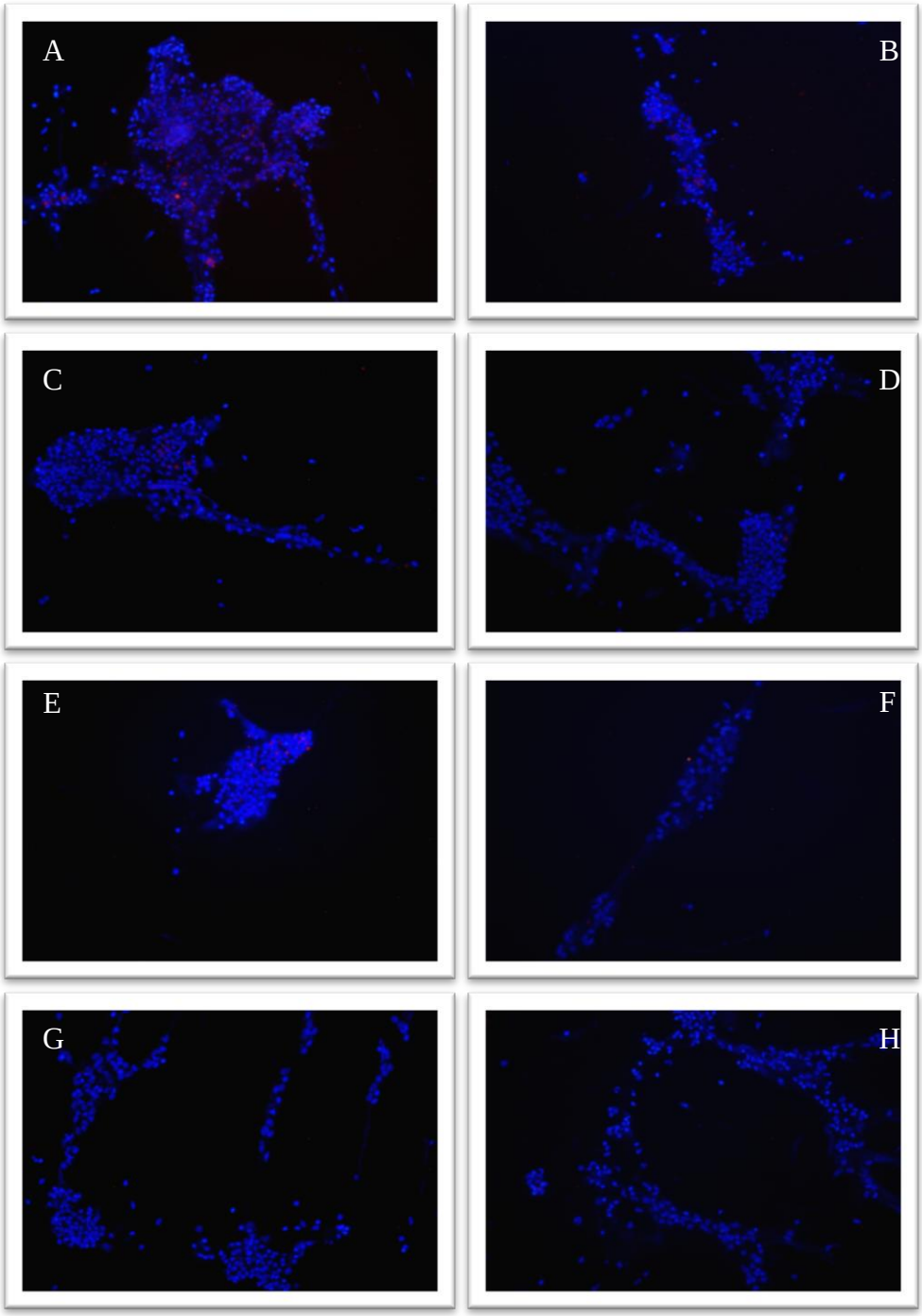


Figure 4.12 The effect of AMH or BMP6 on SMAD 1/5/8 and SMAD 4 interaction using PLA in 24h cultured TC with or without DMH1.
The cells were NT, DMSO only, or 0.5μM DMH1 for 1 h. AMH (A), BMP6 (B), AMH in DMSO (C), AMH and DMH1 (D), BMP6 in DMSO (E), BMP6 and DMH1 (F), DMSO with no stimulant (G), DMH1 with no stimulant (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x200 magnification.

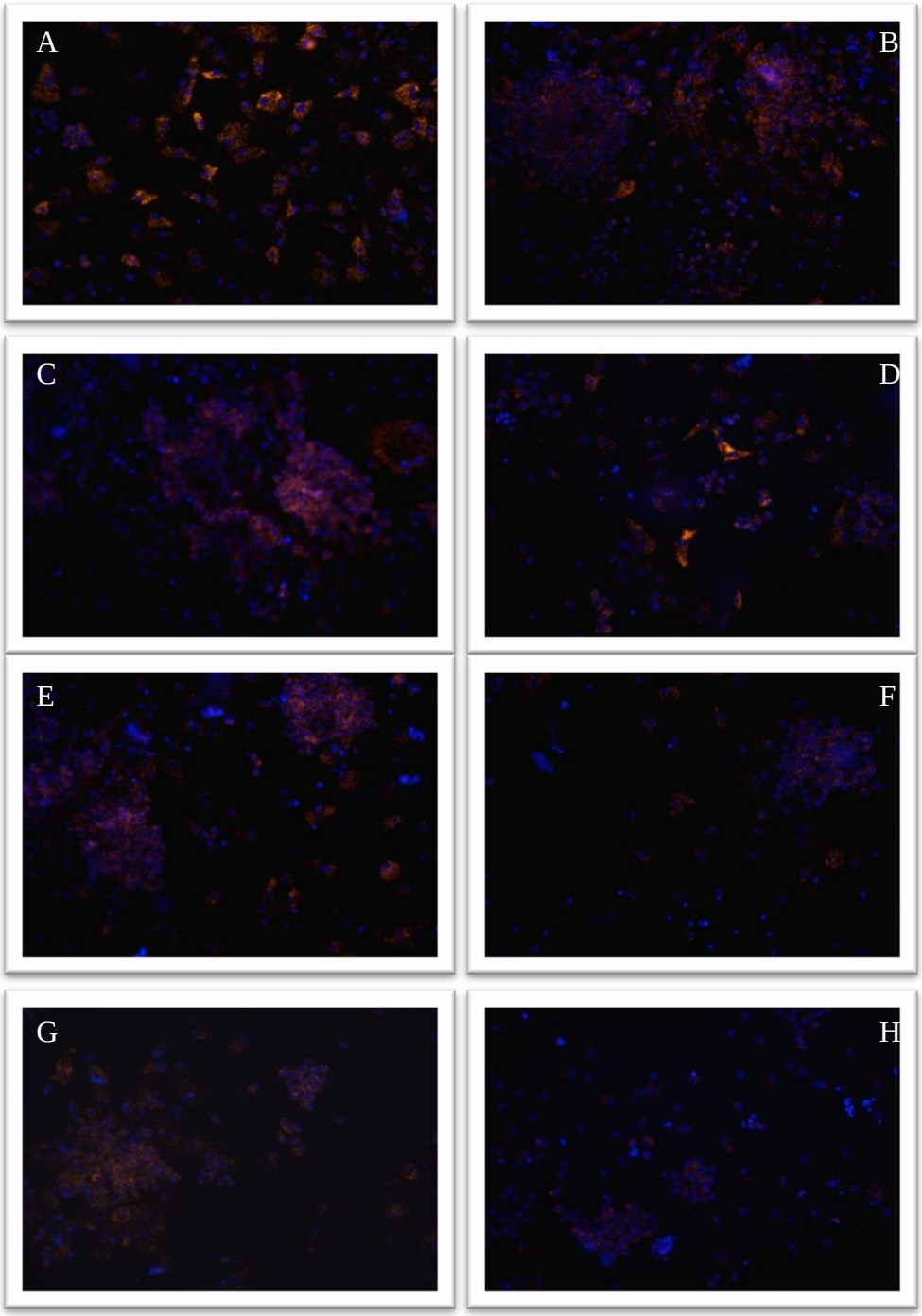


Figure 4.13 The effect of AMH or BMP6 on SMAD 1/5/8 and SMAD 4 interaction using PLA in 144h cultured GC with or without DMH1.
The cells were NT, DMSO only, or 0.5μM DMH1 for 1 h. AMH (A), BMP6 (B), AMH in DMSO (C), AMH and DMH1 (D), BMP6 in DMSO (E), BMP6 and DMH1 (F), DMSO with no stimulant (G), DMH1 with no stimulant (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x200 magnification.

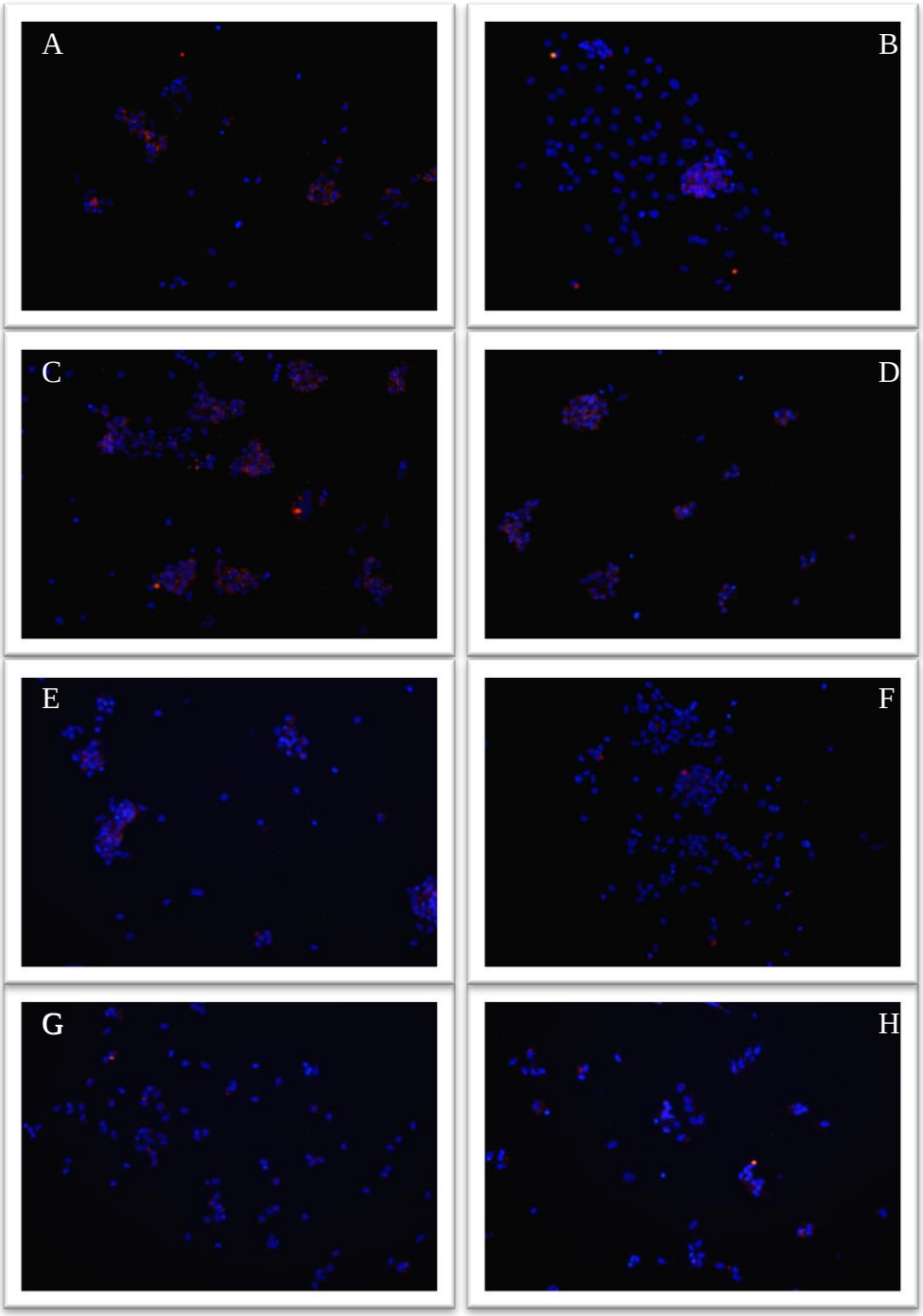


Figure 4.14 The effect of AMH or BMP6 on SMAD 1/5/8 and SMAD 4 interaction using PLA in 144h cultured TC with or without DMH1.
The cells were NT, DMSO only, or 0.5μM DMH1 for 1 h. AMH (A), BMP6 (B), AMH in DMSO (C), AMH and DMH1 (D), BMP6 in DMSO (E), BMP6 and DMH1 (F), DMSO with no stimulant (G), DMH1 with no stimulant (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x200 magnification.

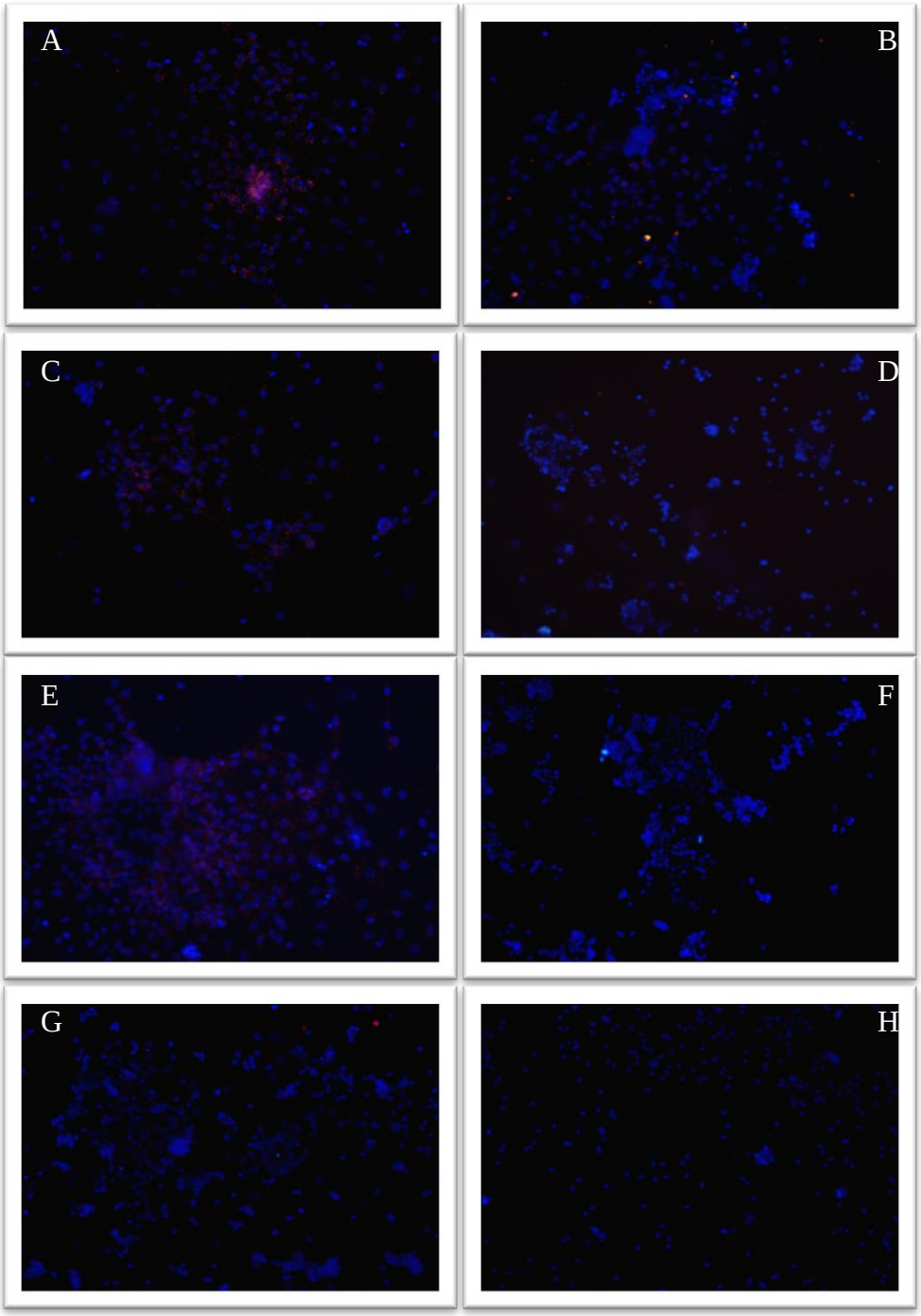


Figure 4.15 The effect of AMH or BMP6 on SMAD 1/5/8 and SMAD 4 interaction using PLA in 144h cultured GC with or without DMH1.
The cells were NT, DMSO only, or 20μM DMH1 for 1 h. AMH (A), BMP6 (B), AMH in DMSO (C), AMH and DMH1 (D), BMP6 in DMSO (E), BMP6 and DMH1 (F), DMSO with no stimulant (G), DMH1 with no stimulant (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x200 magnification.

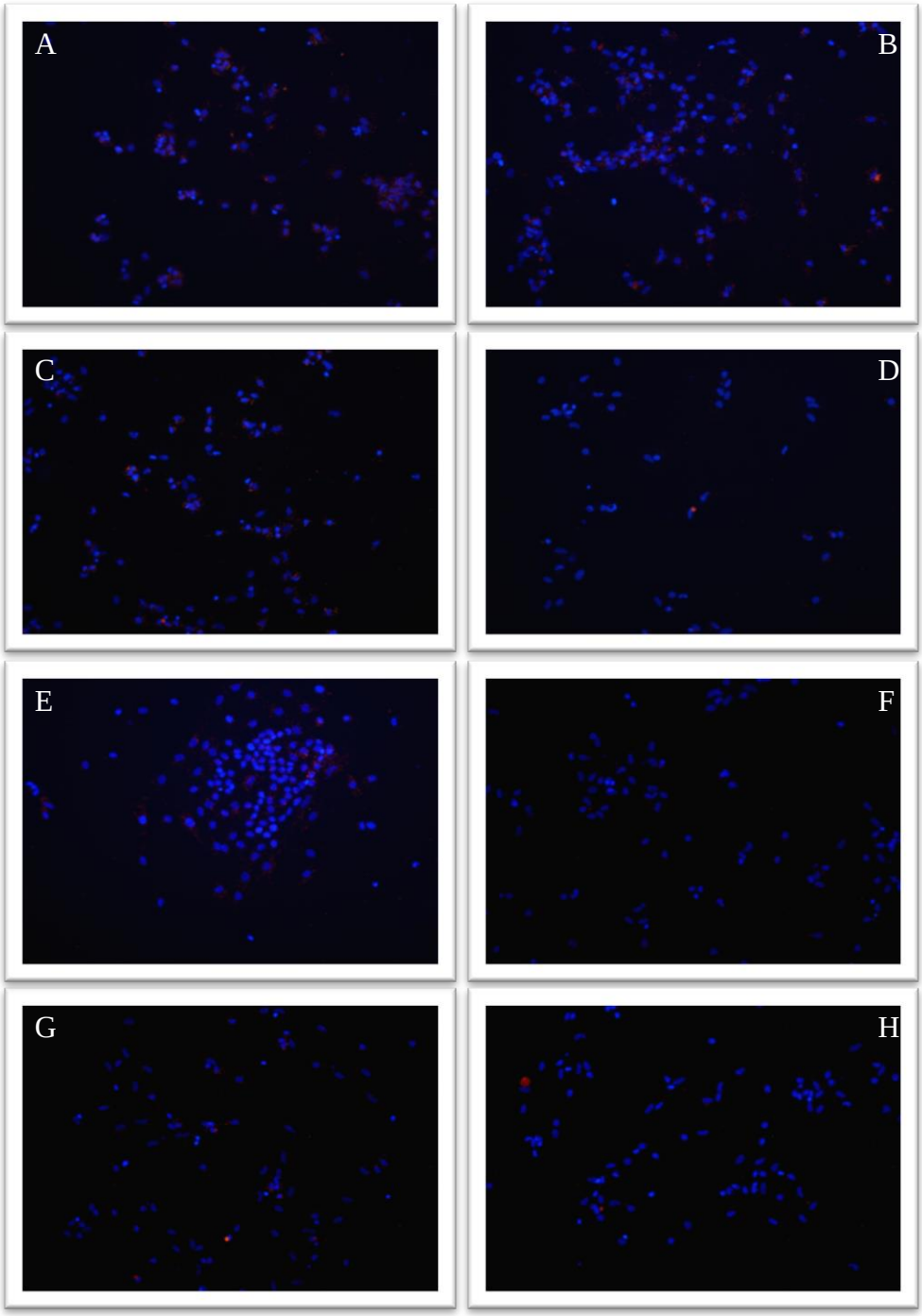


Figure 4.16 The effect of AMH or BMP6 on SMAD 1/5/8 and SMAD 4 interaction using PLA in 144h cultured TC with or without DMH1.
The cells were NT, DMSO only, or 20μM DMH1 for 1 h. AMH (A), BMP6 (B), AMH in DMSO (C), AMH and DMH1 (D), BMP6 in DMSO (E), BMP6 and DMH1 (F), DMSO with no stimulant (G), DMH1 with no stimulant (H). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x200 magnification.

