Anti-Müllerian Hormone:

Function and Molecular Mechanism of Action in the Ovary

Layout and Cover Design: Mieke de Been

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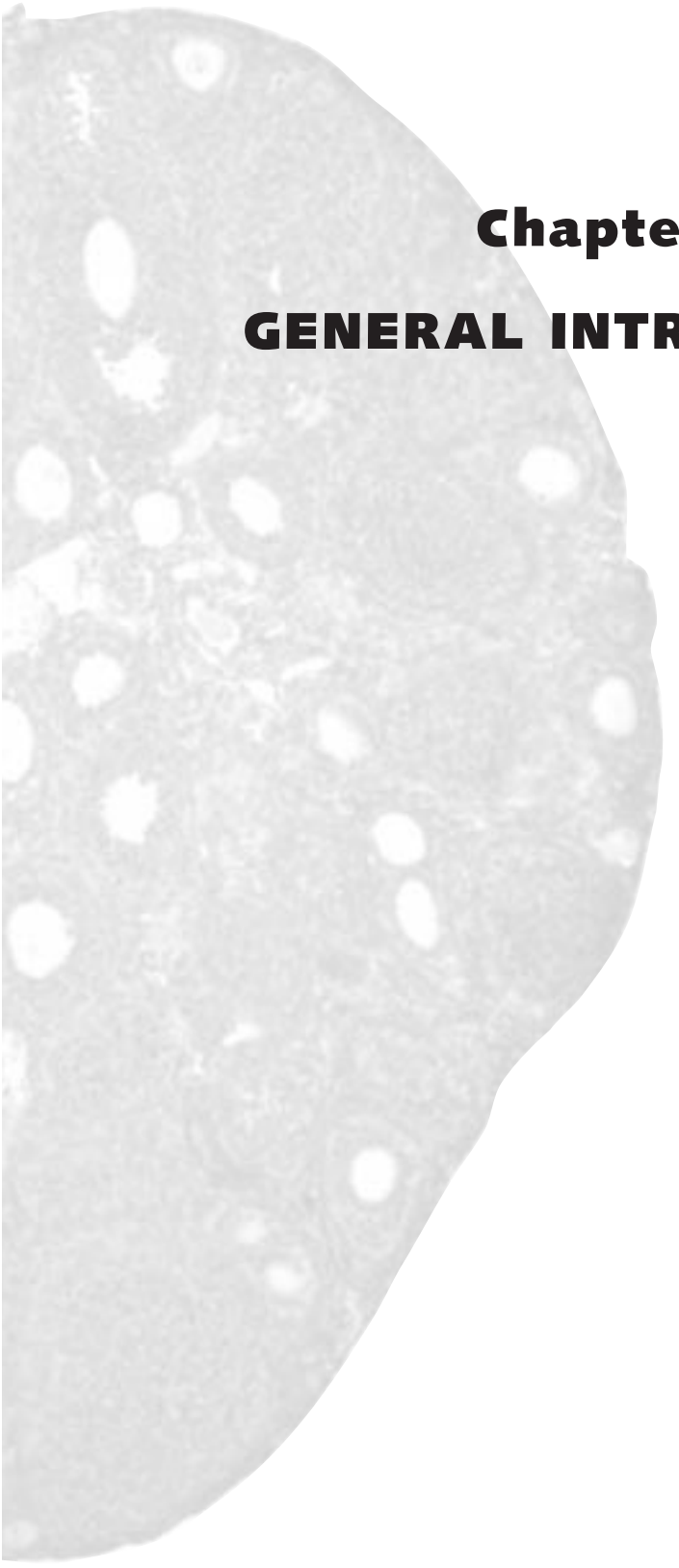
ABBREVIATIONS

3β-HSD	3β-hydroxysteroid dehydrogenase
ActRII	activin receptor type II
ALK	activin-receptor like kinase
AMH	anti-Müllerian hormone
AMHRII	anti-Müllerian hormone type II receptor
AMSH	SH3 domain of a signal-transducing adaptor molecule
BAMBI	BMP and activin membrane-bound inhibitor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BMPRII	bone morphogenetic protein type II receptor
Cryab	crystallin alpha B
CYP11A	cytochrome P450 cholesterol side-chain cleavage
CYP17	P450 17α-hydroxylase/C17-20 lyase
CYP19	aromatase
DES	diethylstilbestrol
DNA	deoxyribonucleic acid
e.g.	for example *(exempli gratia)*
EGF	epithelial growth factor
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GDF	growth and differentiation factor
G-protein	guanine-nucleotide binding protein
GnRH	gonadotropin-releasing hormone
hCG	human chorionic gonadotropin
Id2	inhibitors of DNA binding or differentiation
I-Smad	inhibitory Smad
IVF	*in vitro* fertilization
JNK	c-JunN-terminal kinase
KL	kit-ligand
LEF1	lymphoid enhancer factor 1
LH	luteinizing hormone
LHR	luteinizing hormone receptor
Lxn	latexin
MAPK	mitogen-activated protein kinase
MH	mad homology
MIS	Müllerian-inhibiting substance
MMP2	matrix metalloproteinase 2
MRKI	MIS receptor knock-in
NGF	nerve growth factor
PBS	phosphate-buffered saline
PCNA	proliferating cell nuclear antigen
Pcolce2	procollagen C-endopeptidase enhancer 2
PCOS	polycystic ovary syndrome
PCR	polymerase chain reaction
PGC	primordial germ cell
PMDS	persistent Müllerian duct syndrome
PP2A	protein phosphatase 2A
ROC	receiver operating characteristic
ROC_{AUC}	calculated areas under the receiver operating characteristic
Rpl24	ribosomal protein L24
R-Smad	receptor-specific Smad
mRNA	messenger ribonucleic acid
Sapks	stress-activated protein kinases
SARA	Smad anchor for receptor activation
SCF	stem cell factor
SD	standard deviation
SEM	standard error of the mean
Smurf	Smad ubiqitination regulatory factor
S/T	serine/threonine
StAR	steroidogenic acute regulator protein
TβRII	transforming growth factor β type II receptor
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TRAP	TβRI-associated protein 1
Trfr	transferrin receptor
TRIP1	TGFβ-receptor interacting protein 1
Tnfaip3	tumor necrosis factor alpha-induced protein 3
WT	wild-type
ZP	zona pellucida



Chapter 1

GENERAL INTRODUCTION

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1.1 INTRODUCTION

Menopause is a major milestone in the aging process in women and starts after the last menstrual cycle. The cessation of ovarian function causes the almost complete absence of female sex steroid hormone secretion by the ovaries in postmenopausal life. Consequently, before and after the menopausal transition very different physiological hormonal regimens prevail, which have a major impact on healthy aging of women and their quality of life. After menopause, due to the loss of exposure to protective female sex steroid hormones, women have an increased risk for different health problems, such as osteoporosis (Gibaldi, 1997), and changes in cognitive function (*e.g.* Alzheimer's disease) (Kesslak, 2002). Furthermore, it has been suggested that the risk for cardiovascular diseases increases after the menopause (Mijatovic, *et al.*, 1999), although these findings are under debate (Tunstall-Pedoe, 1998). Since the age at menopause affects the length of the postmenopausal period, it is of utmost importance to understand the process leading to menopause.

Menopause is caused by the exhaustion of the follicle stock in the ovaries. The limited supply of follicles is formed during fetal life, from the 1000-2000 germ cells which migrate from the allantois to the gonadal ridge to populate the ovary. The germ cells divide 10-15 times until the maximal number of oocytes, about 7 million, is reached at 20 weeks of gestation. From that moment on the number of oocytes decreases in a logarithmic fashion and at birth about 1 million oocytes remain present. A further decrease of the number of oocytes occurs during childhood, resulting in 300,000-500,000 oocytes at menarche (te Velde, *et al.*, 1998; Macklon and Fauser, 1999). Loss of primordial follicles, which form the limited resting follicle stock, continues with approximately 1000 follicles/month by primordial follicles entering the growth phase or undergoing atresia. This rate of loss may even increase after the age of 35 years (Gougeon, *et al.*, 1994; Gougeon, 1996; Macklon and Fauser, 1999). Only about 400 oocytes are destined to full maturation and ovulation during female reproductive life in the human (Macklon and Fauser, 1999).

At menopause, the size of the follicle pool is approximately 100-1000 and a permanent cessation of menstrual cycles occurs because of this exhaustion of the follicle pool. This number of follicles is unable to maintain the hormonal concentrations necessary for normal menstrual cycles (te Velde and van Leusden, 1994). The median age at menopause in the Western world is 51 years, with a variation of 40-60 years (te Velde, *et al.*, 1998; Macklon and Fauser, 1999). Menopause is preceded by a premenopausal transition period of 15-20 years. During this transition period fertility decreases, culminating through sub-fertility into complete infertility 10 years before menopause. There are indications that these intervals are fixed, implying that early infertility may indicate early menopause. In general, this means that optimal fertility in women occurs up to 30 years of age and decreases thereafter (te Velde, *et al.*, 1998).

Since the size of the primordial follicle pool is determined during embryogenesis and is not replenished at any time thereafter, the size of this pool and its rate of depletion determine the end of fertility and the timing of onset of menopause. Thus, in view of early infertility and the health problems of women associated with menopause, it is of key importance to understand the mechanisms that determine the early growth and differentiation of primordial follicles. In the remainder of this Chapter, primordial follicle recruitment and effects of members of the transforming growth factor β (TGFβ) superfamily of growth and differentiation factors, which play an important role in this early follicular development, are described in more detail.

1.2 FOLLICULOGENESIS: A MORPHOLOGICAL OVERVIEW

In this section, the histological changes during follicle development from the primordial to the pre-ovulatory stage are described. These histological characteristics are important as they are used to describe effects of hormones and growth factors on different types of follicles. The histological changes during folliculogenesis will be described for both mouse and human, since there are important differences between these species in the histological characteristics of the follicles and the developmental stage they have reached. A summary of these characteristics combined with the nomenclature is given in Table 1.1 (mouse) and Table 1.2 (human). In addition, Figure 1.1 shows an overview of follicle development and effects on follicle types of LH, FSH and TGFβ superfamily members.

Table 1.1: Summary of nomenclature and characteristics of follicular development in the mouse. (Pedersen and Peters, 1968).

Type:	Diameter oocyte (µm):	Number of granulosa cells:	Characteristics of the follicle:	Other nomenclature:		
1	20	0		small	naked oocyte	
2	20	few	flat granulosa cells	small	primordial	primordial
3a	≥20	≤20	cuboidal and flat granulosa cells	small	intermediary	
3b	20-70	21-60	one layer of cuboidal granulosa cells	medium	(small) pre-antral	primary
4	20-70	61-100	two layers of granulosa cells	medium	pre-antral	secondary
5a	≥70	101-200	three layers of granulosa cells	medium	pre-antral	secondary
5b	70	201-400	first theca cells are recruited	large	pre-antral	secondary
6	70	401-600	scattered areas of fluid	large	(small) antral	tertiary
7	70	>600	one fluid cavity, cumulus oophorus, but no stalk	large	antral	tertiary
8	70	>600	cumulus stalk	large	pre-ovulatory	tertiary

Table 1.2: Summary of nomenclature and characteristics of follicular development in the human. (Gougeon, 1996).

Class:	Diameter follicle: (µm):	Number of granulosa cells:	Characteristics of the follicle:	Other nomenclature:		
	<80	<15	flat granulosa cells	resting follicle	primordial	
	<80	<15	cuboidal and flat granulosa cells	resting follicle	intermediary	
	<80	<15	one layer of cuboidal granulosa cells	resting follicle		small primary
	<80	15	1-2 layers of granulosa cells, oocyte diameter >19 µm	growing follicle		secondary
	80-100	15-600	own blood supply	growing follicle		secondary
1	0.1	600	first theca interna cells, 3-6 layers of granulosa cells	growing follicle	pre-antral	tertiary
2	0.2	$3\text{-}5\times10^3$		growing follicle	early antral	tertiary
3	0.4	15×10^3		growing follicle	antral	tertiary
4	0.9	75×10^3		growing follicle	antral	tertiary
5	2	375×10^3	recruitable	growing follicle	antral	tertiary
6	5	1.87×10^6	selected	growing follicle	antral	tertiary
7	10	9.4×10^6		growing follicle	early preovulatory	tertiary
8	16-20	47×10^6		growing follicle	preovulatory	tertiary

At birth, the ovaries of mice only contain naked oocytes (type 1 follicles), which are enclosed by pregranulosa cells into primordial follicle structures one day later (Peters, 1987). Oocytes which fail to recruit (pre)granulosa cells and to form a follicle will degenerate (Byskov, 1986). As soon as the primordial follicles arise, the basement membrane, which consists of collagen, laminin and fibronectin, is formed around the granulosa cells (Hirshfield, 1991). The primordial follicle pool forms the resting stock of follicles in the ovary. Start of growth of primordial follicles occurs at different times during the life of the animal.

The histological feature, that characterizes the first growth of the primordial follicle (type 2 follicle) in rodents is an increase in the size of the oocyte. Thereafter, granulosa cells proliferate and transform from squamous to cuboidal cells. From this moment on, follicles are called type 3a or intermediary follicles. When the oocyte is surrounded by one complete layer of cuboidal granulosa cells the follicle is referred to as type 3b. As soon as the oocyte starts to grow, the zona pellucida is formed. This extracellular glycoprotein matrix is located between the oocyte and granulosa cells and consists of three zona pellucida (ZP) proteins (ZP1, ZP2, ZP3), all produced by the oocyte. Granulosa cells probably do not contribute to the formation of this matrix (Zamboni and Upadhyay, 1983). During the development from type 3b to type 5a follicles, granulosa cells proliferate extensively. In mice, oocyte growth is completed in type 5a follicles when the follicle contains three layers of granulosa cells. These follicles contain the first theca cells recruited from the stromal cells (Pedersen and Peters, 1968). Theca and granulosa cells are separated by the basement membrane and both cell types proliferate during further development, although theca cells not as extensively as granulosa cells (Peters, 1969). Scattered areas of fluid are found in type 6 follicles, which will form the antrum in type 7 follicles (Pedersen and Peters, 1968). As soon as fluid spaces are found in the follicle, it is called an antral follicle. The follicle diameter further increases due to granulosa- and theca cell proliferation. At this point theca cells also undergo a morphological and functional change. Theca cells lying next to the basement membrane (theca interna) acquire morphological hallmarks of steroidogenesis, like smooth endoplasmatic reticulum and mitochondria with tubular cristae, while theca cells that are located more peripheral (theca externa) stay small and compact (Stevens and Lowe, 1992). The pre-ovulatory follicle (type 8) is characterized by an oocyte that is surrounded by specialized granulosa cells called cumulus cells (Pedersen and Peters, 1968). The oocyte and cumulus cells are located in the antrum and connected to the follicle wall through a stalk. Granulosa cells of the pre-ovulatory follicle stop dividing in response to the luteinizing hormone (LH) surge and initiate a program of final differentiation (Hirshfield, 1991). In response to the LH peak the oocyte with cumulus cells will be ovulated. The remaining cells form the corpus luteum which plays an important role as a steroid hormone producing structure.

In women, folliculogenesis occurs along a similar pathway. However, some differences in timing of histological changes between woman and mouse have been described. Primordial follicle formation occurs already during the fetal period in women (Gougeon, 1996), while in mice this occurs after birth. In addition, histological changes of the primordial follicles are described before the follicles enter the growing phase. The flat granulosa cells of primordial follicles become cuboidal (intermediary follicle) and proliferate until one layer of cuboidal granulosa cells is formed. However, this class 3a follicle is still considered a resting follicle (Gougeon, 1996). From the moment on which 1-2 layers of granulosa cells surround the oocyte, the follicle is called “growing”.

A small antrum is formed in class 2 follicles. In mice, antrum formation coincides with follicle stimulating hormone (FSH)-dependency and selection of follicles for pre-ovulatory growth. This is different from human follicular development where antrum formation occurs in class 2 follicles, while class 5 follicles are selected for pre-ovulatory growth by FSH. In women the oocytes of class 1 to class 8 follicles continue to grow while in mice the oocytes stop growing in type 5a follicles (Gougeon, 1996).

1.3 FOLLICULOGENESIS: REGULATION BY LH AND FSH

Folliculogenesis describes the development of primordial follicles to pre-ovulatory follicles in the ovary. Two important regulatory points are found during folliculogenesis: recruitment and selection (Figure 1.1). At recruitment, primordial follicles enter the growth phase from the dormant follicle pool, while at selection follicles are selected for growth until the pre-ovulatory stage. Folliculogenesis is a tightly regulated process in which factors produced by the oocyte, granulosa- and theca cells have important roles to fulfill. In addition, the gonadotropins LH and FSH produced by the pituitary gland, play a dominant role during follicular development.

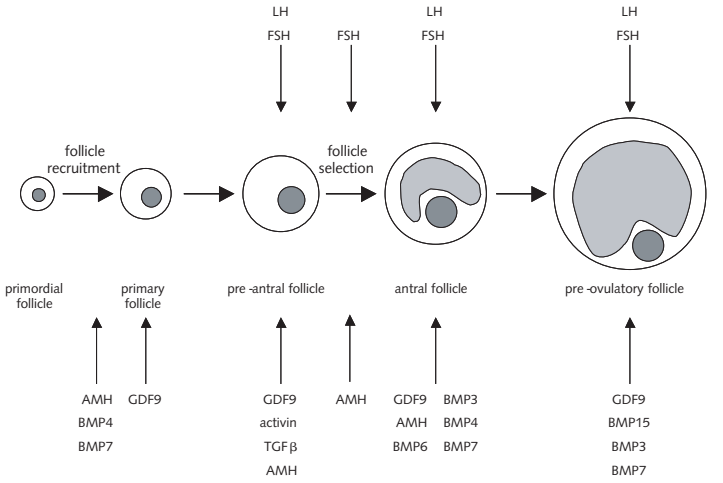


Figure 1.1: Overview of follicle development and effects of LH, FSH and TGFβ superfamily members in mammals. TGFβ superfamily members, LH and FSH influence the growth and/or differentiation of follicles from the primordial to the pre-ovulatory stage.

No direct effects of gonadotropins on primordial follicle recruitment have been found, although indirect effects have been described. Granulosa cell proliferation and growth of pre-antral follicles is stimulated by FSH. After selection of follicles on basis of their FSH-sensitivity, follicles become dependent of FSH for survival, growth, antrum formation and oocyte maturation. LH is essential for successful ovulation of the oocyte. The effects of FSH and LH on follicle recruitment are partly mediated through estrogens. LH stimulates synthesis of testosterone in the theca cells. Testosterone is converted by aromatase, which is expressed in the granulosa cells, to estrogens. This conversion is

stimulated by FSH through upregulation of the aromatase enzyme (Xiao, *et al.*, 1990, 1992; Burns, *et al.*, 2001). Thus, FSH and LH play an important role in folliculogenesis and their effects will be described in more detail in the following sections.

1.3.1 Primordial follicle recruitment and early follicular development

Primordial follicles enter the growing pool at an unknown rate and in an ordered sequence. Entering the growing pool commits the follicle to grow to the pre-ovulatory stage or to atresia. Some primordial follicles start growing immediately after they are formed, but most of them stay dormant for months (mice) or years (human). FSH is not necessary for primordial follicle recruitment since FSHβ *null* mice still contain growing follicles (Kumar, *et al.*, 1997) and *in vitro* culture of ovaries of several species results in recruitment of primordial follicles into the growing pool without addition of gonadotropins (Eppig and O'Brien, 1996; Braw-Tal and Yossefi, 1997; Mayerhofer, *et al.*, 1997). In addition, *in vitro* experiments showed that FSH did not stimulate primordial follicle recruitment (Braw-Tal and Yossefi, 1997; Mayerhofer, *et al.*, 1997; Fortune, *et al.*, 1998; Vitt, *et al.*, 2000b). These results are supported by the observation that FSH and LH receptors are not expressed in primordial follicles (Rannikki, *et al.*, 1995; Teerds and Dorrington, 1995; O'Shaughnessy, *et al.*, 1996, 1997; Oktay, *et al.*, 1997).

Although no direct effect of FSH on primordial follicle recruitment is found, indirect stimulatory effects of FSH and LH on primordial recruitment have been described. Low FSH and LH levels after hypophysectomy or gonadotropin-releasing hormone (GnRH)-agonist treatment, result in a larger pool of primordial and less growing follicles (Jones and Krohn, 1961; Ataya, *et al.*, 1989, 1995; Wang and Greenwald, 1993). In addition, higher FSH and LH serum levels stimulate the recruitment of primordial follicles into the growing pool (Richardson, *et al.*, 1987; Faddy, *et al.*, 1992; Meredith, *et al.*, 1992; Marcus, *et al.*, 1993; Gougeon, *et al.*, 1994; Flaws, *et al.*, 1997). For example, in humans and rats the depletion of the resting follicle pool accelerates when FSH and LH levels start to rise as a result of aging (Richardson, *et al.*, 1987; Faddy, *et al.*, 1992; Marcus, *et al.*, 1993; Gougeon, *et al.*, 1994). These effects of LH and FSH on primordial follicles are indirect, *e.g.* through pre-antral or antral follicles which do express FSH and LH receptors. These follicles in turn probably produce one or more factors that have an effect on the primordial follicle pool.

FSH and LH stimulate the growth of small follicles, although these hormones are not essential. Ovaries of mice and rats exposed to low FSH and/or LH serum levels contain all types of follicles until the early antral stage (Lane and Greep, 1935; Paesi, 1949; Halpin and Charlton, 1988; Wang and Greenwald, 1993), like for example the FSHβ *null* mice (Kumar, *et al.*, 1997). However, a decreased serum FSH level leads to a decreased number of pre-antral follicles (van Cappellen, *et al.*, 1989; McGee, *et al.*, 1997). Furthermore, high FSH serum levels stimulate pre-antral follicle development and the number of pre-antral follicles present in the ovary (Pedersen, 1969; Dullaart, *et al.*, 1975; Uilenbroek, *et al.*, 1976; Hage, *et al.*, 1978; McGee, *et al.*, 1997; Vitt, *et al.*, 2000b). In addition, also LH stimulates growth of pre-antral follicles cultured *in vitro* (Cortvrindt, *et al.*, 1998; Wu, *et al.*, 2000).

FSH stimulates the production of estrogen in granulosa cells, which is important for (late) folliculogenesis. Both granulosa and theca cells are necessary for production of estrogens. Theca cells produce androgens and this production is stimulated by LH and inhibin (Hsueh, *et al.*, 1987; Hillier, 1991; Wrathall and Knight, 1995). FSH induces the expression of aromatase (Xiao, *et al.*, 1990, 1992; Burns, *et al.*, 2001), LH receptor (LHR) (Richards, *et al.*, 1976) and inhibin production in granulosa cells

(Richards, *et al.*, 1976; Lee, *et al.*, 1982; LaPolt, *et al.*, 1989; Xiao, *et al.*, 1990, 1992; Campbell, *et al.*, 1996; Harlow, *et al.*, 2002). In this way, FSH stimulates the production of estrogens . Estrogen and FSH together stimulate expression of FSH receptor (FSHR) in granulosa cells.

In addition, estrogens, like FSH, stimulate granulosa cell proliferation by increasing expression of cyclin D_2 expression, which is an important regulator of cell cycle progression (Robker and Richards, 1998).

Estrogens have no important role in primordial follicle recruitment or pre-antral follicle development since ovaries of the aromatase *null* mice contain pre-antral follicles, just like the mice lacking the estrogen receptor α and β (ERα, ERβ) (Fisher, *et al.*, 1998; Couse, *et al.*, 1999). However, effects of estrogen on early folliculogenesis have been described. Estrogens stimulate the expression of epithelial growth factor (EGF) receptor in pre-antral granulosa cells (Garnett, *et al.*, 2002). EGF and transforming growth factor α (TGFα) are signaling through the EGF receptor and both growth factors are involved in early folliculogenesis. Fertility of female TGFα overexpressor mice is severely decreased after two pregnancies, which suggests that TGFα is involved in regulation of follicle recruitment (Ma, *et al.*, 1994). Furthermore, a seven-fold increase in the number of antral follicles, caused by higher FSH levels or a decrease of apoptosis, is found in ovaries of these mice (Tilly, *et al.*, 1992; Ma, *et al.*, 1994). EGF induces, after binding to the EGF receptor, granulosa cell proliferation and pre-antral follicle growth (Roy and Greenwald, 1991a, 1991b; Roy, 1993; Gutierrez, *et al.*, 2000; Saha, *et al.*, 2000). These results show a role of estrogen in early follicular development. Interestingly, also androgens stimulate pre-antral follicle development of *in vitro* cultured follicles since non-aromatizible androgens stimulate follicle diameter and antrum development (Murray, *et al.*, 1998).

In summary, FSH and LH have no direct effect on primordial follicle recruitment, but stimulate pre-antral follicle growth and development. Production of estrogen is stimulated by FSH, however estrogen does not seem to play a very important role in early folliculogenesis.

1.3.2 Selection and late follicular development

All large pre-antral and small antral follicles will undergo atresia, except for a proportion of follicles that will be rescued by FSH and selected to grow until the pre-ovulatory stage. This process is referred to as selection. Ovaries of FSHβ and FSHR *null* mice contain no antral follicles, indicating the essential role of FSH for follicular growth after the pre-antral stage (Kumar, *et al.*, 1997; Dierich, *et al.*, 1998; Burns, *et al.*, 2001). In addition, higher FSH levels increase the number of follicles selected for pre-ovulatory growth (Lunenfeld and Insler, 1993). Ovaries of LHR *null* mice contain follicles until the antral stage but no pre-ovulatory follicles or corpora lutea, indicating that LH becomes essential in pre-ovulatory follicle development (Lei, *et al.*, 2001; Zhang, *et al.*, 2001). Indeed, LH as well as FSH inhibits atresia of large pre-antral and antral follicles cultured *in vitro* (Hartshorne, *et al.*, 1994; Gaytan, *et al.*, 1996; Cortvrindt, *et al.*, 1998; Mao, *et al.*, 2002).

FSH stimulates granulosa cell proliferation as shown by the larger diameter of *in vitro* cultured follicles when FSH is added to the culture medium (Hartshorne, *et al.*, 1994; Itoh, *et al.*, 2002; Mao, *et al.*, 2002). This effect is at least partly caused by the regulation of cyclin D_2 expression. Cyclin D_2 expressed in granulosa cells is an important positive regulator of cell cycle progression (Xiong, *et al.*, 1992; Robker and Richards, 1998). Ovaries of cyclin D_2 deficient mice contain no follicles beyond the pre-antral stage, illustrating the important role of cyclin D_2 in late follicular development (Sicinski, *et al.*, 1996). *In vivo* experiments showed that FSH stimulates expression of cyclin D_2 and inhibits expression of

$p27^{kip1}$, which in turn inhibits cell cycle progression by binding to the cyclin D_2-cdk4/6 complex. The LH peak terminates granulosa cell proliferation by stimulating expression of $p27^{kip1}$ and $p21^{cip1}$, both inhibitors of cell proliferation (Robker and Richards, 1998).