4.4.2.2 The Effect of SMAD Signalling Inhibition on Ovarian Steroidogenesis

E2 and A4 were measured using ELISA in the culture media from both GC and TCs cultures respectfully. Steroid production was observed to be gradually increasing across each time point, from 24 hours until reaching a maximum production at 192 hours ($p<0.001$) and ($p<0.001$) respectively (Figure 4.17 and Figure 4.18).

4.4.2.2.1 E2 production by GC following 0.5μM DMH1 treatment and AMH stimulation

AMH stimulation with or without DMH1 resulted in no statistical difference in GC E2 production between the treatment groups until 144 hours of culture. However at 192 hours of culture, stimulation of GC with AMH, that were in only DMSO, resulted in a statistically significant decrease ($p<0.01$) in E2 production when compared to the control group (NT DMSO). At this same time point, no statistically significant change was observed in E2 production following DMH1 treatment in either the AMH treated or in the NT group when compared to the control (Figure 4.17).

4.4.2.2.2 A4 production by TC following 0.5μM DMH1 treatment and AMH stimulation

Minimal differences in A4 production were evident between different treatment groups in TC that were cultured at 24 and 48 hours. However, following 96 hours of culture, AMH treatment in only DMSO caused a statistically significant decrease in TC A4 production when compared to the control group (NT DMSO) ($p<0.01$), while other groups showed no significant differences from the control. At 144h of TC culture,

AMH stimulation caused a statistically significant decrease in A4 production both in the DMH1 treated group ($p<0.01$) and in the DMSO treatment group ($p<0.01$) when compared to the control group (NT DMSO). This difference was less evident in the TCs that were treated with DMH1 when compared to TCs that were exposed to only DMSO (Figure 4.18). Similarly, at 192 hours of culture, AMH in DMSO caused a statistically significant decrease in A4 production ($p<0.01$) when compared to the control, while AMH and DMH1 treatment also caused a significant decrease in A4 production ($p<0.05$) when compared to the control but again this effect was less than the AMH in DMSO treatment group (Figure 4.18).

4.4.2.2.3 E2 production by GC following 20μM DMH1 treatment and AMH stimulation

A substantial increase in GC production of E2 was seen after 144 and 192 hours of culture. At these same time points, exposure of GC with AMH that is in DMSO only, resulted in a statistically significant decrease in E2 production when compared to the control group (NT DMSO) ($p<0.05$ and $p<0.01$ respectively). However, when the cells were stimulated with AMH in the presence of the high dose of DMH1, no significant difference was observed when compared to the control group (NT DMSO). Interestingly, at these same two time points, cells that were not stimulated with AMH but treated with DMH1 resulted in a statistically significant increase ($p<0.05$ and $p<0.01$ respectively) in the production of E2 when compared to the control (NT DMSO) (Figure 4.19).

4.4.2.2.4 A4 production by TC following 20μM DMH1 treatment and AMH stimulation

Minimal differences in A4 production were evident between different treatment groups in TC that were cultured at 24 and 48 hours. At 96 hours of TC culture, AMH stimulation caused a statistically significant decrease in A4 production both in the DMH1 ($p<0.01$) and DMSO ($p<0.01$) treatment groups when compared to the control group (NT DMSO). Similar to what was seen in GC at 144 and 192 hours, stimulation of TC with AMH that were in DMSO, resulted in a statistically significant decrease in A4 production when compared to the control group($p<0.05$ and $P<0.05$ respectively). However, when the cells were stimulated with AMH in the presence of DMH1, no significant difference was observed when compared to the control group (NT DMSO). Likewise, at these same time points, cells that were not stimulated with AMH but treated with DMH1 resulted in a statistically significant increase in the production of A4 when compared to the control (p,0.05 and P,0.01 respectively) (Figure 4.20).

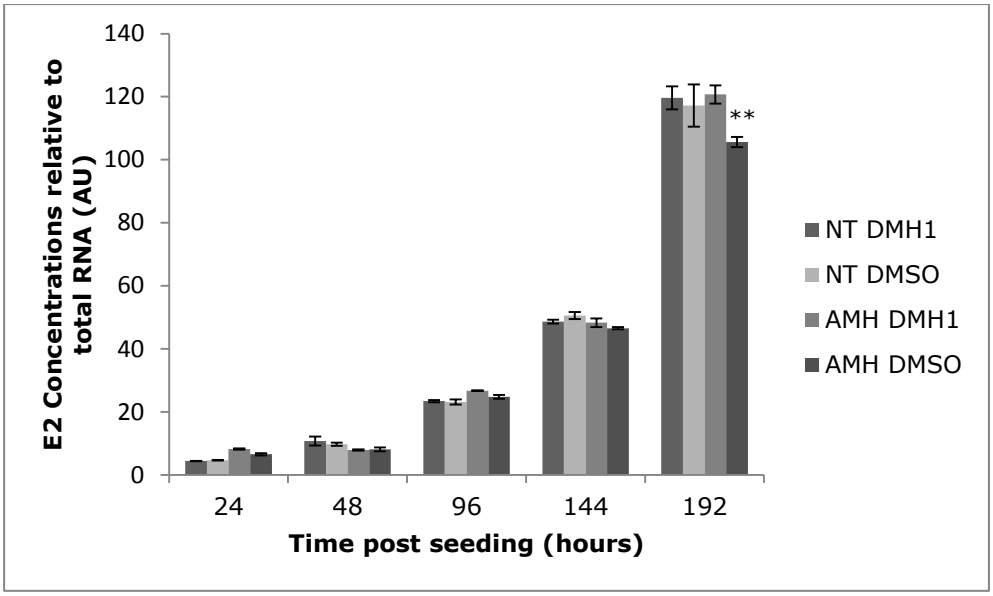


Figure 4.17 The effect of 0.5μM DMH1 for 1h with or without AMH stimulation for 30min on the E2 production by GC at different time points.

Values represent mean ± SEM. *, P<0.05, and **, P< 0.01 indicates a significant difference from NT and DMSO at the same treatment duration.

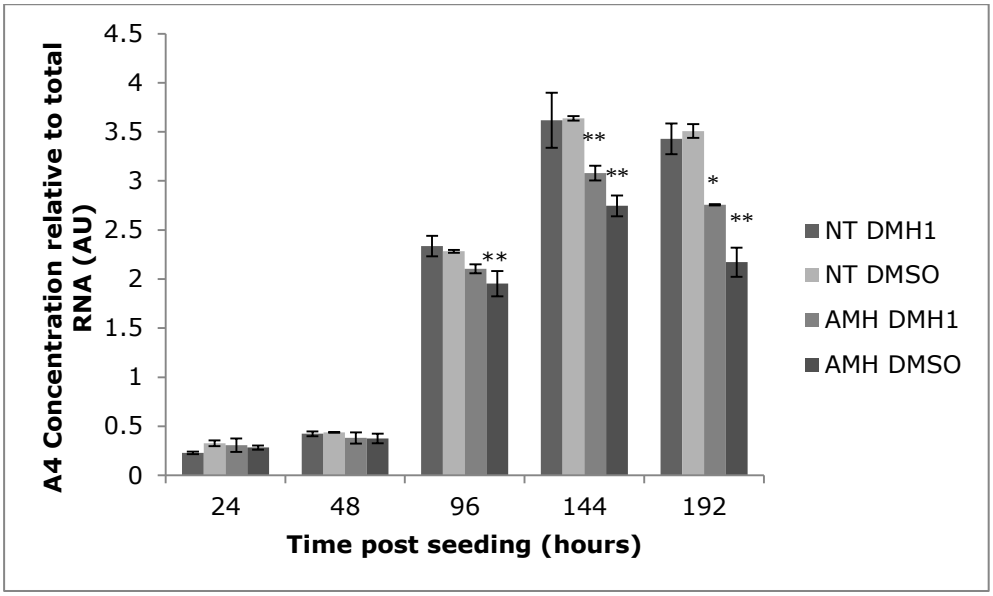


Figure 4.18 The effect of 0.5μM DMH1 for 1h with or without AMH stimulation for 30min on the A4 production by TC at different time points.

Values represent mean ± SEM. *, P<0.05, and **, P< 0.01 indicates a significant difference from NT and DMSO at the same treatment duration.

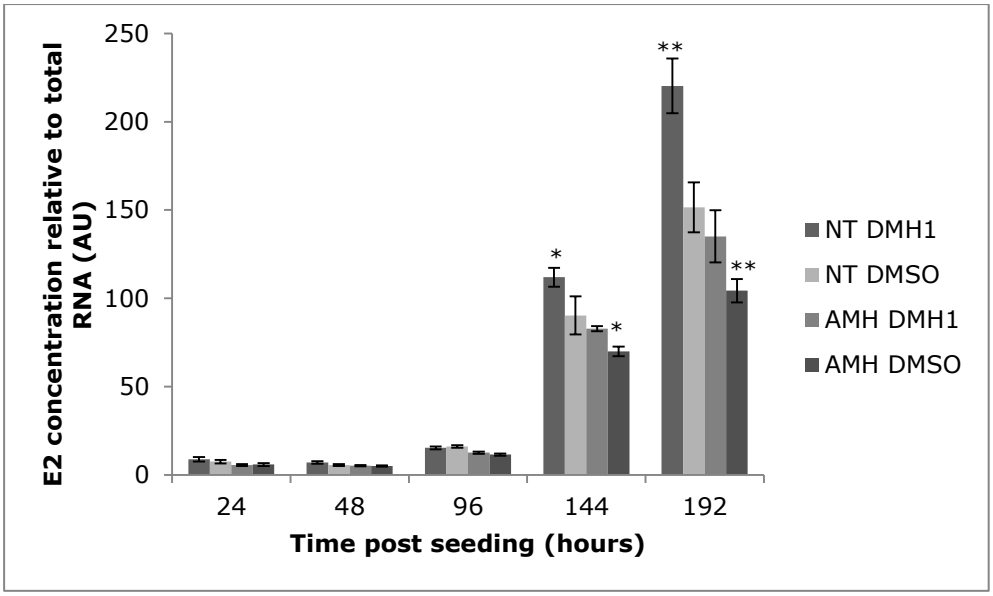


Figure 4.19 The effect of 20 μM DMH1 with or without AMH stimulation for 24h on the E2 production by GC at different time points.

Values represent mean ± SEM. *, P<0.05, and **, P< 0.01 indicates a significant difference from NT and DMSO at the same treatment duration.

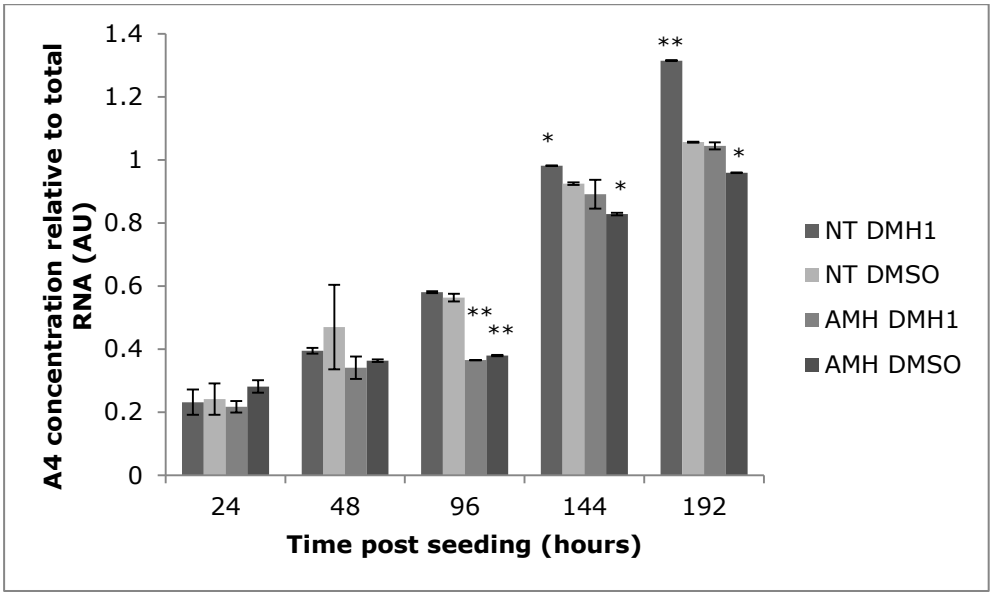


Figure 4.20 The effect of 20μM DMH1 with or without AMH stimulation for 24h on the A4 production by TC at different time points.

Values represent mean ± SEM. *, P<0.05, and **, P< 0.01 indicates a significant difference from NT and DMSO at the same treatment duration.

4.4.2.3 The Effect of AMH with or without DMH1 treatment on the MAPK signalling pathway

In the reporter gene transfected GC and TCs that were stimulated with 50ng/ml AMH and treated with or without a low (0.5μM) or high (20μM) dose of DMH1, MAPK signalling pathway activity, as measured by production of luciferase from the (SRE)-responsive luciferase construct showed no significant statistical difference between these treatment groups and the control (unstimulated cells in DMSO). (Figure 4.21 and Figure 4.22). Positive controls were not used.

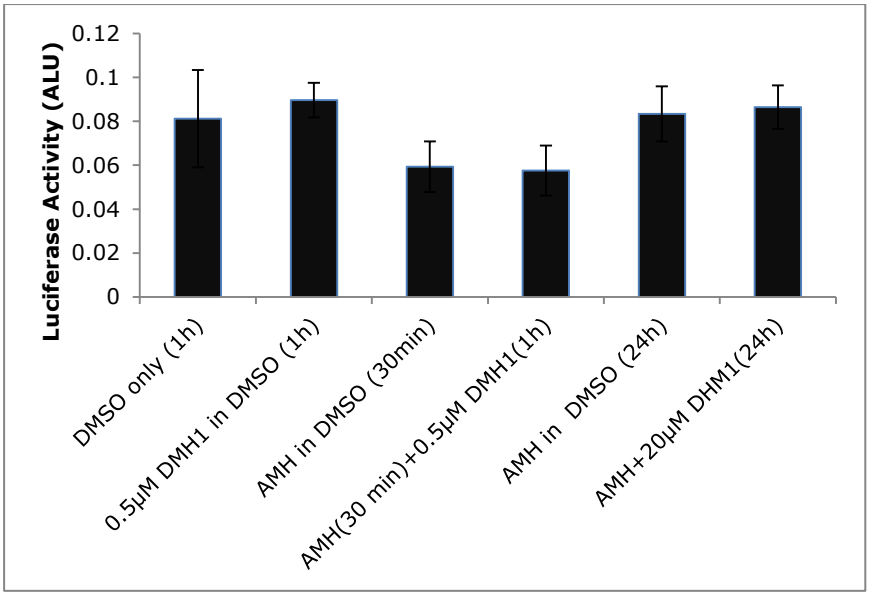


Figure 4.21 The effect of DMH1 with or without AMH stimulation on the activation of MAPK signalling pathway in 96 hour cultured GC.
Data expressed as mean + SEM. Significance was set at P<0.05.

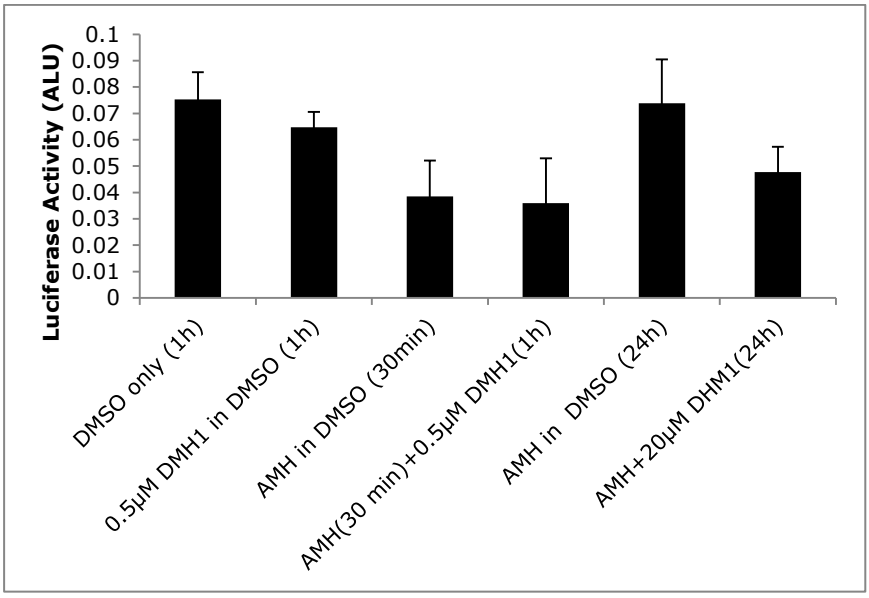


Figure 4.22 The effect of DMH1 with or without AMH stimulation on the activation of MAPK signalling pathway in 96 hour cultured TC.
Data expressed as mean + SEM. Significance was set at P<0.05.

4.5 Discussion

Although AMH has been extensively studied in recent years, its regulatory role and its signal transduction pathway by which it acts remains poorly elucidated. In rat GCs it is established that AMH acts through the AMHR2 to exert its action in an autocrine manner. However little is known about the signal transduction pathway in monovular species. Further, recently AMH has been shown to affect LH induced TC androgens (Campbell *et al.* 2012), indicating to the possibility of a paracrine effect of AMH on TCs. Therefore, the goal of this study was to further elucidate the signal transduction pathway of AMH in GCs in a monovular species and determine if TCs possess a similar signalling pathway through which AMH can act on.

Although AMH has been shown to be expressed by GC of growing follicles (Durlinger *et al.* 2002a, Weenen *et al.* 2004, Themmen 2005), and that the AMHR2 is in fact expressed by GC and TCs, little evidence in the literature exists that shows that AMH does interact with the activated receptor triggering a downstream signalling pathway that may convey the effect of AMH on these cells, which made this a research priority that was investigated in this piece of work. Using PLA, results showed that AMH/AMHR2 interactions were seen in both cell types at 24 and 144 hours, which suggested that both cell types may possess a signal transduction pathway for AMH. The downside of this was that as a result of levels being below western blot sensitivity it was not possible to quantify the signal obtained to determine whether a clear difference was evident between GC and TC at these different time points and therefore limited the analysis to detection rather than quantification. However, this novel finding opened the door,

for further unanswered questions of whether AMH utilises the SMAD 1/ 5/8 pathway in both of these cell types.

In order to determine if AMH causes an activation in the SMAD 1/5/8 pathway in 144 hour culture ovine GC and TC, either a post treatment increase in the levels of phosphorylated SMADs needed to be obtained or visualisation of the interaction between the SMAD 1/5/8 with SMAD 4, whereby only an activated SMAD1/5/8 complex can interact with SMAD 4, signifying phosphorylation. As PLA was used and optimised in this study, this technique was chosen over the other. Previous studies have demonstrated the activation of the BMP signalling pathway by BMP 6 (the chosen control) and AMH in mouse and human granulosa tumour cell lines (Dutertre *et al.* 2001, Moore *et al.* 2003, Sedes *et al.* 2013). In this study, Both AMH and BMP6 caused activation of the BMP-like ligand signalling pathway in GC via activation of SMAD 1, 5 or 8. This is in agreement with the earlier studies but extends them by showing novel evidence that like BMP 6, AMH activates the BMP signalling pathway in a transient manner. The findings suggest that the peak time of activation was between 10 and 20 minutes following treatment, indicated by the maximum SMAD 1/5/8 complex – SMAD 4 interaction. In TC, a similar pattern of complex formation between SMAD 1/5/8 complex and SMAD 4 was seen to that of GC appearing transient and optimal formation between 10 to 20 minutes following AMH treatment. It is therefore safe to suggest that for the first time that TC has been shown to utilise the same BMP signalling pathway as that seen in GC.

Although these studies showed that AMH and BMP 6 were able to activate the BMP signalling pathway via the SMAD 1, 5 or 8, they were unable to determine the specific R-SMADs being

activated. However, previous studies performed by the same team, that examined the changes in SMAD 1 and 5 mRNA expression following treatment with AMH and BMP 6 at different culture time points in GC, suggest that both of these SMADs may be used in the AMH and BMP6 activated BMP signal transduction pathway and that SMAD 1 was the predominant SMAD utilised (unpublished data, Smith, H., Elfituri, A., Marsters, P. & Campbell, B.K.). Unfortunately due to unavailability, the extent of SMAD 8's involvement in this pathway in comparison to SMAD 1 and 5 was not examined, which necessitates further studies. This is in agreement with a recent publication that showed that AMH activated both SMAD 1 and 5, predominately SMAD 1, in mice GC, while SMAD 8 was not shown to be activated by AMH (Sedes *et al.* 2013). Another interesting finding was that AMH and BMP 6 treatment induced a higher SMAD 1 and 5 mRNA expression level at the early time points (24 and 48 hours), as opposed to the later time points (96 and 144 hours). It is therefore speculated that the AMH and BMP 6 mediated signal through the BMP signalling pathway may be more pronounced at the earlier time points i.e. up to 48 hours in comparison to the later time points.

The time point chosen in this study was based on findings of previous work (Campbell *et al.* 2012) which showed an AMH treatment response at the same time point. However, it is tempting to speculate that if the experiments in this study were performed at an earlier time, a larger response may have been observed. This may be supported by earlier findings of high levels of the AMH receptor being expressed in the early time points when compared to the later time points. Unfortunately due to technical difficulties, the effect of AMH on

the specific SMADs mRNA expression was not assessed and is therefore an area for future work.

To strengthen the results obtained above, a study was conducted to further elucidate the signalling pathway of AMH in 144 hour cultured ovine GC and TC, looking at the interaction between the AMH receptor complex and the SMAD 1/5/8 complex. Results were supportive of the above findings showing that the receptor-SMAD interaction complex was evident in GC and TC with the signal reaching its peak following 10-20 minutes of AMH treatment. This novel finding, added further backing to the proposed theory, of a similar signal transduction pathway for AMH in TC as that seen in GC.

Finally, an experiment was conducted to assess the BMP responsive element activation by AMH using a luciferase reporter gene, in an attempt to further elucidate the transduction pathway of AMH in GC and TCs and whether it has the capability of transducing its signal to the nucleus of these cells to stimulate the BMP responsive element indicated by an increase in the luciferase activity. Results showed that AMH and BMP 6 (positive control) stimulated the BRE-Luc reporter gene expression which was indicated by an increase in the luciferase activity in both GC and TCs. In GCs, despite the transient nature of BMP6 and AMH in activating the SMAD complexes seen at around 10-20 minutes of treatment, a significant higher luciferase activity when compared to the non-treated cells was detected up to 135 minutes of BMP 6 treatment and up to 180 minutes of AMH treatment. This can be explained due to the reported half-life of luciferase which can continue to report up to 3 hours following activation (Thompson *et al.* 1991). Despite the BRE-Luc reporter gene activation was evident following 45 minutes of BMP 6 and AMH

treatment, the initial activation of the BMP signalling pathway must have occurred earlier than this, and therefore, in order to determine the time point of initial activation further investigations may be needed to measure the luciferase activity following treatment at earlier time points, which would be expected to occur, in this case, at around 10-20 minutes after treatment. In TCs, a similar pattern of BRE-Luc reporter gene activation was seen following AMH treatment. However, BRE-Luc reporter gene activation following BMP 6 treatment showed a minor difference to what was seen in GC. The activation levels were evident following 45 minutes of BMP6 treatment but decreased then after to non-treatment levels by 90 minutes of treatment. Again the time of initial activation was not clearly defined, but what can be concluded from this experiment is that AMH may possess a similar transduction pathway in TC to that seen in GC. However, BMP6 may cause an earlier BRE-Luc reporter gene activation in TC to that of GC due to the earlier drop seen in activation levels.

As previously stated, previous work has shown that AMH may have a functional role in ovine TC, whereby it causes a dose dependent inhibition in the LH induced A4 production (Campbell *et al.* 2012). It is therefore clear that AMH signals and mediates an effect in TC. However, up until now, the exact pathway utilised by AMH to mediate this effect has yet to be identified. It is still unclear whether the effect of AMH seen on TC is via the BMP like signal transduction pathway, utilising the SMAD 1/5/8 complex or through an alternative undefined non SMAD signalling pathway.

In order to test this theory, a highly specific BMP signal transduction pathway inhibitor known as DMH1, that targets SMAD dependent but not SMAD independent signalling (Hao *et*

al. 2010), was used to determine if the response seen by AMH on GCs and more importantly TCs in terms of steroid production was attenuated or lost in the presence of the inhibitor DMH1 and therefore providing evidence that AMH may in fact utilise the SMAD 1/5/8 pathway in its inhibitory effect. Results showed that in 24 hour cultured ovine GC, as previously illustrated, AMH and BMP6 (positive control) treatment, stimulated the BMP signalling pathway via the SMAD 1/5/8 complex. However this signal was greatly diminished following inclusion of DMH1, confirming the inhibitory effect of DMH1 on the SMAD pathway. Interestingly, similar findings were seen in ovine TC culture at the same time point. This provides further evidence to support that AMH may utilise this pathway at this earlier time point. This experiment was repeated for both cell types that were cultured to a later time point (144 hours). Results showed similar findings to that seen after 24 hours of culture. AMH stimulated the SMAD pathway in both GC and TC, which was only slightly attenuated when a low dose of DMH1 was used. However, when repeated with a higher treatment dose of DMH1, the AMH inhibitory effect was diminished and therefore supported the inhibitory effect of DMH1 on the SMAD pathway, even at this later time point. Evidence from this experiment, point to the possibility that AMH utilises this BMP signalling pathway at both the early (24 hours) and late (144 hours) culture time points. However, it provides little information on whether this pathway is utilised by AMH to exert its inhibitory function on steroid production by GC and TC. A further experiment was therefore conducted to test this hypothesis. Results of this work showed that, as to be expected, differences in steroid production were mainly seen in the later time points, as the cells differentiate to become

prominent steroid producing cells (Campbell *et al.* 1996, Campbell *et al.* 1998). In GC, AMH had a significant inhibitory effect on E2 production at 192 hours, which is supported by previous work (Campbell *et al.* 2012). However, with the addition of DMH1 this effect was diminished. This is a very important finding which showed that, for the first time, AMH may in fact signal through the SMAD pathway in ovine GC at these later time points. Similarly, in TC A4 production was attenuated by AMH in cells cultured past 96 hours, which corresponds to previous research findings (Campbell *et al.* 2012). This effect was however reversed by the addition of DMH1 but to lesser extent to that seen in GC. This may therefore indicate to the possibility of AMH utilising the SMAD pathway shown above in exerting its effect on steroid production. In an attempt to strengthen the results obtained, a higher nontoxic dose of DMH1 (Hao *et al.* 2010) was used to supress the SMAD pathway. This showed similar findings in GC those previously observed. However in TC, DMH1 was able to attenuate the inhibitory effect of AMH on steroid production to non-treatment levels. Interestingly, non-stimulated GC and TC treated with the high doses of DMH1 showed an increase in the steroid production at these later time point. This may point to the possibility of an endogenously produced activator of the SMAD pathway shared by both cell types being produced that has the capability of also inhibiting steroid production. Unfortunately, due to time constraints, this has yet to be identified and therefore warrants further investigation.

These results may therefore increase our knowledge of how AMH may signal in GC and TC of this monovular species. However due to the complexity of this process this does not

exclude the possibility of other elements being implemented in the regulation of this AMH SMAD pathway and this therefore needs to be further studied.

AMH has been known to have a multitude of roles in ovarian functions, and while evidence now suggests that, AMH utilises the SMAD 1/5/8 pathway in mediating its effect during folliculogenesis. It is therefore plausible that it may rely on other transduction pathways in both GC and TC. The MAPK signalling pathway is known to have a role in cell growth, differentiation and proliferation, which are very important in the processes of folliculogenesis. BMP signals have been shown to activate the MAPK p38 in a SMAD independent manner (Nohe *et al.* 2004, Yu *et al.* 2008). The MAPK pathway has previously been shown to be involved in AMH mediation in the early stages of rodents follicular growth at the time of primordial to primary follicle transition (Nilsson *et al.* 2007). Further, activated extracellular signal regulated kinases 1 (Erk 1: p44 MAPK), (Erk 2: p42 MAPK) and protein kinase B (also known as Akt), which are all known to be part of the MAPK signal transduction pathway, have been found in greater concentrations in late stages of ovine follicular development when compared to subordinate follicles (Evans & Martin 2000). These studies collectively indicate that AMH may have a role in mediating the activation of the MAPK pathway in sheep antral follicles. Results of this study showed that no difference in the MAPK signalling pathway represented by the measured luciferase activity was seen between stimulated and non-stimulated GC and TC in the presence or absence of the already characterised SMAD 1/5/8 pathway. This may indicate to a number of possibilities. Firstly, that AMH does not utilise the MAPK pathway in conveying its signal in these cell types.

Secondly, an alternative explanation may be that neither the follicular stage of small antral follicles nor the timing of the culture performed at 96 hours were optimal to mediate the effect of AMH in activating the MAPK pathway, as the study above showed that greater concentrations of the activated MAPK signalling cascade was observed at the late stages of the follicular development as the follicle was reaching dominance. This may therefore indicate that the optimal time to study the effect of AMH on the follicle would be at this stage. However, as previously stated, *in vivo* and *in vitro* studies have shown that at this later time AMH declines dramatically as the follicle reaches dominance which further raises the question of AMH involvement in this pathway. The only other explanation may be that AMH has an inhibitory effect on the pathway and the decrease in AMH at this stage results in the activation of the pathway. To consolidate this contention, positive controls need to be added to the experiment to validate the activity of this pathway and to confirm findings of this study which due to time constraints was not performed which is a limitation of this study and therefore need further investigation.

In conclusion, results of this work suggest that AMH act through the SMAD 1/5/8 pathway to modulate steroid production in both GC and TCs, and there was no evidence of involvement of the MAPK pathway in the culture period and treatment conditions employed in this study.

Chapter 5 The Role of Androgens in the Control of Granulosa Cell Function

5.1 Introduction

Follicular development is an intricate process involving, not only a complex interaction between gonadotrophic hormones, sex steroids and a range of growth factors, but many other local signals between the developing oocyte and its surrounding somatic cells (Gougeon 1996, McNatty *et al.* 2000, Eppig *et al.* 2002, Matzuk *et al.* 2002, Webb & Campbell 2007). The roles of sex steroids in ovarian function have been the focus of a great deal of research interest, with increasing attention being directed toward androgen hormones and their roles in follicular development and female fertility.

It is now well-known that FSH-stimulated GCs are able to process androgens to produce E2 and many cellular responses to androgens during follicular development have been linked to this E2 biosynthesis pathway (Hewitt et al. 2005, Gervasio et al. 2014). However, it has also been shown (Mangelsdorf *et al.* 1995, Rosenfeld *et al.* 2006) that, androgens have roles beyond that of a substrate for E2, as with other members of the steroid hormone family, androgens can utilise specific nuclear receptors (androgen receptors, AR) to transduce their cellular effects. Studies have shown that these signalling pathways involve intracellular cascades of events that culminate in the increase or decrease of transcription of target genes (Mangelsdorf *et al.* 1995, Rosenfeld *et al.* 2006). In addition, androgens have also been found to exert their actions by interacting with receptors located on the cell membrane. These too may initiate a series of intracellular

events but generally result in rapid non-genomic actions such as, assisting in the activation of intermediaries involved in other signalling pathways (Kousteni et al. 2001, Lösel et al. 2003). With there being several different ways in which androgens elicit cellular responses it seems likely that the regulation of their signalling pathways in GCs is critical in balancing E2 production with other androgenic intra-ovarian functions.

However, though there have been many advances in the characterisation and understanding of androgen signalling pathways, their involvement in the regulation and maintenance of folliculogenesis remains to be fully clarified (Gervasio *et al.* 2014). A review of outcomes from early studies suggested that androgens were associated with infertility as they appeared to be detrimental to ovarian function (Prizant *et al.* 2014). However there have now been a number of studies which appear to contradict this idea. These have produced evidence that at optimal levels receptor-mediated androgen signalling is essential for normal ovarian follicular development (Murray *et al.* 1998a, Wang *et al.* 2001a, Yeh *et al.* 2002, Hu *et al.* 2004, Shiina *et al.* 2006, Lenie & Smitz 2009, Sen & Hammes 2010). It thus seems likely that the regulation of the various androgen intra-cellular signalling pathways must be finely balanced in order to maintain the essential levels for normal ovarian follicle growth and function, and prevent the detrimental effect of excess androgenic conditions. Perhaps the most well documented disorder that is characterised by increased ovarian androgen production is PCOS. In this disorder, in addition to high levels of LH and AMH production, there is a hyperandrogenic microenvironment within the ovary, and this is thought to

negatively affect follicular development, promote follicular stagnation at the early antral stage of follicular development, decreasing the possibility of a dominant and ovulatory follicle development and therefore resulting in anovulation and infertility. On the other hand, low levels of androgens have been associated with, perturbation of follicular development, low functional ovarian reserve, and ovarian insufficiency which can thereby, also disturb normal female fertility (Prizant et al. 2014).

In PCOS women with increased levels of testosterone and/or LH there is frequently an accompanying high serum level of AMH and it is suggested that this may cause impaired follicle and oocyte development, and impinge on subsequent embryo quality (Dumesic et al. 2008, Franks et al. 2008, Patel & Carr 2008). However, high serum AMH level may not necessarily signify high intra-follicular AMH. In reinforcement of these findings a recent publication has shown that prenatal treatment with testosterone, stimulated AMH expression in preantral and antral follicles from adult ovine ovaries (Veiga-Lopez *et al.* 2012). This also concurred with the finding that in PCOS women AMH expression is elevated in GCs (Pellatt *et al.* 2007). Collectively these publications are all in agreement with the hypothesis that the disruption in folliculogenesis in PCOS may be mediated, at least in part, by AMH. However, it is still unclear whether the androgen-mediated changes in ovarian AMH are transduced via the androgen receptor or are an androgen-aromatised oestrogen effect. Consequently this can be considered a research priority, necessitating further investigation. Further, while many correlation studies over the years have proposed the idea that androgens may regulate AMH mRNA expression and/or protein levels (Lebbe &

Woodruff 2013), direct evidence of androgen induced AMH expression and its underlying actions in GCs are still lacking.

A key aim of this present study was to utilise aromatisable and non-aromatisable androgens in order to determine the effect of these androgens on FSH-stimulated GCs in terms of their production of two markers of follicular development, E2 and Inhibin A. An extension of this aim was to determine the effect FSH/IGF had on this process by using optimal and low levels. A further aim was to also study the direct effect of these androgens on AMH production and to further clarify whether AMH has a direct role in modulating E2 production in these cultured cells. The study was designed to provide some additional clarity concerning androgen signal transduction pathway activation and its effects on aromatase mRNA expression in these cultured cells.

5.2 Experimental Design

5.2.1 The Effect of T4 on E2 and Inhibin A Production in GC in the Presence of Normal or Low Levels of FSH and IGF

An *in vitro* approach was utilised in order to shed light on androgen stimulated signalling pathways in GCs. GCs from small (>3 mm in diameter) antral follicles were seeded as previously described in the 'Methods' chapter (Section 2.1.2.) on to a 96 well plate. The cells were then treated with 4 different concentrations of testosterone (100ng/ml, 10ng/ml, 1ng/ml and 0ng/ml). The culture media in one half of the plate contained complete GC culture media, while in the other half the culture media contained only 10% of IGF and FSH (10ng/ml and 1ng/ml). The cells were cultured for 192 hours with the media obtained at 6 different time points (16h, 24h, 48h, 96h, 144h and 192h). The spent media was collected and stored at -20^{o}C for ELISA. At the end of the culture period GC numbers were estimated using the neutral red method as previously described in chapter 2 (section 2.1.3). The accumulative E2 and Inhibin A concentrations were measured by ELISA as described in the general method section (chapter 2 section 2.3.2).

5.2.2 The Effect of T4 and DHT on E2 Production in Cultured GC in the Presence or Absence of AMH

The effect of aromatisable (T4) and non-aromatisable (DHT) androgens on E2 production in cultured GCs in the presence or absence of AMH was measured in ovine GC cultures which had been set up in 96 well plates at a seeding concentration of 75,000 cells/well and cultured for 48, 96 and 144 hours. The cells were treated with either an aromatisable (100ng/ml

testosterone) or non- aromatisable (100ng/ml DHT) androgen or in combination of both androgens. Selected wells were also challenged with 50ng/ml AMH in the presence of both androgens. Non treated cells were used as a control. The levels of steroids in the medium were assayed at each time point utilising the general ELISA methodology as earlier detailed (Section 2.3.2.). At the end of the culture period GC numbers were estimated using the neutral red method as previously described in in the 'Methods' chapter (Section 2.1.3.).

5.2.3 The Effect of Androgens on AMH Production

Two parallel experiments were conducted to study the effect of androgens on AMH production in cultured ovine GCs. Cell cultures were set up in 24-well plates and seeded at 500, 000 cells/well. In the first experiment, the cells were treated with different concentrations of testosterone (1000 ng/ml, 100ng/ml, 10ng/ml, and non-treated). The cells were incubated for 5 different time points (24, 48, 96, 144, and 192 hours). At the end of each time point spent media was collected, stored at -20C^{o} and later analysed using ELISA to determine the levels of AMH and E2 as described in the general methodology chapter (Section 2.3.2). The cells were harvested by lysing and stored as previously described (Section 2.3.1.1.) for future analysis. In the second experiment, cells were treated with different concentrations of DHT (1000 ng/ml, 100ng/ml, 10ng/ml, and non-treated) in the presence of a constant level of testosterone (50ng/ml). As above, cells were incubated for 5 different time point and at the end of the culture period, the collected media was analysed using ELISA to measure the level of AMH and E2

production by the GCs at each time point. In both experiments, none treated cells were used as a control.

5.2.4 The Effect of Androgens on Aromatase Gene Expression

Lysed cells that were obtained from the previous experiments were used to estimate the aromatase gene expression in GCs that were either treated with different concentrations of testosterone or DHT, utilising the general RT-PCR methodology as earlier detailed (Section 2.3.1). The primers used for the genes of interest are illustrated in (Table 5.1) and 18s rRNA was used as an endogenous control.

Table 5.1 18S and Aromatase primers used.

Target Gene	Forward Primer Sequence (Sense)	Reverse Primer Sequence (Antisense)
18S	5'-TTGACTCAACACGGGAAACCT-3'	5'-AGAAAGAGCTATCAATCTGTCAATCCT-3'
Aromatase	5'-GCGCATGGTACCATCTGT-3'	5'-CACATAGCCCAAGTCATTGCA-'3

5.2.5 The Effect of T4 and DHT on the Activation of the AR Pathway

Finally, an experiment was conducted using the Cignal AR Reporter Assay (CCS-1019L) to determine if androgens (testosterone and DHT) stimulate the activation of the Testosterone Response Element (TRE)-responsive luciferase construct in ovine GCs, a response that relies on the activation of the AR signal transduction pathway.

GCs were cultured in 24 well plates for 48 hours in complete GC media that was devoid of Bovine Serum Albumin and antibiotics. The transfection mix was prepared for the TRE

responsive luciferase reporter gene plasmid. A transfection reagent FuGENE6 was then added to the mix at a ratio of 3:1 FuGENE6 to plasmid DNA. Existing media was removed from the all the wells and the transfection mix added to each well. In addition, selected wells remained untreated but received the same volume of medium per well. After 48 hours of transfection, the media was removed from each well and replaced with GC media containing different concentrations of androgens (testosterone and DHT). The cells were then further incubated for 2 hours. Following the incubation period, the cells were lysed and frozen at -20C^{o} prior to performing the dual luciferase reporter assay as described in the methods chapter 2 (section 2.6).

5.3 Statistical Analysis

Unless otherwise stated, data from this chapter are presented as mean +/- SEM of at least three different experiments conducted in triplicate. Statistical significance was assumed at $p<0.05$. Data were tested for normality before using a two way ANOVA on SPSS to determine sample group differences of E2, Inhibin A and AMH; and the aromatisable and non-aromatisable androgen induced activation of the AR signalling pathway in GCs. ELISA plates inter-and intra-assay coefficients of variation were all within 10%. Cell-numbers were consistent throughout the culture period unless otherwise stated where cell-number differences were normalised against total RNA amounts for the same sample and relative quantities measured in arbitrary units (AU). Results of the Aromatase qPCR were normalised against the cell number by utilising an endogenous control (18S mRNA) and analysed relative to a standard curve according to the software provided by Applied Biosystem, version 2. Statistical significance was compared using a two way ANOVA analysed on SPSS.

5.4 Results

5.4.1 The Effect of T4 on E2 and Inhibin A Production in GC in the Presence of Normal or Low Levels of FSH and IGF

5.4.1.1 E2 Production:

In the presence of normally optimised FSH/IGF levels (10.0 ng/mL / 1.0 ng/mL), increasing the concentration of T4 had no effect on E2 production over the first 48 hours of culture (Figure 5.1 A). Thereafter, while oestradiol in the cells treated with 1ng/ml testosterone did not rise above basal levels, a marked effect of T4 was noted at higher doses. Cells challenged with T4 at a concentration of 100ng/ml showed a significant increase in E2 production from 48 hours onwards ($p<0.001$), while cells treated with only 10ng/ml of testosterone significantly elevated E2 production after 144 hours of culture ($p<0.01$) (Figure 5.1 A).

Similarly, T4 had no effect on GCs that were cultured in a low FSH/IGF environment (1ng/ml /0.1ng/ml) in the first 48 hours of culture (Figure 5.1 B). However, as the culture progressed, cells that were treated with 100ng/ml of T4 showed a significant rise ($p<0.05$) in the production of E2 after 96 hours, while cells that were subjected to lower concentrations of T4 did not impact E2 production when compared to the control (Figure 5.1 B).

E2 production in ovine GCs cultured in the presence of normally optimised FSH/IGF and T4 (100ng/ml) was significantly higher ($p <0.05$) then cells cultured with FSH/IGF at a low concentration but with the same testosterone level (100ng/ml) after 144 hours of culture. On the other hand, no

significance was observed between the normal and low FSH/IGF treat groups at lower concentrations of testosterone throughout the culture period.