Not only granulosa cell proliferation, but also antrum formation and oocyte maturation are under control of FSH. Ovaries of FSHβ *null* mice contain no antral follicles (Kumar, *et al.*, 1997) and antrum formation of *in vitro* cultured pre-antral follicles is stimulated by FSH (Hartshorne, *et al.*, 1994; Itoh, *et al.*, 2002; Mao, *et al.*, 2002; Mitchell, *et al.*, 2002). Furthermore, LH enhances FSH-induced antrum formation *in vitro* (Cortvrindt, *et al.*, 1998). During late follicular development, oocytes complete the first meiotic division up to metaphase II of meiotic stage. This process is stimulated by FSH or LH and a synergistic effect is found when FSH and LH both are present (Cortvrindt, *et al.*, 1998).

The stimulation of estrogen synthesis by FSH and LH, like in the pre-antral follicles, is also important for late follicle development. Ovaries of aromatase-deficient mice contain almost no antral follicles, which demonstrates the importance of estrogen in late follicular development (Fisher, *et al.*, 1998). A similar phenotype is found in ovaries of mice deficient for both ERα and ERβ (Couse, *et al.*, 1999).

The effects of LH on late follicular development can be partly explained through its effect on estrogen biosynthesis. However, also estrogen-independent effects of LH are described. In rats, treated with a GnRH-antagonist and in which also oestradiol synthesis was inhibited by an aromatase inhibitor, FSH and LH treatment resulted in a larger increase in the number of antral follicles compared to animals which did not receive LH (Uilenbroek, *et al.*, 1997). Thus, during the last phase of folliculogenesis, LH is involved in successful maturation of the follicles. Furthermore, LH is essential for ovulation of the oocyte (Filicori, e*t al.*, 2002).

In summary, follicular development after selection is dependent on FSH. FSH rescues follicles from atresia, stimulates antrum formation, granulosa cell proliferation, LHR expression, oocyte maturation and estrogen production. LH inhibits granulosa cell proliferation but enhances other effects of FSH. In addition, LH is necessary for ovulation of the oocyte.

The systemic factors LH and FSH are very important for follicle development from small pre-antral to pre-ovulatory follicles, but they are not the only factors involved. As described above, follicular development until the antral stage can occur without these gonadotropins and this implies a role for local factors. Indeed, several growth factors are produced by the oocyte, granulosa- and theca cells. These growth factors can play an important role, often by modulating the effects of FSH and LH, and folliculogenesis can be disregulated when such a growth factor is absent. The next section describes the effects of members of the TGFβ superfamily during folliculogenesis.

1.4 FOLLICULOGENESIS: ROLE OF MEMBERS OF THE TGFβ SUPERFAMILY

The transforming growth and differentiation factor β (TGFβ) family is the largest family of growth and differentiation factors. This family can be divided in several subfamilies, such as the TGFβs (TGFβ1-5), activins (inhibin α, βA-C, E) and bone morphogenetic proteins (*e.g.* BMP1-15, GDF1-9) (reviewed by Massagué, 1998), which have a broad range of functions in cell cycle, differentiation, cell adhesion and morphology.

The next sections describe the roles of TGFβ superfamily members, expressed by oocytes, theca- and granulosa cells during folliculogenesis. Since the focus of experiments described in this thesis (Chapters 3-7) is on primordial and pre-antral follicles, the effects of TGFβ family members on early folliculogenesis will be described in more detail compared to the effects on antral and pre-ovulatory follicles. Furthermore, the signal transduction characteristics of the TGFβ superfamily members are described in Chapter 2. Here, only the localization of the expression of receptors in the ovary is summarized (Table 1.3).

Tabel 1.3: Summary of TGFβ superfamily ligand-receptor combinations and the localization of expression in the ovary. For more detailed information about signaling of TGFβ superfamily members, see Chapter 2.

Ligand:	Receptor:	Localization of expression of the receptor:	References:
GDF9	BMPRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Shimasaki *et al.*, 1999; Vitt *et al.*, 2002; Erickson and Shimasaki, 2003
BMP15	BMPRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Shimasaki *et al.*, 1999; Moore *et al.*, 2002; Erickson and Shimasaki, 2003
BMP6	BMPRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Ebisawa *et al.*, 1999; Shimasaki *et al.*, 1999; Erickson and Shimasaki, 2003
	ActRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Cameron *et al.*, 1994; van den Hurk and Van de Pavert, 2001; Drummond *et al.*, 2002; Pangas *et al.*, 2002
BMP3	BMPRII? ActRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Shimasaki *et al.*, 1999; Erickson and Shimasaki, 2003
BMP4	BMPRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Koenig *et al.*, 1994; ten Dijke *et al.*, 1994; Yamaji *et al.*, 1994; Nohno *et al.*, 1995; Shimasaki *et al.*, 1999; Erickson and Shimasaki, 2003
BMP7	BMPRII	• oocyte of all follicle types • granulosa cells all of growing follicles	ten Dijke *et al.*, 1994; Rosenzweig *et al.*, 1995; Shimasaki *et al.*, 1999; Erickson and Shimasaki, 2003
BMP2	BMPRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Koenig *et al.*, 1994; ten Dijke *et al.*, 1994; Yamaji *et al.*, 1994; Nohno *et al.*, 1995; Shimasaki *et al.*, 1999; Erickson and Shimasaki, 2003
activin	ActRII	• oocyte of all follicle types • granulosa cells all of growing follicles	Cameron *et al.*, 1994; van den Hurk and Van de Pavert, 2001; Drummond *et al.*, 2002; Pangas *et al.*, 2002
	ActRIIB	• oocyte of all follicle types • granulosa cells all of growing follicles	Cameron *et al.*, 1994; Drummond *et al.*, 2002; Pangas *et al.*, 2002
TGFβ	TβRII	• oocyte of primordial and primary follicles • granulosa cells • theca cells	Roy and Kole, 1998; Qu *et al.*, 2000
AMH	AMHRII	• granulosa cells of pre-antral, small antral and probably also primordial follicles. • theca cells of pre-antral and small antral follicles	Baarends *et al.*, 1994; di Clemente *et al.*, 1994; Baarends *et al.*, 1995; Ingraham *et al.*, 2000

1.4.1 TGFβ superfamily members expressed by the oocyte

Folliculogenesis is a tightly regulated process in which the interactions between oocyte, granulosa- and theca cells play an important role. At least three members of the TGFβ superfamily are expressed in the oocyte: growth and differentiation factor-9 (GDF9), bone morphogenetic protein-15 (BMP15) and bone morphogenetic protein-6 (BMP6). A summary of the effect of these TGFβ family members expressed by the oocyte is given in Table 1.4 and Figure1.1.

Table 1.4: Overview of the effects on follicle development of TGFβ family members expressed by the oocyte.

Family member:	Site of action:	Effects:	References:
GDF9	granulosa cells oocyte?	• obligatory for growth of primary follicles	Dong *et al.*, 1996
		• stimulates granulosa cell proliferation, synergistic effect with FSH	Elvin *et al.*, 1999b; Hayashi *et al.*, 1999; Vitt *et al.*, 2000b; Nilsson and Skinner, 2002
		• downregulates KL and inhibin α-subunit expression in small primary follicles but upregulates KL and inhibin α-subunit expression in granulosa cells of larger follicles	Dong *et al.*, 1996; Carabatsos *et al.*, 1998; Elvin *et al.*, 1999b; Hayashi *et al.*, 1999; Nilsson and Skinner, 2002; Roh *et al.*, 2003
		• survival factor for *in vitro* culture of follicles	Hreinsson *et al.*., 2002
		• (indirectly) involved in theca cell recruitment	Elvin *et al.*, 1999b; Vitt *et al.*, 2000b
		• inhibits FSH-induced differentiation in antral follicles, by inhibiting progesterone and estrogen synthesis, LHR expression and stimulating inhibin A and B production	Elvin *et al.*, 1999a; Vitt *et al.*, 2000a; Roh *et al.*, 2003
		• regulates expression of several key granulosa cell enzymes necessary for normal ovulation	Elvin *et al.*, 1999a, 1999b
BMP15	granulosa cells oocyte?	• stimulates granulosa cell mitosis	Otsuka *et al.*, 2000
		• stimulates KL mRNA expression	Otsuka and Shimasaki, 2002
		• inhibits FSH induced differentiation by inhibiting FSHR mRNA expression and FSH-induced progesterone production	Otsuka *et al.*, 2000, 2001b
		• involved in ovulation	Yan *et al.*, 2001
BMP6	granulosa cells oocyte?	• inhibits FSH-induced differentiation by inhibiting FSH-induced progesterone production and FSHR mRNA expression	Otsuka *et al.*, 2001a

Growth and Differentiation Factor-9

Growth and differentiation factor-9 (GDF9) protein and messenger RNA (mRNA) is expressed in oocytes from early primary follicles onward with most extensive staining in primary and pre-antral follicles in rat (Hayashi, *et al.*, 1999; Jaatinen, *et al.*, 1999), human (Aaltonen, *et al.*, 1999) and mouse (Elvin, *et al.*, 1999a). The type II receptor for GDF9, BMP receptor II (BMPRII), is expressed in granulosa cells and oocytes (Vitt, *et al.*, 2002; Erickson and Shimasaki, 2003). This pattern of receptor expression suggests that GDF9 acts on granulosa cells and oocytes. It is unclear which type I receptors are involved in GDF9-signaling. Binding to the BMPRII suggests involvement of ALK2, ALK3 or ALK6 during signal transduction (see Chapter 2). However, signaling through these type I receptors can be questioned, since Smad2 is activated in GDF9-signaling (Roh, *et al.*, 2003).

Studying the ovaries of GDF9 *null* mice revealed that GDF9 is not necessary for primordial follicle recruitment. These mice are infertile due to a block in folliculogenesis from stage 3b onwards after normal recruitment of primordial follicles (Dong, *et al.*, 1996). In addition, *in vitro* culture of 4-day-old neonatal rat ovaries in the presence of GDF9 had no effect on primordial follicle recruitment (Nilsson and Skinner, 2002). Interestingly, the number of primordial follicles decreased by 29% in ovaries of rats treated with GDF9 (Vitt, *et al.*, 2000b). This effect on primordial follicle recruitment can be explained by the enhancement of primary follicle progression which could lead to an indirect stimulation of primordial follicle recruitment (Hirshfield, 1994).

Primary follicle development is dependent on GDF9, since development of primary follicles is blocked in GDF9 *null* mice ovaries (Dong, *et al.*, 1996). Furthermore, GDF9 stimulates primary follicle development as indicated by an increase in mature primary and a decrease in early primary follicle numbers in ovaries cultured in the presence of GDF9 (Nilsson and Skinner, 2002). In addition, treatment of immature rats with GDF9 caused an increase in the number of primary and secondary follicles by 30% and 60% respectively (Vitt, *et al.*, 2000b). Furthermore, GDF9 stimulates proliferation of granulosa cells since a low expression of the proliferation markers proliferating cell nuclear antigen (PCNA) and Ki-67 in granulosa cells of GDF9 *null* mice was found (Elvin, *et al.,* 1999b). In addition, culturing pre-antral rat follicles in the presence of GDF9 increases their diameter, caused by the proliferation of granulosa cells, of these follicles to the same extent as FSH treatment. GDF9 and FSH together have a synergistic effect on granulosa cell proliferation (Hayashi, *et al.*, 1999).

How GDF9 exerts its effect on proliferation of the granulosa cells is still unclear because cyclin D_2, which is the most important cyclin for entering S-phase, is normally expressed in GDF9 *null* mice. Also $p27^{kip1}$ and $p21^{cip1}$, which bind to cyclin D_2-cdk4/6 complexes and prevent their activation and thus cell cycle progression, are expressed normally (Elvin, *et al.*, 1999b). It is likely that GDF9 influences other cell cycle regulators, which are necessary for normal cell division.

Interestingly, GDF9 is not only involved in growth of follicles but also in their survival and death. *In vitro* culture studies of human primordial follicles revealed that GDF9 acts as a survival factor since less atretic follicles were detected in ovarian tissue cultured with added GDF9 (Hreinsson, *et al.*, 2002). Furthermore, GDF9 is also necessary for apoptosis of granulosa cells as almost no apoptotic cells are found in GDF9 *null* mice. Follicular structures do not disappear after the oocyte has degenerated, but the granulosa cells form a steroidogenic cluster, which is positive for antral granulosa cell markers like the inhibin α-subunit and aromatase and for periovulatory and luteal granulosa cell markers like LHR and P450 side chain cleavage (P450scc). These results suggest that the expression of LHR and P450scc, which are normally only expressed in corpora lutea (LHR and P450scc) or granulosa cells of pre-ovulatory follicles (LHR), are probably inhibited by the oocyte through GDF9 (Elvin, *et al.*, 1999b). The different effects of GDF9 on apoptosis, survival and proliferation could be explained by differences in expression of transcription factors, co-activators or co-repressors, which result in differences in gene transcription by GDF9.

Maturation of oocytes of type 3b follicles in GDF9 *null* mice is comparable to that of oocytes found in antral follicles of heterozygote mice. Nevertheless, *in vitro* maturation of homozygous GDF9 knockout oocytes revealed that the majority of oocytes fail to complete meiotic maturation. This discrepancy can be explained by a defect in communication between granulosa cells and oocyte. Indeed, modified interactions between these cells were found using electron and fluorescence microscopy. Although the follicles of GDF9 *null* mice stop growing at the early primary stage, the oocytes continue to grow as can be seen by the changes in germinal vesicle chromatin patterns (Carabatsos, *et al.*, 1998).

GDF9 regulates mRNA expression of kit-ligand (KL) and inhibin α-subunit. GDF9 *null* mice overexpress KL and this probably results in faster and larger growing oocytes compared to their wild-type littermates (Dong, *et al.*, 1996; Carabatsos, *et al.*, 1998; Elvin, *et al.*, 1999b). Follicles with dying oocytes in GDF9 *null* mice express an even higher level of KL, but this expression disappears together with the oocyte (Elvin, *et al.*, 1999b). Like KL, the inhibin α-subunit also is expressed at a higher level

in ovaries of GDF9 *null* mice (Elvin, *et al.*, 1999b). The effect of GDF9 on KL and inhibin α-subunit expression is dependent on the developmental stage of the follicle. GDF9 stimulates KL and inhibin α-subunit expression in rat ovaries, which contain larger follicles than type 3b, in an *in vitro* culture system (Hayashi, *et al.*, 1999; Nilsson and Skinner, 2002; Roh, *et al.*, 2003). The GDF9-related gene BMP15 is not differentially expressed in GDF9 *null* mice ovaries and therefore abnormalities in these ovaries are not caused by a changed expression of this gene (Yan, *et al.*, 2001).

Theca cell recruitment also appears to be dependent on GDF9, since theca cell markers such as 17α-hydroxylase (P450c17), LHR and c-kit are not expressed in the stromal cells surrounding the follicles in GDF9 mice (Elvin, *et al.*, 1999b). In addition, treatment of immature rats with GDF9 results in an increase of 17α-hydroxylase mRNA, a typical theca cell marker, in the stromal cells surrounding the granulosa cells (Vitt, *et al.*, 2000b). The effects of GDF9 on theca cells are indirect, because theca cells do not express BMPRII, which is the type II receptor for GDF9 (Erickson and Shimasaki, 2003).

In antral follicles, GDF9 acts as an inhibitor of luteinization because it stimulates granulosa cell proliferation and inhibits FSH-induced differentiation of granulosa cells. FSH-induced LHR expression and production of estrogen and progesterone are inhibited by GDF9 in granulosa cells cultured *in vitro* (Vitt, *et al.*, 2000a). Interestingly, GDF9 alone stimulates estrogen synthesis in granulosa cell culture of antral follicles and progesterone synthesis in granulosa cell culture of pre-ovulatory follicles (Elvin, *et al.*, 1999a; Vitt, *et al.*, 2000a). Granulosa cells cultured *in vitro* can produce progesterone because they express P450scc, which is not expressed in granulosa cells of pre-antral and antral follicles *in vivo* (Elvin, *et al.*, 1999b; Otsuka, *et al.*, 2001b). However, the stimulatory effect on estrogen and progesterone synthesis changes to inhibition when FSH (5 ng/ml) is present (Vitt, *et al.*, 2000a). Therefore, during folliculogenesis GDF9 would probably have an inhibitory effect on progesterone and estrogen production, because serum FSH levels higher than 5 ng/ml are commonly present. Only after ovulation and before implantation, when the oocyte and cumulus cells are free floating within the oviduct and not connected to the systemic blood supply, GDF9 could stimulate progesterone synthesis in the surrounding cumulus cells (Elvin, *et al.*, 1999a). Furthermore, expression of the inhibin α- and β-subunit and thereby production of inhibin A and B are stimulated by GDF9 alone, while GDF9 enhances FSH-induced production of inhibin A and inhibin B (Roh, *et al.*, 2003). In this way, GDF9 is involved in negative feedback regulation of FSH-release.

In the pre-ovulatory follicle, GDF9 plays an important role in the regulation of normal ovulation, by stimulating cumulus expansion and regulating expression of several genes in the cumulus cells, like hyaluran synthase 2, cyclooxygenase 2, urokinase plasminogen activator, LHR and steroidogenic acute regulator protein (StAR) (Elvin, *et al.*, 1999a, 1999b).

In summary, GDF9 plays an important role during folliculogenesis from primary follicle development until ovulation.

Bone morphogenetic protein-15

Bone morphogenetic protein-15 (BMP15), a GDF9-related gene, is co-expressed with GDF9 in mouse oocytes of primary and growing follicles, albeit with a lower expression level (Aaltonen, *et al.*, 1999; Jaatinen, *et al.*, 1999; Otsuka, *et al.*, 2000). Like GDF9, BMP15 signals by binding to BMPRII, and acts on the granulosa cells and possibly also on the oocyte (Table 1.3) (Moore, *et al.*, 2002; Vitt, *et al.*, 2002).

Ovaries of BMP15 *null* mice contain all stages of follicular development, which shows that BMP15 is not obligatory for primordial follicle recruitment or follicular development (Yan, *et al.*, 2001). In addition, we have found that ovaries of prepuberal and adult BMP15 *null* mice show a normal number of growing follicles but a decrease in the primordial follicle stock, suggesting the involvement of BMP15 in migration and/or proliferation of primordial germ cells, primordial follicle formation or follicle survival (unpublished observations). Although early folliculogenesis is normal in the absence of BMP15, *in vitro* studies showed a stimulatory effect of BMP15 on granulosa cell mitosis (Otsuka, *et al.*, 2000). Interestingly, this stimulation of granulosa cell proliferation by BMP15 is, at least partly, achieved in cooperation with KL/c-kit-signaling. A c-kit neutralizing antibody inhibited BMP15 induced proliferation of granulosa cells in a co-culture of granulosa cells and oocytes (Otsuka and Shimasaki, 2002). In addition, BMP15 stimulates expression of KL1 and KL2 mRNA, thereby forming a negative feedback loop because BMP15 mRNA expression itself is inhibited by KL (Otsuka and Shimasaki, 2002).

Additionally, BMP15 inhibits FSHR mRNA expression in *in vitro* cultured granulosa cells and as a consequence the FSH-induced expression of LHR, inhibin α-, βA-, βB-subunits, StAR, P450scc and 3β-hydroxysteroid dehydrogenase (3βHSD) but not aromatase. It is unclear why the aromatase expression is not decreased after reduction of FSHR mRNA expression. However, the mRNA expression of aromatase could be inhibited when no androstenedion was present in the culture medium. Consistent with the BMP15 effects on mRNA expression of several steroidogenic enzymes, BMP15 inhibits the FSH-induced progesterone, but not estrogen synthesis when androstenedion is present (Otsuka, *et al.,* 2001b). If FSH-induced estrogen synthesis *in vivo* is inhibited by BMP15 needs to be determined. However, the follicular fluid in dominant follicles contains a high concentration of androstenedion, suggesting that BMP15 is unable to inhibit estrogen production in these follicles (Otsuka, *et al.*, 2000, 2001b). Thus, BMP15 inhibits luteinization of the follicle by stimulating granulosa cell proliferation and by suppressing FSH-induced progesterone, but probably not estrogen production.

BMP15 does not seem to play an essential role in early folliculogenesis, but is important for ovulation. A reduced number of ovulated oocytes was found in superovulated BMP15 *null* mice and some oocytes remained trapped in follicles. The impaired ovulation and fertilization of oocytes in BMP15 *null* mice could explain the reduced litter size in these homozygous knockout females (Yan, *et al.*, 2001). However, the reduced fertility in BMP15 null mice could not be completely explained by impaired ovulation and fertilization, since also fewer litters per month are born (Yan, *et al.*, 2001). This suggests that BMP15 also has an effect on the length of the estrous cycle.

In sheep, it was found that normal BMP15 expression is obligatory for early follicular development. Several breeds of sheep have a high incidence of twinning, indicating that often more than one follicle is selected. Two of these breeds, the Inverdale ($FecX^{I}$) and Hanna ($FecX^{H}$) sheep have been found to carry a naturally occurring inactivating mutation in the BMP15 gene. Loss of one normal gene copy results in a lower BMP15 expression and a high incidence of twinning in these sheep. Homozygous mutants are infertile since follicle development beyond the primary stage is impaired. In these mutants, oocytes grow in the absence of granulosa cell proliferation until they cannot be supported by the residual granulosa cells whereupon they degenerate (Galloway, *et al.*, 2000). Recently, it was shown that the phenotype of Inverdale sheep could be explained by the absence of GDF9 protein rather than of BMP15. BMP15 and GDF9 can form heterodimers and the processing of GDF9 homodimers is severely impaired when GDF9 is expressed in a cell line together with the mutated

BMP15 construct of the Inverdale sheep (Liao, *et al.*, 2003). This reduced GDF9 protein expression could explain that the phenotype of homozygous Inverdale sheep is more similar to the phenotype of GDF9 *null* mice than of the BMP15 *null* mice.

In conclusion, BMP15 is not necessary for primordial follicle recruitment in mice and stimulates granulosa cell proliferation, inhibits FSH-induced progesterone, but not estrogen synthesis and stimulates expression of KL. In this way, BMP15 inhibits luteinization of follicles. Furthermore, BMP15 is involved in ovulation. In sheep, homozygous mutations in the BMP15 gene result in a block in folliculogenesis after the primary follicle stage. This block in folliculogenesis is probably caused by a decreased GDF9 homodimer production in these mutants.

Bone Morphogenetic Protein-6

Bone morphogenetic protein-6 (BMP6) is a third TGFβ family member expressed by oocytes. In contrast to GDF9 and BMP15, BMP6 is also expressed in granulosa cells of secondary and tertiary follicles (Erickson and Shimasaki, 2003). The type II receptor for BMP6 is BMPRII and this receptor is expressed in the granulosa cells and oocytes (Table 1.3) (Ebisawa, *et al.*, 1999; Erickson and Shimasaki, 2003).

The role of BMP6 is not clear, since BMP6 *null* female mice are fertile, have a normal littersize and a normal reproductive life-span (Solloway, *et al.*, 1998). The reason for the absence of a phenotype in BMP6 *null* mice could be due to the expression in the ovary of related BMPs, which compensate for the absence of the BMP6 (redundancy). Alternatively, it is possible that BMP6 evokes no biological effects in the ovary *in vivo*.

However, *in vitro* studies revealed that BMP6 could influence granulosa cell function. FSH- and forskolin-induced FSHR mRNA accumulation is inhibited by BMP6. In addition, FSH-induced progesterone production is inhibited by BMP6 through inhibition of StAR and P450scc mRNA expression. No influence of BMP6 was found on granulosa cell proliferation, on basal FSHR mRNA expression, on FSH-induced aromatase expression or estrogen production. Since BMP6 does not inhibit effects of 8-bromo-cAMP but inhibits effects of FSH and forskolin on progesterone production and FSHR expression, it has been suggested that the underlying mechanisms involve the downregulation of adenylate cyclase activity (Otsuka, *et al.*, 2001a). This mechanism of action is different from that of BMP15, which also inhibits progesterone secretion, but achieves this effect by inhibiting FSHR mRNA expression (Otsuka, *et al.*, 2001b).

In summary, BMP6 inhibits FSH-induced progesterone but not estrogen synthesis and this leads to the hypothesis that BMP6, like BMP15, is an inhibitor of luteinization.

1.4.2 TGFβ superfamily members expressed by theca cells

Theca cells are recruited from the interstitium of the ovary adjacent to the pre-antral follicle. The most important function of theca cells is the production of androgens. The granulosa cells convert androgens to estrogens, which are important for follicle development. At least three members of the TGFβ superfamily are expressed by the theca cells: bone morphogenetic proteins-3, -4 and -7. A summary of the effects of TGFβ family members expressed by the theca cells is given in Table 1.5 and Figure 1.1.

Table 1.5: Overview of the effects on follicle development of TGFβ family members expressed by theca cells.
Effects on folliculgenesis of BMP2 and AMH, both expressed by theca- and granulosa cells, is described in Table 1.6.

Family member:	Site of action:	Effects:	References:
BMP3	theca cells	• probably effects in late follicular development	Jaatinen *et al.*, 1996
BMP4	oocyte	• stimulates primordial follicle recruitment	Nilsson and Skinner, 2003
	granulosa cells	• inhibits FSH-induced luteinization by inhibiting FSH-induced progesterone but stimulating estrogen synthesis	Shimasaki *et al.*, 1999; Mulsant *et al.*, 2001
		• survival factor for follicular and non-follicular ovarian cells	Nilsson and Skinner, 2003
BMP7	oocyte	• stimulates development from primordial to primary, pre-antral and antral follicles	Lee *et al.*, 2001
	granulosa cells	• stimulates granulosa cell proliferation	Lee *et al.*, 2001
		• inhibits FSH-induced luteinization by inhibiting FSH-induced progesterone but stimulating estrogen synthesis	Shimasaki *et al.*, 1999

Bone Morphogenetic Protein-3

Bone morphogenetic protein-3 and its related protein BMP3b (also called GDF10) are expressed by theca cells of tertiary follicles (Erickson and Shimasaki, 2003). Furthermore, it has been reported that human luteal-granulosa cells express BMP3 and that this expression is inhibited by human chorionic gonadotropin (hCG) (Jaatinen, *et al.*, 1996). These results suggest a role for BMP3 and BMP3b in late follicular development.