5.4.1.2 Inhibin A Production:

Inhibin A production in response to different T4 treatments in the presence of a normal or low dose of FSH/IGF followed a similar trend to that found with E2 with cells producing very minimal inhibin A before 48 hours and then significantly increasing over time thereafter (Figure 5.2).

In the presence of optimised FSH/IGF, cells treated with very low testosterone (1ng/ml) showed no rise in Inhibin A production over the baseline levels throughout the culture period, while cells challenged with 10ng/ml of T4 significantly elevated ($p < 0.05$) inhibin A production only after 96 hours of culture. However, a highly significant ($p<0.001$) increase in production in inhibin A was observed in cells treated with the optimal T4 concentration (100ng/ml) after 96 hours (Figure 5.2 A). On the other hand, no statistically significant differences were determined between T4 treatments at any time point when less FSH/IGF (0.1 ng/ml) was used (Figure 5.2 B).

FSH/IGF treatment levels had an effect on Inhibin A production levels. GCs treated with optimal levels of FSH/IGF showed a significantly higher ($p<0.001$) Inhibin A production when compared to cells treated with low levels of FSH/IGF with all testosterone treatment groups. This difference was observed early in cells that were treated with 100ng/ml and 10ng/ml testosterone starting from 48 hours onwards. While, the difference was seen later at 96 hours onwards in cells treated with low levels of testosterone.

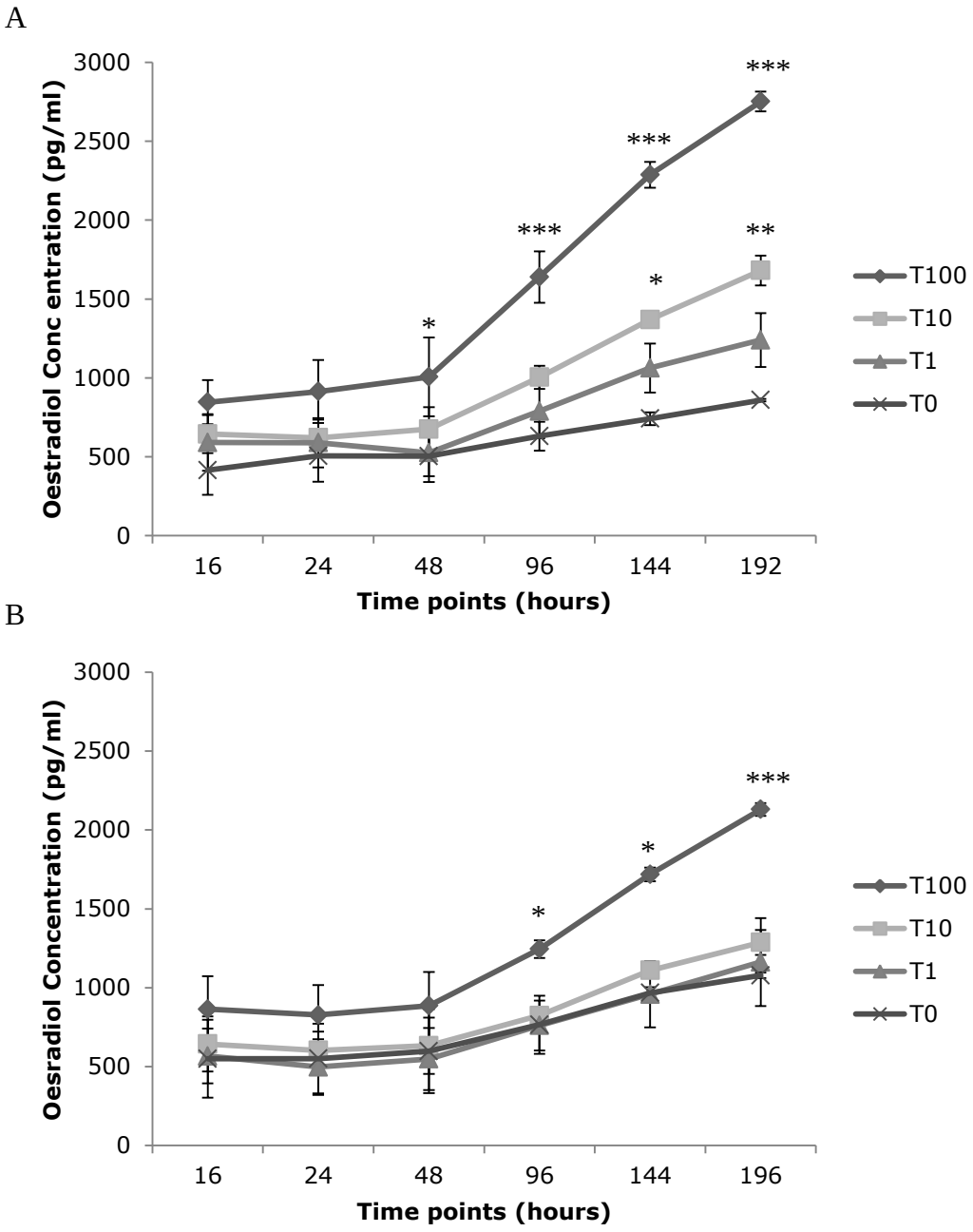
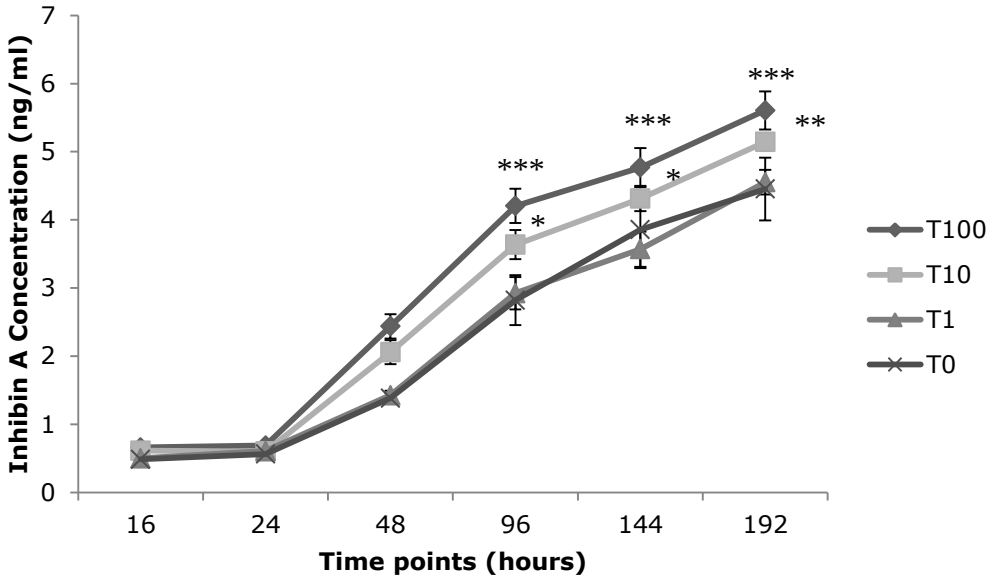


Figure 5.1 E2 production over time in ovine GC challenged with different concentrations of T4 in the presence of normal or low levels of FSH/IGF.

Cells were treated with normal FSH/IGF culturing concentration (A), or at lower FSH/IGF culturing concentration (B). Values represent mean ± SEM. *, P<0.05, and ***, P< 0.001 indicates a significant difference from NT at each time point.

A



B

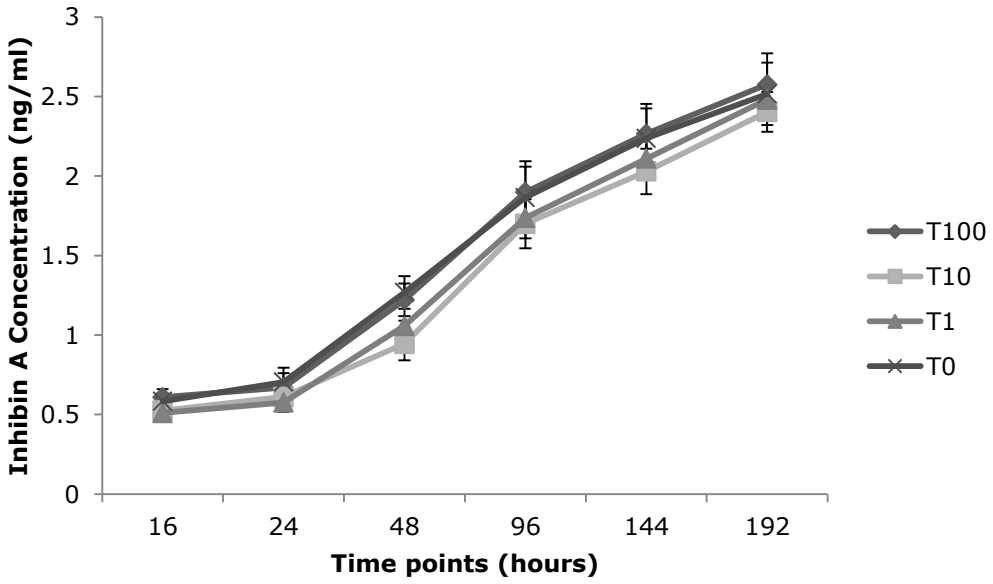


Figure 5.2 Inhibin A production over time in ovine GC challenged with different concentrations of T4 in the presence of normal or low levels of FSH/IGF.

Cells were treated with normal FSH/IGF culturing concentration (A), or at lower FSH/IGF culturing concentration (B). Values represent mean ± SEM. * P<0.05, and *** P< 0.001 indicates a significant difference from NT at each time point.

5.4.2 The Effect of Aromatisable (T4) and Non-Aromatisable (DHT) Androgens on E2 Production in Cultured GC in the Presence or Absence of AMH

E2 production by GCs treated with an aromatisable androgen (T4), a non-aromatisable androgen (DHT) or the combination of both with or without AMH challenge was compared over time. As previously illustrated, all treatment groups that were cultured in the presence of T4 showed a significant increase ($p<0.001$) in E2 production after 48 hours of culture. As it is not an aromatisable androgen, DHT did not cause an increase in E2 production when included in culture alone. Cells that were treated with both t4 (100ng/ml) and DHT (100ng/ml) showed a significant increase ($p<0.001$) in E2 production when compared to cells treated with only T4 at the same time point. This effect was reversed when AMH was included in the culture causing a statistically significant inhibition ($p<0.001$) to E2 production at all-time points studied (Figure 5.3), findings that are in agreement with previously presented work (Campbell *et al.* 2012).

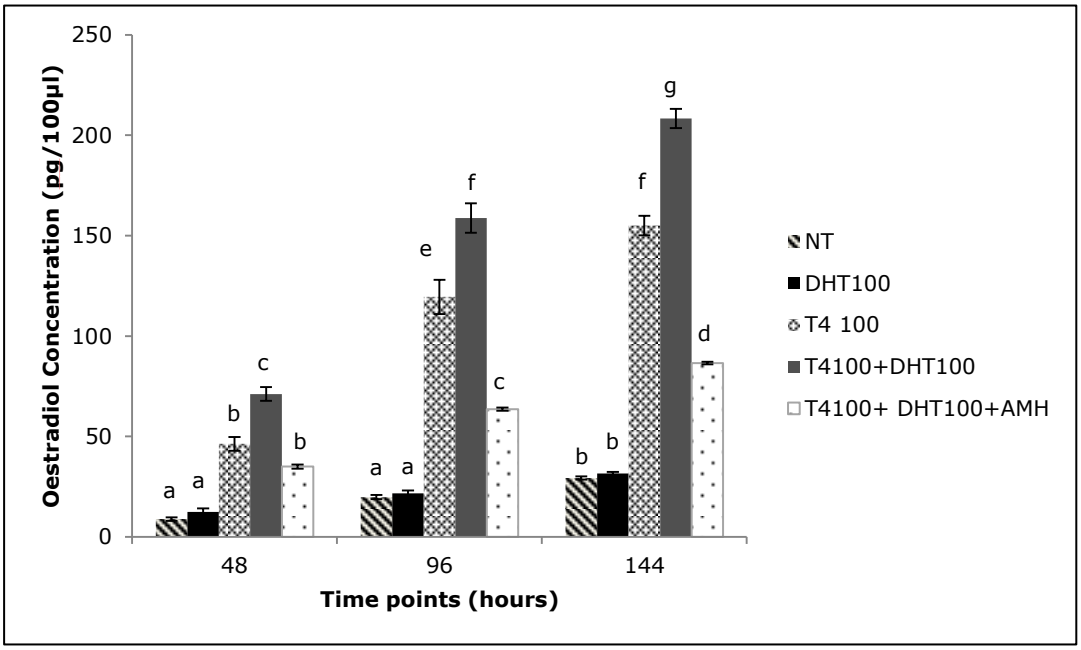


Figure 5.3 E2 Production over time in ovine GC treated with T4, DHT, or the combination of both +/- AMH.
Values represent mean ± SEM. Different letters indicates significant differences ($p<0.05$).

5.4.3 The Effect of Androgens on AMH Production

5.4.3.1 Testosterone:

AMH production increased with time throughout the culture period in all treatment groups. However no significant difference in production was observed between cells that were treated with low levels of T4 when compared to the control throughout the culture period. Cells treated with normal levels also showed no significant rise in production from the baseline until 144 hours, but a significant difference ($p<0.01$) in secretion was seen late in the culture at 192 hours (Figure 5.4A). Interestingly, a completely different picture was seen in cells that were treated with very high levels of T4 (Figure 5.4A). These cells showed a dramatic increase in AMH production throughout the culture period that started from the early time points reaching highly significant levels ($p<0.001$).

As shown in (Figure 5.4 B), E2 production showed a normal trend with a similar pattern of expression as that shown earlier (Figure 5.1). Production increased throughout the culture period in all treatment groups reaching its peak at 192 hours. However cells that were treated with high concentrations of T4 (1000ng/ml) exhibited a dramatically significant increase ($p>0.001$) in E2 production starting from the very early time points, while cells treated with the normal levels of T4 (100ng/ml) resulted in a significant increase ($p>0.01$) in E2 production only from 144 hours of culture. On the other hand, as expected cells that were treated with low levels of T4 did not show any rise in production when compared to the control (Figure 5.4B).

5.4.3.2 DHT:

AMH production increased steadily as the culture progressed. However, DHT (Figure 5.5 A) had a significantly lower impact on AMH production when compared to that seen by T4 (Figure 5.4 A). Only in cells treated with very high levels of DHT (1000ng/ml), was AMH significantly increase, which was only seen in the later time points (144 and 192 hours) ($p<0.01$ and $P<0.001$ respectively). While DHT, at lower concentrations did not have a significant impact on AMH production throughout the culture period (Figure 5.5 A).

Examining Figure 5.5 B, it is evident that as T4 (50ng/ml) was included in the culture media, GCs exhibited a similar pattern of E2 production as previously illustrated, where the secretion levels were seen to increase as the culture progressed. However, DHT treatment at the level of 10ng/ml and 100ng/ml apparently did not cause an increase E2 production at each time point until 144 hours, where thereafter cells treated with 100ng/ml DHT had a significant effect on production ($p<0.01$) at 192 hours (Figure 5.5 B). On the other hand, cells treated with high levels of DHT caused a highly significant effect on E2 production ($p<0.001$) throughout the culture period.

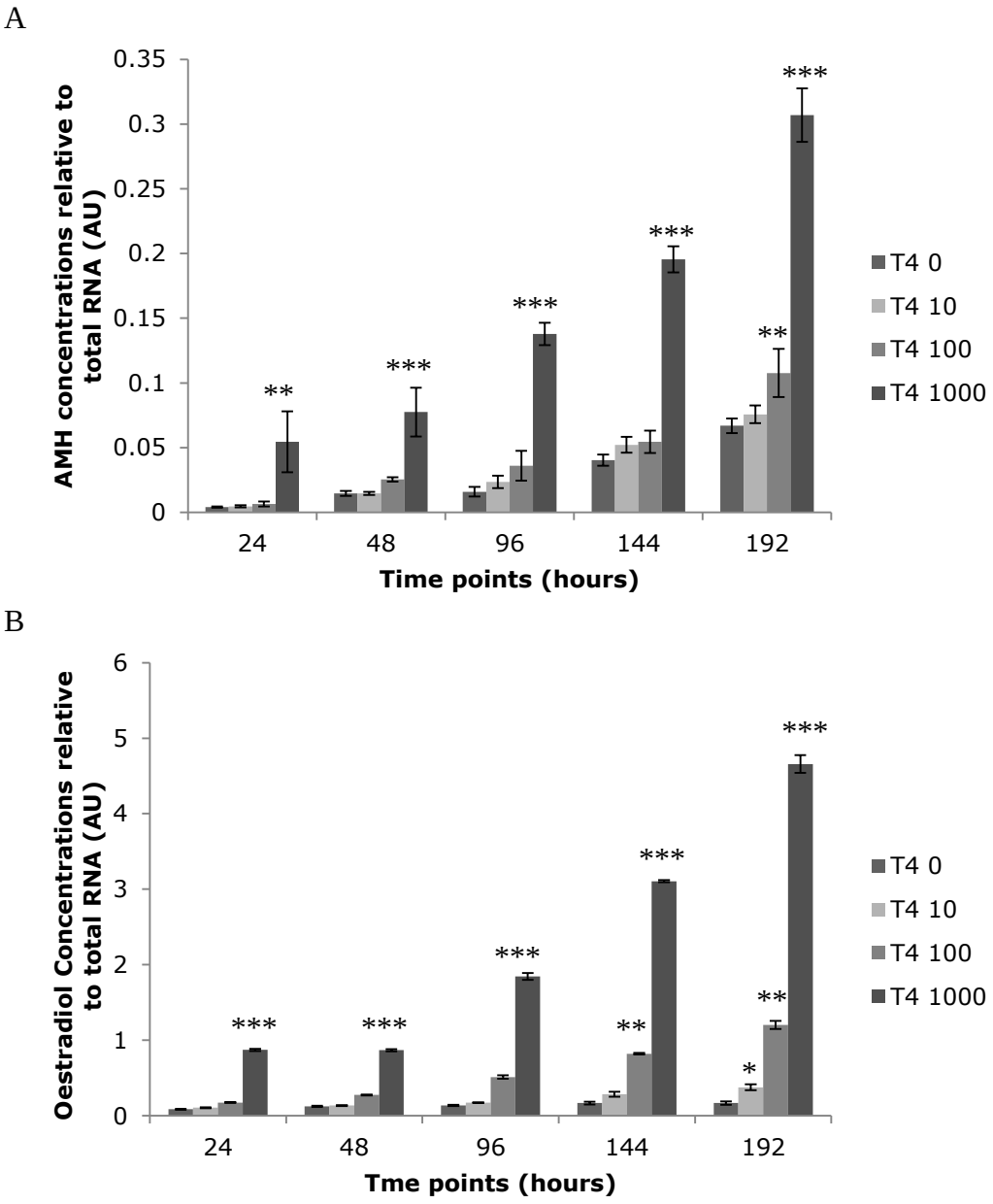


Figure 5.4 Production of AMH and E2 over time in ovine GC challenged with different concentrations of T4.

Production of AMH (A) and Oestradiol (B). Values represent mean ± SEM. * P<0.05, ** P<0.01, and *** P< 0.001 indicates a significant difference from NT at each time point.