Bone Morphogenetic Protein-4

Bone morphogenetic protein-4 (BMP4) is expressed by theca-interstitial cells from primary follicles onwards, with increasing intensity when the follicles become larger (Shimasaki, *et al.,* 1999; Erickson and Shimasaki, 2003). The receptor for BMP4 is BMPRII and is expressed in the oocyte and granulosa cells, which suggests that this factor acts on these two cell types (Table 1.3) (Koenig, *et al.*, 1994; ten Dijke, *et al.*, 1994; Yamaji, *et al.*, 1994; Nohno, *et al.,* 1995; Shimasaki, *et al.*, 1999; Erickson and Shimasaki, 2003).

In vitro culture of granulosa cells of antral follicles showed an inhibitory effect of BMP4 on FSH-induced progesterone secretion and a stimulatory effect on FSH-induced estrogen secretion (Shimasaki, *et al.*, 1999; Mulsant, *et al.*, 2001). In this way, BMP4 inhibits luteinization of antral follicles.

Recently, a stimulatory function of BMP4 on primordial follicle recruitment was discovered. Neonatal rat ovaries cultured in the presence of BMP4 contained less primordial and more growing follicles than the controls (Nilsson and Skinner, 2003). Furthermore, ovaries cultured in the presence of a BMP4 neutralizing antibody were smaller than controls. This was caused by loss of oocytes and primordial follicles, an increase in cellular apoptosis and loss of normal stromal ovarian tissue (Nilsson and Skinner, 2003). These results suggest that BMP4 acts as a survival factor for both follicular and non-follicular cell types in the neonatal ovary.

Bone Morphogenetic Protein-7

Similar to BMP4, bone morphogenetic protein-7 (BMP7) is expressed in theca cells of secondary and tertiary follicles and it receptor (BMPRII) is expressed in the granulosa cells and oocytes (Table 1.3) (ten Dijke, *et al.*, 1994; Rosenzweig, *et al.*, 1995; Shimasaki, *et al.*, 1999; Erickson and Shimasaki, 2003).

Some BMP7 effects are similar to the effects of BMP4. Like BMP4, BMP7 inhibits FSH-induced progesterone production and stimulates FSH-induced estrogen secretion (Shimasaki, *et al.*, 1999). These effects are achieved through inhibition of FSH-induced StAR expression and stimulation of aromatase mRNA expression (Lee, *et al.*, 2001). The inhibition of progesterone synthesis might explain the reduced number of ovulated oocytes from ovaries treated with BMP7 (Lee, *et al.*, 2001). Like BMP4, BMP7 facilitates transition of primordial follicles to primary, pre-antral and antral follicles in rats treated with BMP7 (Lee, *et al.*, 2001). This stimulation of outgrowth of primordial follicles can be explained by the increased growth of pre-antral follicles which produce factors that regulate primordial follicle recruitment (Hirshfield, 1994). Furthermore, granulosa cell proliferation is stimulated in a dose dependent manner by BMP7, independent of FSH (Lee, *et al.*, 2001).

Thus, BMP7 has several effects throughout follicular development. At the start of follicular development BMP7 facilitates primordial follicle outgrowth, and at the end of follicular development it inhibits luteinization and ovulation.

1.4.3 TGFβ superfamily members expressed by granulosa cells

Granulosa cells surround the oocyte and play an important role in the regulation of follicular development. Granulosa cells are the most important cells through which FSH acts and regulates folliculogenesis. At least five members of the TGFβ superfamily are expressed by these granulosa cells: bone morphogenetic protein-2, inhibins, activins, TGFβ and the anti-Müllerian hormone. A summary of the effect of TGFβ family members expressed by the granulosa cells is given in Table 1.6 and Figure 1.1.

Bone Morphogenetic Protein-2

Bone morphogenetic protein-2 (BMP2) is expressed by granulosa cells from primary follicles onwards. In addition, BMP2 is also expressed by theca cells, but at a lower level compared to the expression in granulosa cells (Erickson and Shimasaki, 2003). BMP2 acts, like BMP4, on granulosa cells and possibly also on oocytes as the type II receptor (BMPRII) for BMP2 is expressed there (Table 1.3) (Koenig, *et al.*, 1994; ten Dijke, *et al.*, 1994; Yamaji, *et al.*, 1994; Nohno, Mizunuma *et al.,* 1999, 1995; Shimasaki, *et al.*, 1999; Erickson and Shimasaki, 2003).

BMP2 enhances FSH-stimulated estrogen production in granulosa cells of immature follicles, without affecting inhibin A production or proliferation of the granulosa cells (Souza, *et al.*, 2002). Furthermore, BMP2 induces inhibin βB-subunit mRNA expression and inhibin B secretion by human luteal granulosa cells and this effect is inhibited by hCG (Jaatinen, *et al.*, 2002). This upregulation of inhibin B and inhibin βB-subunit mRNA expression by BMP2 could be relevant *in vivo* since inhibin B is the predominant form of inhibin in the pre-ovulatory follicle and its concentration in follicular fluid is much higher than inhibin A (Groome, *et al.*, 1996).

In conclusion, BMP2 stimulates differentiation of granulosa cells by enhancing the effect of FSH on estrogen production and could be involved in regulation of expression of inhibin B protein and inhibin βB-subunit RNA.

Table 1.6: Overview of the effects on follicle development of TGFβ family members expressed by granulosa cells. Effects on folliculogenesis of BMP6, expressed in granulosa cells and oocytes, is described in Table 1.4.

Family member:	Site of action:	Effects:	References:
BMP2	granulosa cells oocyte?	• enhances FSH-induced estrogen production • induces inhibin βB and inhibin B production in human luteal granulosa cells	Souza *et al.*, 2002 Jaatinen *et al.*, 2002
inhibin	granulosa cells theca cells oocyte?	• stimulates androgen production in theca cells • antagonizes activin and possibly also BMPs	Findlay, 1993; Hillier and Miro, 1993; Wiater and Vale, 2003
activin	granulosa cells oocyte	• induces together with FSH follicular structure reorganisation of granulosa and oocytes *in vitro* • activin produced by larger pre-antral follicles inhibits growth of smaller pre-antral follicles *in vitro* • immature animals: stimulates FSH sensitivity of follicles by stimulating FSH-induced FSHR expression and inhibiting FSH-induced downregulation of FSHR • immature animals: activin stimulates granulosa cell proliferation of pre-antral follicles and inhibin and estradiol production • adult animals: activin inhibits FSH-induced estrogen, inhibin production and follicle growth • suppresses theca cell androgen production	Li *et al.*, 1995 Mizunuma *et al.*, 1999 Hasegawa *et al.*, 1988; Xiao *et al.*, 1992; Nakamura *et al.*, 1993 LaPolt *et al.*, 1989; Li *et al.*, 1995; Miro and Hillier, 1996; Yokota *et al.*,1997; Liu *et al.*, 1998, 1999; Mizunuma *et al.*, 1999 Yokota *et al.*, 1997; Liu *et al.*, 1998, 1999 Findlay, 1993; Hillier and Miro, 1993
TGFβ	theca cells oocyte?	• stimulates follicular growth, inhibin and estradiol production of pre-antral follicles of adult but not immature mice	Roy and Kole, 1998; Liu *et al.*, 1999; Mizunuma *et al.*, 1999
AMH	granulosa cells	• inhibits primordial follicle recruitment • inhibits FSH sensitivity of pre-antral and antral follicles	Durlinger *et al.*, 1999, 2002 Durlinger *et al.*, 2001

Inhibins and activins

Activins and inhibins are dimeric glycoproteins formed from the inhibin subunits α, βA and βB. The inhibin α- together with the inhibin βA-subunit forms the inhibin A protein, while inhibin B consists of an inhibin α- and an inhibin βB-subunit. Homo- and heterodimerization of the inhibin β-subunits gives rise to three forms of activin, referred to as activin A (βA-βA), activin AB (βA-βB) and activin B (βB-βB). Inhibins inhibit the secretion of FSH from the pituitary and FSH stimulates the inhibin production by the ovary (Drummond, *et al.*, 2000). In this way, inhibins are involved in the feedback system between ovary and pituitary gland. Activins stimulate FSH secretion from the pituitary. However, it is unlikely that activins produced in the ovaries are responsible for this effect, because in the circulation activin is tightly bound to follistatin which neutralizes its biological activity (Woodruff, 1998). Instead, locally produced activin could be responsible for FSH secretion in the pituitary (Besecke, *et al.*, 1997). Activins and inhibins also directly influence folliculogenesis in the ovary, and these effects will be described below.

Expression of the inhibin α-subunit is found in granulosa cells of primary, pre-antral and antral follicles of several species (Meunier, *et al.*, 1988; Tebar, *et al.*, 1997; McNatty, *et al.*, 1999; Pangas, *et al.*, 2002). Expression of inhibin βA- and βB-subunits is detected in granulosa cells of pre-antral and antral follicles and conflicting data were reported about their expression in theca cells (Meunier,

et al., 1988; Roberts, *et al.*, 1993; Tebar, *et al.*, 1997; van den Hurk and Van de Pavert, 2001; Pangas, *et al.*, 2002). As expected from the mRNA expression pattern, inhibin α-subunit protein is detected in granulosa cells of primary to pre-ovulatory follicles while the inhibin βA- and βB-subunit proteins are detected in granulosa cells of secondary and tertiary follicles (Meunier, *et al.*, 1988). Furthermore, activin A is detected in granulosa cells of pre-antral and antral follicles and in theca cells of small antral follicles in porcine (van den Hurk and Van de Pavert, 2001). Activins signal through two receptors, activin receptor type II (ActRII) and type IIB (ActRIIB). ActRII is expressed in oocytes of all follicle types and in pre-antral and antral granulosa cells (Cameron, *et al.*, 1994; van den Hurk and Van de Pavert, 2001; Drummond, *et al.*, 2002; Pangas, *et al.*, 2002). ActRIIB is expressed by granulosa cells and oocytes (Cameron, *et al.*, 1994; Drummond, *et al.*, 2002; Pangas, *et al.*, 2002). These expression patterns suggest that activins act on oocytes and granulosa cells (Table 1.3). Until now, no inhibin-specific receptors have been identified. Betaglycan, a TGFβ type III receptor, enhances the binding of inhibin to the ActRII and the heteromerization of activin type I and type II receptors is inhibited. In this way, the activin-opposing actions of inhibin could be explained (Lebrun and Vale, 1997; Martens, *et al.*, 1997; Lewis, *et al.*, 2000).

No effect of activin or inhibin on primordial follicle recruitment has been described but an important role was found in early follicle development. *In vitro* culture of granulosa cells obtained from diethylstilbestrol (DES)-treated immature rats, inhibin production is stimulated by FSH and activin enhances the sensitivity of pre-antral follicles for FSH. This effect is achieved by stimulating FSHR mRNA expression in granulosa cells and preventing FSH-induced downregulation of FSHR number (Hasegawa, *et al.*, 1988; Xiao, *et al.*, 1992; Nakamura, *et al.*, 1993). In general, most actions of inhibin are explained by its activin antagonizing effects (Lebrun and Vale, 1997; Martens, *et al.*, 1997). However, recently it was shown that inhibin also could antagonize signaling of BMPs by binding to BMPRII (Wiater and Vale, 2003). Since BMPs play an important role in folliculogenesis, actions of inhibins in the ovary could be more widespread.

FSH and activin are also involved in follicular structure formation. A monolayer of granulosa cells and oocytes of primary follicles of immature rats cultured *in vitro*, forms follicle structures under the influence of activin and FSH. Addition of LH to the culture medium induces dissociation of these follicle-like structures, in a process resembling ovulation (Li, *et al.*, 1995). Furthermore, there are indications that activin, produced by secondary follicles, causes small pre-antral follicles to remain dormant. A co-culture of small and larger pre-antral follicles caused an inhibition of growth of the smaller follicles. Addition of follistatin to the culture medium resulted in an increase in diameter of small and larger pre-antral follicles. This indicates that the growth inhibition of smaller follicles could be caused by activin or BMPs (Mizunuma, *et al.*, 1999).

Opposite effects of activin on granulosa cells of pre-antral follicles from immature and adult animals cultured *in vitro* have been found. Activin A stimulates the proliferation of granulosa cells of pre-antral follicles of immature rats and mice, measured by ^{3}[H]thymidine uptake and follicle diameter. Furthermore, FSH-stimulated growth of granulosa cells is enhanced by activin while inhibin has no effect (Li, *et al.*, 1995; Miro and Hillier, 1996; Yokota, *et al.*, 1997; Liu, *et al.*, 1998, 1999; Mizunuma, *et al.*, 1999). In contrast, *in vitro* growth of pre-antral follicles of adult mice is not stimulated by activin and FSH-stimulated growth is inhibited (Yokota, *et al.*, 1997; Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999).

Furthermore, activin stimulates production of inhibin and estradiol by pre-antral follicles of immature but not of adult mice (Yokota, *et al.*, 1997; Liu, *et al.*, 1998, 1999). In addition, activin A stimulates expression and biosynthesis of the inhibin α-subunit in cultured granulosa cells of immature rats (LaPolt, *et al.*, 1989). I*n vivo* data showed an opposite effect from *in vitro* data. Inhibin stimulates growth of follicles of immature rats *in vivo* and activin causes follicular atresia (Woodruff, *et al.*, 1990), while *in vitro* inhibin has no effect and activin stimulates follicular growth (Li, *et al.*, 1995; Miro and Hillier, 1996; Yokota, *et al.*, 1997; Liu, *et al.*, 1998, 1999; Mizunuma, *et al.*, 1999).

During follicular development, the effect of activin A on steroidogenesis changes. Basal steroidogenesis in an undifferentiated granulosa cell culture from immature rats is not affected by activin, but the FSH-induced estrogen and progesterone synthesis is enhanced. In differentiated and pre-ovulatory granulosa cells activin stimulates basal as well as FSH-induced estrogen production. Basal progesterone production is stimulated in differentiated and inhibited in pre-ovulatory granulosa cells by activin, whereas FSH-induced progesterone synthesis is inhibited in both differentiated and pre-ovulatory granulosa cell cultures (Hutchinson, *et al.*, 1987; Miro, *et al.*, 1991). These results show a role for activin in modulating steroidogenesis during follicular development. In theca cells, inhibin enhances LH-induced production of androgens, while activin suppresses this production (Findlay, 1993; Hillier and Miro, 1993).

In conclusion, activin has multiple roles in pre-antral follicle development as it stimulates granulosa cell proliferation in follicles of immature mice and rats, enhances FSH-responsiveness, inhibin and estrogen production in granulosa cells and inhibits androgen production by theca cells. Furthermore, activin is involved in follicle formation of pre-antral follicles. In addition, activin produced by larger pre-antral follicles can inhibit growth of smaller pre-antral follicles. Inhibin stimulates LH-induced androgen synthesis of theca cells and is an antagonist of activin- and possibly also BMP-action.

Transforming Growth Factor β

The expression pattern of transforming growth factor β (TGFβ) and its receptors suggests a role for TGFβ in early folliculogenesis. Three isoforms of TGFβ can be distinguished, TGFβ1, 2 and 3. TGFβ2 expression is found in monkey granulosa cells of non-growing follicles, while no expression is found in small growing follicles (Gougeon and Busso, 2000). In the hamster, expression of TGFβ2 also declines after follicles have started to grow, although this drop occurs only after 2-3 layers of granulosa cells have formed (Roy and Hughes, 1994). In contrast, no expression of TGFβ1 is found in non-growing and small growing monkey (Gougeon and Busso, 2000), rat (Teerds and Dorrington, 1992) or marmoset follicles (Wehrenberg, *et al.*, 1998). In porcine and bovine follicles, the theca cells seem to be the source of TGFβ1 protein expression in antral follicles (Skinner, *et al.*, 1987; May, *et al.*, 1996). TGFβ receptor II is found in oocytes of primordial and primary follicles and a weak staining is present in theca- but not granulosa cells (Qu, *et al.*, 2000) while another group reported also expression in granulosa cells (Table 1.3) (Roy and Kole, 1998). This TGFβ receptor II expression in granulosa cells can be stimulated by EGF and FSH in human pre-antral follicle culture (Roy and Kole, 1998).

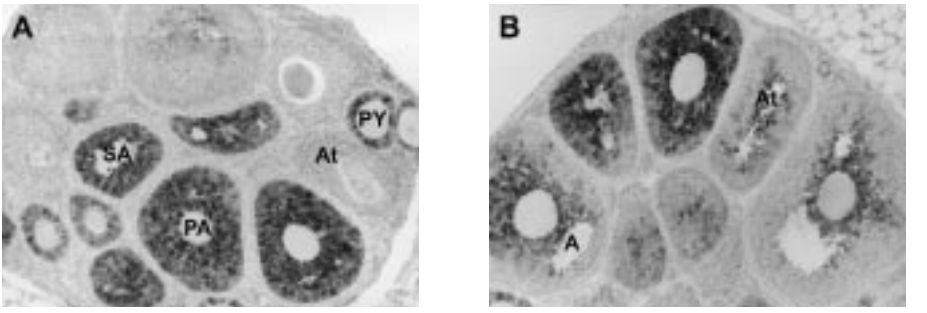
TGFβ stimulates follicle growth, inhibin and estradiol production in *in vitro* culture of pre-antral follicles of adult (Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999) but not in immature mouse (Liu, *et al.*, 1999) or human pre-antral follicles (Roy and Kole, 1998). In contrast, in vitro proliferation of bovine

granulosa cells was inhibited by TGFβ (Skinner, *et al.*, 1987). Thus, although the expression pattern of TGFβ and its receptor suggests a role of TGFβ in early folliculogenesis, the functional role of TGFβ still needs to be determined.

Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) is secreted by the testis during male sex differentiation and is responsible for the regression of the Müllerian ducts, which form the *anlagen* of the oviduct, uterus and the upper part of the vagina in females. In human girls, AMH is expressed after week 36 of gestation (Rajpert-De Meyts, *et al.*, 1999), while in mice AMH production starts in the days immediately following birth (Chapter 3). In females, AMH is only expressed in the ovary and its mRNA and protein are found in granulosa cells of small growing (i.e. pre-antral and small antral) non-atretic follicles in rat and mouse (Ueno, *et al.*, 1989; Hirobe, *et al.*, 1992; Baarends, *et al.*, 1995a and Chapter 3 of this thesis). The pregranulosa cells of primordial follicles do not express AMH, but when primordial follicles are recruited into the growing pool, granulosa cells start to express AMH mRNA and protein (Münsterberg and Lovell-Badge, 1991; Hirobe, *et al.*, 1992; Taketo, *et al.*, 1993; Baarends, *et al.*, 1995a and Chapter 3 of this thesis). The highest level of AMH expression is found in the granulosa cells of large pre-antral and small antral follicles (Figure 1.2). Two studies have reported expression of AMH in rat pre-ovulatory follicles (Ueno, *et al.*, 1989; Hirobe, *et al.*, 1994). However, these results were not confirmed by other studies, which showed disappearance of AMH expression from antral stage onward and its absence in pre-ovulatory follicles and corpora lutea (Figure 1.2) (Hirobe, *et al.*, 1992; Baarends, *et al.*, 1995a; Durlinger, *et al.*, 2002). In addition, AMH expression disappears when a follicle becomes atretic (Hirobe, *et al.*, 1992; Baarends, *et al.*, 1995a; Durlinger, *et al.*, 2002). AMH expression is not found in theca cells, oocytes and interstitial cells of the ovary (Ueno, *et al.*, 1989; Hirobe, *et al.*, 1992; Hirobe, *et al.*, 1994; Baarends, *et al.*, 1995a and Chapter 3 of this thesis).

Figure 1.2: Immunohistochemical localization of AMH in Bouin-fixed ovaries of adult mice.
A. AMH protein is expressed in granulosa cells of small growing follicles, like primary (PY), pre-antral (PA) and small antral (SA) follicles, but not in atretic follicles (At).
B. AMH protein expression disappears in larger antral follicles (A) and in atretic follicles (At).



Like other members of the TFGβ family, AMH signals by binding to a type II transmembrane serine/threonine (S/T) kinase receptor which forms a complex with and subsequently activates a type I S/T kinase receptor by phosphorylation (see also Chapter 2).

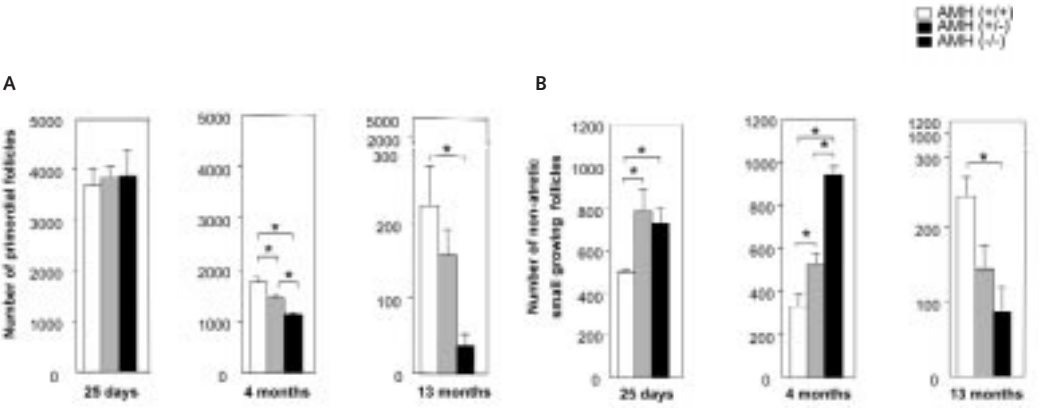
The AMH-specific type II receptor (AMHRII) has been cloned and its mRNA expression is colocalized with AMH in the granulosa cells (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b). It is likely that the AMHRII is also expressed in the pregranulosa cells of primordial follicles because ovarian expression of AMHRII is already found during the fetal period and remains present after birth when the ovary mainly contains primordial follicles (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b, Chapter 5). In addition, it has been suggested that AMHRII is also expressed in theca cells of pre-antral and small antral follicles in rat ovaries (Table 1.3) (Ingraham, *et al.*, 2000).

Although AMH *null* female mice did not show an obvious phenotype (Behringer, *et al.*, 1994), a more extensive study of the total follicle population in AMH *null* mice and their wild-type littermates revealed that AMH has an effect on initial follicle development (Durlinger, *et al.*, 1999). Ovaries of prepubertal (25-day-old) and adult (4-month-old) AMH *null* mice contained more growing follicles than ovaries of their wild-type littermates (Figure 1.3) (Durlinger, *et al.*, 1999). In addition, ovaries of AMH *null* adult mice contained less primordial follicles than control ovaries. These results indicate that in the absence of AMH more primordial follicles are recruited into the growing pool. Interestingly, heterozygous AMH *null* mice have a phenotype between those of wild-type and homozygous mice (Figure 1.3). This AMH gene dose-dependency suggests that AMH production and secretion depend on the intrinsic activity of the gene itself and is not under stringent feedback control (Durlinger, *et al.*, 1999).

Figure 1.3: Follicle population in 25-day-old, 4-month-old and 13-month-old AMH(+/+), AMH(+/-) and AMH(-/-) mice.

A. Primordial follicle population in ovaries of 25-day-old, 4-month-old and 13-month-old AMH(+/+), AMH(+/-) and AMH(-/-) mice.

B. Non-atretic small growing follicle population in ovaries of 25-day-old, 4-month-old and 13-month-old AMH(+/+), AMH(+/-) and AMH(-/-) mice. Data represent the mean ± SEM (n=4-5). An *asterisk* indicates a statistically significant difference (P≤0.05). Data taken and adapted from: Durlinger A.L.L., Kramer P., Karels B., de Jong F.H., Uilenbroek J.Th.J., Grootegoed J.A. and Themmen A.P.N.; Control of primordial follicle recruitment by Anti-Müllerian hormone in the mouse ovary; Endocrinology; Vol. 140; No. 12; 5789-5796; 1999; copyright owner, The Endocrine Society.



As a result of the absence of AMH, and thus the increased recruitment of primordial follicles, aged 13-month-old AMH *null* ovaries were almost completely depleted of their primordial follicles. As a consequence, these ovaries hardly contained any growing follicles (Figure 1.3). This was reflected by the fact that 56% of the AMH null females at 16-17 months of age had stopped ovulating compared to only 18% of the aged wild-type females (Durlinger, *et al.*, 2002). Thus, by inhibiting initial recruitment AMH prevents early depletion of the follicle pool in the ovary. This hypothesis was confirmed by *in vitro* studies (see Chapter 3).

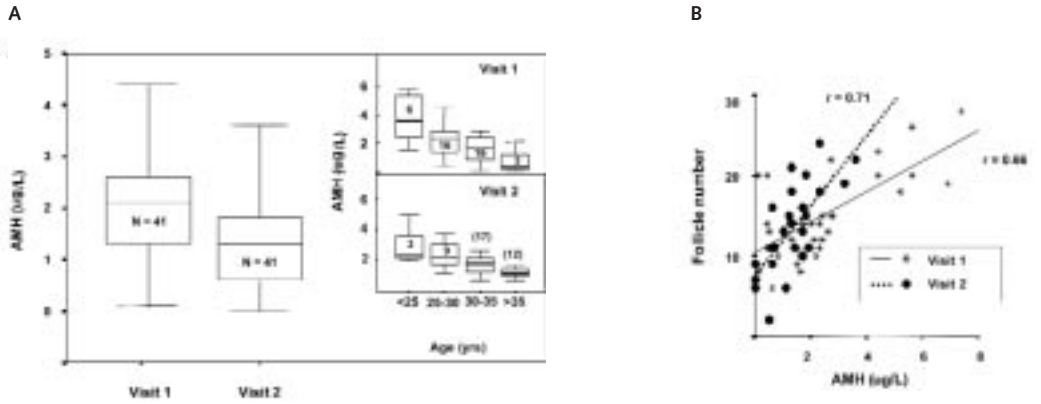
Not only does AMH play a role in initial follicle recruitment, but AMH is also involved in the regulation of selection of follicles for pre-ovulatory growth as shown by experiments in AMH *null* mice (Durlinger, *et al.*, 1999). The initial finding that led us to hypothesize that AMH might modulate the FSH-sensitivity of follicles was that, despite lower FSH serum levels, ovaries of AMH *null* mice contained more growing follicles compared to their wild-type littermates. *In vitro* culture of mouse pre-antral follicles showed that AMH is able to inhibit FSH-stimulated growth of these follicles after four and five days of culture. This was reflected by a decreased follicle diameter of follicles cultured in the presence of AMH compared to controls. This growth inhibition is probably caused by a decrease in the rate of granulosa cell proliferation (Durlinger, *et al.*, 2001). In contrast, McGee *et al.* found a stimulatory effect of AMH on the growth of *in vitro* cultured rat pre-antral follicles (2001). Although in both studies pre-antral follicles were used, species difference and culture conditions (presence/absence of serum and thus growth factors) may explain these opposite results (McGee, personal communication).