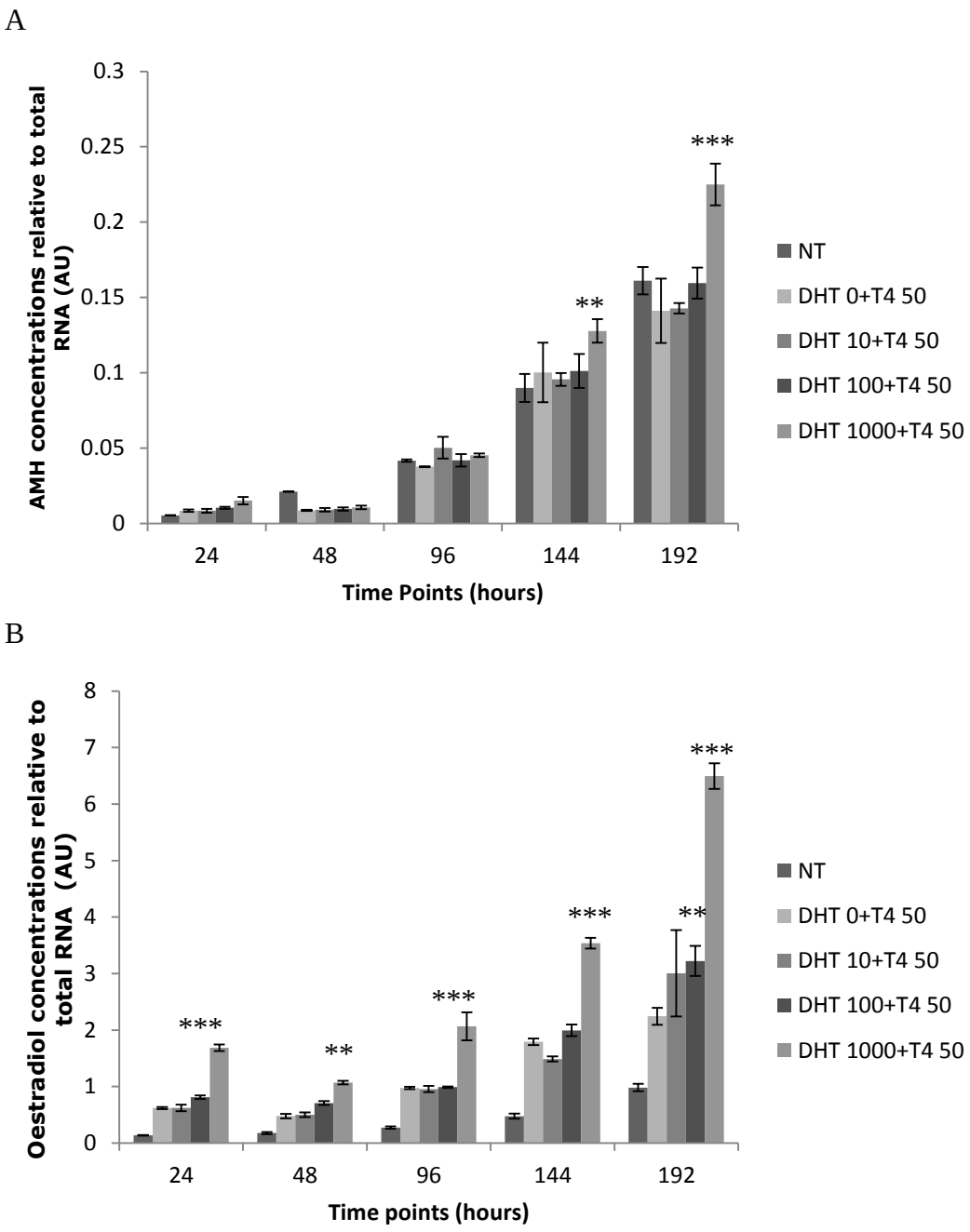


Figure 5.5 Production of AMH and E2 over time in ovine GC challenged with different concentrations of DHT in the presence of 50ng/ml T4.

Production of AMH (A) and Oestradiol (B). Values represent mean ± SEM. *, P<0.05, **, P<0.01, and ***, P< 0.001 indicates a significant difference from NT at each time point.

5.4.4 The Effect of Androgens on Aromatase Gene Expression

5.4.4.1 Testosterone:

As shown in Figure 5.6, a gradual drop in aromatase gene expression was evident throughout the culture period. No significant difference in expression was seen between the T4 treated groups from 48 hours of culture onwards. At 24 hours of culture, testosterone had an effect on aromatase gene expression resulting in a dose dependant decrease in expression ($p<0.01$ with 10ng/ml T4) and ($p<0.001$ with 100 and 1000ng/ml T4).

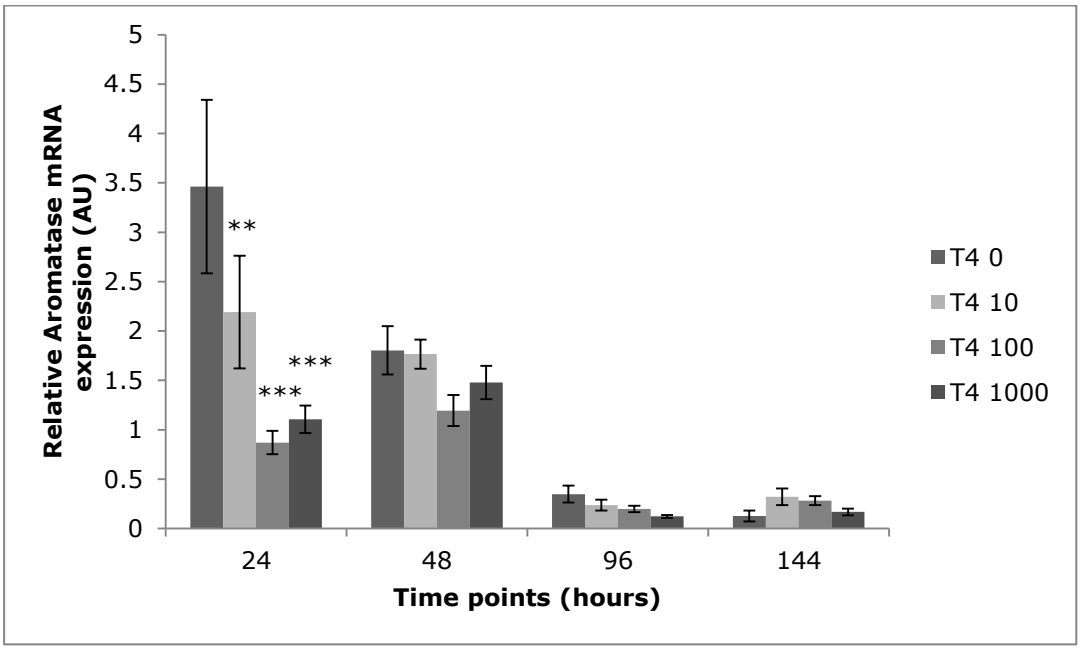


Figure 5.6 Aromatase mRNA expression over time in ovine GC challenged with different concentrations of T4.
Values represent mean ± SEM. **, P<0.01, and ***, P< 0.001 indicates a significant difference from NT at each time point.

5.4.4.2 DHT:

Similar to what was observed in the previous experiment (Figure 5.6), Aromatase gene expression gradually decreased with time (Figure 5.7). However, DHT had no effect on aromatase expression at all the time points. A significant difference in aromatase expression ($p<0.05$) was seen in cells treated with the constant concentration of T4 (50ng/ml) compared to the cells that were not treated with T4 at 24 hours (Figure 5.7), which is in agreement with the prior experiment.

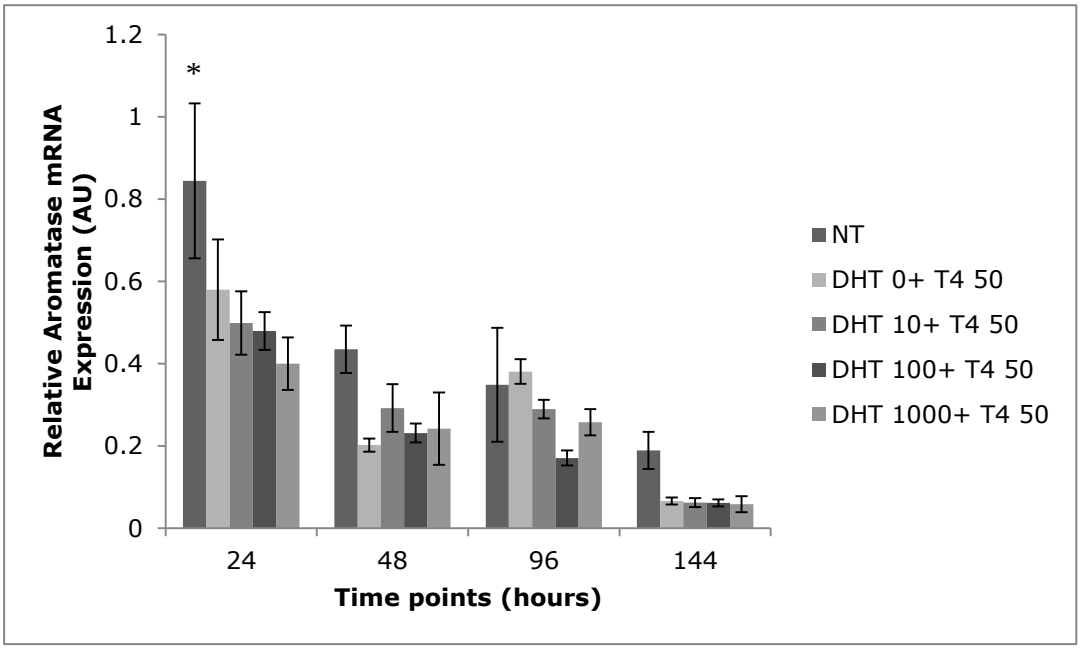


Figure 5.7 Aromatase mRNA expression over time in ovine GC challenged with different concentrations of DHT in the presence of 50ng/ml T4.

Values represent mean ± SEM. *, $P<0.05$, indicates a significant difference from cells treated with (DHT0 +T4 50).

5.4.5 The Effect of Aromatisable and Non-Aromatisable Androgens on the Activation of the AR Pathway

In GCs with transfected reporter gene that were stimulated with an aromatisable androgen (T4), AR pathway activity, as measured by production of luciferase from TRE-responsive luciferase construct showed that as the concentration of T4 was increased so did the activity levels, reaching a peak in cells treated with 50ng/ml T4 ($p<0.001$). However, at this same time point, AR pathway activation decreased to control levels in cells treated with 100ng/ml and 1000ng/ml (Figure 5.8).

Figure 5.9 shows that in GCs stimulated with a non-aromatisable androgen (DHT) in conjunction with a constant level ofT4, the highest level of luciferase activity was evident in cells that were treated with 50ng/ml of T4 without DHT ($p<0.001$). As the DHT levels were increased the activity declined, reaching control levels at doses of DHT greater than 100ng/ml. Interestingly, cells treated with only DHT resulted in significantly high activity levels ($p<0.05$) when compared to the control.

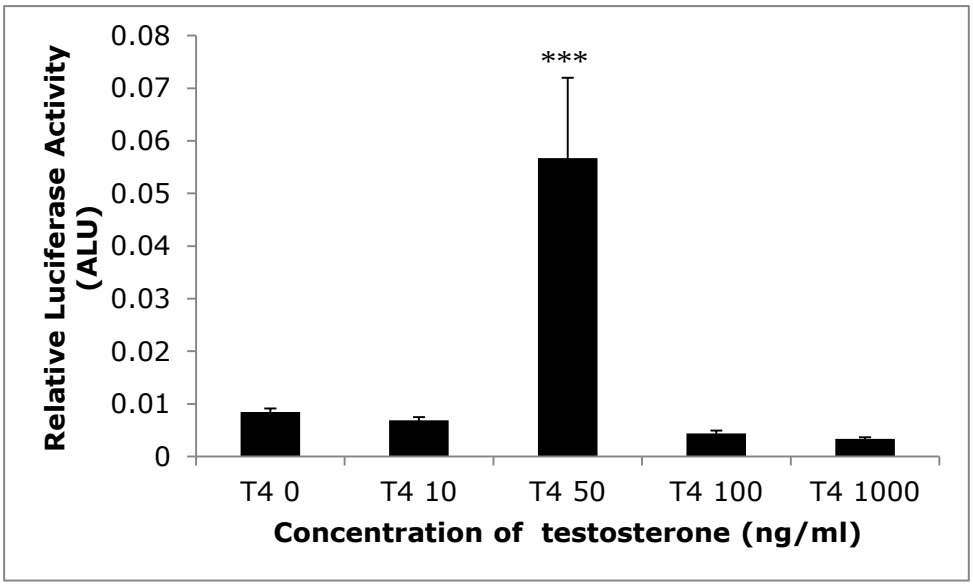


Figure 5.8 The effect of T4 on the activation of the AR pathway by assessing T4 responsive element activation, in ovine GC challenged with different concentrations of T4.
Values represent mean ± SEM. ***, P< 0.001 indicates a significant difference from NT.

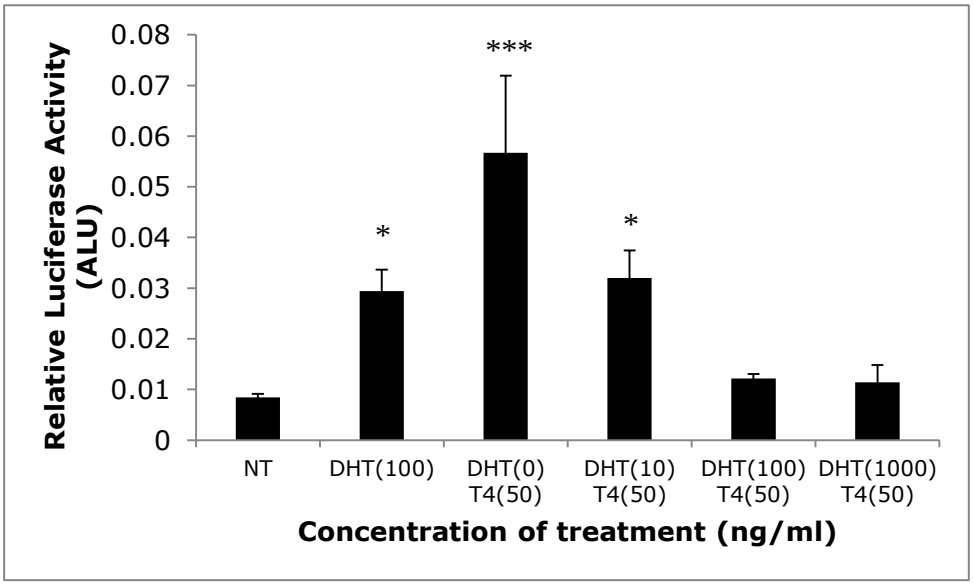


Figure 5.9 The effect of DHT on the activation of the AR pathway by assessing T4 responsive element activation, in ovine GC.
The cells were challenged with either DHT 100ng/ml or different concentrations of DHT (0, 10, 100, 1000 ng/ml) in the presence of 50ng/ml testosterone and compared to non-treated cells. Values represent mean ± SEM. *, P<0.05, and ***, P< 0.001 indicates a significant difference from NT.

5.5 Discussion

The results from this study support the concept that androgens not only regulate E2 production but may also have a wider effect on follicular development. This present study showed in cultured GC that the important marker of follicular cell differentiation (Inhibin A) was upregulated by T4 in a process that was fine-tuned by an optimal level of FSH and IGF. Further, this study also showed that GCs treated with a non-aromatisable androgen (DHT) in conjunction with T4 resulted in an increase in E2 concentrations when compared to cells treated with T4 alone, and this effect was reversed when AMH was added to the culture, a novel finding that to the best of knowledge has not been shown before. Results from this work also showed that androgens may also have an important role in regulating AMH production.

In order to facilitate the study of the effect of the androgen T4 on cultured ovine GCs, a specialised ovine granulosa serum-free culturing system was used to offer the necessary conditions for gonadotropin-mediated cell differentiation and steroid production. The cells were grown in either an optimal FSH/IGF environment (Campbell *et al.* 1996), as described in this chapter (Section 5.2.) or a low suboptimal FSH/IGF level. Results showed that GCs remained undifferentiated and produce low amounts of steroids until around 48 hours of culture as previously shown by other researchers (Campbell *et al.* 1996). However, E2 production increased thereafter and was majorly influenced by the concentration of T4 in culture with levels increasing as T4 concentration was increased. Further, as expected FSH/IGF concentrations had an impact on E2 production especially at the later time points where a significant higher production was observed in cells that were

treated with the optimal FSH/IGF levels. All these results were to be expected. However, when analysing the results of Inhibin A production following the same treatment layout, it was evident that the effect observed could not have been purely due to the substrate product effect of T4 on GCs. Inhibin A, like oestradiol, showed an increase with time but was influenced again by the treatment concentration of testosterone. And likewise, normal FSH/IGF treated cells produced significantly higher levels of Inhibin A than those treated with low levels of FSH/IGF from early on in the culture period. As Inhibin A is an important FSH responsive marker of GC differentiation (Campbell & Baird 2001), therefore we can conclude that androgens, and in this case the aromatisable androgen T4, may have a direct effect on follicular growth irrespective of its role as a steroid precursor in monovular species, in this case the sheep. Another important observation to note is that by increasing the dose of T4, there was a notable increase in the production of oestradiol and Inhibin A in cells that were treated with normal FSH and IGF concentrations. Although still just a speculation necessitating further investigation, T4 may have a regulatory effect on FSH function. These results are in agreement with many published animal (Harlow *et al.* 1988, Vendola *et al.* 1998a, Vendola *et al.* 1999b, Weil *et al.* 1999a, Wang *et al.* 2001b, Hamel *et al.* 2005) and human (Fabregues *et al.* 2009, Gleicher *et al.* 2011) studies that suggest that androgens may augment the function of FSH and IGF (Gervasio *et al.* 2014) in the ovary. Another recent study also showed that aromatisable and non-aromatisable androgens promoted FSH receptor protein expression in primary mouse GCs (Sen *et al.* 2014).

To consolidate our results above, an experiment was conducted to indirectly test the effect of androgens on folliculogenesis by measuring E2 in cultured ovine GCs treated with a non-aromatisable androgen (DHT) in the presence of an aromatisable androgen (T4) as a substrate for E2 production. Results were promising, showing that E2 increased over time as expected, but when DHT was added it caused a significant increase in E2 production in all the tested time points, indicating that androgens may have a role in E2 production and follicular development besides being a substrate for E2. The necessity of adding an aromatisable androgen was clearly evident by showing that cells treated with only DHT caused minimal E2 production at a level similar to cells that were none treated with androgens. Overall, these findings complement recent publications in disputing the long held belief that implements androgens including DHT as inhibitors of antral follicular development (Bagnell *et al.* 1982, Billig *et al.* 1993). These studies suggest that androgens may have a growth promoting role in folliculogenesis which depends on the stage of follicular development. The administration of DHT or T4 to adult rhesus monkeys resulted in a significant increase in the number of preantral and small antral follicles and promoted GC and TC proliferation (Vendola *et al.* 1998b). *In vitro* studies showed that DHT and other androgens had a stimulatory role within the mice developing follicles (Murray *et al.* 1998b), DHT and T4 enhanced human ovarian follicular tissue survival (Otala *et al.* 2004), DHT caused a significant increase in preantral follicle growth in rats (Orisaka *et al.* 2009) and DHT was also shown to enhance procine GC proliferation, facilitated by the presence of GDF9 (Hickey *et al.* 2005). Collectively, these studies suggest that androgens, and in this case DHT, exert a direct stimulatory action on the

follicular development, especially during the preantral-early antral stage transition.

The way DHT, a non aromatisable androgen, causes the increase in E2 production must have been different to simple aromatisation, most likely due to stimulation of the AR. Support for this idea comes from a study that showed that the androgens action in inducing follicular growth in the presence of GDF9 was suppressed with the addition of flutamide, a specific androgen receptor antagonist (Orisaka *et al.* 2009).

The role of AMH during folliculogenesis is poorly understood. What we know from polyovular species is that AMH may have a role in the inhibition of primordial follicle recruitment (Durlinger *et al.* 2002b), may inhibit gonadotropin dependent growth of preantral follicles (Durlinger *et al.* 2002a, Themmen 2005) and attenuates the FSH-dependent increase in aromatase activity and LH receptor expression (di Clemente *et al.* 1994). However, in monovulatory species the evidence from the literature for the role of AMH during folliculogenesis is less clear. Recent studies in monovulatory sheep have shown a negative association between AMH and GCs aromatase activity (Campbell *et al.* 2012). In this study the results concur with these previous findings and extend them by showing that in ovine GCs from small antral follicles, AMH caused a significant decrease in E2 production at all tested time points.

Reviewing the literature, the effect of androgens on GC produced AMH in a monovular species in an *in vitro* setting, to our knowledge, has never been studied. Therefore, this novel piece of work may be of great value as it sheds light on the effect of androgens on ovine GCs in a controlled culture

environment that can produce results that can be easily interpreted. Results showed that ovine GCs treated with abnormally high T4 concentrations caused a significant rise in the production of AMH throughout the culture period with secreted levels reaching its peak at the later time points, while normal T4 concentrations only caused an increase in AMH production only after 192 hours of culture. On the other hand, while still showing the same trend, DHT's effect on AMH production by GCs was not as potent as that of T4. The effect was only observed in cells that were treated with significantly high levels of DHT and only in the later time points, while normal and low levels of DHT had not significant effect on AMH production throughout the culture period.

These results may therefore provide further evidence that high levels of androgens may directly cause an increase in AMH production, and collectively, *in vivo* studies conducted in PCOS women (Pigny *et al.* 2003, Laven *et al.* 2004, Eldar-Geva *et al.* 2005) and this *in vitro* experiment may indicate to the likely involvement of androgens and/or AMH in the aetiology of PCOS especially in women with hyperandrogenism. This high androgenic environment seen in this experiment at the later time points may therefore be consistent with the observation *in vivo* that shows that high androgen levels may maintain the high AMH production even in the late stages of the follicle development, while in normal physiological conditions AMH secretion diminishes as the follicle approaches ovulation (Andersen & Lossl 2008). Further, the evidence of an AMH pathway shown in the previous chapter could lend support to the a paracrine effect of AMH on theca cells, which may indicate to possible mechanisms of theca cell dysregulation seen in PCOS due to the disruption of the regulatory loop

between GC produced AMH and the TC produced androgens (Figure 5.10). However, this needs to be tested directly in women with PCOS.