The inhibitory effect of AMH on FSH-sensitivity was confirmed by *in vivo* studies using AMH *null* mice. In the presence of low FSH serum levels, induced by GnRH-antagonist treatment, ovaries of AMH *null* mice contained more growing follicles than the wild-type ovaries under the same experimental conditions. Comparable results between wild-type and AMH *null* mice ovaries were found when FSH serum levels were artificially increased (Durlinger, *et al.*, 2001). Taken together, these studies show that AMH inhibits selection of follicles for pre-ovulatory growth by decreasing their sensitivity for FSH. In this way AMH is able to control the number of large pre-antral and small antral follicles that continue to grow to the pre-ovulatory stage.

The usefulness of AMH as a new marker for female fertility was studied. Fertility in women is determined by the ovarian reserve, which reflects both the quantity and quality of the ovarian follicle pool. The decline of the ovarian reserve is called ovarian ageing. Besides aspects such as oocyte quality, the number of primordial follicles that is left in the ovary is also an important parameter for ovarian reserve (te Velde and Pearson, 2002). Although it is difficult to directly measure the size of the primordial follicle stock in women, it appears that the number of growing follicles correlates with the size of the primordial follicle stock from which they are recruited (Gougeon, *et al.*, 1994; Scheffer, *et al.*, 1999). Therefore, a marker that reflects all follicles that have made the transition from the primordial follicle pool to the growing pool may be a good indirect marker of the quantitative aspect of the ovarian reserve. Since AMH is produced by growing follicles, the hypothesis whether AMH is a candidate marker of ovarian reserve has been tested by measuring the AMH serum levels of 41 healthy normo-ovulatory women at two visits to the clinic with an average interval of 2.6 $\pm$ 1.7 years. AMH serum levels significantly decreased between the two visits, and within each visit a negative correlation was found between age and AMH levels (Figure 1.4A). In addition, the number of growing antral

follicles was counted using ultrasonography in the same women and FSH, inhibin B and estrogen levels were determined. Of the serum levels measured, only those of AMH showed a strong correlation with the number of antral follicles (Figure 1.4B) (de Vet, *et al.*, 2002). These results indicate that AMH, indeed, may be used as a marker for ovarian ageing.

Figure 1.4: Results of measurements of serum AMH at two different time points in 41 healthy normo-ovulatory women, with an average time interval between two visits of 2.6 years ± 1.7 years.
The average age at visit 1 was 29 ± 4 years and at visit 2 32 ± 4 years. This figure shows the correlation of AMH with age at the two visits (A) and of AMH with the number of antral follicles (B), with the AMH level at visit 1 (closed circles, solid line) and visit 2 (open circles, dotted line) (de Vet, *et al.*, 2002). Reprinted by permission from the American Society for Reproductive Medicine (Fertility and Sterility, 2002; Vol 77; 357-362).



The usefulness of AMH serum levels as a measure for the ovarian reserve was tested further in a cohort of women undergoing *in vitro* fertilization (IVF) treatment. Hormonal parameters and antral follicle count using transvaginal ultrasonography were determined on the third day of the menstrual cycle in 119 IVF patients, not more than three months preceding the IVF treatment (van Rooij, *et al.*, 2002). The measured parameters were analyzed after division of the patients into two groups on the basis of the number of oocytes retrieved after IVF treatment: normal responders (four or more retrieved oocytes) and poor responders (less than four retrieved oocytes or cancellation of IVF treatment). Ovaries of normally responding women contained significantly more growing antral follicles than ovaries of women with a poor response to IVF treatment. In addition, AMH serum levels in these poorly responding women were lower compared to those in normally responding women (Table 1.7). Serum AMH levels correlated strongly with the antral follicle count, the number of follicles retrieved, age, inhibin B and FSH (van Rooij, *et al.*, 2002). In addition, logistic regression analysis used to predict poor or normal response showed that both antral follicle count and AMH serum levels were equally important for prediction, since the calculated areas under the receiver operating characteristic (ROC) curve (ROC_{AUC}) were 0.86 and 0.85, respectively (van Rooij, *et al.*, 2002). The ROC_{AUC} determines the sensitivity of the diagnostic test and may vary between 0.5 (no discriminative power) to 1.0 (perfect discrimination) (Harrell, *et al.*, 1996). These data support the observations made in another study where also a correlation was found between AMH serum levels and the number of retrieved oocytes in women undergoing IVF treatment (Seifer, *et al.*, 2002). However, not only low AMH serum levels

but also high AMH levels correlate with a poor response in IVF treatment. In patients with polycystic ovary syndrome (PCOS), higher AMH serum levels were found than in normo-ovulatory women. In these PCOS patients a higher number of immature oocytes was found after ovarian stimulation for IVF treatment (Fallat, *et al.*, 1997). Thus, AMH serum levels can give a prediction for the success of oocyte retrieval during IVF treatment.

Although the serum level of AMH may not be the only marker of ovarian reserve, the relatively small fluctuations in AMH serum levels during the menstrual cycle (Cook, *et al.*, 2000) indicate that a blood sample may be drawn at any moment during the menstrual cycle, to predict the ovarian reserve. This further supports the advantage of the use of AMH as a marker for ovarian reserve over antral follicle count using ultrasound.

Table 1.7: Results of ovarian reserve test in IVF treated patients, divided in normal and poor responders.
AMH levels are lower in poor responders than in normal responders. The number of antral follicles found at day 3 of the cycle is higher in normal responders than in poor responders (van Rooij *et al.*, 2002).

Variables:	Total (n=119):	Normal responders (n=84):	Poor responders (n=35):	p:
Age (years)	33.8 (22.3-44.0)	33.8 (24.4-44.0)	36.3 (22.3-43.3)	0.09
AMH (μg/L)	0.9 (0.0-6.2)	1.4 (0.0-6.2)	0.2 (0.0-1.7)	<0.001
Antral follicles (n)	8 (0-35)	11 (0-35)	4 (0-15)	<0.001
Number of oocytes (n=96)	7 (1-28)	9 (4-28)	2 (1-3)	not applicable

1.5 AIM OF THIS STUDY

Age at menopause, and therefore also the age of infertility and the length of postmenopausal life, is determined by the efficiency by which the primordial follicle stock is utilized. Since AMH is involved in the regulation of primordial follicle recruitment, this hormone is likely to play a role in the determination of the onset of menopause. Therefore, it is important to understand the mechanisms by which AMH mediates its effects. In this thesis, the mechanisms (signal transduction and regulated genes) by which AMH exerts its effects in the ovary (inhibition of primordial follicle recruitment) are investigated. Furthermore, expression of several growth factors, which are also involved in follicle recruitment, has been investigated.

To understand the signaling mechanism, which might be used by AMH, Chapter 2 reviews the TGFβ superfamily signal transduction mechanisms.

AMH *null* mice ovaries contained less primordial and more growing follicles than their wild-type littermates. These results suggest that AMH inhibits recruitment of primordial follicles. To examine the ability of AMH to inhibit primordial follicle recruitment *in vitro* we used an ovary organ culture system. The results of this study are described and discussed in Chapter 3.

In the Müllerian ducts, AMH signals through the AMHRII. In Chapter 4 we investigated if AMH also signals through this receptor in the ovary by studying the ovarian phenotype inof AMHRII *null* mice.

The signaling pathway used by AMH in the ovary is unknown. Therefore, we identified the AMH-signaling pathway and the expression and localization of candidate receptors and downstream mediators for AMH signaling in the ovary (Chapter 5).

Primordial follicle recruitment is regulated by several growth factors produced by growing follicles. In Chapter 6 we studied the expression and localization of growth factors, involved in primordial follicle recruitment. The results give more knowledge about the role in primordial follicle recruitment of several growth factors produced by growing follicles.

To understand how AMH achieves its effect in the ovary, we initiated a search for AMH-regulated genes in the ovary. Therefore we used the mouse granulosa cell line KK-1 and mouse neonatal ovary culture. Chapter 7 describes the first results of this study.

In the general discussion the mechanisms by which AMH achieves its effects is discussed. In addition, the role of AMH in follicle development compared to the role of systemic factors LH and FSH and the clinical relevance of AMH are discussed.

Chapter 2

TGFβ SUPERFAMILY:

LIGANDS, RECEPTORS AND SIGNAL TRANSDUCTION PATHWAYS

Chapter 2
TGFβ SUPERFAMILY: LIGANDS, RECEPTORS AND SIGNAL TRANSDUCTION PATHWAYS

2.1 INTRODUCTION

The transforming growth and differentiation factor β (TGFβ) superfamily is the largest family of growth and differentiation factors which is presently known. Family members have a broad range of functions in mesenchymal-epithelial interactions, cell cycle regulation, lineage determination, cell differentiation, motility, adhesion and death. The TGFβ superfamily can be divided in several subfamilies, such as the TGFβs (TGFβ1-5), activins (inhibinα, βA-C, E) and bone morphogenetic proteins (e.g. BMP1-15, GDF1-9). Furthermore, anti-Müllerian hormone (AMH) is a distant member of the TGFβ superfamily (Figure 2.1A) (Chang, *et al.*, 2002). The important roles of several TGFβ superfamily members during folliculogenesis have been described in Chapter 1. This chapter describes the characteristics of the TGFβ superfamily members and the signaling mechanisms by which they achieve their effects. In addition, the signaling mechanisms of AMH, a member of the TGFβ superfamily and the focus of this thesis, and AMH-regulated genes will be described in more detail.

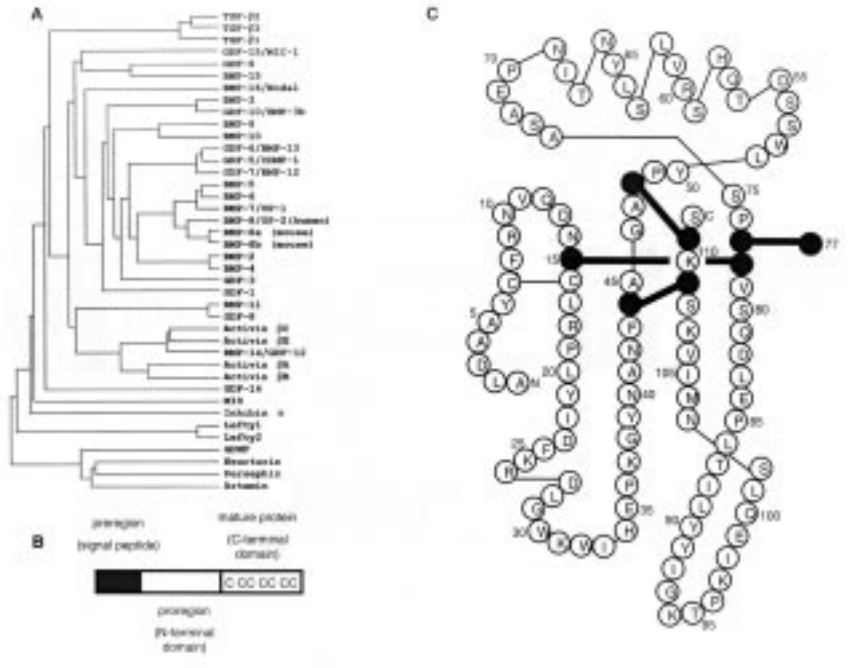


Figure 2.1: The TGFβ superfamily of growth and differentiation factors.

A. Similarity of amino acid sequences from mature proteins between the members of the TGFβ superfamily of growth and differentiation factors (Chang *et al.*, Genetic analysis of the mammalian transforming growth factor-beta superfamily. Copyright 2002, The Endocrine Society).

B. Schematic representation of processing of TGFβ superfamily members monomers. Family members are produced as preproproteins. The preregion is the signal peptide. The mature protein is released after cleavage from the proregion.

C. Two-dimensional structure of the TGFβ2 monomer. Amino-acids are indicated by their one-letter code. The six conserved cysteine residues (Cys 15, 44, 48, 77, 78, 109 and 111) are reflected by squares. Furthermore, the disulfide bonds formed by these cysteine residues are drawn. Cys 77 forms the disulfide bond with the other monomer. Reprinted by permission from Nature (Schlunegger and Grutter), Copyright 1992, Macmillan Publishers Ltd.

2.2 CHARACTERISTICS OF TGFβ FAMILY MEMBERS

TGFβ family members are synthesized as large precursor proteins which are able to form homo- or heterodimers. All precursor proteins contain a mono- or dibasic cleavage site located approximately 110 amino acids from the C-terminus. After cleavage, the biologically active dimeric C-terminal protein (mature protein) and the N-terminal protein are formed (Figure 2.1B) (Gentry and Nash, 1990; Massagué, 1990). For all members of the TGFβ superfamily cleavage of the precursor protein is necessary for biological activity, since the uncleaved protein is inactive (Gentry, *et al.*, 1988). The N-terminal fragment is involved in proper folding and secretion of the mature TGFβ1 protein and it is suggested that this is applicable to all TGFβ superfamily members (Gray and Mason, 1990; Sha, *et al.*, 1991). Dissociation of this N-terminal domain from the mature protein is necessary for full biological activity of this protein. This dissociation is a regulated process and provides a mechanism of controlling the bioactivity of the protein (Gentry and Nash, 1990). This is shown by the TGFβ protein where the dimer of the N-terminal domain interacts with the mature hormone, thereby preventing binding of TGFβ to its receptor and thus biological activity (Gentry and Nash, 1990). AMH is the only known TGFβ family member where continuous association between the mature and N-terminal domain after cleavage is necessary for full biological activity. The mature protein alone has a decreased bioactivity *in vitro* and this bioactivity can be normalized by adding the N-terminal domain of AMH in the culture medium. Presumably the N-terminal part of AMH stabilizes the proper conformation of this hormone (Wilson, *et al.*, 1993). Mutations in this N-terminal domain are found in persistent Müllerian duct syndrome (PMDS) patients (see section 2.5), suggesting that these mutations result in an inactive hormone *in vivo* (Imbeaud, *et al.*, 1994).

The biologically active C-terminal domains of all family members share a high percentage of homology (23-80%). Furthermore, the mature protein is characterized by a conserved motif of seven cysteine residues (Wilson, *et al.*, 1993), except in growth and differentiation factor (GDF) 9, GDF3 (McPherron and Lee, 1993) and bone morphogenic protein (BMP) 15 (Dube, *et al.*, 1998), which only contain six cysteine residues. Crystallography of transforming growth factor β2 (TGFβ2) revealed that the cysteines are involved in proper folding of the hormones. One cysteine forms a disulfide bond between the two mature proteins. The other six cysteines are responsible for the intrachain folding through the formation of disulfide bridges in a region known as the "cysteine knot" (Figure 2.1C) (Daopin, *et al.*, 1992; Schlunegger and Grutter, 1992).

2.3 SIGNALING OF TGFβ FAMILY MEMBERS

2.3.1 Characteristics of serine/threonine kinase receptors and signaling

Members of the TGFβ superfamily mediate their actions by binding to serine/threonine (S/T) kinase receptors. These receptors are divided in three types of S/T receptors based upon their relative molecular weights of 55 kDa (type I), 80 kDa (type II) and 300 kDa (type III) found in binding studies (Cheifetz, *et al.*, 1987; Massagué, 1990, 1992). The type I and type II receptors bind the ligand and mediate downstream signaling while the type III receptor modulates signaling by binding the ligand

and presenting it to the type II receptor (see Section 2.4). The type II and type I receptors share a similar overall structure consisting of a N-terminal signal sequence, a short cysteine-rich extracellular region (cysteine box), a single hydrophobic membrane-spanning domain and a cytoplasmic region containing a kinase domain. Furthermore, the type II receptors contain a S/T-rich C-terminal tail, which is absent in type I receptors. Another difference between type I and type II receptors is a highly conserved region of 30 amino acids in the juxtamembrane region, named the GS box based on a GSGSG motif. This GS box is found in the type I, but not in the type II receptors and contains five clustered S/T residues which are essential for signaling (Massagué, 1992; Wrana, *et al.*, 1994).

Binding of TGFβ family members to the type II receptor induces the formation of a hetero-tetrameric complex containing two type II and two type I receptors (Figure 2.2) (Wrana, *et al.*, 1994; Heldin, *et al.*, 1997; Kirsch, *et al.*, 2000). The kinase domain of type II receptors is highly phosphorylated. TGFβ type II receptor (TβRII) studies showed that this phosphorylation of the kinase domain is ligand-independent due to autophosphorylation and, possibly, due to the activity of other intracellular kinases (Wrana, *et al.*, 1994; Chen and Weinberg, 1995). After type I/type II receptor complex formation, the constitutively active type II receptors phosphorylate the type I receptors at the GS box (Wrana, *et al.*, 1994; Chen and Weinberg, 1995; Weis-Garcia and Massagué, 1996). For type I receptor activation, at least two of the S/T residues in the GS box have to be phosphorylated (Wieser, *et al.*, 1995). Next, downstream intracellular substrates are phosphorylated by the type I receptor. Thus, the type I receptors acts as a downstream substrate of the type II receptors and therefore determine the specificity of downstream signals induced by ligands of the TGFβ superfamily (Wrana, *et al.*, 1994; Chen and Weinberg, 1995).

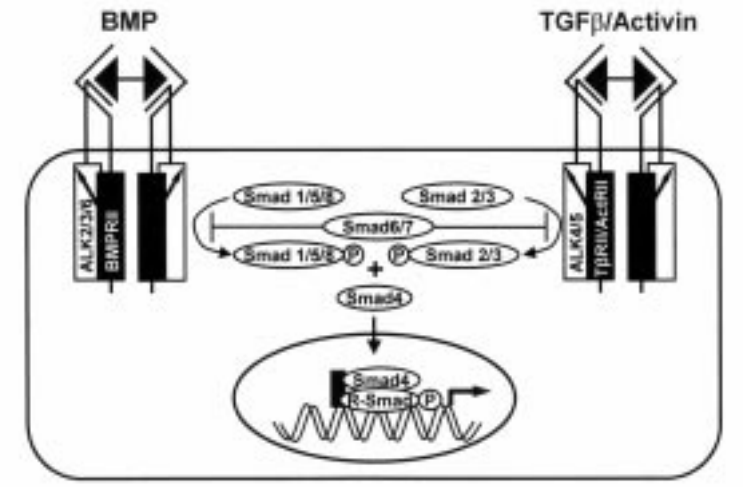


Figure 2.2: Signaling of TGFβ family members through the TGFβ/activin-like and BMP-like signaling pathway. TGFβ and activin activate Smad2 and/or Smad3 after binding to their type II receptors (TβRII, ActRII or ACTRIIB) which form a complex with ALK5 or ALK4. BMPs activate Smad1, Smad5 or Smad8 through a receptor complex containing BMPRII and ALK2, ALK3 or ALK6. Upon activation of the receptor-specific Smads (R-Smads), a complex with Smad4 is formed. This complex translocates to the nucleus and regulates gene transcription. The inhibitory Smad6 and Smad7 antagonize signaling. Smad7 inhibits both signaling pathways, whereas Smad6 preferentially inhibits BMP-like signaling. (Figure adapted from J.A. Visser.)

Until now, five type II en seven type I receptors have been identified in mammals. Table 2.1 shows an overview of ligands and their receptors. TGFβs signal through the TGFβ type II receptor (TβRII) (Attisano, *et al.*, 1993; Franzen, *et al.*, 1993), while the activin type II receptors type II and type IIB (ActRII and ActRIIB) are used by activins and BMPs (Attisano, *et al.*, 1992, 1993; Yamashita, *et al.*, 1995). In contrast, the BMP type II receptor (BMPRII) specifically signals for BMPs (ten Dijke, *et al.*, 1994; Rosenzweig, *et al.*, 1995; Yamashita, *et al.*, 1995). The type II receptor for AMH (AMHRII) is the fifth known type II receptor (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b).

Table 2.1: Ligand-receptor combinations of the TGFβ superfamily.

Ligand:	Type II receptor:	Type I receptor:	References:
TGFβs	TβRII	ALK5	Attisano *et al.*, 1993; Franzen *et al.*, 1993;
		ALK1	Chen and Massagué, 1999; Oh *et al.*, 2000
activins	ActRII	ALK4	Attisano *et al.*, 1992, 1993; Franzen *et al.*, 1993;
	ActRIIB	(ALK2)	Yamashita *et al.*, 1995
nodal	ActRIIB	ALK4	Reissmann *et al.*, 2001
		ALK7	
BMPs	ActRII	ALK2	Attisano *et al.*, 1992, 1993; Koenig *et al.*, 1994;
	ActRIIB	ALK3	ten Dijke *et al.*, 1994; Rosenzweig *et al.*, 1995;
	BMPRII	ALK6	Yamashita *et al.*, 1995
AMH	AMHRII	ALK2	Baarends *et al.*, 1994; di Clemente *et al.*, 1994;
		ALK3	Gouédard *et al.*, 2000; Clarke *et al.*, 2001; Visser *et al.*, 2001;
		ALK6	Jamin *et al.*, 2002

Based on similarities of the amino acid sequences, three different groups can be distinguished within the seven type I receptors. Activin-receptor like kinase (ALK)4 (also termed ActRIB), -5 (also termed TβRI) and –7 form one group. ALK4 signals for activins and nodal proteins which play an important role in mesendoderm formation and left-right patterning during vertebrate development, whereas ALK7 only signals for nodal (Attisano, *et al.*, 1992, 1993; Franzen, *et al.*, 1993; Reissmann, *et al.*, 2001). ALK5 serves as a type I receptor for TGFβs (Franzen, *et al.*, 1993). The second group is formed by ALK3 and ALK6 (also known as BMPRIA and BMPRIB respectively) which both are involved in BMP and AMH-signaling (Koenig, *et al.*, 1994; ten Dijke, *et al.*, 1994; Gouédard, *et al.*, 2000; Jamin, *et al.*, 2002). Finally, ALK1 and ALK2 form the third group of type I receptors. ALK2 (ActRIA) is a type I receptor for BMPs and AMH (ten Dijke, *et al.*, 1994; Rosenzweig, *et al.*, 1995; Yamashita, *et al.*, 1995; Clarke, *et al.*, 2001; Visser, *et al.*, 2001), while ALK1 signals for TGFβs in endothelial cells (Chen and Massagué, 1999; Oh, *et al.*, 2000).

2.3.2 Downstream mediators of TGFβ family signaling

The major downstream signaling molecules in TGFβ-signaling are the Smad proteins. These proteins can be divided into three subclasses: receptor-specific Smads (Smad1, 2, 3, 5 and 8), common-partner Smads (Smad4) or inhibitory Smads (Smad6 and 7). Receptor-specific and common-mediator Smad proteins contain two regions of homology, the N-terminal Mad homology 1 (MH1) domain and the C-terminal Mad homology domain 2 (MH2). The two domains are separated by a proline-rich region, named the linker region. Inhibitory Smads only contain the MH2 domain and linker region (Heldin, *et al.*, 1997).

Smad activation and signaling

The MH2 domain of receptor-specific Smads contains a conserved Ser-Ser-X-Ser motif which is phosphorylated by the type I receptor (Macias-Silva, *et al.*, 1996; Kretzschmar, *et al.*, 1997; Souchelnytskyi, *et al.*, 1998). Phosphorylation of this motif disrupts the interaction between the MH1 and MH2 domains, thereby activating the protein. The activated receptor-specific Smad forms a hetero-oligocomplex with the common-mediator Smad4 and translocates to the nucleus where it regulates gene transcription either through direct binding to specific DNA-sequences or through interaction with other DNA-binding proteins (Baker and Harland, 1996; Lagna, *et al.*, 1996; Liu, *et al.*, 1996, 1997; Hata, *et al.*, 1997; Meersseman, *et al.*, 1997; Wu, *et al.*, 1997, 2001; Kawabata, *et al.*, 1998; Lebrun, *et al.*, 1999; Masuyama, *et al.*, 1999; Qin, *et al.*, 2001, 2002). The MH1 domain acts as a negative regulator of Smad activation since its interaction with the MH2 domain prevents binding of the non-phosphorylated receptor-specific Smad to a common-mediator Smad. Furthermore, the MH1 domain is involved in maintaining a cytoplasmic localization of non-activated receptor-specific Smads, because receptor-specific Smads lacking the MH1 domain translocate to the nucleus without being phosphorylated (Baker and Harland, 1996).

Based upon which Smad is used by TGFβ superfamily members, two main signaling pathways can be distinguished. Smad2 and Smad3 are activated by TGFβs and activins through ALK4, ALK5 or ALK7 (TGFβ/activin-like signal pathway). On the other hand, Smad1, Smad5 and Smad8 are activated by BMPs through ALK2, ALK3 and ALK6, referred to as the BMP-like signal pathway (Figure 2.2). The only known exception is ALK1, a type I receptor for TGFβ, which activates Smad1 instead of Smad2 or Smad3. Activation of Smad1 by ALK1 is possible due to sequences in the kinase domain of this receptor, which also can be found in ALK2 (Chen and Massagué, 1999). Furthermore, recently it has been reported that GDF9 binds to the BMPRII while activating Smad2 (Vitt, *et al.*, 2002; Kaivo-Oja, *et al.*, 2003; Roh, *et al.*, 2003). Until now, the type I receptor involved in GDF9-signaling has not been identified. However, these results suggest that type I receptors signaling in the BMP-like signaling pathway can phosphorylate Smad2 or that GDF9 recruits a type I receptor from the TGFβ-like signaling pathway into a complex with BMPRII.

Inhibitory Smads

The signaling of TGFβ family members through receptor-specific and common-mediator Smads is antagonized by the inhibitory Smad6 and Smad7. Smad7 is a general inhibitor of TGFβ/activin-like and BMP-like signaling pathways (Hayashi, *et al.*, 1997; Nakao, *et al.*, 1997; Itoh, *et al.*, 1998; Ishisaki, *et al.*, 1999; Hanyu, *et al.*, 2001), while Smad6 preferentially inhibits the BMP-like signaling pathway (Figure 2.2) (Hata, *et al.*, 1998; Itoh, *et al.*, 1998; Ishisaki, *et al.*, 1999; Hanyu, *et al.*, 2001).

The antagonistic effect of Smad6 and Smad7 on signaling is achieved by binding of these inhibitory Smads to the activated type I receptor, in this way preventing recruitment and phosphorylation of receptor-specific Smads (Hayashi, *et al.*, 1997; Imamura, *et al.*, 1997; Nakao, *et al.*, 1997; Souchelnytskyi, *et al.*, 1998; Lebrun, *et al.*, 1999; Hanyu, *et al.*, 2001). However, Smad6 also blocks Smad-signaling by forming a complex with the receptor-activated Smad1, thereby inhibiting the Smad1-Smad4 complex formation (Hata, *et al.*, 1998). In addition, Smad6 inhibits transcriptional activity of receptor-specific Smads by interacting with transcription factors (Bai, *et al.*, 2000).