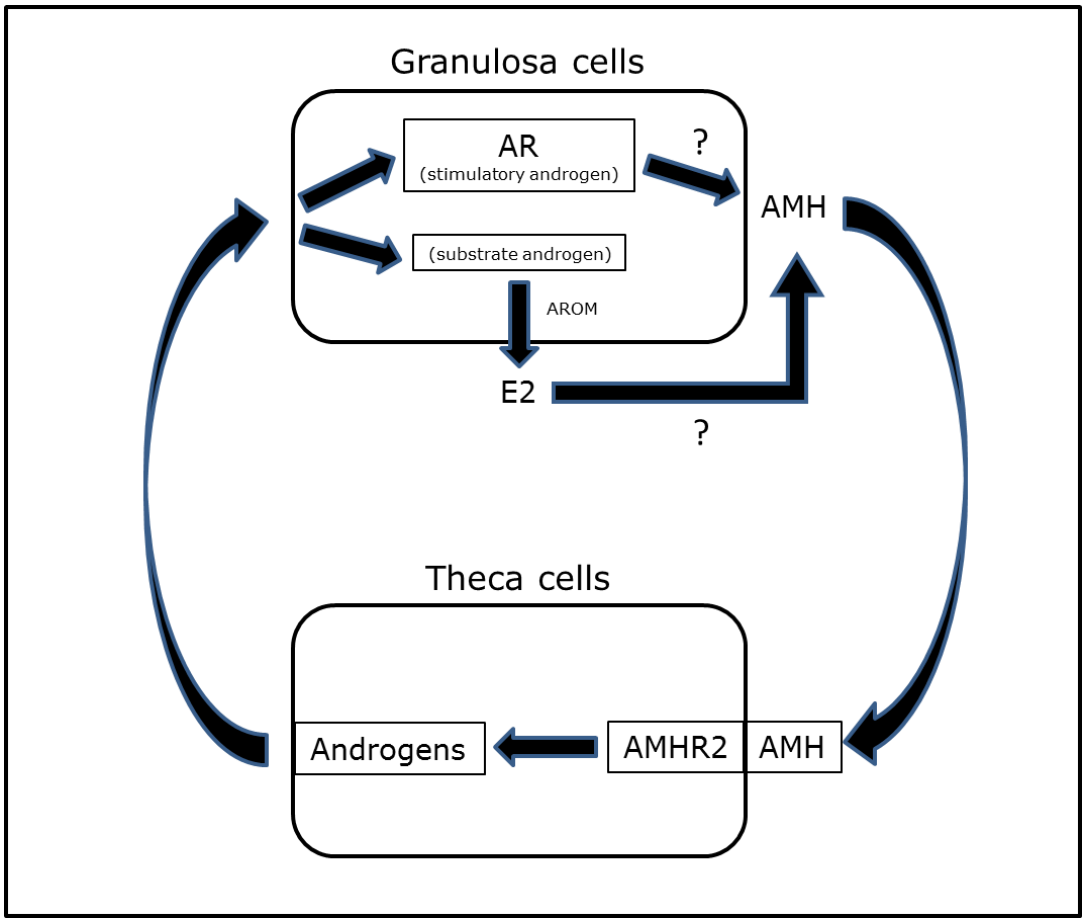


Figure 5.10 The possible regulatory loop between GC produced AMH and TC produced androgens.

As previously discussed, AMH and aromatase have an inverse relationship (Campbell *et al.* 2012). In this study we further confirmed these findings by showing that aromatase expression decreased as the culture progressed. A result that is in agreement with previous studies on ovine tissue (Campbell *et al.* 2012).

One of the crucial events during normal follicular development is the differentiation of GCs to produce E2 (Robertson &

Burger 2002), a process known to be regulated by the production of the aromatase protein (Steinkampf *et al.* 1987) which is further regulated by the level of aromatase mRNA expression (Lephart *et al.* 1992). The way this tightly regulated mechanism is controlled is poorly understood. Reviewing our results, it is evident that androgens have a role in this regulation.

In this study, aromatase gene expression was profiled in GCs treated with androgens at different concentrations for different time periods, in an attempt to expand our knowledge of how androgens effect this regulation. Two main pathways have been suggested. The first was the indirect effect of aromatised androgens to E2 that then affect aromatisation and the other is a direct effect of androgens through the AR which thereafter may affect aromatase expression. Results suggest that aromatase expression is at its highest when E2 production in GCs is low, during the first 48 hours of culture. As E2 production increases thereafter aromatase expression decreases, which may indicate to a local feedback loop that exists between the two. An interesting finding at the early time points showed that there was a difference between aromatase expression in cells that were treated with low T4 when compared with cells that were treated with normal or supra-physiological levels of T4. This again may indicate the possibility that these cells, in a low oestrogenic environment, are stimulated to express higher levels of aromatase to enhance E2 production. However, the decrease in aromatase gene expression in conjunction with the corresponding higher E2 production in cells treated with T4 may indicate an effect on aromatase activity rather than only affecting production. This is supported by results that showed that DHT, a non

aromatisable androgen, caused an increase in GCs E2 production, while not affecting aromatase gene expression, a novel finding that has not been shown before. Support from the literature comes from a study that showed that E2 production increased in the presence of DHT by increasing aromatase activity though binding with non-active sites on the aromatase enzyme (Kirilovas *et al.* 2003). These researchers also suggested the possible mechanism of action of DHT to be through DHT AR aromatase interactions to stimulate aromatase. Further, GCs that were treated with flutamide, an AR antagonist, caused an inhibition in aromatase activity (Hillier & De Zwart 1981).

As demonstrated earlier, GCs produce E2 depending on the availability of aromatisable androgens. It is also suggested above that the androgen signalling pathway may be involved in the regulation of GC production of E2. To elucidate this effect, we studied the effect of T4 and DHT on the AR signalling pathway by utilising a luciferase reporter gene system. Results showed that androgens in general may possess a bimodal effect on the activation of the AR pathway. The results indicate an accumulative effect of androgens, on the AR pathway activity rather than an individual androgen effect, which may indicate that both aromatisable and non-aromatisable androgens are capable of activation of the AR pathway at different potencies. Once the maximum activation level is reached, irrelevant of the type of androgen, this effect may be reversed, causing an inhibition of activation. This study also showed that T4 is a more potent activator of the AR pathway than DHT, whereby 50ng/ml of T4 caused nearly double the activity of when 100ng/ml DHT was used. However, this effect may be due to the suppressive effect of

DHT and therefore further investigation using similar treatment doses of both androgens are needed to confirm these findings.

In conclusion, the results presented in this chapter provide novel and convincing evidence to support the hypothesis that androgens may at least in part be involved in the regulation of folliculogenesis and AMH production. It seems feasible that a finely tuned regulatory loop between GC produced AMH and TC produced androgens exists that is vital for normal follicular development and perturbations in this may result in abnormal conditions such as PCOS.

Chapter 6 Further Elucidation of Two Novel Putative Mechanisms for AMH Regulation in Granulosa cells

6.1 Introduction

AMH is known to have imperative roles during folliculogenesis (Durlinger *et al.* 2002a) but it seems equally important that its production steadily decreases during the antral stages of normal follicular development (Pellatt *et al.* 2007), such that it is absent in the follicular fluids of large pre-ovulatory follicles. It is clear that AMH production must be under tight regulation as high levels in large follicles are synonymous with infertility disorders (Fallat *et al.* 1997, Ocal *et al.* 2011) such as PCOS and OHSS. The mechanisms underlying this regulation are, to date, poorly understood and thus their elucidation remains an important research goal.

It was hypothesised that VEGF could be linked to AMH regulation, as though it appears to have a vital angiogenic role in folliculogenesis (Zimmermann et al. 2001), it has been noted to be, like AMH, also markedly elevated in both PCOS and OHSS (Kamat *et al.* 1995, Pellicer *et al.* 1999). Also, due to the characteristic avascular property of GCs, it seems likely that the high levels of VEGF may be important to other non-angiogenic mechanisms involved with the development of the follicle. Furthermore, VEGF has been linked to the aetiology of one of the undesirable side effects of IVF treatment, OHSS (McClure et al. 1994, Agrawal et al. 1998, Garcia-Velasco & Pellicer 2003). An exuberant multifollicular response that occurs during ovarian stimulation with exogenous

gonadotropins, and found, to be even more profound in patients with PCOS/PCO, in which VEGF levels have been found to be elevated (Kamat et al. 1995). OHSS has a reported prevalence for women that have PCOS who over respond to stimulation and develop OHSS to reach 38-100% (Peitsidis & Agrawal 2010). This not only increases VEGFs research importance but may also offer further clues to its mechanism of action. Strong evidence now suggested that in patients that suffer from OHSS, VEGF facilitates widespread ovarian vascular leakage and an acute fluid shift into the extravascular spaces (Rizk et al. 1997). It is excessively produced and secreted into the follicular fluid of the increased number of antral follicles in OHSS (Rizk et al. 1997). The predominant form of VEGF found in the ovarian follicles is VEGF-A (Greenaway et al. 2004, Marsters et al. 2014). The most abundant forms of VEGF-A isoforms identified have been $VEGF_{121}$ and $VEGF_{165}$ with the designated number referring to the number of amino acids that make up the protein expression product. Therefore, the aim of this piece of work was to determine the effect of VEGF on AMH utilising the two recombinant VEGF-A isoforms ($VEGF_{121}$ and $VEGF_{165}$) on ovine GCs grown *in vitro*. This study also aimed to elucidate the role of VEGF on folliculogenesis, specifically, exploring the effect of VEGF on steroid production in cultured ovine GCs. Finally, the aim of this study was to investigate the prevalence of VEGF receptors type 1 and 2 in these cultured cells and the effect exogenous VEGF treatment had on their expression.

A second aim of this section of this present study was to investigate a potential regulatory role in AMH production, for SOX transcription factors. Recently, members of the SOX family have drawn researcher's attention, where they have

been found to play a role in gonadal development (Chaboissier et al. 2004, Koopman 2005). Recent evidence now indicates that oocyte somatic cell interactions may play a vital role in regulation of follicular development (Webb & Campbell 2007). Based on this assumption, this project was constructed to examine the hypothesis that members of the SOX family of transcriptional factors may play a role in regulating the expression of somatic cell factors like AMH and effect folliculogenesis directly or indirectly. Support of this hypothesis comes from murine studies that showed that SOX8, a member of this family, which can substitute SOX9 in the transactivation of AMH in sertoli cells, has been found to be expressed in the oocyte of preantral follicles and in the oocyte and cumulus cells of pre-ovulatory follicle (Salmon *et al.* 2005). Further support comes from a recent publication that showed that the cumulus cell layer in sheep continues to express AMH even in pre-ovulatory follicles, while the membrana GC layer showed no AMH expression and high aromatase levels (Campbell et al. 2012). Using a SOX8 knock-down immortalised human granulosa cells (KGN cells) model, the aim to this study was to determine the effect of the SOX8 protein on AMH expression which may therefore provide further evidence of the seemingly closely regulated intraovarian interactions.

6.2 Experimental Design

6.2.1 The Effect of VEGF on AMH Production, E2 production and VEGF Receptor Expression in Cultured GC.

Harvested GCs were obtained from small antral follicles as previously described in chapter 2 (section 2.1.2). Two sets of experiments were then conducted.

The first involved culturing ovine GCs in 24 well plates in treatment free media over a 192 hours culture period. The media was collected at different time points (24, 48, 96, 144, and 192 hours) and stored at -20^{o}C. At each time point the cells were harvested, lysed and stored at -20°C for later use in RNA extraction and preparation of cDNA libraries for RT-PCR. Specific fragments of VEGFR1 and VEGFR2 genes were amplified from each of the cDNA libraries, utilising quantitative real-time PCR, as fully described in the 'Methods chapter (Section 2.3.1). Outcomes relative to an endogenous control (18S) were compared. The primers used for the genes of interest are illustrated in (Table 6.1).

In the second set of experiments GCs were treated with either 10ng/ml of $VEGF_{121}$, $VEGF_{165}$ or a combination of both isoforms (R & D Systems). A non-treated group was also included as a control. The cells were cultured over a period of time and media was retrieved and stored for later analysis at three different time points (24, 48, and 96 hours). At each of these time points, following removal of the media, the GCs were lysed as above and reserved at -20°C for later use in RT-PCR. The accumulative AMH and oestradiol concentrations were measured by ELISA as described in the general method section (Chapter 2 Section 2.3.2.). Lysed cells that were

obtained were used to estimate the VEGFR 1 gene expression in each of the treatment groups utilising the general RT-PCR methodology as earlier detailed (Section 2.3.1.). Unfortunately, due to technical difficulties and time constraints, we were unable to measure VEGFR 2 expression in these cells. The same primers were used as above (Table 6.1).

Table 6.1 Forward and reverse primer sequences for internal standard (18S), and VEGF receptor type 1 and 2.

Target Gene	Forward Primer Sequence (Sense)	Reverse Primer Sequence (Antisense)
18 S	5'-TTGACTCAACACGGGAAACCT-3'	5'-AGAAAGAGCTATCAATCTGTCAATCCT-3'
VEGFR-1	5’-CACCAAGAGCGACGTGTG-3’	5’- AAGAAGTCCTCGGAGAAGGC-3’
VEGFR-2	5’-AGACTGGTTCTGGCCCAAC-3’	5’-GAAGCCTTTCTGGCTGTC-3’

6.2.2 The Expression of SOX8 in Primary Ovine GC

Fluorescent ICC studies were conducted to study the expression of SOX8 protein in primary 144 hours cultured ovine GCs obtained from small antral follicles using the technique described in the general methods chapter (Section 2.7.). A primary SOX8 rabbit polyclonal antibody (ab104245; abcam) and a fluorescent anti rabbit secondary antibody (CF 555, biotium) were used. Negative controls that were devoid of primary antibody were included in each slide for background control.

6.2.3 The Effect of SOX8 Knock Out, and Exogenous SOX8 Protein Substitution, on AMH and E2 production in KGN cells

Human granulosa-like tumour cell line (KGN), a cell line that originated from a stage 3 GC carcinoma which was removed from a 63 year old Japanese lady in 1984 (Nishi *et al.* 2001), was used in this experiment due to its close resemblance to normal GCs. This cell line was found to maintain most of the physiological activities seen in normal GCs including expression of functional FSH receptors and the same pattern of steroidogenesis (Nishi *et al.* 2001), which made it an attractive candidate in this study.

The KGN cell line was obtained from the Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Japan. KGN cells were maintained in DMEM/F12 medium that was supplemented with Fungizone (2.5µg/ml), Penicillin (50U/ml) /streptomycin (50µg/ml) cocktail solution and Foetal Calf Serum (10%v/v). The cells were incubated at 37^{o}C in 95%O_2 and 5% CO_2. The grown cells underwent a maximum of 10 passages before

being discarded and the cells used in this experiment were between 5 to 10 passages.

KGN cells were cultured for 24 hours. After this culture period, SOX8 was knocked out by transfecting the cells with SOX8 siRNA. The transfection mix was prepared on the day of transfection that was composed of the transfection reagent Dharmafect and the SOX8 siRNA, all mixed in a 5% FCS and antibiotic free culture media. Following a further 48 hours of culture, the cells were then treated with different concentrations of human SOX8 protein (abcam) for 1 hour (NT, 100nM, 1μM or 10μM). After the spent treatment period, all the media was removed and stored at -20^{o}C for later ELISA analysis. The cells were lysates and stored at -20^{0}C for later use in RT-PCR as described in chapter2 (section 2.3.1.1). The expression level of SOX8 in each treatment group was compared to mRNA expression levels in KGN cells that had SOX8 knocked out but were devoid of any treatment. The primers used for the genes of interest are illustrated in (Table 6.2) and 18s rRNA was used as an endogenous control.

Table 6.2 Forward and reverse primer sequences for internal standard (18S), and SOX8.

Target Gene	Forward Primer Sequence (Sense)	Reverse Primer Sequence (Antisense)
18 S	5'-TTGACTCAACACGGGAAACCT-3'	5'-AGAAAGAGCTATCAATCTGTCAATCCT-3'
SOX8	5′ – CGTGTACAAGGCTGAAGCAG – 3′	5′ – GGTCTGCCCTGTGTGGTC – 3′

The stored media was analysed using ELISA to determine the effect of SOX8 knockout in KGN cells and subsequent SOX8 treatment at different concentrations (NT, 100nM, 1μM or 10μM) on the levels of AMH and E2 production as previously described in the general methodology section (Chapter2 section 2.3.2).

6.3 Statistical analysis

Data from all experiments in this chapter were presented as mean +/- SEM of at least three different experiments conducted in triplicates. The results of the semi-quantitative comparison of the target genes were normalised against the cell number utilising 18S as an endogenous control and analysed relative to a standard curve in accordance with the Applied Biosystem provided software. PCR and ELISA data were tested for normality before the use of a two way ANOVA to determine the level of significance between the different results. In all experiments significance was set as $p < 0.05$.

6.4 Results

6.4.1 The Effect of VEGF on AMH Production in Cultured Ovine GC

GC AMH production was shown to increase over time in both treated and non-treated groups. VEGF isoforms treatment showed a noticeable effect on ovine GC AMH production when compared to the control. Minimal differences in AMH production were noted between treatment groups following 24 hours of culture. However, at 48 hours, $VEGF_{121}$ treatment caused a significant reduction ($p<0.05$) in AMH production when compared to the control. This was even more pronounced with $VEGF_{165}$ treatment ($p<0.01$), and an even greater reduction was evident when both isoforms were added to the culture ($p<0.01$). Similarly, VEGF isoforms caused a greater reduction ($p<0.001$) in AMH production in GCs at 96 hours of culture, following the same trend as that seen at 48 hours (Figure 6.1).

6.4.2 The Effect of VEGF on E2 Production in Cultured Ovine GC

As previously illustrated, non-treated GC E2 production increased over time. Treatment of ovine GCs with either of the VEGF isoforms, $VEGF_{121}$ or $VEGF_{165}$, had no significant effect on ovine GC E2 production at any of the time points. The combination of both isoforms had no noticeable effect on E2 production at 24 or 48 hours, however, E2 production following 96 hours of culture was shown to be significantly higher ($p<0.05$) when compared to the control at the same time point (Figure 6.2).

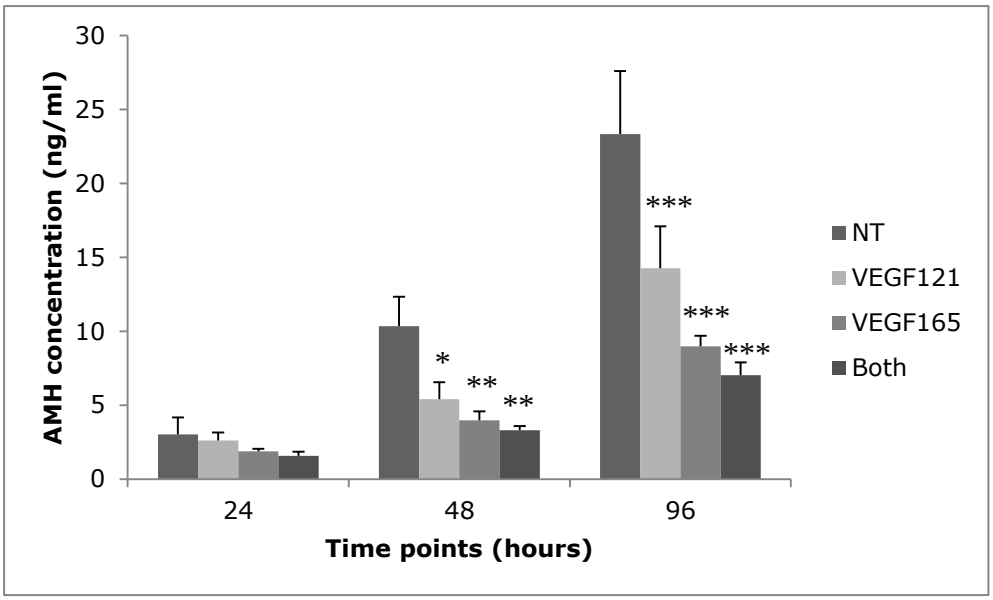


Figure 6.1 Production of AMH over time in ovine GC cultured in the presence of $VEGF_{121}$, $VEGF_{165}$ or both.
Values represent mean ± SEM. *, P<0.05, **, P<0.01, and ***, P<0.001 indicates a significant difference from non-treated cells at the same time point.

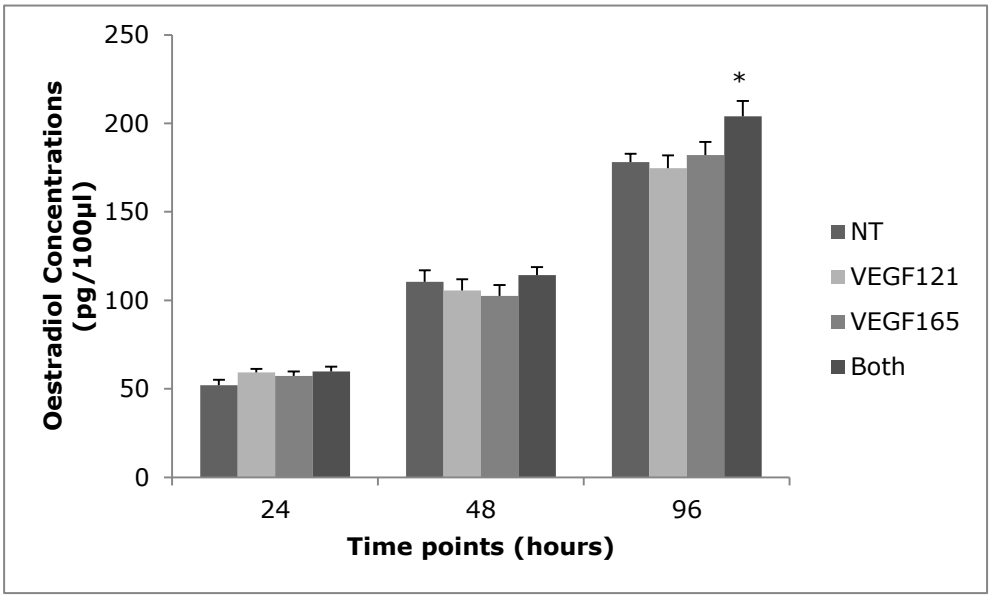


Figure 6.2 Production of E2 over time in ovine GC cultured in the presence of $VEGF_{121}$, $VEGF_{165}$ or both.
Values represent mean ± SEM. *, P<0.05 indicates a significant difference from non-treated cells at the same time point.