Smad6 and Smad7 have an important role in the control of signaling of TGFβ superfamily members. Overexpression of these inhibitory Smads is found in cancer cells and expression level of Smad7 is a prognostic marker in colorectal cancer (Kleeff, *et al.*, 1999a, 1999b; Ozawa, *et al.*, 2001; Boulay, *et al.*, 2003; Cerutti, *et al.*, 2003). The expression of both inhibitory Smads is stimulated by TGFβ superfamily members and in this way a negative feedback loop is formed (Nakao, *et al.*, 1997; Afrakhte, *et al.*, 1998; Ishisaki, *et al.*, 1998, 1999; Itoh, *et al.*, 1998; Takase, *et al.*, 1998).

Other downstream mediators of TGFβ superfamily signaling

In addition to the Smad proteins as downstream mediators, TGFβ superfamily members may also signal through other signaling pathways. The most important alternative signaling pathway is the mitogen-activated protein kinase (MAPK) pathway, which is involved in cell proliferation and differentiation (Mulder, 2000). TGFβ can activate all three distinct groups of MAPKs, the extracellular signal-regulated kinases (ERKs), c-JunN-terminal kinases/stress-activated protein kinases (JNKs/Sapks) and p38 MAPK (Mulder, 2000), while BMPs can activate the p38 MAPK- and ERK-signaling pathway (Gallea, *et al.*, 2001; Goswami, *et al.*, 2001; Lai and Cheng, 2002; Lee, *et al.*, 2002). However, the exact mechanism of activation of MAPKs by TGFβ superfamily members remains unknown. Furthermore, also signaling through trimeric guanine-nucleotide binding proteins (G-proteins) has been described (Howe, *et al.*, 1989; Kataoka, *et al.*, 1993).

Recently, the β-catenin signaling pathway was identified as a signaling pathway through which AMH, a member of the TGFβ superfamily, mediates its effect. During Müllerian duct regression (see section 2.5), β-catenin accumulates in the cytoplasm of mesenchymal cells which are exposed to AMH (Allard, *et al.*, 2000). Furthermore, it is suggested that β-catenin together with lymphoid enhancer factor 1 (LEF1) may influence gene expression in the mesenchymal cells of the Müllerian ducts (Allard, *et al.*, 2000). However, the exact mechanism of the activation of β-catenin by AMH still needs to be elucidated.

2.4 MODULATION OF TGFβ-SIGNALING

Signaling of TGFβ superfamily members can be regulated, both positively and negatively, at several levels. Regulation by Smad6 and Smad7 at the receptor level has already been described in section 2.3.2. However, also binding of the ligand to its receptor can be regulated as well as signaling downstream of the receptor (Figure 2.3).

Ligand binding of TGFβ superfamily members to the receptor can be inhibited as well as facilitated. Several antagonists of TGFβ superfamily members, such as follistatin, noggin, chordin and members of the DAN family (e.g. cerberus, drm/gremlin, dante) have been identified. These proteins achieve their antagonistic effect by binding to the ligand, thereby preventing binding to the receptors. Follistatin and follistatin-like protein are glycoproteins that bind activins and BMPs (de Winter, *et al.*, 1996; Iemura, *et al.*, 1998; Tsuchida, *et al.*, 2000). Noggin, chordin and DAN family members block interaction of BMPs but not of TGFβs or activins to their receptor (Piccolo, *et al.*, 1996, 1999; Zimmerman, *et al.*, 1996; Hsu, *et al.*, 1998; Yokouchi, *et al.*, 1999). Interestingly, the antagonistic effect of chordin can be overcome by bone morphogenetic protein 1 (BMP1, also known as Xolloid in Xenopus).

BMP1 is a metalloproteinase that cleaves free chordin and chordin bound to BMPs, thereby releasing the BMPs (Piccolo, *et al.*, 1997).

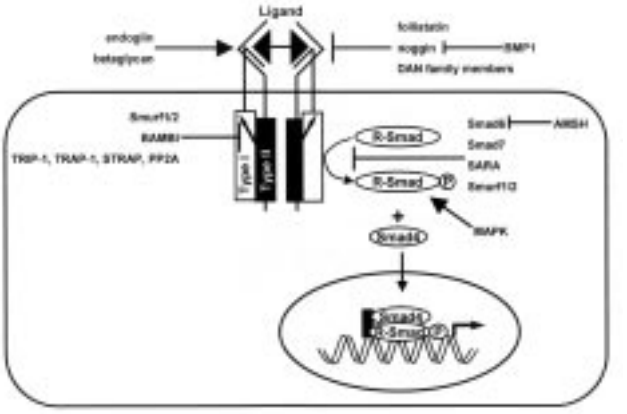


Figure 2.3: Modulation of signaling by TGFβ superfamily members.
Signaling can be modulated at three different levels. Binding of the ligand to the receptor is stimulated by endoglin and betaglycan, while follistatin, noggin and DAN family members inhibit ligand-receptor binding. The inhibitory effect of noggin can be antagonized by BMP1. Activation of the type I receptor is inhibited by Smurf proteins, BAMBI and TRIP-1, TRAP-1, STRAP or PP2A. Furthermore, activation of the receptor-specific Smads (R-Smads), which results in gene transcription regulation, can be modulated. Smad6, Smad7, Smurf proteins and SARA inhibit activation of receptor specific Smads. AMSH antagonizes the action of Smad6. In contrast, MAPK has shown to be involved in activation of R-Smads.

The binding of ligand to the receptors is positively regulated by endoglin and betaglycan (also called TGFβ type III receptor). Both endoglin and betaglycan are membrane bound proteins which enhance TGFβ binding to the type II receptor by binding the ligand and presenting it to the type II receptor. Endoglin may be a specific accessory protein for ALK1 because mutations in these genes result in similar phenotypes. However, the ligand for endoglin and ALK1 may not have been identified yet, since only a weak interaction of TGFβ is found for these receptors (Massagué, 1998).

At the receptor level, signaling is negatively regulated by binding of Smad6 and Smad7 to the type I receptor, thereby preventing phosphorylation of Smad proteins (see section 2.3.2). Furthermore, Smad6 and Smad7 inhibit TGFβ-signaling by inducing ubiquitination of the type I receptors. This is achieved by binding of Smad6 and Smad7 to Smad ubiqitination regulatory factor 1 and 2 (Smurf1 and Smurf2). This Smurf-Smad complex moves to the membrane and binds to the type I receptor, where Smurf induces degradation of the type I receptor by ubiquitination of this receptor (Kavsak, *et al.*, 2000; Ebisawa, *et al.*, 2001; Hanyu, *et al.*, 2001). Interestingly, an antagonist of Smad6 has been identified, which is called associated molecule with SH3 domain of a signal-transducing adaptor molecule (AMSH). Its antagonistic effect is achieved by binding Smad6, thereby preventing Smad6 to bind to the activated type I receptor (Itoh, *et al.*, 2001) and regulates the inhibitory effect of Smad6. Another protein which inhibits downstream signaling is BMP and activin membrane-bound inhibitor (BAMBI). This protein has a high homology with the TGFβ superfamily type I receptors but lacks the intracellular kinase domain, which is necessary for type I receptor activation. Therefore, BAMBI inhibits signaling of TGFβ superfamily members by forming a complex with type I receptors

(except ALK2) and thereby preventing formation of active receptor complexes (Onichtchouk, *et al.*, 1999). In addition, several other receptor-interacting proteins have been found which negatively modulate signaling of TGFβ superfamily members, such as TGFβ-receptor interacting protein 1 (TRIP-1), TβRI-associated protein 1 (TRAP-1), serine/threonine kinase receptor-associated protein (STRAP) and a regulatory subunit of protein phosphatase 2A (PP2A). However, little is known about their mechanisms of action (reviewed by Massagué and Chen, 2000).

Downstream of the receptors, signaling is regulated by affecting the access of Smads to the receptor. Smad anchor for receptor activation (SARA) binds receptor-specific Smad proteins and presents these Smads to the activated receptor complex. In this way, phosphorylation of receptor-specific Smads is increased, as was shown in cells overexpressing SARA (Tsukazaki, *et al.*, 1998). In addition, Smad levels in the cell are regulated by the ubiquitin ligases Smurf1 and Smurf2. Smurf1 interacts preferentially with Smads which signal in the BMP-like signaling pathway (Smad1, 5 and 8), whereas Smurf2 binds Smads signaling in the TGFβ/activin-like signaling pathway (Smad2 and 3). Upon the formation of the receptor-specific Smad/Smurf complex, Smad proteins are ubiquitinated and degraded (Zhu, *et al.*, 1999; Bonni, *et al.*, 2001; Ebisawa, *et al.*, 2001). Furthermore, it has been shown that the MAPK pathway modulates TGFβ-signaling at the level of Smad proteins. The Smad1 protein contains four ERK consensus phosphorylation sites, which are necessary for activation of the SBE-Luc promotor construct by TGFβ (Mulder, 2000).

2.5 AMH SIGNAL TRANSDUCTION

AMH plays an important role during folliculogenesis in the ovary, since it inhibits primordial follicle recruitment and lowers the sensitivity of large pre-antral and antral follicles for FSH (see Chapter 1). However, until recently little was known about the signaling pathway by which AMH achieves these effects. The only identified mediator in AMH-signaling was the AMHRII (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b). Most knowledge about the AMH-signaling pathway was obtained by studying Müllerian duct regression.

The Müllerian ducts , the *anlagen* of the female genital tract, are formed during embryogenesis. AMH, produced by the Sertoli cells in the fetal testis, causes the regression of the Müllerian ducts by inducing apoptosis of the epithelial cells (Jost, 1947; Visser, *et al.*, 1998; Roberts, *et al.*, 1999; Allard, *et al.*, 2000). However, the AMHRII is expressed by the mesenchymal and not by the epithelial cells of the Müllerian ducts (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b), suggesting that AMH induces a mesenchymal signal to the epithelial cells, which causes apoptosis in these cells. Indeed, results of *in vitro* co-culture of mesenchymal and epithelial cells of Müllerian ducts suggest that AMH stimulates the expression of a death factor or represses the expression of a survival factor in the mesenchymal cells which, after being secreted, influences the survival of the epithelial cells (Figure 2.4) (Roberts, *et al.*, 1999).

Ultimate proof that the AMHRII is the true type II receptor for AMH, was obtained by the identification of several mutations in AMHRII gene in persistent Müllerian duct syndrome (PMDS) patients which phenotype is similar to mutations in the AMH gene (Knebelmann, *et al.*, 1991; Carré-Eusèbe, *et al.*, 1992; Imbeaud, *et al.*, 1994, 1995, 1996).

PMDS is a rare form of male pseudohermaphrodism in which the Müllerian ducts have failed to regress. Furthermore, male mice in which the AMHRII gene was disrupted, retain their Müllerian ducts, similar to the AMH *null* mice (Behringer, *et al.*, 1994; Mishina, *et al.*, 1996). In addition, Müllerian ducts regress in female mice which overexpress AMH during embryogenesis (Behringer, *et al.*, 1990). These *in vivo* data show that the AMHRII is the type II receptor for AMH in Müllerian duct regression.

Recent studies have shed a light on the identity of the AMH type I receptor(s) and its downstream signaling components. Results of these studies are described below.

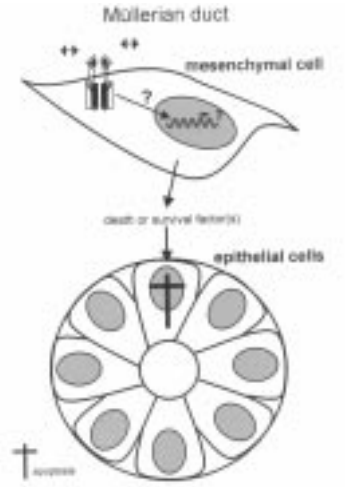


Figure 2.4: AMH induces apoptosis of epithelial cells in Müllerian duct regression.
AMHRII is expressed by the mesenchymal cells of the Müllerian ducts. By stimulating the expression of a death factor or by inhibiting the expression of a survival factor in the mesenchymal cells, apoptosis in the epithelial cells is induced. (Figure adapted from Roberts et. al., Copyright 2002, The Company of Biologists Ltd).

2.5.1 AMH-signaling in vitro

As described in section 2.3, two main signaling pathways can be distinguished for TGFβ family members: the TGFβ/activin-like or BMP-like signaling pathway. Recently, it was found that also AMH signals through the BMP-like signaling pathway (Visser, *et al.*, 2001). In the mouse embryonic carcinoma cell line P19, AMH stimulates the BMP-responsive mouse Tlx-2 promoter reporter but is unable to activate the TGFβ/activin-responsive A3-Luc promoter construct (Visser, *et al.*, 2001). Similar results were obtained by two other groups who found that AMH stimulates the BMP-responsive Xvent promotor construct in P19 cells and not the TGFβ/activin-responsive 3TP-Luc construct (Gouédard, *et al.*, 2000; Clarke, *et al.*, 2001). The stimulation could only be achieved upon cotransfection of the AMHRII but not with other type II receptors like BMPRII or ActRII. These *in vitro* data clearly show that AMHRII is the only type II receptor through which AMH signals (Visser, *et al.*, 2001).

Three type I receptors ALK2, ALK3 and ALK6 have been identified as candidate AMH type I receptors by several studies. Transfection of all type I receptors in P19 cells together with the Tlx2-Luc reporter revealed that cotransfection of ALK2 enhanced AMH-signaling. Cotransfection of ALK6 only showed a slight increase in AMH-signaling whereas ALK3 even had an inhibitory effect on AMH-signaling (Visser, *et al.*, 2001).

The preference for ALK2 in AMH-signaling was supported in a dominant-negative strategy in P19 cells. Increasing concentrations of dominant-negative ALK2 or ALK6 receptors, which cannot be activated due to a truncated kinase domain, were transfected in P19 cells. Increasing amounts of dominant-negative ALK2 inhibited AMH induced Tlx2-Luc activation, while the dominant-negative ALK6 had no effect (Visser, *et al.*, 2001). Similarly, the truncated ALK2, but not ALK3 or ALK6, was able to inhibit AMH-signaling in P19 cells using the Tlx2-Luc promoter (Clarke, *et al.*, 2001). These results suggest that ALK2 could be a type I receptor involved in AMH-signaling.

However, also ALK6 has been suggested to be an AMH type I receptor. An AMH-dependent interaction between AMHRII and ALK6 was observed in the CHO-3W cell line, whereas no interaction between AMHRII and ALK2 or ALK3 was found. Furthermore, transfection of a dominant-negative ALK6 in CHO-3W cells inhibited AMH-induced stimulation of the Xvent-Luc reporter (Gouédard, *et al.*, 2000) suggesting a role for ALK6 in AMH-signaling. However, a potential problem with overexpression of type I receptors in cell lines is the formation of non-specific receptor interactions. Indeed, overexpression of AMHRII results in ligand-independent interactions with all type I receptors (Visser, 2003). The P19 cell line contains an endogenous type I receptor for AMH, since AMH can activate the Tlx2-Luc reporter after transfection of AMHRII in these cells (Visser, *et al.*, 2001). Therefore, in order to determine whether ALK2 or ALK6 is a AMH type I receptor, these cells were treated with antisense oligos for ALK2 or ALK6. In agreement with the results of the dominant-negative type I receptor experiments in P19 cells, the morpholino antisense ALK2 oligomers completely inhibited AMH-induced Tlx-2 promoter activity, whereas antisense ALK6 treatment had no effect (Visser, *et al.*, 2001). These results prove that ALK2, besides being a BMP type I receptor, also functions as a AMH type I receptor in P19 cells.

As described in section 2.3.2, BMP-like signaling is mediated through the downstream signaling molecules Smad1, Smad5 and Smad8. Therefore, these Smads are also candidate downstream mediators in AMH-signaling. Indeed, Smad1 and Smad5 augmented AMH-signaling in P19 cells and this signaling was inhibited by dominant-negative Smad5. In contrast, Smad2, a TGFβ/activin-like signaling Smad, was unable to affect AMH-signaling in P19 cells (Visser, *et al.*, 2001). Using the Gal4-Smad1 fusion protein with a Gal4-reporter construct, Clarke and colleagues showed that AMH-signaling can be mediated through Smad1 (Clarke, *et al.*, 2001). Furthermore, phosphorylation of Smad1 was shown in Sertoli- and Leydig derived cell lines upon AMH exposure (Gouédard, *et al.*, 2000). These results show that Smad1 and Smad5 are AMH downstream mediators.

In conclusion, *in vitro* experiments have shown that AMH signals through the BMP-like but not the TGFβ/activin-like signaling pathway. In the next section experiments are described in which AMH-signaling in vivo was investigated.

2.5.2 AMH-signaling in vivo

The ultimate proof for a role of the candidate type I receptors in AMH-signaling might be obtained from knockout mice. Unfortunately, genetic disruption of most type I receptors results in early embryonic lethality. Thus, no information in Müllerian duct regression or gonadal function could be obtained from these mice. One exception is the ALK6 *null* mice, which are viable but display major defects in the appendicular skeleton (Yi, *et al.*, 2000). No abnormalities in Müllerian duct regression in male ALK6 *null* mice are found (Clarke, *et al.*, 2001). Furthermore, follicular development seems

to proceed normally up to the pre-ovulatory stage where the cumulus expansion is impaired, causing the infertility of ALK6 *null* female mice (Yi, *et al.*, 2001). In Booroola sheep, a naturally occurring mutation in the kinase domain of the ALK6 gene was found, which results in an increased ovulation rate and litter size (Davis, *et al.*, 1982; Piper and Bindon, 1982; Souza, *et al.*, 2001). The ALK6-mutation in Booroola sheep causes a less active ALK6, resulting in a decreased signaling of BMPs (Fabre, *et al.*, 2003). This could explain the differences found in phenotype with the ALK6 *null* mice, which do not contain a (partially) active ALK6 receptor. Ovaries of homozygous carriers of the Booroola mutation contain the same number of antral and non-atretic follicles compared to non-carriers. However, the number of pre-ovulatory follicles is increased. Furthermore, these pre-ovulatory follicles are smaller and contain less granulosa cells than those in control animals (McNatty, *et al.*, 1985). Thus, the mutation in the ALK6 gene mainly affects the pre-ovulatory follicles. Therefore, like the ALK6 *null* mice, this sheep model does not suggest involvement of ALK6 in AMH-signaling, since AMH-effects are found on primordial and small non-selected follicles.

The conclusion that ALK6 is not involved in Müllerian duct regression, was confirmed by urogenital ridge culture. Tsuji and colleagues showed that treatment of female embryonic urogenital ridges with AMH resulted in the regression of the Müllerian ducts (Tsuji, *et al.*, 1992). Inhibition of the ALK6 expression by morpholino antisense ALK6 did not prevent the regression of the Müllerian ducts, which shows that ALK6 expression is not necessary for this regression (Visser, *et al.*, 2001). However, the regression of the Müllerian ducts could be prevented using a morpholino antisense ALK2, suggesting that ALK2 is involved in Müllerian duct regression and thus AMH-signaling (Visser, *et al.*, 2001).

To circumvent embryonic lethality an ALK3 conditional knockout mouse has been developed. This conditional knockout mouse was generated using the *Cre-loxP* system. An AMHRII-Cre mouse, carrying the Cre gene under control of the AMHRII promoter, was crossbred to a mouse carrying the ALK3 gene flanked by *loxP* sites (Jamin, *et al.*, 2002). Thus, in the conditional knockout mice, cells which express the AMHRII do not express ALK3 and this results in male mice with PMDS showing that ALK3 is necessary *in vivo* for Müllerian duct regression (Jamin, *et al.*, 2002). However, ALK3 is not the only type I receptor through which Müllerian duct regression can be induced. When the ALK3 conditional knockout mouse is crossbred to a mice overexpressing AMH no PDMS is found anymore, suggesting that another type I receptor may partially rescue the PMDS phenotype (Jamin, *et al.*, 2003). One candidate is ALK2, since this receptor is involved in Müllerian duct regression *in vitro* cultured urogenital ridges (Visser, *et al.*, 2001).

In summary, the *in vivo* and *in vitro* data show that AMH may signal through ALK2 and ALK3, but not through ALK6 in the Müllerian ducts. In the ovary, the AMH-signaling pathway still needs to be determined. However, based on the ovarian phenotype in ALK6 *null* mice, it is unlikely that ALK6 is the type I receptor involved in AMH-signaling. Future studies should reveal the type I receptor(s) for AMH in the ovary.

2.5.3 Expression of type I receptors and Smads in AMH target tissue

Involvement of candidate type I receptors and Smad proteins in AMH-signaling requires its expression in AMH target tissue. AMHRII is expressed in the mesenchymal cells of the Müllerian ducts and in the granulosa cells of healthy primary and pre-antral follicles in mouse and rat ovary (Baarends, *et al.*, 1994;

di Clemente, *et al.*, 1994b). Furthermore, expression of AMHRII is found in theca cells of rat pre-antral and small antral follicles (Ingraham, *et al.*, 2000).

Initial studies mainly focused on expression patterns of AMH candidate type I receptors in the Müllerian ducts. ALK2 and ALK6 are both expressed in the Müllerian ducts, however in different cell types. *In situ* hybridizations showed ALK2 expression in the mesenchymal cells surrounding the Müllerian ducts in mice and rats, thereby colocalizing with the AMHRII (He, *et al.*, 1993; Clarke, *et al.*, 2001; Visser, *et al.*, 2001). In contrast, ALK6 mRNA expression is detected in the epithelial layer and not in the mesenchymal cells of the Müllerian ducts (Dewulf, *et al.*, 1995; Clarke, *et al.*, 2001). Furthermore, ALK2 is detected in male and female urogenital ridges and in fetal gonads of mice, while ALK6 expression is absent in fetal gonads and is expressed at a lower level in the urogenital ridge (Visser, *et al.*, 2001). Thus, also based on the expression patterns in the Müllerian ducts, ALK2 is more likely to be involved in AMH-signaling than ALK6. The expression pattern of ALK3 in the Müllerian ducts still needs to be determined. However, based on the ALK3 conditional knockout mice, this receptor is at least expressed by the mesenchymal cells in the Müllerian duct, which also express the AMHRII (Jamin, *et al.*, 2002).

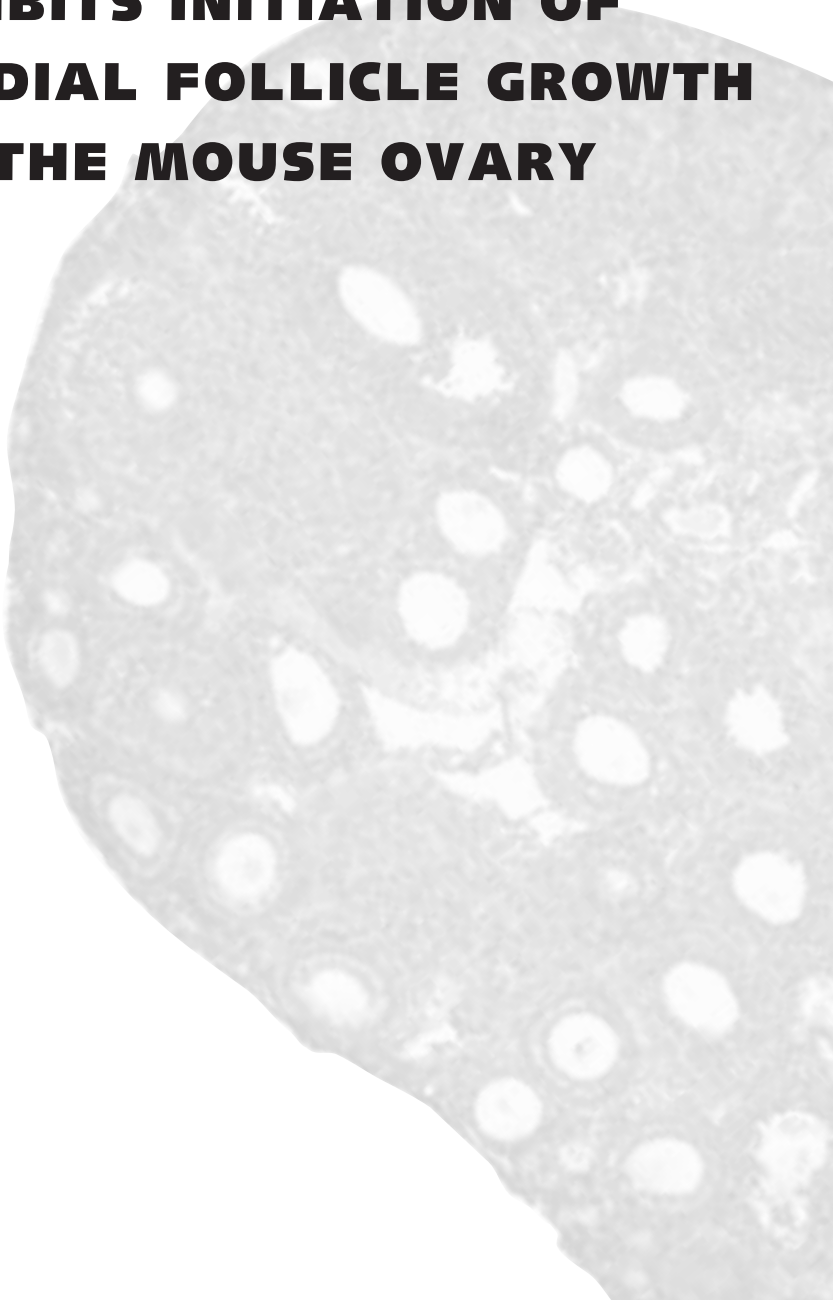
In the ovary, ALK6 is expressed in oocytes of primordial and growing follicles and is highly expressed in granulosa cells of antral follicles (Yi, *et al.*, 2001; Erickson and Shimasaki, 2003). In addition, a low expression of ALK6 is detected in theca cells (Erickson and Shimasaki, 2003). This expression pattern differs from AMHRII, which is expressed in granulosa cells of healthy primary and pre-antral follicles (Baarends, *et al.*, 1995b) or in the theca cells of pre-antral and antral follicles (Ingraham, *et al.*, 2000). ALK3 is expressed in granulosa cells of primary follicles, secondary and tertiary follicles, while oocytes of all follicles express this type I receptor. Furthermore, expression of ALK3 in theca cells from pre-antral and larger follicles has been described (Erickson and Shimasaki, 2003, see also Chapter 5). Based on this expression pattern, ALK3 could mediate AMH-signaling, since it is coexpressed with AMHRII. The expression pattern of the third BMP-like signaling type I receptor, ALK2, still needs to be determined in the ovary (see Chapter 5).

The AMH downstream signaling proteins Smad1, Smad5 and Smad8 are expressed by the mesenchymal cells of the Müllerian ducts, albeit that Smad5 is expressed at a lower level than Smad1 or Smad8 (Clarke, *et al.*, 2001). Interestingly, a higher expression level of Smad8 mRNA was found in mesenchymal cells of the Müllerian ducts in male compared to female mice fetuses, suggesting that AMH may positively regulate its downstream signaling component (Clarke, *et al.*, 2001). Thus, based on the expression patterns, all three Smad proteins can be involved in AMH-signaling. The expression patterns of the AMH receptor-specific Smad proteins in fetal and adult gonads still needs to be determined.

In conclusion, the expression patterns of the type I receptors involved in the BMP-like signaling pathway, suggest that ALK2 and ALK3 are strong candidate type I receptors for AMH-signaling. Involvement of ALK6 in mediating the AMH signal cannot completely be excluded, but is less likely. These findings are in agreement with observations from several *in vitro* and *in vivo* data. In addition, all three Smad proteins involved in the BMP-like signaling pathway (Smad1, 5 and 8) are candidate downstream mediators for AMH-signaling.

Chapter 3

ANTI-MÜLLERIAN HORMONE INHIBITS INITIATION OF PRIMORDIAL FOLLICLE GROWTH IN THE MOUSE OVARY



Chapter 3
ANTI-MÜLLERIAN HORMONE INHIBITS INITIATION OF PRIMORDIAL FOLLICLE GROWTH IN THE MOUSE OVARY

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ABSTRACT

Recruitment of primordial follicles is essential for female fertility, however, the exact mechanisms regulating this process are largely unknown. Earlier studies using anti-Müllerian hormone-deficient mice, suggested that anti-Müllerian hormone (AMH) is involved in the regulation of primordial follicle recruitment. We tested this hypothesis in a neonatal ovary culture system, in which ovaries from 2-day-old C57Bl/6J mice were cultured for 2 or 4 days in the absence or presence of AMH. Ovaries from 2-day-old mice contain multiple primordial follicles, some naked oocytes, and no follicles at later stages of development. We observed that in the cultured ovaries, either non-treated or AMH-treated, follicular development progressed to the same extent as in *in vivo* ovaries of comparable age, confirming the validity of our culture system. However, in the presence of AMH, cultured ovaries contained 40% fewer growing follicles compared to control ovaries. A similar reduction was found after 4 days of culture. Consistent with these findings, we noted lower inhibin α-subunit expression in AMH-treated ovaries compared to untreated ovaries. In contrast, expression of AMHRII and the expression of oocyte markers GDF9 and ZP3 were not influenced by AMH.

Based on the results, we suggest that AMH inhibits initiation of primordial follicle growth and therefore functions as an inhibitory growth factor in the ovary during these early stages of folliculogenesis.

INTRODUCTION

For female mammals, the finite number of primordial follicles available during reproductive life is established during fetal development (human) (Peters, 1969) or just after birth (mouse, rat) (Peters, 1978; Ueno, *et al.*, 1989). Factors involved in the initiation of primordial follicle growth, a process also known as initial recruitment (McGee and Hsueh, 2000), have long remained unknown. Recently, however, it was shown that stem cell factor (SCF, or kit-ligand) (Parrott and Skinner, 1999), growth and differentiation factor 9 (GDF9) (Vitt, *et al.*, 2000b), basic fibroblast growth factor (bFGF) (Nilsson, *et al.*, 2001) and nerve growth factor (NGF) (Dissen, *et al.*, 2001) promote initiation of primordial follicle growth. Also anti-Müllerian hormone (AMH) has been implicated in primordial follicle recruitment from analysis of AMH null ovaries (Durlinger, *et al.*, 1999). AMH is a member of the transforming growth factor-β (TGFβ) superfamily of growth and differentiation factors (Cate, *et al.*, 1986) and is responsible for Müllerian duct regression during male fetal development (Jost, 1947). This ligand-receptor system is also found in the ovary with expression of both the AMH ligand and its type II receptor (AMHRII) postnatally in granulosa cells of mainly non-atretic pre-antral and small antral follicles (Hirobe, *et al.*, 1992; Baarends, *et al.*, 1995a). In addition, AMHRII is also expressed in the prenatal ovary (Baarends, *et al.*, 1994). Examination of ovarian follicles in AMH-deficient female mice revealed lower numbers of primordial follicles and more growing follicles compared to wild-type littermates. These findings led to the proposal that AMH inhibits primordial follicle recruitment (Durlinger, *et al.*, 1999).

In this study, we examined the ability of AMH to inhibit primordial follicle recruitment in cultured neonatal mouse ovaries. To this end, we developed a neonatal ovary culture system, in which follicular development progresses to the same extent as in *in vivo* ovaries of comparable age. In this system we characterized primordial follicle recruitment by the determination of the number of primordial and growing follicles in neonatal ovaries after 2 and 4 days of culture in the presence or absence of recombinant AMH. The effects of AMH on primordial follicle recruitment was also investigated by assessing AMHRII and inhibin α-subunit transcript expression and additional oocyte-specific markers, such as growth and differentiation factor 9 (GDF9) and zona pellucida protein 3 (ZP3).

Collectively, results from these studies suggest that AMH is a potent inhibitor of primordial follicle recruitment.

MATERIALS AND METHODS

AMH production

Human embryonic kidney 293 (HEK293) cells were stably transfected with a cDNA encoding His-tagged rat AMH inserted in the pRc/CMV expression vector. The AMH cDNA contained an optimized cleavage site that yields maximal amounts of cleaved mature AMH (Nachtigal and Ingraham, 1996; Ingraham, *et al.*, 2000). HEK293 cells were cultured in DMEM/F-12 (Gibco, Life Technologies Ltd, Paisley, Scotland) supplemented with 5% fetal calf serum (FCS), penicillin (400 IU/ml), streptomycin (0.4 mg/ml) and neomycin G418 (0.4 mg/ml). At a cell confluence of about 80-90%, the medium was replaced by medium without FCS.

After 4 days the medium was collected and proteins with a molecular mass above 10 kDa were concentrated approximately 35-fold using a Centriprep10 filter system (Millipore Corp., Bedford, MA).

The amount of AMH was measured by ELISA making use of the His-tag, with the Biorad TMB Peroxidase ElA substrate kit (Biorad, Life Science Groups, Hercules, CA). The primary antibody, pentaHis monoclonal antibody (raised in rabbit; Qiagen GmbH, Hilden, Germany), was used at 100 ng/ml. The secondary antibody, a goat-anti-mouse IgG peroxidase conjugate (Sigma Chemical Co., St. Louis, MO), was used at a 1:1000 dilution. The amount of AMH was calibrated using the same standard preparation of His-tagged AMH in every ELISA and was expressed in arbitrary units. The concentrated supernatant of wild-type HEK293 cells was used as control medium.

The presence or absence of AMH in the media used in the experiments was investigated by Western blotting using a primary polyclonal antibody against AMH (raised in goat; C-20, Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:2000 dilution and a secondary peroxidase-conjugated mouse-anti-goat/sheep antibody (Sigma) at a dilution of 1:10000.

The AMH preparations (800 U/ml) were shown to be bioactive using a Müllerian duct regression assay (Nachtigal and Ingraham, 1996; Ingraham, *et al.*, 2000, data not shown).

Animals and ovary organ culture

C57Bl/6J female mice were used in all experiments described in this report. For the ovary organ culture experiments ovaries of 2-day-old females were used, since these ovaries predominantly contain primordial follicles, some naked oocytes and no growing follicles. Day of birth was denoted as day 1. The animals were obtained from the Animal Facility (EDC) of the Faculty of Medicine and Health Sciences of the Erasmus University Rotterdam and were kept under standard animal housing conditions in accordance with the NIH guidelines for the Care and Use of Experimental Animals. Animals were sacrificed by decapitation.

Ovaries of 2-day-old mice were either immediately fixed in Bouin's fixative for morphological examination and follicle counting, or snap-frozen in liquid nitrogen for RNA isolation, or placed in culture. An organ culture method was used as described by Cooke (Cooke, *et al.,* 1987), which was modified in our laboratory with the help of Dr. Judith Emmen (Emmen, *et al.*, 2000). The ovaries, together with some surrounding tissue, were removed from the abdomen under a dissection microscope. The tissue was placed in watch-glasses containing phosphate-buffered saline (PBS). With the help of two syringe needles the tissue surrounding the ovaries, including the capsule in which the ovary is enclosed (peri-ovarian sac), was removed. Since large inter-animal variation in the number of follicles per ovary was observed (see Results section), ovaries of each animal were matched and one was used for control cultures and the other for treated cultures. Ovaries were cultured in a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA). Each well contained 0.5 ml DMEM/F-12 medium with GLUTAMAX-I (Gibco) supplemented with 1% (v/v) charcoal-stripped fetal calf serum (CS-FCS), insulin (10 µg/ml) and transferrin (10 µg/ml; Sigma). Penicillin (250 IU/ml), streptomycin (0.25 mg/ml) and fungizone (3.1 µg/ml) (Bio Whitaker, Walkersville, MD) were added to the culture medium to prevent bacterial contamination. No effect of the stripped FCS was found in the sensitive fetal Müllerian duct regression assay, while by Western blotting no AMH could be detected in the stripped CS-FCS. Each ovary was placed in a small droplet of medium (approximately 15 µl) on a piece of 0.4 µm Millicel-CM filter (Millipore) of approximately 1 cm^2, floating on the culture medium.

The ovaries were cultured at 37C in a humidified atmosphere containing 5% CO_2 for 2 or 4 days. Of each pair of ovaries (derived from one animal) one ovary was cultured in medium containing concentrated medium of wild-type HEK293 cells (control), while the other ovary was cultured in medium containing concentrated medium of HEK293 cells producing rat AMH (800 U/ml).

After 2 days of culture the medium was removed and fresh medium was added. At the end of the culture period, the ovaries were either fixed for 2 hours in Bouin's fixative or snap frozen in liquid nitrogen and stored at –80C. Ovaries of 2-, 4- and 6-day-old C57Bl/6J mice were used for comparison of the development of follicles in the culture experiments, hereby focusing on the developmental stage of the follicles. No comparison was made regarding the number of primordial and growing follicles between cultured ovaries and freshly isolated ovaries of comparable age.

Ovarian histology and follicle counting

To investigate the development of ovarian follicles in the cultured ovaries and to compare it with follicle development *in vivo*, fresh and cultured ovaries were fixed for 2 h in Bouin's fixative, embedded in paraffin after routine histological procedures, and 8 μm serial sections were mounted on slides and stained with hematoxylin and eosin.

Primordial follicles are non-growing follicles and consist of an oocyte partially or completely encapsulated by flattened squamous pregranulosa cells. Early primary follicles have initiated development and contain at least one cuboidal (enlarged) granulosa cells (Pedersen and Peters, 1968). In addition, primordial follicles and early primary follicles were distinguished by size: follicles with a mean diameter $\leq$ 20 μm were classified as primordial follicles, while follicles with a mean diameter $>$ 20 μm were classified as growing follicles (primary and secondary follicles). This borderline of 20 μm was established in a pilot experiment. The mean diameter of the follicles was determined by measuring two perpendicular diameters (van Cappellen, *et al.*, 1989). All primordial follicles were counted in every second section, while in every second section of the growing follicles only those in which the nucleus of the oocyte was clearly visible were counted. We did not attempt to distinguish between non-atretic and atretic follicles, since at this stage of follicle development, atresia is not apparent by histology. Both 2 and 4 day culture experiments were performed twice.

Immunohistochemistry of AMH and inhibin α-subunit

To determine the onset of AMH and inhibin α-subunit protein expression *in vivo* and in cultured neonatal mouse ovaries, immunohistochemistry was performed on freshly isolated 1- to 6-day-old mouse ovaries and on 2-day-old mouse ovaries cultured for 2 or 4 days. Of every group, 4 ovaries were examined, which were derived from 2 different animals.

The ovaries were fixed for 2 h in Bouin's fixative, embedded in paraffin and 8 μm sections were made. Sections were mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma). After deparaffinisation, sections were quenched for 12 min in 3% H_2O_2 /methanol solution to block endogenous peroxidase activity and transferred to PBS. For AMH immunohistochemistry, the sections were microwaved for 3x5 min at 700 W in 0.01 M citric acid monohydrate buffer, pH 6.0 (Merck, Darmstadt, Germany), cooled down to room temperature (RT) and subsequently rinsed in PBS. For AMH immunohistochemistry the sections were preincubated with normal rabbit serum in 5% (w/v) BSA (Dako, Glastrup, Denmark) for 15 min at RT, and for inhibin α-subunit immunohistochemistry

the sections were preincubated with normal swine serum in 5% BSA (Dako) for 15 min at 37C. For AMH immunohistochemistry the preincubation step was followed by incubation at 4C overnight with primary polyclonal antibody against MIS (C-20; Santa Cruz), diluted 1:1000 in 5% BSA in PBS. The antibody was raised against a peptide mapping at the carboxy terminus of human AMH, which differs from the corresponding mouse sequence, by a single amino acid. For inhibin α-subunit immunohistochemistry the preincubation step was followed by incubation at 37C for 30 min with primary polyclonal antibody AS173-11, diluted 1:40 in 5% BSA in PBS. This primary polyclonal antibody was raised against amino acids 37-55 of the rat inhibin pre-pro-α-subunit. After incubation, the sections were rinsed in PBS and subsequently treated for 30 min at RT either with biotinylated rabbit-anti-goat antibody (dilution: 1:400; Dako) for AMH immunohistochemistry or with biotinylated swine-anti-rabbit antibody (dilution 1:400; Dako) for inhibin α-subunit immunohistochemistry. Finally, a treatment with streptavidin-biotin-peroxidase complex (ABC; diluted 1:200 in PBS; Dako) for 30 min at RT followed. Peroxidase activity was developed with 0.07% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Fluka, Basel, Switzerland) for 5-7 min. In each experiment control sections were incubated with 5% BSA/PBS in the absence of the primary antibody (negative control). Hematoxylin/eosin was used for counterstaining. Ovaries from 30-day-old mice, containing many AMH and inhibin α-subunit positive follicles, were used as a positive control.

RNA isolation and RNase protection assay

Freshly isolated 2-, 4- and 6-day-old ovaries or 2-day-old ovaries that were cultured for 2 or 4 days in the absence or presence of added AMH were snap frozen in liquid nitrogen and stored at -80C. Two separate RNase protection assays were performed. In each assay different sets of total RNA were used. For each assay total RNA was isolated from 20 ovaries per culture condition, 20 ovaries of 2-, 4- and 6-day-old mice and 5 ovaries of 30-day-old. Total RNA was isolated using Trizol Reagent (Gibco), which is an improvement of the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). The isolated RNA was dissolved in 10 µl deionized H_2O and stored by –80C.

Mouse zona pellucida protein 3 (ZP3) and mouse inhibin α-subunit DNA templates for *in vitro* transcription were generated by RT-PCR. The RT-PCR reaction was carried out on 2.5 µg total RNA, extracted from 30-day-old mouse ovaries, using random hexameres. A sample of the reverse transcription reaction product was used in the PCR reaction. With the help of ZP3 primers 5'-GAC TTC CAC GGT TGC CTT G-3', annealing to nucleotide sequence 735-753 of the *Zp3* gene (numbering according to GenBank sequence), and 5'-GCA GTC CAG CCT TCC ACA G-3', annealing to antisense sequence 1174-1156, a 440 bp fragment was generated. For the inhibin α-subunit PCR reaction primers 5'-GCC ATC CCA ACA CAT ACG-3', annealing to sense sequence 374-391, and 5'-GAA ACT GGG AGG GTG TAC G-3', annealing to antisense sequence 884-866, were used to generate a 511 bp fragment. These fragments were inserted into the PCR2.1-TOPO vector. A fragment of 521 bp of the PCR2.1-TOPO vector containing the ZP3 PCR product was removed from PCR2.1-TOPO vector by cutting the plasmid with BamHI and XhoI, while a fragment of 538 bp containing inhibin α-subunit PCR product was removed by cutting the PCR2.1-TOPO vector with EcoRI. Both fragments were subsequently subcloned in pBluescript KS and used to generate a [^{32}P]-UTP-labeled anti-sense RNA probe.

Mouse GDF9 DNA template (Laitinen, *et al.*, 1998) was kindly donated by Dr. O. Ritvos (Helsinki). This template was used to obtain a PCR fragment of 411 bp, containing a part of 293 bp of mouse GDF9 construct and a part of pGEM-T vector containing the T7 polymerase site. The primers used in this PCR reaction were primer 5'-CAGGGTTTTCCCAGTCAC-3', annealing to the sense sequence 2937-2954 located within the pGEM-T vector and primer 5'-CTG CCA TGG AAC ACT TGC TC-3', annealing to the antisense sequence 490-471 located within the GDF9 construct. This PCR product was used as template for the construction of a GDF9 [^{32}P]-UTP-labeled anti-sense RNA probe. Mouse AMHRII antisense probe was generated as described before (Visser, *et al.*, 1998). The control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA probe was synthesized using a construct containing 163 bp AccI-Sau3AI fragment of the rat GAPDH cDNA.

RNase protection assays were performed on total RNA, extracted from fresh and cultured mouse ovaries, as described by Baarends (1994). GAPDH was used as a control for RNA loading. The relative amount of protected mRNA band was quantified through exposure of the gels to a phosphor screen (Molecular Dynamics, B & L Systems, Zoetermeer, The Netherlands) and using Phosphor-Imager, and Image Quant (Molecular Dynamics) as computer analysis software. The mRNA levels were normalized to those for GAPDH mRNA and are expressed as a percentage of the mRNA level of 2-day-old ovaries, which was considered 100%.

Two separate assays were performed, using RNA derived from two separate culture experiments. In every assay, 5 µl of the RNA samples was hybridized with inhibin α-subunit and GAPDH probes, and the remaining 5 µl with probes for AMHRII, GDF9, ZP3 and GAPDH. Total RNA of 30-day-old mouse ovaries was used as a control.

Statistical analysis

Results are presented as the mean ± SEM. The data were evaluated for statistical differences by an independent samples T-test using SPSS9.0 (SPSS, Inc., Chicago, IL) computer software. Differences were considered significant at $P \leq 0.05$.

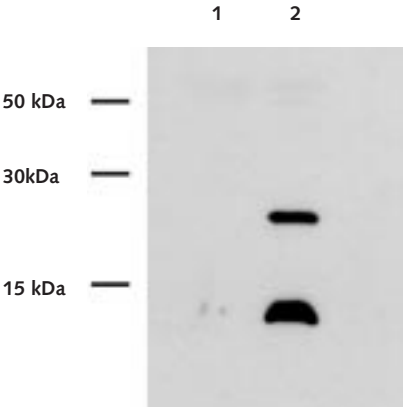
RESULTS

AMH production

To ensure that no AMH is present in the concentrated control medium, and that AMH is present in the concentrated medium of rat AMH-producing HEK293 cells, the concentrated media were examined by Western blot analysis using an AMH antibody. Figure 3.1 shows the result of this analysis. There is no AMH in lane 1, containing concentrated control medium, whereas two bands representing AMH protein can be seen in lane 2 containing concentrated medium of rat AMH-producing HEK293 cells. The lower band is the monomeric form of mature AMH (12 kDa), while the top band is non-reduced AMH dimer (24 kDa). The absence of larger bands indicates complete cleavage of the proform of AMH.

Culture medium from both control and AMH-treated cultures was also examined by Western blot analysis to check for the presence of endogenous AMH and to make sure that the added rat AMH is not degraded during the culture. No AMH was found in the control cultures, while non-degraded AMH was present in the AMH-treated cultures (results not shown).

Figure 3.1: Western blot analysis of concentrated wild-type HEK293 and rat AMH-producing HEK293 cells. No AMH protein expression is found in concentrated medium of wild-type HEK293 cells (lane 1), while AMH protein is found in concentrated medium of rat AMH-producing HEK293 cells (lane 2).



Ovary organ culture and ovarian morphology

The culture system was evaluated by comparing the morphology of 2-day-old ovaries cultured for 2 or 4 days with the morphology of *in vivo* ovaries of corresponding age, which were isolated from 4- and 6-day-old mice, respectively. This evaluation focussed on the developmental stage of the follicles, while no comparison was made regarding the number of different stages of follicles.

Ovaries of 2-day-old mice, which were used for the ovary culture experiments, predominantly contain primordial follicles, some naked oocytes and no growing follicles (Figure 3.2A). Histological examination of the ovaries after 2 or 4 days of culture indicated that the culture conditions had no pronounced detrimental effect on the ovarian tissue, *i.e.* almost no necrosis or apoptotic cells were found (Figure 3.2B). After 2 days of culture the ovaries contained many primordial follicles but also some primary follicles of different sizes (Figure 3.2D), while after 4 days of culture, the ovaries contained in addition to primordial and many primary follicles also some early secondary follicles (Figure 3.2F). Most primordial follicles were located in the cortical part of the ovary, while the primary and secondary follicles (further referred to as growing follicles) were mostly found in the central part of the ovary (Figure 3.2B). As a further validation of the culture system we compared ovaries of similar ages, *i.e.* ovaries cultured for 2 or 4 days were compared with ovaries of 4- or 6-day-old mice respectively. The most significant difference was a larger diameter of primary follicles in ovaries cultured for 2 days than primary follicles in 4-day-old ovaries (Figure 3.2C), while follicle development in ovaries cultured for 4 days were not different from ovaries from 6-day-old mice (Figure 3.2E). It also seems if more follicles reach the primary stage in ovaries cultured for 2 days than in 4-day-old ovaries. However, no direct statements regarding the numbers can be made since no follicle counting was performed in the freshly isolated ovaries.

The presence of AMH in the culture medium did not have detrimental effect on the tissue of the cultured ovaries (results not shown).

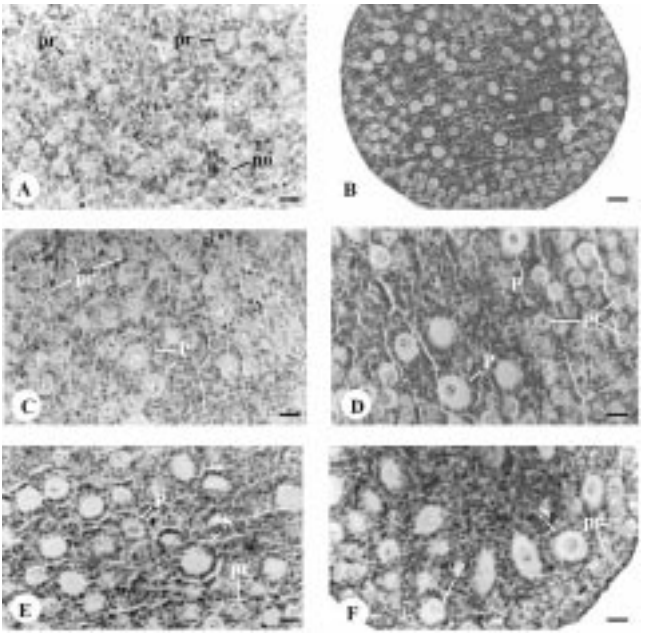


Figure 3.2: Photomicrographs of cultured and non-cultured neonatal mouse ovaries.

A. Section of an ovary of a 2-day-old mouse. Many primordial follicles (pr) and some "naked" oocytes (no) are present. Scale bar 20 µm.

B. Section of a 2-day-old mouse ovary cultured for 2 days. The present culture conditions had no detrimental effect on tissue quality, since almost no necrotic and apoptotic cells were found. Most primordial follicles are located in the cortex of the ovary, while the primary and secondary follicles are predominantly found in the central part of the ovary. Scale bar 50 µm.

C. Section of an ovary of a 4-day-old mouse. Many primordial (pr) and some primary (P) follicles are found. Scale bar 20 µm.

D. Section of a 2-day-old mouse ovary cultured for 2 days. After 2 days of culture the ovaries contained many primordial follicles (pr) and primary follicles (P) of different sizes. Scale bar 20 µm.

E. Section of an ovary of a 6-day-old mouse. Besides primordial (pr) and primary (P) follicles, also some early secondary (S) follicles are present. Scale bar 20 µm.

F. Section of an ovary of a 2-day-old mouse cultured for 4 days. In addition to primordial (pr) and primary (P) follicles, also some early secondary follicles (S) are found. Scale bar 20 µm.

AMH and inhibin α-subunit immunohistochemistry

To further evaluate the condition of the cultured ovaries and to determine the onset of AMH and inhibin α-subunit protein expression *in vivo*, an immunohistochemical study was performed on freshly isolated 1- to 6-day-old mouse ovaries, and in ovaries from 2-day-old mice cultured for 2 or 4 days.

In freshly isolated ovaries of 2-day-old mice, containing naked oocytes, primordial follicles or interstitial tissue no specific AMH protein expression was detected (Figure 3.3A). *In vivo* AMH protein expression was first found in a few granulosa cells of the smallest primary follicles from 4-day-old mice (Figure 3.3C), while in larger primary follicles all granulosa cells show AMH protein expression. High AMH protein expression was found in the granulosa cells of primary and early secondary follicles of 6-day-old mice (Figure 3.3E).

Cultured ovaries exhibited an identical AMH expression pattern compared to freshly isolated ovaries, *i.e.* expression was found in granulosa cells of primary and secondary follicles. However, the expression level of AMH was higher in the primary follicles of ovaries cultured for 2 days than that in freshly isolated ovaries of 4-day-old animals (compare Figures 3.3C and 3G). No difference was found in AMH protein expression level between freshly isolated 6-day-old ovaries and ovaries cultured for 4 days (results not shown). The negative control showed that some background staining was found in the oocytes and in the interstitium (Figure 3.3I).

The inhibin α-subunit protein expression pattern was identical to the AMH protein expression both *in vivo* and *in vitro* (Figures 3.3B-J), although the inhibin α-subunit protein expression was somewhat less abundant. Very low amount of background staining was found in the oocytes (Figure 3.3J).

The presence of AMH in the culture medium did not affect the expression of either AMH or inhibin α-subunit (results not shown).

Histological and immunohistochemical evaluation of cultured and freshly isolated ovaries of comparable age revealed that follicle development *in vivo* and *in vitro* are similar and therefore our neonatal ovary organ culture system was applied to examine the effect of AMH on the initiation of primordial follicle recruitment.

Figure 3.3: Immunohistochemical localization of AMH and inhibin α-subunit in freshly isolated (control) and cultured neonatal C57Bl/6J mouse ovaries. (For a color version of this figure, see page 169.)

A-B. Section of a 2-day-old control mouse ovary. No AMH (A) and inhibin α-subunit (B) protein expression is found in naked oocytes (no), primordial follicles (pr) and interstitial tissue (I). Scale bar 20 µm.

C-D. Section of a 4-day-old control mouse ovary. AMH protein expression (C) and inhibin α-subunit protein expression (D) are found in several granulosa cells of early primary follicles. Scale bar 20 µm.

E-F. Section of a 6-day-old control mouse ovary. Abundant AMH protein expression (E) and inhibin α-subunit protein expression (F) are found in granulosa cells of primary follicles (P) and early secondary follicles (S). Scale bar 20 µm.