6.4.3 VEGFR-1 and VEGFR-2 Expression Levels in Ovine GCs

This experiment was conducted to study the expression levels of both VEGFR 1 and 2 over time in non-treated ovine GCs. Both receptors were found to be expressed in GCs of ovine small antral follicles and followed the same trend of expression (Figure 6.3 and Figure 6.4). Levels were low at 16 hours of culture, followed by a sharp transient increase in expression reaching a peak at 24 hours of culture and decreasing back to the control levels thereafter.

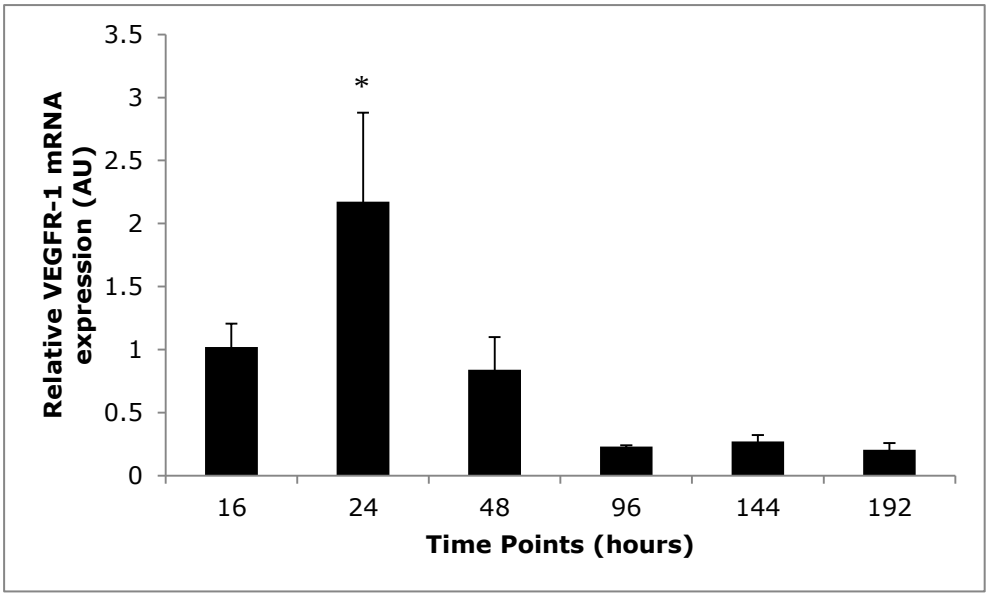


Figure 6.3 VEGFR 1 expression over time in cultured GC.
Values represent mean + SEM. *, P<0.05 indicates a significant difference from VEGFR1 expression at 16 hours of culture.

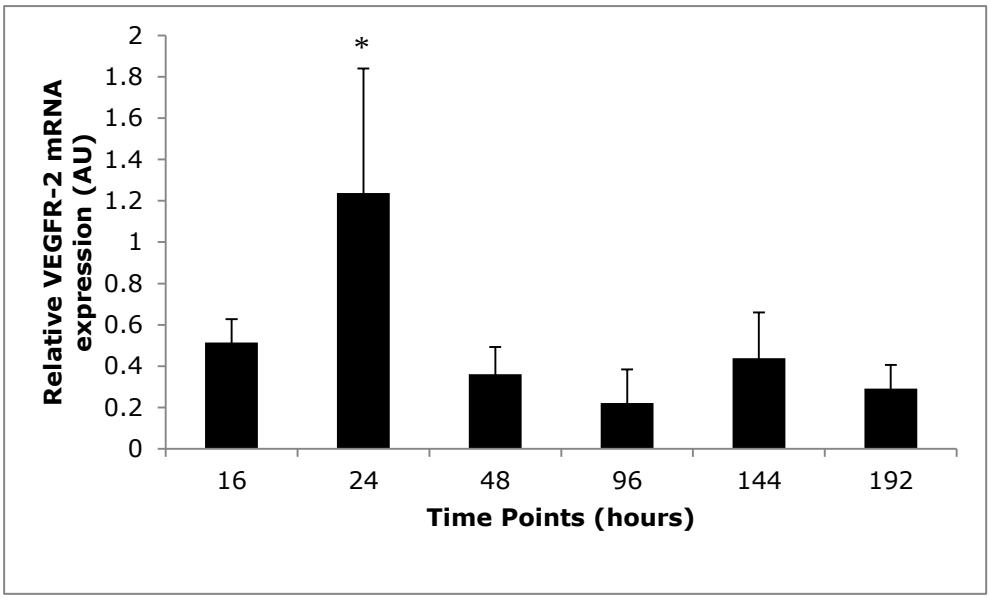


Figure 6.4 VEGFR 2 expression over time in cultured GC.
Values represent mean + SEM. *, P<0.05 indicates a significant difference from VEGFR 2 expression at 16 hours of culture.

6.4.4 The Effect of VEGF on VEGFR Expression in Cultured Ovine GCs

The aim of this experiment was to profile the expression levels of VEGFR 1 and 2 over time following treatment of the GCs with the different VEGF isoforms ($VEGF_{121}$, $VEGF_{165}$ or both). However, due to technical difficulties, only VEGFR1 expression was quantified.

Results for VEGFR1 expression showed that $VEGF_{121}$ treatment had no effect on the receptors expression at any of the time points. However, treatment with $VEGF_{165}$ or treatment with the combination of both isoforms caused a significant reduction ($p<0.001$) in the expression of the receptor at 24 hours, but had no effect at later time points (Figure 6.5).

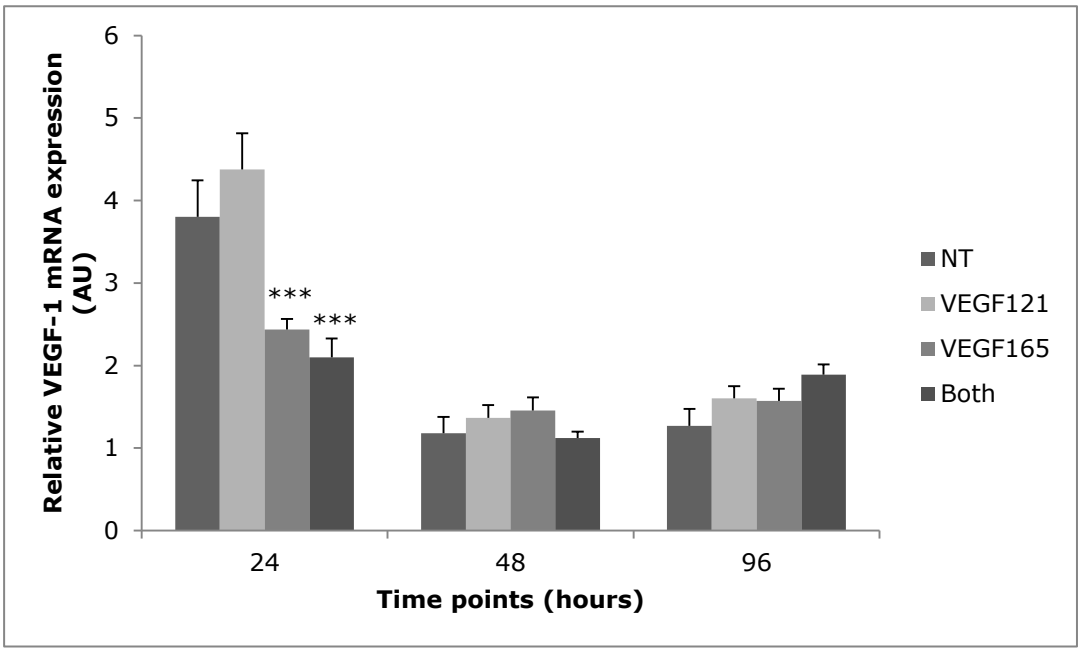


Figure 6.5 VEGFR 1 expression over time in cultured ovine GC in the presence of $VEGF_{121}$, $VEGF_{165}$ or both.
Values represent mean + SEM. ***, P<0.001 indicates a significant difference from non-treated cells.

6.4.5 SOX8 Expression Levels in Cultured Ovine GC

Fluorescent ICC studies showed that SOX8 was heavily expressed in differentiated primary ovine GCs obtained from antral follicles (Figure 6.6).

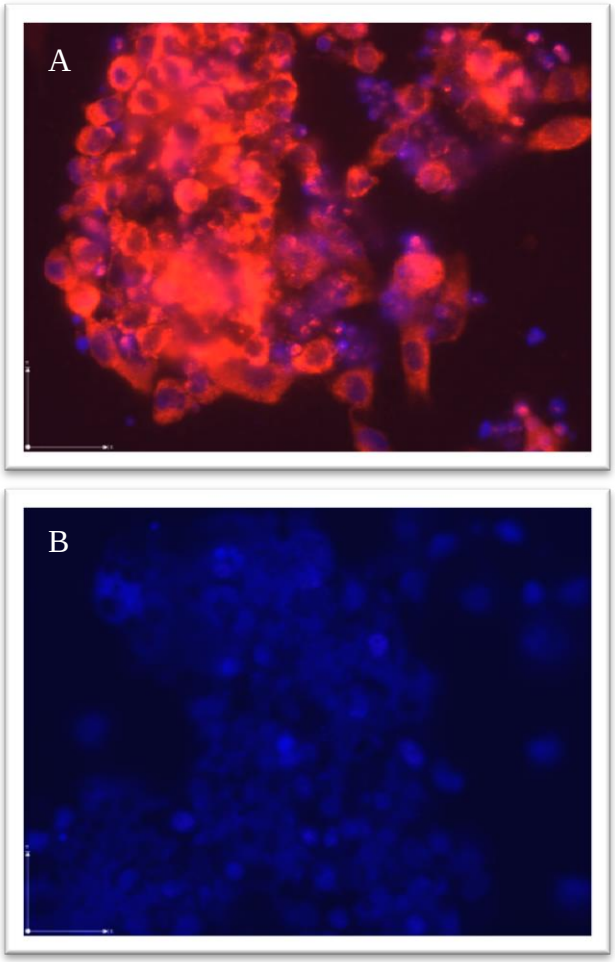


Figure 6.6 SOX8 expression levels in 144 hour cultured ovine GC using fluorescent ICC studies.
A rabbit polyclonal to SOX8 was used (A) and negative control with no primary antibody (B). In all cases, the cell nuclei were counterstained with DAPI (blue) and the cells visualised using a fluorescent microscope at x400 magnification.

6.4.6 Confirmation of SOX8 Knockout by Measuring SOX8 mRNA Expression in KGN cells

The aim of this experiment was to determine whether the siRNA knockout protocol used in this study had the ability to affect the expression level of SOX8 mRNA in cultured KGN cells. Results showed that SOX8 knockout caused a significant attenuation ($p<0.01$) in the expression levels of SOX8 mRNA in these cells. However, as expected, the addition of exogenous SOX8 had no significant effect on the expression of SOX8 mRNA in this cell type (Figure 6.7).

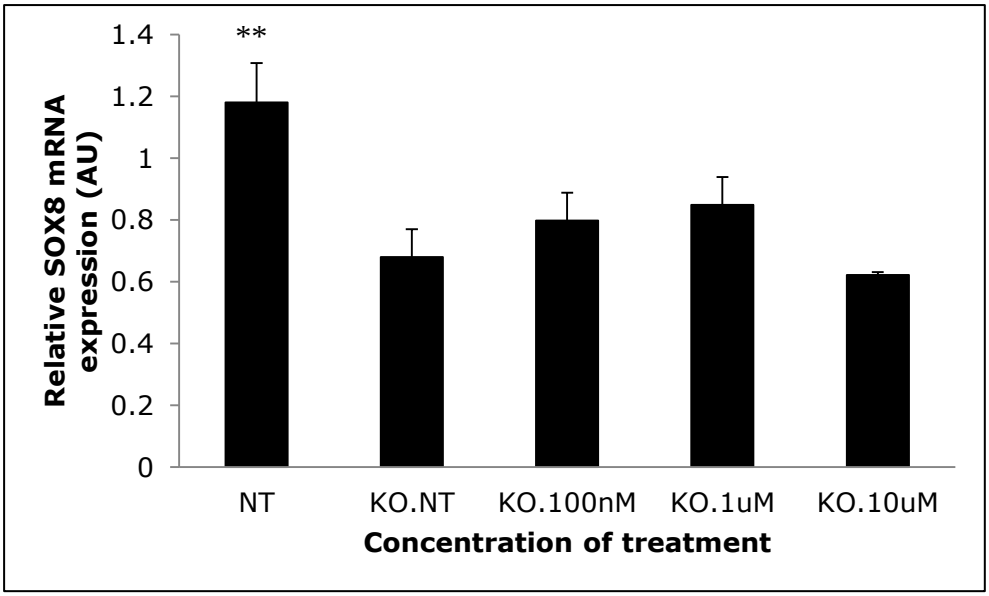


Figure 6.7 SOX8 mRNA expression in 72 hour cultured KGN cells that were subjected to SOX8 (KO) and then treated with different concentrations of SOX8 for 1 hour.
Values represent mean ± SEM. **, P<0.01 indicates a significant difference from KO NT.

6.4.7 The Effect of SOX8 Knock Out, and Exogenous SOX8 Protein Substitution, on E2 and AMH Production in KGN cells

Sox8 knockout resulted in a significant increase ($p<0.001$) in E2 production in non-treated KGN cells. On the other hand, while low doses of exogenous SOX8 treatment had no effect on E2 production in SOX8 knockout KGN cells, the highest dose (10μM) resulted in a significant inhibition in E2 production ($p<0.05$). Although this inhibition did not attenuate E2 production levels to non-treated with SOX8 knockout KGN E2 production levels (Figure 6.8).

In this same experiment, AMH levels were measured in all the above mentioned treatment groups. Results showed that, although levels were very low, neither SOX8 knockout nor exogenous SOX8 treatment had any effect on AMH production in cultured KGN cells (Figure 6.9).

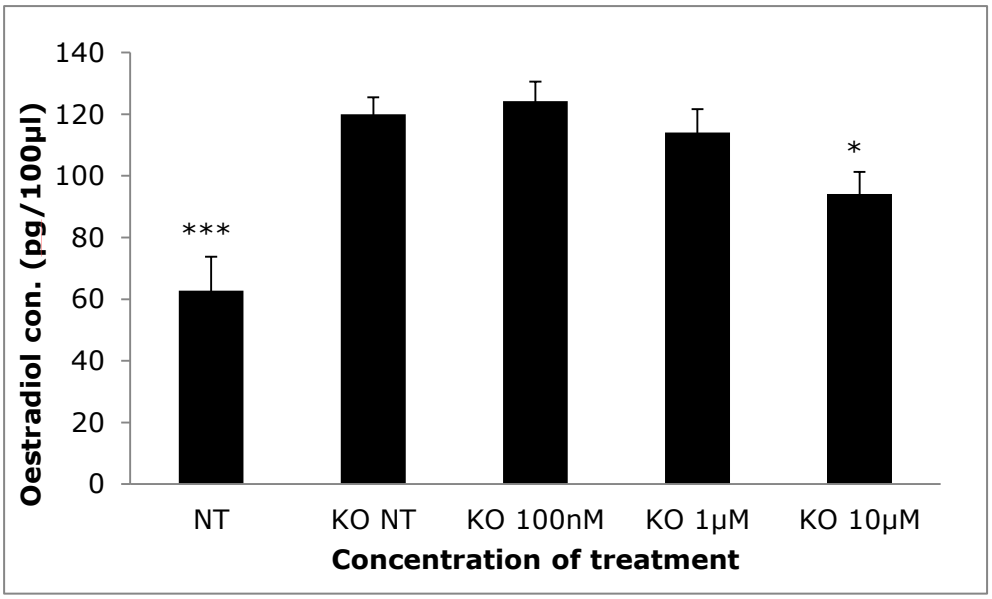


Figure 6.8 E2 production in 72 hour cultured KGN cells that were subjected to SOX8 (KO) and then treated with different concentrations of SOX8 for 1 hour.
Values represent mean ± SEM. *, P<0.05, and ***, P< 0.001 indicates a significant difference from KO NT.

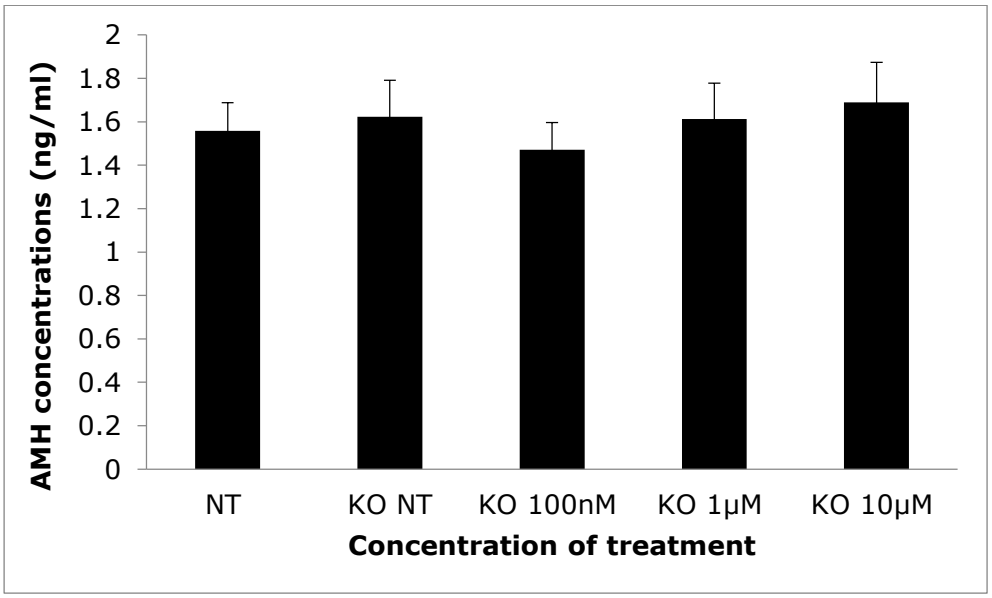


Figure 6.9 AMH production in 72 hour cultured KGN cells that were subjected to SOX8 (KO) and then treated with different concentrations of SOX8 for 1 hour.
Values represent mean ± SEM. No statistical significance was detected between treatment groups which was set as P<0.05 and compared to KO NT.

6.5 Discussion

In order to test the effect of VEGF on AMH production, this present study utilised two variant forms which according to Marsters et al., (2014), are the prominent VEGF-A isoforms found in ovine GCs. These were $VEGF_{120}$ (121 in humans) and $VEGF_{164}$ (165 in humans). Results showed that treatment with VEGF variants (VEGF 165 and 121) either isolated or in combination, caused a significant inhibition in AMH production, following 48 hours of culture. Although at first it seemed that the results from this study contradict the findings of Thomas and colleagues on adult female common marmoset monkeys (Thomas *et al.* 2007) which suggested a regulatory link between VEGF and AMH in the ovary. This study showed that inhibition of ovarian angiogenesis using VEGF trap resulted in a decrease in AMH mRNA expression in preantral follicles *in vivo* (Thomas *et al.* 2007). However, it is possible that the effect seen in this study may in fact complement their results. Thomas and colleagues used an interesting compound called VEGF trap which is basically a decoy receptor formed by fusing the extracellular domains of the human VEGF receptors type 1 and 2 to the Fc portion of a human immunoglobulin, which blocks the action of VEGF. However, in the current study exogenous VEGF treatments were used. It is unknown which of the VEGF variants may have an effect on AMH, as the paper produced by (Thomas *et al.* 2007) was conducted *in vivo* and blocked the most commonly used VEGF receptors. It is therefore possible that the effect seen on AMH may not be due to these variants (165 and 121) but exogenous treatment with them may result in the attenuation of the endogenously produced variants that affect AMH and therefore act in the same manner as that seen with the VEGF trap. However, this

is still speculative which demands further research, but the overall conclusion is that the VEGF pathway may be utilised to modulate ovarian AMH.

Further, it has been shown that VEGF enhanced survival of bovine GCs (Greenaway *et al.* 2004) and induced proliferation of porcine GCs (Grasselli *et al.* 2002), while inhibition of VEGF action resulted in decreased proliferation of GCs, high rates of atresia and failure of follicles to reach ovulation (Wulff *et al.* 2002). Therefore, disruption of this pathway may result in the alteration of AMH production, an important process during normal follicle development. However, the mechanism by which VEGF affects AMH is still unknown. This may possibly be due to a disruption in the normal endocrine control in the follicle, directly disturbing AMH expression or affecting other local factors that might disturb normal AMH expression. Nevertheless, all these remain theories and can only be explained with further research.