G-H. Section of a 2-day-old mouse ovary cultured for 2 days in the absence of added AMH. High AMH protein expression (G) and inhibin α-subunit protein expression (H) are found in granulosa cells of primary follicles (P). Scale bar 20 µm.

I-J. Section of a 4-day-old control mouse ovary. For AMH some background staining is found in the interstitium (I) and the oocytes, while for inhibin α-subunit very low background staining is only found in the oocytes (J). Scale bar 20 µm.

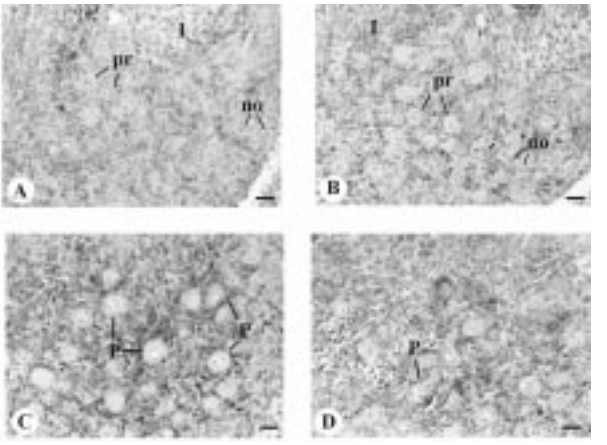
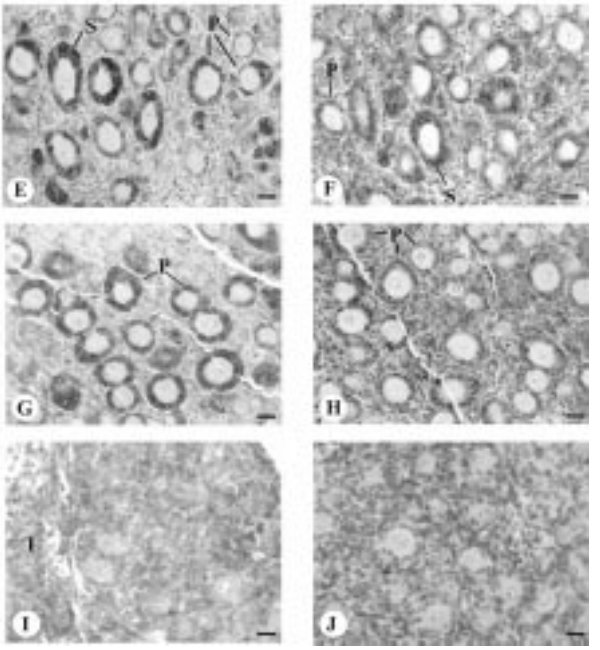


Figure 3.3:



Effect of AMH on initiation of primordial follicle growth

To determine the effect of AMH on the recruitment of primordial follicles, the numbers of primordial, primary and secondary (growing) follicles were counted in ovaries cultured for 2 or 4 days in the absence or presence of AMH.

In the ovarian culture experiment the effect of different culture conditions (+/- AMH) were tested by comparing the number of primordial and growing follicles between ovaries derived from the same animal (paired ovaries), since no large differences exist in the number of primordial follicles between two ovaries derived from the same animal (results not shown). To optimize the contact between the ovarian tissue and the culture medium the peri-ovarian sac was removed before the 2-day-old ovaries were used in the ovarian culture experiments. To test whether the removal of the peri-ovarian sac has any impact on the number of primordial follicles the number of primordial follicles was counted in paired ovaries of two 2-day-old mice of which of one of the ovaries the peri-ovarian sac was removed. Removal of the peri-ovarian sac resulted in the loss of primordial follicles since approximately 3000 (2982 ± 53) primordial follicles were found in 2-day-old ovaries with the peri-ovarian sac in place, and about 1400 (1384 ± 37) after removal of the peri-ovarian sac.

After 2 or 4 days of culture no difference was found in the total number of follicles per cultured ovary, when the ovaries were derived from the same female (results not shown). However, variation did occur between cultured ovaries isolated from different animals. This inter-animal variation was mainly due to a difference in the number of primordial follicles, ranging from 691 to 2281 primordial follicles per ovary. Moreover, primordial follicles were also lost during the culture period, since significantly more primordial follicles were found in the ovaries after 2 days of culture (1893 ± 104) than after 4 days of culture (1401 ± 79).

Table 3.1: The number of primordial and growing follicles after 2 and 4 days of culture in the absence or presence of added AMH.

The numbers of follicles found in two independent experiments are pooled and shown as mean ± SEM (P<0.05). [a] indicates a significant difference from the control. N is the number of ovaries examined.

Days of culture	-/+ AMH	# Primordial follicles	# Growing follicles
2 (N=9)	- AMH	1968 ± 82	81 ± 4
2 (N=9)	+ AMH	1808 ± 141	50 ± 2 [a]
4 (N=11)	- AMH	1369 ± 148	153 ± 8
4 (N=11)	+ AMH	1434 ± 155	97 ± 8 [a]

The total numbers of primordial and growing follicles after 2 and 4 days of culture in the presence or absence of AMH are shown in Table 3.1. After both 2 and 4 days of culture no effect of AMH on the number of primordial follicles was found. However, significantly fewer growing follicles were found in the AMH-treated ovaries compared to the control ovaries. After normalization to percentages, we found that after 2 days of culture the number of growing follicles cultured in the presence of AMH was approximately 59 ± 4% of the control (Figure 3.4A), while after 4 days of culture this was approximately 66 ± 6% (Figure 3.4B).

After 2 days of culture, for both treatment groups, most growing follicles reached a diameter of 30 μm and some a diameter of 40 μm. After 4 days of culture, most growing follicles reached a diameter of 40 μm and some a diameter of 50 μm. The presence of AMH did not affect the maximal diameter reached by the follicles during the culture period.

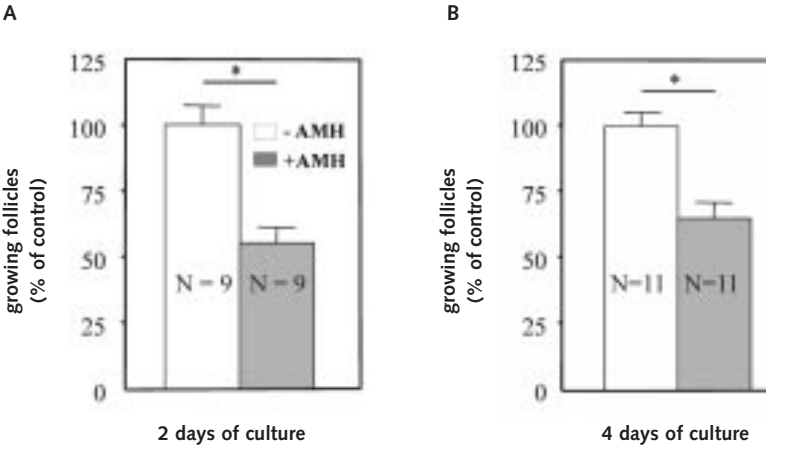


Figure 3.4: Growing follicles as a percentage of the control in 2-day-old mouse ovaries cultured for 2 days (A) or 4 days (B) in the absence or presence of AMH.

The number of growing follicles found in ovaries cultured in the presence of AMH is shown as a percentage of the control. N represents the number of ovaries that were investigated. Data represent the mean ± SEM. The *asterisks* indicate a statistically significant difference (P ≤ 0.05).

RNase protection assay

To investigate whether the decrease in number of growing follicles was reflected in the levels of expression of mRNA for markers of follicle differentiation, the mRNA expression level for the granulosa cell markers, inhibin a-subunit and AMHRII, and for the oocyte-specific markers, GDF9 and ZP3, in freshly isolated 2-, 4- and 6-day-old ovaries and in cultured ovaries was examined using RNase protection assay. Total RNA from 30-day-old mice was used as a control.

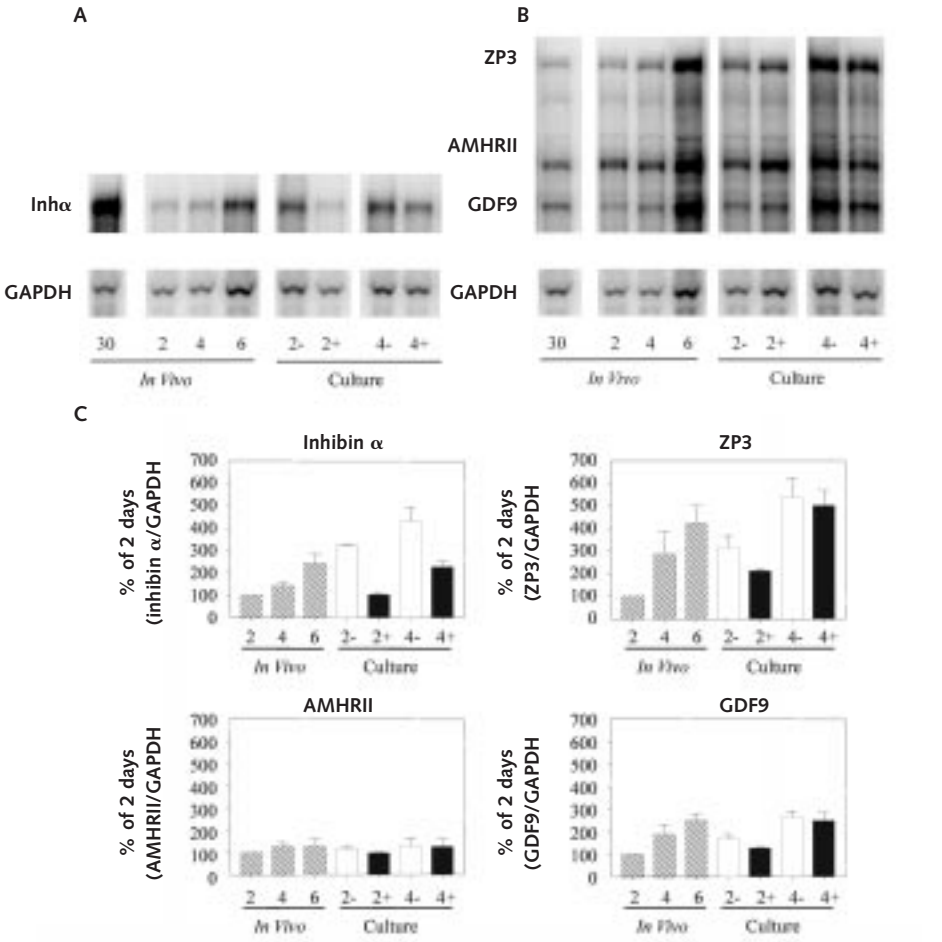


Figure 3.5: Effect of AMH on expression of GDF9, ZP3, AMHRII and inhibin α-subunit mRNAs.

A-B. Phosphor-Image analysis of a representative RNase protection assay for inhibin α-subunit and GAPDH mRNA expression **(A)** and for ZP3, AMHRII, GDF9 and GAPDH mRNA expression **(B)** in neonatal ovaries of 2-, 4- and 6-day-old control mice, and in 2-day-old ovaries cultured for 2 or 4 days in the presence (+) or absence (-) of AMH. RNA from 30-day-old mice is used as a control.

C. Quantification of inhibin α-subunit, ZP3, AMHRII and GDF9 mRNA levels in 2-, 4- and 6-day-old control mouse ovaries, and in 2-day-old mouse ovaries cultured for 2 or 4 days in the absence or presence of added AMH. All these mRNA levels were normalized to that of GAPDH mRNA. Values are represented as a percentage of 2-day-old ovaries (considered 100%) and are the mean ± SEM of two independent experiments. For every culture condition total RNA of 20 ovaries was pooled, while total RNA of 20 ovaries of 2-, 4- and 6-day-old animals and of 5 ovaries of 30-day-old animals were pooled.

Phosphor-Image analysis of the RNase protection assay is shown in Figures 3.5A and 3.5B. Quantitative analysis revealed that AMHRII mRNA expression is present in all ovaries examined and does not change with age or under different culture conditions (Figure 3.5C). The mRNA expression level of inhibin α-subunit increased from day 2 to day 6. In the ovaries cultured for 2 days in the presence of AMH, a decrease in inhibin α-subunit expression level of 68% was found, compared to the ovaries cultured in the absence of AMH. After 4 days of culture this decrease was 46% (Figure 3.5C).

In the freshly isolated ovaries an increase in the mRNA expression level from day 2 to day 6 was found for both oocyte-specific markers (GDF9 and ZP3). No difference was found in mRNA expression level between ovaries cultured in the presence or absence of added AMH, although a slight decrease was found for ZP3 expression after 2 days of culture in the presence of AMH (Figure 3.5C).

DISCUSSION

In the mouse, primordial follicles are formed just after birth, and this pool of primordial follicles constitutes the complete supply of ovarian follicles during reproductive life. Initiation of primordial follicle growth occurs from the moment that these follicles arise in the ovary, although not all primordial follicles start to grow at the same time and some will remain dormant for months in rodents (Peters, 1978; Ueno, *et al.*, 1989) and even for years in the human (Peters, 1969). The factors that regulate this initiation of primordial follicle growth are still largely unknown. Recently, it was shown in a culture system for neonatal rat ovaries that SCF and bFGF can stimulate the recruitment of primordial follicles (Parrott and Skinner, 1999; Nilsson, *et al.*, 2001), while *in vitro* treatment with GDF9 enhances the progression of primordial and primary rat follicles into small pre-antral follicles (Vitt, *et al.*, 2000b). Studying the follicle population of nerve growth factor (NGF)-deficient mice revealed that in these mice fewer primary follicles were formed, whereas an almost normal complement of primordial follicles existed, suggesting that NGF is important for the initiation of primordial follicle growth (Dissen, *et al.,* 2001). These are the first studies, which show that ovarian growth factors play an important role in initiation of primordial follicle growth. In our study of ovaries from AMH-deficient females it became evident that also AMH affects initiation of primordial follicle growth (Durlinger, *et al.*, 1999).

In the present study, the effect of AMH on primordial follicle recruitment was tested using ovaries from 2-day-old mice in a neonatal ovary organ culture system. Ovaries of 2-day-old mice provide a useful system for the study of factors that influence the initiation of primordial follicle growth, since they contain many primordial follicles, only few naked oocytes, and no growing follicles. In this culture system, not all primordial follicles started to grow, but similar to observations *in vivo* (Byskov and Lintern-Moore, 1973) and in another ovary culture system (Eppig and O'Brien, 1996), some of the primordial follicles located in the central part of the ovary were the first to initiate growth.

After 2 and 4 days of culture, fewer growing follicles were found in ovaries cultured in the presence of AMH than in the control ovaries, which indicates that AMH indeed inhibits initial recruitment. By immunohistochemistry it was shown that AMH is already produced by early primary follicles, making AMH a very early marker of ovarian follicle growth. This means that from postnatal day 4, about one day after the first primary follicles are formed in mouse ovaries, these ovaries produce a small amount of AMH. Also in the cultured ovaries endogenous AMH was found in the early primary

follicles which arise after 1 day of culture. Therefore, the effect of AMH found in the culture model may be an underestimation of the actual effects.

To exclude the possibility that the lower number of growing follicles found in AMH-treated ovaries is caused by an AMH-induced retardation of early primary follicle growth, we also looked at the maximal mean diameter that was reached in control and AMH-treated ovaries. Since under both culture conditions the growing follicles reached the same maximal diameter we conclude that AMH has no effect on the pace of early primary follicle growth.

The inhibition of initiation of primordial follicle growth is probably due to a direct effect of AMH on the primordial follicle. Using RNase protection assay we show that AMHRII mRNA expression, essential for an effect of AMH, is present in ovaries of 2-day-old mice. In both mouse and rat, ovarian AMHRII mRNA expression is even found before birth (our observations; Baarends, *et al.*, 1994, 1995a). In addition, *in situ* hybridization studies showed ovary-specific AMHRII expression in neonatal ovaries. However, exact localization of the radio-active AMHRII probe to the pregranulosa cells was not possible since this probe is best visualized using a microscope with darkfield whereby it is very difficult to discern specific structures or cell types in neonatal ovaries (Baarends, *et al.*, 1995a; A.L.L. Durlinger, unpublished observations). At later stages of ovarian development, AMHRII expression is limited to the granulosa cells of mainly non-atretic pre-antral and small antral follicles (Baarends, *et al.*, 1995a; A.L.L. Durlinger, unpublished observations), although recently AMHRII transcripts have also been detected in the internal theca cell layer of pre-antral follicles (Ingraham, *et al.*, 2000).

As soon as primordial follicle growth is initiated, as indicated by a change in appearance of the pregranulosa cells, not only AMH, but also α-inhibin protein is produced by the granulosa cells of the early primary follicles and the inhibitory effect of AMH on primordial follicle recruitment is reflected by a decrease in α-inhibin, used as a marker of early follicle development. However a functional role of α-inhibin produced by these primary follicles is unclear at the present. More insight in the role of inhibin in early follicle development could come from detailed analysis of the follicle population of inhibin-deficient females, before they develop ovarian tumors (Matzuk, *et al.*, 1996).

The number of primordial follicles that start to grow within a certain time period was not dependent on the total number of primordial follicles present in the ovary at the beginning of the culture experiment. It appears that within a certain time period the same number of primordial follicles starts to grow, irrespective of the size of the pool of primordial follicles. The same result was found in another *in vitro* study in which prenatal and neonatal mouse ovaries were placed in culture (Byskov, *et al.*, 1997; Krarup *et al.*, 1969) however, state that their *in vivo* study shows that the fraction of a pool of primordial follicles that initiate growth does depend on the actual size of the pool primordial follicles (Krarup, *et al.*, 1969). The discrepancy between our results and of Byskov *et al.* and the results of Krarup *et al.* could be explained by the fact the ovarian culture experiments are *in vitro* studies, in which many possible factors influencing the initiation of primordial follicle growth are eliminated, while Krarup *et al.* performed an *in vivo* study.

Although the amount of AMH (800 U/ml) used in the herein described culture experiments caused complete Müllerian duct regression *in vitro* (A.L.L. Durlinger, unpublished observations), the recruitment of primordial follicles was not completely blocked. This observation is in accordance with the findings in AMH-deficient females, in which not all primordial follicles initiated growth. Thus, AMH is not the only regulator of initial recruitment. Indeed, the stimulatory effect of SCF (Parrott and Skinner, 1999),

GDF9 (Vitt, *et al.*, 2000b), bFGF (Nilsson, *et al.*, 2001) and NGF (Dissen, *et al.*, 2001) on the initiation of primordial follicle growth show that activation of primordial follicle growth is under the influence of both stimulatory and inhibitory regulation. It would be interesting to culture neonatal ovaries with SCF, GDF9, bFGF or NGF in the presence of AMH to study the possible functional interactions on initial recruitment. Indications for an interaction between SCF and GDF9 already exist, since SCF mRNA expression is highly elevated in mice lacking GDF9, suggesting that GDF9 is one of the oocyte-secreted factors that negatively regulate SCF expression (Elvin, *et al.*, 1999b). Besides AMH, SCF, GDF9, bFGF and NGF probably many other, as yet unidentified, factors are involved, whose role in initial recruitment is still not established. Identification of receptors for hormones and growth factors expressed by either the oocyte or the pregranulosa cells of primordial follicles will give more insight in the process of initial recruitment.

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Chapter 4

THE ROLE OF THE ANTI-MÜLLERIAN HORMONE TYPE II RECEPTOR IN OVARIAN FUNCTION

Chapter 4
THE ROLE OF THE ANTI-MÜLLERIAN HORMONE TYPE II RECEPTOR IN OVARIAN FUNCTION

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ABSTRACT

Anti-Müllerian hormone (AMH) is a member of the TGFβ superfamily of growth and differentiation factors. In the ovary, AMH is produced by granulosa cells of primary, pre-antral and small antral follicles and inhibits recruitment of primordial follicles into the growing pool. Furthermore, AMH decreases the sensitivity of large pre-antral and small antral follicles for FSH. These effects are thought to be mediated through the AMH type II receptor (AMHRII).

The function of the AMHRII in the ovary was studied using the AMHRII LacZ knock-in mice (MRKI(-/-)). The ovaries of 4-month-old MRKI(-/-) mice contained an increased number of small growing and atretic follicles. The number of primordial follicles in the ovary was decreased in the MRKI(-/-) mice compared to wild-type (WT) mouse. These results are similar to the results obtained from the ovaries of 4-month-old AMH null (AMH(-/-)) mice. However, the decrease of primordial follicle numbers was more pronounced in the MRKI(-/-) than in the AMH(-/-) ovaries. The ovarian phenotype of the MRKI(+/-) was similar to the WT, showing that one allele for the AMHRII is enough for normal AMH-signaling in the ovary. In the MRKI(-/-) mice, the FSH level was decreased and inhibin levels were increased compared to the WT, albeit not significant.

We conclude that the AMHRII plays a role in primordial follicle recruitment. These effects can be explained by the absence of AMH action. However, the decrease of the total number of follicles, mainly caused by the decrease of primordial follicle numbers, in the 4-month-old MRKI ovaries, shows an additional effect for the AMHRII in primordial follicle recruitment and/or follicle formation. These additional effects could indicate a role of AMHRII in signaling through constitutive activity of the AMHRII in absence of the ligand. Future studies of ovaries of neonatal and/or fetal mice should reveal whether the absence of AMHRII is involved in decreased primordial follicle formation or is responsible for increased recruitment of primordial follicles.

INTRODUCTION

Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance (MIS), is a member of the transforming growth factor β (TGFβ) superfamily which is composed of several subfamilies, such as the TGFβs (TGFβ1-5), activins (inhibin α, βA-C,E) and bone morphogenetic proteins (e.g. BMP1-15, GDF1-9). Members of this family play an important role in mesenchymal-epithelial interactions, cell growth, extracellular matrix production and tissue remodeling (Massagué, 1998). Jost showed in 1947 that AMH expressed by the testis during male sex differentiation, is responsible for regression of the Müllerian ducts, the *anlagen* of female genital tract (Jost, 1947). In the rodent ovary, AMH expression is found in granulosa cells of primary, pre-antral and small antral follicles, but not of large antral, pre-ovulatory or atretic follicles (Chapter 3; Münsterberg and Lovell-Badge, 1991; Hirobe, *et al.*, 1992; Taketo, *et al.*, 1993; Baarends, *et al.*, 1995b; Durlinger, *et al.*, 2002). AMH plays an important role in follicle development, since it inhibits recruitment of primordial follicles and decreases the sensitivity of large pre-antral and small antral follicles for FSH (Chapter 3; Durlinger, *et al.*, 1999, 2001).

Like other members of the TGFβ family, AMH signals by binding to a type II transmembrane serine/threonine (S/T) kinase receptor which forms a complex with and then activates a type I S/T kinase receptor by phosphorylation. The AMH-specific type II receptor (AMHRII) has been identified (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b) and its mRNA expression is colocalized with AMH in the granulosa cells of healthy primary and pre-antral follicles (Baarends, *et al.*, 1995b). It is likely that the AMHRII is also expressed in the pregranulosa cells of primordial follicles because expression of AMHRII is already found during the fetal period and remains present after birth when the ovary mainly contains primordial follicles (Durlinger, *et al.*, 2002; our unpublished data). In addition, it has been suggested that AMHRII is also expressed in theca cells of pre-antral and small antral follicles in rat ovaries (Ingraham, *et al.*, 2000).

To study the function of AMHRII, AMHRII *null* mice were generated (Mishina, *et al.*, 1996). Like the AMH(-/-) mice, male AMHRII(-/-) mice suffered from persistent Müllerian duct syndrome (PMDS) and showed Leydig cell hyperplasia in the testes (Behringer, *et al.*, 1994; Mishina, *et al.*, 1996). Furthermore, no additional phenotype was observed in the AMH/AMHRII double mutant (Mishina, *et al.*, 1996). The phenocopy of the AMH(-/-) and AMHRII(-/-) mice suggests that AMH is the only ligand for the AMHRII. However, the phenotype of the ovary of AMHRII(-/-) mice has not been determined. Here, we studied the ovarian phenotype of mice where a LacZ reporter cassette was introduced into the AMHRII gene (MIS-receptor knock-in mouse (MRKI)) (Arango, *et al.*, 1999). Like the AMHRII null mice, these mice do not express the AMHRII, as becomes clear from the fact that in males the Müllerian ducts did not regress. Here, we report the phenotype of the ovaries of these MRKI(-/-) and MRKI(+/-) mice and compared this with the ovarian phenotype of the AMH *null* mice, in order to gain more information about the role of the AMHRII in the ovary.

MATERIALS AND METHODS

Animals

MIS Receptor Knock-In (MRKI) mice were maintained under standard animal housing conditions in accordance with NIH guidelines for the care and use of experimental animals and were housed in the animal facility (EDC) of the Erasmus MC. For this study 4-month-old female MRKI(-/-) (n=6), MRKI(+/-) (n=4) and their wild-type (WT) (n=2) siblings were used. Mice were sacrificed by decapitation at the day of estrus, after which blood was collected. The stage of estrus cycle was determined by microscopical examination of vaginal smears according to the criteria of Allen (1922). Body weight was determined and blood was collected. Serum samples were stored at –20C until assayed for FSH and inhibin A and inhibin B. The ovaries were isolated, weighed, fixed overnight in Bouin's fixative, embedded in paraffin and sectioned at 8 µm. In addition, the uterus was also isolated and weighed.

The genotype of the MRKI mice was determined using a PCR primerset for the wild-type and the null allele. Wild-type allele PCR primers (forward primer: 5'-AGG TGG GTC AGA CCC AGA GCC-3' and reverse primer 5'-GCA TGA CCT CCT TCC TGG ATT-3') were used in a concentration of 0.5 pmol/µl and the reaction was performed in 50 µl in a PCR buffer containing 1.2 mM DTT, 50 mM KCl, 10 mM TrisHCl, 0.25 mM dNTP (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 3.0 mM $MgCl_2$ and 0.05 units/µl Supertaq (Sphearo Q, Leiden, The Netherlands). The null allele was detected using LacZ primers (forward primer: 5'-GCA TCG AGC TGG GTA ATA AGG GTT GGC AAT-3' and reverse primer: 5'-GAC ACC AGA CCA ACT GGT AAT GGT AGC GAC-3'). Null allele PCR primers were used in a concentration of 100 ng/µl and the reaction was performed in 50 µl in the amplitaq PCR buffer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) containing 15 mM $MgCl_2$, 0.25 mM dNTP (Amersham Pharmacia Biotech), and 1.5 U/µl amplitaq (Applied Biosystems). The PCR reaction was performed using an annealing temperature of 65C and 35 PCR cycles. DNA for these PCRs was isolated as described by Durlinger (1999).