Another aspect of this study was to investigate the effect of VEGF isoforms, 121 and 165, on steroidogenesis, namely E2, in cultured ovine GCs, in order to better understand the role of VEGF in folliculogenesis in this monovular species. As to be expected, results showed that VEGF variants had minimal effect in the first 48 hours of culture. However, 96 hour cultured cells treated with both variants simultaneously showed a significant increase in oestradiol production when compared to the non-treated control cells. This is in agreement with a study conducted on porcine GCs which showed that cells treated with exogenous VEGF obtained from small antral follicles resulted in a dose dependent increase in E2 production, while VEGF treatment had an inhibitory effect on E2 production in cells obtained from large antral follicles

(Grasselli *et al.* 2002). Further, a study on cattle showed that $VEGF_{120}$ and $VEGF_{165}$ expression in GCs, and the concentration of E2 in follicular fluid were higher in large post selection follicles when compared to the pre selection follicle (Shimizu *et al.* 2007b). On the other hand, ovine follicles that were undergoing atresia were shown to have a decreased expression of VEGF 121 and 165 isoforms, and associated with a decreased concentration of follicular fluid E2 (Rosales-Torres *et al.* 2010). Finally, a study on cultured sheep GCs, showed that cells cultured in high density, which are known to upregulate VEGF 121 and 165 isoforms, significantly affected E2 production when compared to production of cells cultured at low density (Marsters *et al.* 2014). Collectively, this indicates that VEGF may have a role in the regulation of E2 during follicular development in an autocrine manner and that this regulation is follicle stage dependent. From the above studies, it can also be speculated that E2 may also have a role in the modulation of VEGF expression resulting in a regulatory loop between E2 and VEGF, however this warrants further investigation.

VEGFR2 is considered the main signalling mediator of VEGF-A action in endothelial and other cell types, while less is known for certain about VEGFR1. Several studies have provided support for the contention that it may act as a decoy receptor with minimal signalling activity (Carmeliet *et al.* 2001, Bates 2010), and thus acts as an attenuator of the type 2 receptor signalling (Ehrbar *et al.* 2004).

This present study's analysis of VEGF type 1 and 2 receptors also provided evidence that, of the two, the type 1 receptor maybe the more dominant one in ovine GC. Though both receptors showed a similar pattern of mRNA expression in

ovine GC cultured over time, the type 1 receptor had higher levels when compared to VEGFR2. This, therefore, adds some further support to an earlier study's findings which suggested that normal VEGF activity relies on a balance between the expression of both receptors and that the overall activity of VEGFR2 is controlled by competing with VEGFR1 (Ehrbar *et al.* 2004).

In this present study it was felt that regulation of the VEGF actions would be at least partially via local regulation of the VEGFR1. Outcomes of this present study's analysis of VEGF suggest that regulation of the VEGFR1 may, in part, be via a locally-derived VEGF variant. Treatment of GCs with exogenous VEGF isoforms demonstrated an effect on VEGFR1 expression, while unfortunately; VEGFR2 expression was not measured due to technical difficulties. Results showed that while non-treated GCs showed the same pattern of VEGFR1 expression seen above, granulosa cell treatment with either $VEGF_{165}$ alone or in combination with $VEGF_{121}$ was associated with a significant reduction in VEGFR1 expression and again its effect was only seen early in the culture period. On the other hand, treatment with $VEGF_{121}$ alone had no effect.

Reviewing the literature, very little research has been conducted in this area, to determine the effect of the different VEGF isoforms on it receptors. However, a study on chicken chorioallantoic membrane (CAM) showed that $VEGF_{121}$ induced vascularisation was associated with a significant decline in VEGFR1 (Ehrbar *et al.* 2004). These findings are in partially agreement with the present study, in which $VEGF_{165}$ caused a similar decline in the expression of this receptor, while $VEGF_{121}$ failed to show a similar effect. This may be attributed to the difference in tissue type between the two studies.

However in both studies, it is assumed that the decline in VEGFR1 may contribute to the stimulation of the type 2 receptor in order to facilitate VEGF function. A recent publication, however, showed conflicting results (Marsters *et al.* 2014). In this study VEGFR1 was increased rather than decreased in ovine GCs that were cultured in high density, which is known to cause an upregulation of the endogenously produced $VEGF_{121}$ and $VEGF_{165}$. The contradicting results of this study can be attributed to the difference in the overall environment of the cell culture. It could also be speculated that in these dense cells, VEGFR2 is heavily expressed and therefore necessitates the upregulation of the type 1 receptor to attenuate the effect of VEGF. Conclusively, there is no right or wrong here, as these receptors can be upregulated or downregulated, in other words fine-tuned, depending on the cell situation and environment.

The expression of VEGF and its receptors in GCs, and the avascular nature of GCs, suggests that VEGF may potentially possess non-angiogenic roles during the development of the follicle. There is a good deal of evidence confirming the broad functionality of VEGF in developing follicles. Research in primates has shown that systemic or intra-follicular injection of VEGF antagonists caused a significant impairment in the normal process of folliculogenesis, ovulation, and subsequent luteal function (Fraser & Wulff 2001). Furthermore, studies on rats showed that intra-ovarian injection of VEGF stimulated follicular development of preantral and antral follicles (Danforth *et al.* 2003, Shimizu *et al.* 2007a). The manner by which VEGF exerts its effect in still not fully understood. Undoubtedly, VEGF has been associated with the induction of angiogenesis in the follicular wall (Doyle *et al.* 2010),

however, studies on ruminant or porcine cells suggest that VEGF may act directly by promoting cell survival, supressing apoptosis, enhancing proliferation, and migration of GCs (Grasselli *et al.* 2002, Greenaway *et al.* 2004, Kosaka *et al.* 2007, Rolaki *et al.* 2007).

A number of studies have shown that VEGF and its receptors, VEGFR 1 and 2, are expressed in GCs and the changes in their expression during follicular development have been shown in many species such as in pigs, cattle, and primates (Fraser & Wulff 2001, Grasselli *et al.* 2002, Greenaway *et al.* 2004), with a consistent increase in VEGF and VEGFR2 expression in dominant follicles (Doyle *et al.* 2010). Further, changes in circulating levels of the VEGF receptor have been reported to regulate VEGF activity (Kuemmel *et al.* 2009). This can be seen during pregnancy where increased circulating levels of VEGFR1 serve to attenuate the effect of high levels VEGF produced by the placenta (Clark *et al.* 1998). Moreover, changes in the level of the VEGF receptors have been associated with OHSS, where a low level of VEGFR2 was linked to the occurrence and severity of the disease (Pietrowski *et al.* 2012).

A further aim was to better understand the role of SOX8 in the regulation of AMH and folliculogenesis. In this study, results showed that this method of knockout using SOX8 siRNA was capable of attenuating SOX8 mRNA expression significantly. However, it should be confirmed that the knock out process actually reduces or completely halts SOX8 protein production by using a technique capable of measuring protein. The knockout of SOX8 had a significant effect on E2 resulting in increased production. This was then gradually inhibited with the addition of SOX8 protein to the media in a dose dependent

manner. However, measuring AMH production showed fewer differences in its production between all these groups. This contradicts previous findings which showed an inhibition in AMH expression following SOX8 knockout by immuno-suppression in cultured KGN cells (unpublished data, Topipat, C., Picton, H. & Campbell, B.K.), however, in this previous study only mRNA was measured while here we have measured actual protein production. It is possible that as these cells are differentiated cells, they may therefore not have the capacity to change the amount of AMH production significantly. Potentially a better model would be to study the effect of SOX8 on AMH in primary cells at a stage of known AMH function and significant AMH production for example at the small antral follicular stage. What can be gained from this study is that SOX8 may have a role in regulating folliculogenesis and altering E2 production, however, it seems unlikely that this effect is mediated primarily by an effect on AMH which is known to have an inhibitory role during folliculogenesis.

In conclusion, this chapter provides evidence that folliculogenesis is not only controlled by systemically produced stimuli, but also involves a finely balanced complex interaction of many local intra-ovarian mechanisms. Evidence from this research also suggests that VEGF but not SOX8 may have a possible role in the regulation of AMH.

Chapter 7 General Discussion

7.1 Introduction

Evidence from recent research suggest that folliculogenesis is regulated by stage specific locally produced growth factors and hormones from both germ and somatic ovarian cells, which have been shown to have roles in proliferation, differentiation and angiogenesis as the growing follicle progresses to become an ovulatory follicle (Knight & Glister 2003, Webb & Campbell 2007, Fraser & Duncan 2009). AMH has been identified as one of these potentially important local factors and in polyovulatory species has been implicated in the regulation of follicle growth (Durlinger *et al.* 2002a). However in monovulatory species, despite measurement of its circulating levels being useful as a marker of ovarian reserve and its apparent link to the aetiology of PCOS, little is known about its roles and how they link to the mechanisms underlying development in the growing follicle.

7.2 AMH-Responsiveness in ovine TC

The approaches utilised in this study were aimed at improving our understanding of the roles and regulatory mechanisms of action of AMH in follicular somatic compartments during the crucial stages of antral follicle development leading to ovulation. Though earlier studies have produced evidence that AMH is produced in the GC compartment (Vigier *et al.* 1984, Rajpert-De Meyts *et al.* 1999, Pellatt *et al.* 2007) the understanding with regards to TC has remained unresolved with reports being quite contradictory (Baarends *et al.* 1995a). In contrast to the previously held view that TC in

monovulatory species may not be responsive to AMH (Baarends *et al.* 1995a), one important novel piece of work carried out in the TC of sheep showed that there was a measurable response to AMH stimulation (Campbell et al 2012). In that study (Campbell *et al.* 2012) showed that AMH had a dose dependant inhibitory effect on theca-derived androstenedione and it was suggested that this may indicate a possible paracrine effect of GC-derived AMH where the previous belief has been that AMH functions only in a autocrine manner (Baarends *et al.* 1995a). The Campbell et al., (2012) work, however, introduces a possible conflict of understanding in that though it has previously been thought that cellular responsiveness to AMH is exclusively mediated by receptor complexes containing the AMH-specific receptor; AMHR2 (Rey *et al.* 2003), only minimal evidence has been reported that this receptor is produced in TC (Baarends *et al.* 1995b, Ingraham *et al.* 2000) and, to date, none confirming that it is produced in the TC of monovulatory species. Therefore it is of particular interest that this present study, using a highly sensitive ICC approach (PLA, Section 2.8.), has produced the first direct evidence that AMHR2 is produced by the TC of a monovulatory species (sheep), and that it does mediate cellular responses to AMH by activation of a BMP-like, SMAD signalling pathway. The validity of these outcomes was reinforced by the use of two separate primary anti-AMHR2 antibodies and the clear detection of downstream SMAD pathway activation after AMH stimulation. The extreme sensitivity required to obtain positive detection suggests that amounts of AMHR2 in ovine TC are very low but, as this present study also found that AMHR2 mRNA appeared to be constitutively expressed, it seems likely that the low steady state levels of the mature receptor in these cells are under

tight, post-transcriptional regulation. However this aspect of AMH-responsiveness in TC requires further investigation.

7.3 A Role for Androgens in the Regulation of AMH

AMH regulation of androgen production in TC (Campbell et al., 2012) is a novel concept in monovulatory species with the prevailing view being that thecal androgens may have regulatory roles in AMH production in GC. Many researchers over the years have explored this notion (Lebbe & Woodruff 2013), however, clear evidence of a direct effect of thecal androgens on GC AMH remains to be categorical defined. Therefore a key aim of this present study was to address this shortfall. As it has been suggested that E2 could act as an intermediary of androgen activity in GC (Adashi & Hsueh 1982) it was necessary to overcome the problem of differentiating between androgens as a substrate for E2 and androgens as stimulators of E2 production. This was achieved by utilising a non-aromatisable androgen together with an aromatisable androgen (T4) which yielded markedly higher amounts of E2 and AMH than T4 alone. This appears to suggest that androgens do have a direct effect on AMH production in the GC of a monovulatory species (sheep). However future studies are needed to ascertain whether T4 or other aromatisable androgens are indeed stimulatory, or were the effects on E2 and AMH, seen in this study, restricted only to the non-aromatisable androgen utilised here (DHT). Likewise without further work it is difficult to speculate on a putative mechanism behind this effect. Unfortunately, time constraints did not allow for further investigation. However one approach that could be utilised is the use of reporter

genes attached to likely candidate pathway response elements.

The greatest difficulty in achieving a better understanding of how androgens regulate follicular development, including an AMH regulatory role, lies in the fact that only a limited number of androgen receptor-mediated signal transduction genes, of importance in ovarian physiology have been identified (Prizant *et al.* 2014). Research focusing on this aspect may not only provide a better understanding of the mechanism behind androgen signalling in the GC compartment but may also provide answers regarding the aetiology and pathophysiology behind many conditions such as low functional ovarian reserve, premature ovarian insufficiency and PCOS.

7.4 The Effect of VEGF and SOX8 on AMH

As, along with AMH, VEGF has also been noted to be present at high levels in the antral follicles of many PCOS cases and in OHSS individuals (Kamat *et al.* 1995, Pellicer *et al.* 1999) it was considered, in this present study, to be a potential focus of interest. It was surmised that it may be active in the regulation of AMH and a novel finding of the study was that in cultured ovine GCs, AMH production was inhibited by both of the predominant VEGF isoforms ($VEGF_{120}$ and $VEGF_{164}$) previously reported in follicular somatic cells (Marsters et al., 2014), with the effect being additive. Owing to time constraints the mechanisms of this VEGF effect were not further explored but it is clear that regulation of AMH is complex and multi-levelled, and that local factors such as the VEGF isoforms may be critical components.

Another family of factors which have been associated with AMH regulation in testes somatic cells (Kent *et al.* 1996, Vidal *et al.* 2001, Chaboissier *et al.* 2004, Jiang *et al.* 2013) are the SOX transcription factors, where it has been suggested SOX9 upregulates AMH. SOX9 does not occur in the somatic compartments of human follicles but another family member SOX8 is prevalent. In this present study a human secondary cell-line, KGN, was used to evaluate SOX8 as a regulator of AMH. However this member of the SOX family, at least, appeared to have no effect on GC production of AMH, although it was noted that it had an inhibitory effect on the steroidogenesis of E2. Thus it could be speculated that SOX8 is involved in the signalling pathway of genes connected to this process, such as aromatase and this now requires further study.

7.5 Summary and Conclusion

One of the important events during normal follicular development is the regression of AMH production which occurs steadily concomitant with the follicle's progression into the terminal stages of follicle growth leading to ovulation. However, the findings of this present study do suggest that the suppression of AMH production is likely to be essential for the elevation in E2 levels, noted to be characteristic of normal antral follicle development, dominant follicle selection and ovulation. This contention is further supported by a recent publication by (Jeppesen *et al.* 2013), which has also demonstrated a strong negative relationship between AMH expression and follicular fluid E2.

Taken together the findings of this present study demonstrate that regulation of AMH is complex and dynamic, involving both systemic and local mediators. From this work it seems clear that AMH regulation is key to normal follicle development. It revealed a possible mechanism for the characteristically high levels of AMH linked to normal or low levels of E2 in PCOS by showing that increased production and accumulation of thecal androgens could stimulate increases in AMH but, that in this scenario, E2 could be prevented from further elevation or may even diminish due to the inhibitory effect of high levels of AMH. Though many question marks remain regarding the terminal stages of follicle development, the present study has provided some useful insights into AMH regulation and function in the somatic compartments of growing follicles and highlighted a number of very interesting future research focusses which could lead to a better understanding of anovulatory infertility and diseases like PCOS.

In conclusion, this current study provides strong evidence that in a monovular species, AMH signals through BMP-like receptor complexes containing the AMHR2 and stimulates a BMP-like, SMAD 1/5/8, signal transduction pathway in both GC and TCs. From this it seems likely that AMH via this specific receptor-mediated pathway may have key roles during follicular development, particularly during the gonadotrophin-responsive to gonadotrophin dependent stages. This study also indicates a possible link, in sheep, between the direct effect of androgens on folliculogenesis and AMH regulation. Collectively, the outcomes of this present study suggest the possibility of a regulatory loop in which GC-produced AMH stimulates TC to produce androgens for utilisation as a GC substrate for aromatisation to E2. Elevated E2 may complete

the feedback loop by inhibiting AMH production. In this putative regulatory loop, systemic or local external stimuli, including VEGF variants and SOX8 may regulate on a fine-tuning bases as their effect appears to be indirect. However, though this present study has produced some novel and revealing outcomes, a deeper understanding of androgen and AMH roles in the regulation of follicle development is essential in order to elucidate fully their involvement in anovulatory infertility.

7.6 Future Work

Like any study, the future impact it has is what drives researchers to conduct and pursue that piece of work, either it may have important implications in terms of potential therapeutic values or it may open new avenues for future work to explore and further ideas to investigate. This study fulfilled both. A better understanding of the AMH and androgen pathways in both GC and TCs, and how they intertwine and are regulated in a monovular species, not only provides a better understanding of folliculogenesis in general but also brings us one step closer to untangling the dilemma of how perturbation of these signalling pathways may contribute to the pathophysiology of many common cause of infertility including PCOS, allowing for targeted therapeutic strategies to manipulate these signalling pathways and ultimately improve the prognosis in these women.

To our knowledge, this study provides novel evidence of the role and control of AMH and androgens during folliculogenesis in a monovular species. However, this has opened many doors

for future work and many unanswered questions that warrant further investigation.

Results from this project have shown that AMH binds the receptor complex containing AMHR2 and signals via the BMP signalling pathway in both GC and TCs. However, due to the myriad of functions of AMH in the ovary it cannot be excluded that AMH may have alternative pathways by which it may convey its signal. Unfortunately, following this study on the effect of AMH on the MAP kinase pathway it seems unlikely that AMH utilises this route and therefore investigating further alternative pathways seems valid. Further, following the interaction of AMH with the AMHR2, this results in the recruitment of one of the three identified AMH type 1 receptors which causes phosphorylation of the BMP-like signalling pathway effector proteins SMAD1, SMAD5 and SMAD8 (Rey *et al.* 2003). It is still unclear which or when a specific type 1 receptor or SMAD protein is used and therefore necessitates further investigation.

Moreover, using a non aromatisable androgen, results from this study point towards a possible signalling pathway for androgens in the ovary that does not necessitate its conversion to E2. The most likely candidate is that it interacts with the AR, based on findings from this study which investigated the effect of androgens on the AR signalling pathway by utilising a luciferase reporter gene system. However, this remains to be speculative and therefore demands further analysis by assessing the effect of androgens on the AR expression by measuring its mRNA levels following treatment or by direct visualisation using immunochemistry studies.

Results from this study also show that VEGF and SOX8, two novel local intra-ovarian stimuli, may have a role in fine tuning folliculogenesis. VEGF has been found to have a regulatory role on AMH; however, the mechanism behind this is still unknown. The possible theories are that it may affect the normal endocrine control in the follicle or alter other local factors and thereby indirectly effecting AMH. All these questions are yet to be answered and demand further work. Using KGN cells this research has shown that SOX8 may have a role in folliculogenesis. However, it failed to determine a clear effect on AMH in this cell type. Based on previous research which has shown it to influence AMH it is possible that the effect on AMH was not apparent due to the differentiated state of these cells. Future work on primary cells at a stage of known AMH function and significant AMH production may provide clarity to the effect of SOX8 on AMH.

Studies conducted on twins and families suggest that PCOS may have a heredity component implicated in the origin of the syndrome. However, genetic approaches to its aetiology have been held back due to the phenotypical heterogeneity of features in families and disunities in diagnostic criteria (Roldán *et al.* 2004). This diverse interaction between multiple genetic variations and environmental factors which may contribute to the complex trait of PCOS (Simoni *et al.* 2008), has made it difficult to define the altered metabolic pathways in patients with PCOS and publications that have focused on this disease have produced conflicting and inconclusive results. Correct PCOS diagnosis and identification may have therapeutic and preventive implication for women and families affected by this syndrome. In the future, therapeutic approaches may rely on knowing the genes associated with the aetiology of PCOS.

Therefore, future studies should aim to address the genetic aspect of PCOS, focusing on genes related to our field of interest that may contribute to the susceptibility of this disease.

7.7 Limitation of this study

One of the major limitations of this study was that these studies were conducted in an *in vitro* environment which may not truly reflect the conditions *in vivo*. While these studies have provided valuable information, it would be even more informative if these studies were paralleled with similar *in vivo* experiments which ultimately would consolidate the present findings. A few technical limitations were also encountered during the course of this project. A number of ingredients were extremely difficult to import due to restrictions including DHT. This meant that a number of studies were held back until approvals were obtained. While no funding was held back, the high cost of performing some of the experiments including both reagents and kits meant that rigorous planning and preparation needed to be achieved before execution to insure the optimal benefit. Finally, optimisation was a major challenge during this project as many of the techniques involved convoluted protocols necessitating surgical accuracy in order to obtain the desired results.

Chapter 8 References

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