Ovarian histology and follicle counting

Serial 8 µm sections of one ovary were used for follicle counting. Follicle counts were performed as described by Durlinger (1999). In summary, primordial follicles were counted in every second section, while growing follicles were divided into two size classes, *i.e.* small and large follicles. The small follicle class contains pre-antral and small antral follicles with a diameter smaller than 310 µm and the large follicle class contains antral follicles with a diameter larger than 310 µm. The diameter was determined by measuring two perpendicular diameters in the sections in which the nucleolus of the oocyte was present. In addition, non-atretic and atretic follicles were counted separately. Furthermore, the number of corpora lutea was determined.

Measurements of serum FSH, inhibin A and inhibin B

Serum FSH was determined by radioimmunoassay using rat FSH as a ligand and antibodies against ovine FSH (Welschen, *et al.*, 1975). All samples were measured in one assay. Serum inhibin A and inhibin B were measured using kits purchased from Serotec Ltd (Oxford, UK) and the hormone measurements were performed in one assay.

Statistical analysis

Results are presented as mean ± SEM. The data were analyzed for statistical differences by an independent samples t-test or by one-way analysis of variance (ANOVA), followed by a Duncan's new multiple range test using SPSS, Inc9.0 (SPSS, Inc., Chicago, IL) computer software. Differences were considered to be significant at $P \leq 0.05$.

RESULTS

Ovarian morphology and follicle counts

The follicle population in the ovaries of MRKI(-/-), MRKI(+/-) mice and their WT littermates was determined. Ovaries of 4-month-old MRKI(-/-) mice contained follicles of all classes and corpora lutea, suggesting an active estrous cycle (Figure 4.1). The numbers of healthy growing and atretic growing follicles were increased over 2-fold in MRKI(-/-) ovaries compared to WT ovaries (Figure 4.2). A decrease was detected in the number of primordial follicles, which resulted in a decrease of the total number of follicles in the MRKI(-/-) ovaries compared to WT (Figure 4.2). The ovaries of MRKI(+/-) ovaries showed a follicle population most similar to WT ovaries. A small number of large follicles was detected in WT, MRKI(+/-) and MRKI(-/-) animals (results not shown). This was expected since the mice were killed on the day of estrous when no large follicles have developed yet (Welschen and Rutte, 1971; Osman, 1985).

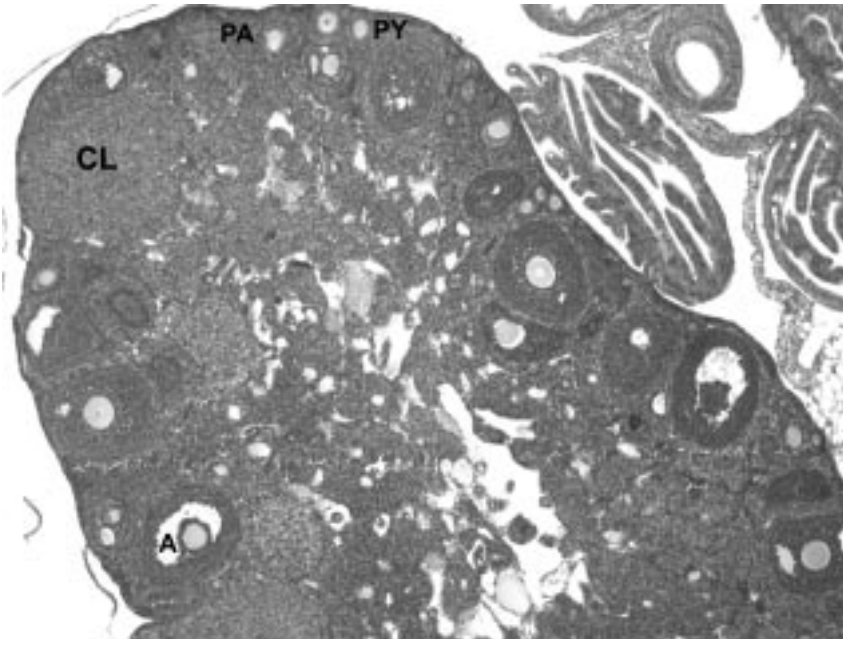

Figure 4.1: Photomicrograph of a 4-month-old MRKI (-/-) mouse ovary.
The ovary contains primary (PY), pre-antral (PA) and antral (A) follicles and corpora lutea (CL). In addition also primordial follicles are present. However, they cannot be seen at this magnification. Magnification x 50.

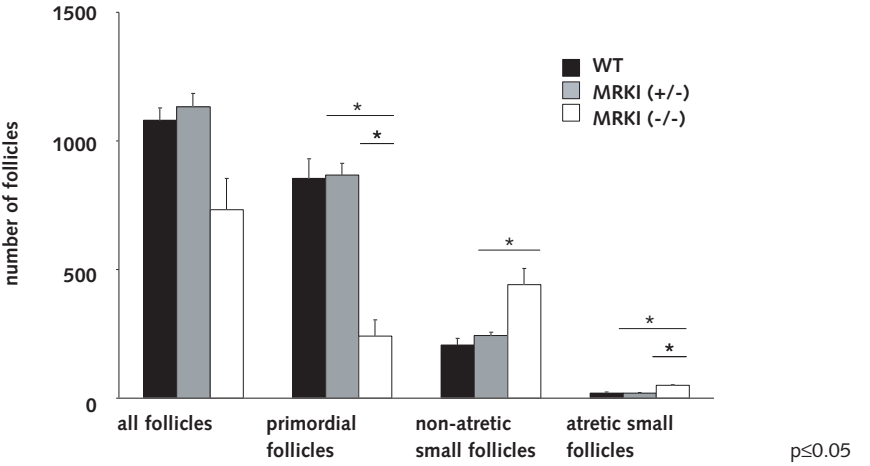

Figure 4.2: Follicle population in 4-month-old WT, MRKI(+/-) and MRKI (-/-) mice ovaries.
From two WT, four MRKI(+/-) and six MRKI(-/-) animals the primordial, non-atretic small and atretic small, non-atretic large and atretic large follicles were counted in a single ovary. The number of large follicles was very low and not presented in this figure. Data represent the mean ± SEM. *Asterisks* indicate a statistically significant difference (P≤0.05).

Weight of animals, ovaries and uterus

The increase in the number of small growing follicles in 4-month-old AMH(-/-) mice resulted in an increase in ovarian weight (Durlinger, *et al.*, 1999). Ovarian weight of 4-month-old MRKI(-/-) mice was increased compared to WT animals, although no significant difference was found (Table 4.1). Like in AMH(-/-) animals, the uterine weight (an estrogen-sensitive parameter) was decreased in MRKI(-/-) mice compared to WT and MRKI(+/-) mice and no difference in body weight between the three groups was detected.

Table 4.1: Weight of 4-months-old MRKI(+/+), MRKI(+/-) and MRKI(-/-) mice and their ovaries and uterus.
Values represent means ± SEM. No significant differences were measured.

	WT (n=2)	MRKI(+/-) (n=4)	MRKI (-/-) (n=6)
Animal weight (g)	21.8 ± 1.4	22.3 ± 0.2	22.1 ± 0.7
Ovary weight (mg)	4.1 ± 0.5	4.6 ± 0.5	6.3 ± 0.6
Uterine weight (mg)	83.0 ± 3.0	64.2 ± 6.8	63.8 ± 7.2

Table 4.2: Serum levels of inhibin A, inhibin B and FSH in 4-months-old WT, MRKI(+/-) and MRKI(-/-) mice.
Values represent the mean ± SEM. No significant differences were measured.

	WT (n=2)	MRKI(+/-) (n=4)	MRKI(-/-) (n=6)
Inhibin A (ng/l)	8.5 ± 6.5	20.8 ± 7.5	23.0 ± 12.1
Inhibin B (ng/l)	36.0 ± 18.0	49.3 ± 15.7	62.2 ± 9.2
FSH (ng/ml)	21.8 ± 2.1	10.5 ± 6.2	13.4 ± 4.3

Measurements of serum FSH, inhibin A and inhibin B levels

Inhibin A and inhibin B levels were increased and FSH levels were decreased in the serum of MRKI(-/-) females compared to WT (Table 4.2), albeit that the low number of WT animals (n=2) precluded significant differences.

DISCUSSION

AMH plays an important role in ovarian development since it inhibits primordial follicle recruitment (Chapter 3; Durlinger, *et al.*, 1999) and lowers the sensitivity of large pre-antral and small antral follicles for FSH (Durlinger, *et al.*, 2001). It is likely that AMH achieves these effects by binding to the AMHRII. By studying the phenotype of the MRKI(-/-) and MRKI(+/-) mice ovaries, we sought to determine the role of AMHRII in the ovary.

Studying the follicle population of 4-month-old MRKI(-/-) mice revealed an ovarian phenotype similar to that of AMH(-/-) mice ovaries. Like the AMH(-/-) mice, MRKI(-/-) ovaries showed an increased number of small growing and small atretic follicles and a decreased number of primordial follicles compared to the WT. These results suggest an increased recruitment of primordial follicles into the growing pool in the 4-month-old MRKI(-/-) ovary. The follicle population in MRKI(+/-) mice ovaries was most similar to that in WT ovaries, suggesting that, although only one AMHRII allele is present in the ovary, AMHRII-signaling is normal. The increase in the number of growing follicles resulted in an increased ovarian weight of MRKI(-/-) ovaries compared to their controls, albeit not significantly due to the small number (n=2) of control animals.

The increase in the number of small growing follicles in ovaries of 4-month-old AMH(-/-) mice resulted in an increase in immunoreactive serum inhibin and a decrease in serum FSH levels (Durlinger, *et al.*, 1999), since pre-antral mouse follicles produce inhibin A and inhibin B (O'Shaughnessy and Gray, 1995; Smitz and Cortvrindt, 1998). The increase in inhibin production resulted in an increased inhibition of FSH synthesis and release from the pituitary gland (de Jong, 1988). This decrease in FSH synthesis subsequently caused a decreased estrogen production by the ovaries resulting in a 1.8-fold (non-significant) decrease in uterine weight (Durlinger, *et al.*, 1999). Similarly as in AMH(-/-) animals, serum levels of inhibin A and inhibin B in MRKI(-/-) mice showed an increase followed by a decreased FSH serum levels compared to the WT animals. The absence of significant difference might be explained by the low number of WT animals, but also other explanations are possible. Determination of estrus by vaginal smears is difficult and the large variation found between the serum FSH, inhibin A and inhibin B levels of WT, MRKI(+/-) and MRKI(-/-) mice suggests that not all animals were killed at estrus. Inhibin measurements in AMH(-/-) mice were performed using an assay which detects levels of immunoreactive inhibin, while in the MRKI(-/-) mice inhibin A and inhibin B levels were measured. Increase of the level of immunoreactive inhibin serum suggests an increase in inhibin A, inhibin B and/or free inhibin α-subunits. Therefore, this method is less specific.

The increased size of the small growing follicle population in MRKI(-/-) mice suggests that the presence of one AMHRII allele in the ovary is sufficient for AMH-signaling, since the MRKI(+/-) ovaries contained similar number of follicles compared to the WT animals. However, the inhibin A and inhibin B serum levels seemed to be in between levels in the WT and MRKI(-/-) animals (Table 4.2).

Furthermore, the FSH serum levels were similar to the levels found in MRKI(-/-) mice. However, the variations in serum levels between animals and the small number of WT animals investigated, show that more research is necessary before drawing any conclusions. Recently, it was described that AMH stimulates the FSHβ mRNA expression in pituitary gonadotropin-derived cell lines (Bédécarrats, *et al.*, 2003). However, if AMH influences FSHβ expression in vivo is uncertain since AMH serum levels hardly change in females during the menstrual cycle (Cook, *et al.*, 2000) and inhibins have a major influence on FSH production and secretion (Drummond, *et al.*, 2000).

The AMHRII is the only described type II receptor for AMH and therefore we hypothesized that the phenotype of AMH(-/-) and MRKI(-/-) ovaries would be similar. To test this hypothesis, we compared the follicle population in ovaries of AMH(-/-) and MRKI(-/-) mice in more detail (Figure 4.3). The ovaries of WT siblings of AMH(-/-) and MRKI(-/-) mice contained similar numbers of primordial, small growing and atretic follicles (results not shown). In addition, no difference in the follicle population could be found between AMH(+/-) and MRKI(+/-) mice (results not shown). Also the number of small growing follicles, atretic follicles (Figure 4.3) and corpora lutea (results not shown) in the MRKI(-/-) mice and the AMH(-/-) mice were similar. However, the number of primordial follicles was significantly decreased in MRKI(-/-) mice, suggesting an increased depletion of the primordial follicle pool. The phenotype of the AMH(+/-) mice ovaries was intermediate between the WT and AMH(-/-) mice. It was suggested that this AMH production and secretion is not under stringent feedback control and that the lower concentration of AMH protein was the cause of the intermediate phenotype (Durlinger, *et al.*, 1999). In contrast, the MRKI(+/-) phenotype is similar to the WT, suggesting that the expression of the AMHRII is more strictly regulated than the AMH expression. That deletion of one receptor gene does not necessarily result in a phenotype is shown by the LH receptor (LHR)(+/-) mice of which the phenotype is similar to that of the WT, while the LHR(-/-) mice do show a phenotype (Zhang, *et al.*, 2001). It is unlikely that differences in AMH-signaling in the ovary would cause changes in expression of the AMHRII, since expression of the AMHRII is not under control of AMH: AMH treatment of neonatal ovaries or the mouse granulosa cell line KK1/AMHRII did not affect AMHRII mRNA expression (unpublished results).

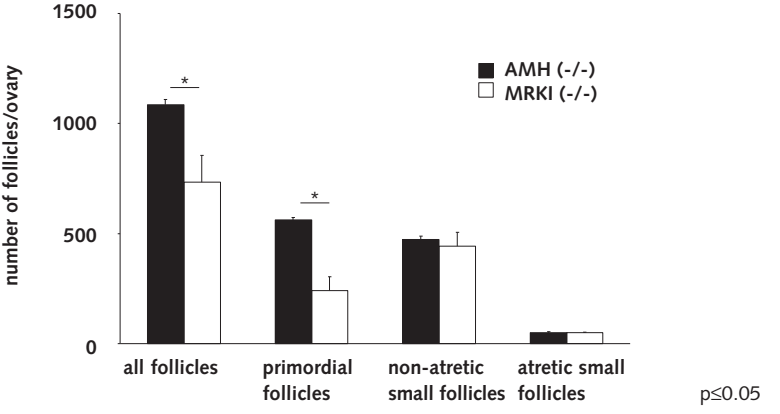

Figure 4.3: Follicle population in 4-month-old MRKI(-/-) and AMH(-/-) mouse ovaries.
Similar number of small growing and atretic follicles and a decreased number of primordial and total follicles was found in MRKI(-/-) compared to AMH(-/-) female ovaries. Data represent the mean ± SEM (n=4-6). *Asterisks* indicate a statistically significant difference (P≤0.05).

The difference found between AMH(-/-) and MRKI(-/-) ovaries might be explained by ligand-independent activity of the AMHRII. Formation of ligand-independent receptor complexes is possible and has been described for BMPRII and ALK3 and ALK6 (Gilboa, *et al.*, 2000). Whether such receptor complexes do signal in the absence of ligand *in vivo* is unclear, but *in vitro* cotransfection of BMPRII with ALK3 or ALK6 showed signaling capabilities of these complexes without ligand (Gilboa, *et al.*, 2000). In addition, cotransfection of ALK4 with ActRII or ActRIIB and ALK2 with ActRIIB resulted in ligand-independent signaling (Wang, *et al.*, 2003) and the AMHRII can form ligand-independent complexes with all type I receptors, when these receptors are overexpressed in cells (Gouédard, *et al.*, 2000; Visser, 2003). Further research is necessary to show if the AMHRII is capable of forming ligand-independent complexes with type I receptors *in vivo* and if these complexes are capable of signaling. On basis of these data, we hypothesize that AMH-signaling in MRKI(-/-) ovaries is blocked completely while in AMH(-/-) ovaries some ligand-independent signaling takes place. Therefore, the ovarian phenotype in MRKI(-/-) mice is similar but more severe than in AMH(-/-) mice.

Another possible explanation for the differences found between AMH(-/-) and MRKI(-/-) phenotypes may be caused by AMH and AMHRII acting independently of each other. All five known type II receptors signaling for TGFβ superfamily members bind more than one ligand, except AMHRII which only binds AMH. AMH signals through the BMP-like signaling pathway (Gouédard, *et al.*, 2000; Clarke, *et al.*, 2001; Visser, *et al.*, 2001) and it can be suggested that some BMPs also signal through the AMHRII. Oppositely, it can be suggested that AMH signals through another type II receptor. However, there are no indications for AMH-signaling through another type II receptor or binding of another ligand to the AMHRII.

An explanation for the decrease of the number of primordial follicles might also be explained by a decrease in primordial follicle formation. Primordial follicles are formed from the germ cells and the surrounding ovarian tissue the day after birth. Progenitors of the germ cells are the primordial germ cells (PGCs), which are first found in E7.5 mice in the allantois (Tam and Snow, 1981; Ginsburg, *et al.*, 1990). At E13, the PGCs have migrated to the ovary and at that time, AMHRII expression is detected in mouse urogenital ridges and gonads and its expression is found until birth (Visser, *et al.*, 2001). It would be interesting to know if the AMHRII is also expressed between E7.5 and E13, since during that period the PGCs proliferate and migrate towards the ovary (Tam and Snow, 1981; Ginsburg, *et al.*, 1990). Involvement of AMHRII by ligand-independent signaling or signaling by other ligands in this process might influence the number of PGCs and thus the number of primordial follicles in the ovary in MRKI(-/-) mice. In addition, AMHRII could be involved in the formation of primordial follicles on the day after birth. At that time, AMHRII is already expressed (Chapter 5) and might be involved in follicle formation.

In conclusion, the phenotype of the MRKI(-/-) ovaries can be explained by the absence of AMH-signaling. The decreased primordial follicle population in 4-month-old MRKI(-/-) compared to the AMH(-/-) mice ovaries might be explained by the absence of ligand-independent signaling of the AMHRII in the MRKI(-/-) ovaries. Thus, the phenotype of the ovaries between AMH(-/-) and MRKI(-/-) mice are similar.

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Chapter 5

ANTI-MÜLLERIAN HORMONE AND ITS SIGNALING PATHWAY IN THE OVARY

Chapter 5
ANTI-MÜLLERIAN HORMONE AND ITS SIGNALING PATHWAY IN THE OVARY

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ABSTRACT

Anti-Müllerian hormone (AMH) is a member of the TGFβ superfamily which regulates follicle development at two regulatory points. It inhibits the recruitment of primordial follicles into the growing pool and decreases the sensitivity of large pre-antral and antral follicles to follicle stimulating hormone (FSH). Thus, AMH plays an important role in determining the number of growing follicles in the ovary. However, the signaling mechanism by which AMH mediates its effect in the ovary is unknown. In the Müllerian ducts, AMH signals through its specific AMH type II receptor (AMHRII) and the type I receptors ALK2, ALK3 and ALK6 have been implicated.

Here, we have investigated the AMH signaling pathway in the ovary. We found that AMH signals through a BMP-like signaling pathway in the mouse granulosa cell line KK-1. In agreement with this, AMH stimulation results in the phosphorylation of Smad1 and not Smad2.

In addition, we determined the expression level and localization of AMH-signaling mediators in the ovary. All candidate type I receptors and downstream Smad proteins are expressed in the ovary albeit at different levels. ALK2 mRNA expression was found in all cell types of the ovary, including the granulosa cells. Interestingly, AMH stimulates the expression of the receptor-specific Smad8 and the inhibitory Smad6 and Smad7 in both cultured neonatal ovaries and KK-1 cells stably expressing the AMHRII.

In conclusion, AMH signals through the BMP-like signaling pathway in the ovary and stimulates an intracellular negative feedback loop formed by upregulation of Smad6 and Smad7 expression. All candidate type I receptors and Smads are expressed in the ovary and therefore can be involved in AMH-signaling.

INTRODUCTION

The ovary provides an environment where oocytes differentiate, mature and are selected for ovulation and fertilization. Primordial follicles form the resting pool of follicles and development of the primordial follicle to a pre-ovulatory follicle is under tight control of several intra-ovarian factors, such as members of the transforming growth factor β (TGFβ) superfamily of growth- and differentiation factors (such as AMH, GDF9, BMP15, inhibin, activin) and systemic factors, like the pituitary-produced gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). Two important sites of regulation during follicular development can be distinguished: recruitment of primordial follicles into the growing pool and selection of large pre-antral and antral follicles for further growth to the pre-ovulatory stage (McGee and Hsueh, 2000). Every primordial follicle that has entered the growing pool will grow to the pre-ovulatory stage or be removed through the process of atresia.

Previously, we have found that anti-Müllerian hormone (AMH), a member of the TGFβ superfamily, is involved at both regulatory sites. Recruitment of primordial follicles is inhibited by AMH, as was shown by the phenotype of AMH *null* mice. Ovaries of adult homozygous AMH *null* mice contained less primordial and more growing follicles than control ovaries (Durlinger, *et al.*, 1999). This AMH effect was confirmed by culturing neonatal ovaries in the presence of AMH (Chapter 3). By inhibiting primordial follicle recruitment, AMH prevents early depletion of the follicle pool in the ovary and partially determines the size of the pool of growing follicles in the ovary. At selection, AMH decreases the sensitivity of large pre-antral and antral follicles to FSH thereby controlling the number of follicles that are selected to grow to the pre-ovulatory stage (Durlinger, *et al.*, 2001). Interestingly, the importance of AMH in the regulation of the growing pool is illustrated by the expression pattern of AMH. AMH is expressed in granulosa cells immediately after recruitment of the follicle and expression disappears after the follicle has been selected for growth to the pre-ovulatory phase (Chapter 3). However, the mechanisms by which AMH inhibits primordial follicle recruitment and decreases the sensitivity of large pre-antral and small antral follicles for FSH are unknown.

Members of the TGFβ superfamily signal by binding to transmembrane serine/threonine (S/T) kinase type I and II receptors (reviewed by Miyazono, *et al.*, 2001). When ligands have formed a complex with the type II receptor and the type I receptor, the type I receptor is phosphorylated at the GS-box. Subsequently, the receptor-specific Smads (R-Smads) are phosphorylated by the type I receptor. The phosphorylated R-Smads form a complex with the common Smad4 and this complex translocates to the nucleus where it regulates gene transcription (reviewed by Miyazono, *et al.*, 2001). Based on the identity of the Smads that are used for downstream signaling, two main signaling pathways can be distinguished. Smad2 and Smad3 are phosphorylated in the TGFβ/activin-like signaling pathway while in the BMP-like signaling pathway Smad1, Smad5 and Smad8 are activated (reviewed by Miyazono, *et al.*, 2001). The inhibitory Smads (I-Smads), Smad6 and Smad7, prevent phosphorylation of the R-Smads and in this way can block signaling of TGFβ superfamily members. Smad7 functions as a common I-Smad and inhibits both BMP- and TGFβ/activin-like signaling whereas Smad6 preferentially inhibits signaling of the BMP-like signaling pathway (Itoh, *et al.*, 1998; Ishisaki, *et al.*, 1999; Hanyu, *et al.*, 2001). Expression of these I-Smads is induced by TGFβ/activin- or BMP-like signaling, thereby providing an intracellular negative feedback mechanism for signaling by TGFβ family members (Nakao, *et al.*, 1997; Takase, *et al.*, 1998).

For AMH, the specific type II receptor (AMHRII) has been identified and cloned (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b). Recently, more knowledge about other components of the AMH-signaling pathway has been obtained. It was found that AMH signals through the BMP-like signaling pathway in non-ovarian cells (Gouédard, *et al.*, 2000; Clarke, *et al.*, 2001; Visser, *et al.*, 2001). The type I receptors ALK2, ALK3 and ALK6 are candidate receptors involved in AMH-signaling. Dominant-negative and antisense approaches in cell lines showed that ALK2 is necessary for AMH-signaling (Clarke, *et al.*, 2001; Visser, *et al.*, 2001). In addition, Müllerian duct regression is inhibited by antisense ALK2 (Visser, *et al.*, 2001). ALK3 also plays a role in AMH-signaling, as indicated by the presence of persistent Müllerian ducts in conditional ALK3 *null* mice (Jamin, *et al.*, 2002). Interestingly, when conditional ALK3 *null* mice are crossbred with mice overexpressing AMH, Müllerian duct regression occurs normally (Jamin, *et al.*, 2003). This suggests that, in the presence of high AMH levels, receptor(s) different from ALK3 can act as AMH type I receptor, at least in the process of Müllerian duct regression. Furthermore, the involvement of ALK6 in AMH-signaling was suggested, based on the observation that AMH-induced a ligand-dependent AMHRII/ALK6 receptor complex (Gouédard, *et al.*, 2000). However, the ALK6 *null* mouse model (Clarke, *et al.*, 2001), the expression pattern of ALK6 in the Müllerian ducts (Dewulf, *et al.*, 1995; Clarke, *et al.*, 2001) and *in vitro* experiments in which the role of ALK6 in AMH-signaling is investigated (Clarke, *et al.*, 2001; Visser, *et al.*, 2001) suggest that ALK6 is not involved in AMH-signaling in Müllerian duct regression, although a role for ALK6 in AMH-signaling in the ovary cannot be ruled out yet.

To determine whether AMH signals through a similar mechanism in the ovary, we have studied the function of its signaling components in this organ. Using the mouse granulosa cell line KK-1, we determined the pathway through which AMH signals in ovarian cells. Moreover, candidate type I receptors for AMH must be co-expressed with AMHRII in ovarian cells. Therefore, we have determined the expression pattern of AMHRII and candidate type I receptors in developing postnatal ovaries. In addition, we measured mRNA expression of candidate AMH-signaling components in these ovaries. We concluded that ALK2 is a candidate type I receptor for AMH in the ovary, since it was expressed by granulosa cells of all follicle types. In addition, we show that AMH stimulates expression of the inhibitory Smad6 and Smad7, thereby forming an intracellular negative feedback loop.

MATERIALS AND METHODS

Animals

Mice were obtained from the Animal Facility (EDC) of the Erasmus MC. Mice were maintained under standard animal housing conditions in accordance with NIH guidelines for the care and use of experimental animals. Ovaries were isolated at 2, 6, 12, 18 and 25 days of age, snap frozen in liquid nitrogen and stored at -80C. Ovaries of 2-day-old AMH *null* mice were cultured under similar conditions as previously described (Chapter 3), using purified rat AMH (533 ng/ml). AMH *null* mouse genotype was determines as described by Durlinger *et al.* (1999).

For *in situ* hybridizations, ovaries of 18-day-old mice were fixed in 4% paraformaldehyde (PFA) at room temperature for 3 hours and subsequently incubated overnight at 4C in 30% sucrose dissolved in phosphate-buffered saline (PBS). Next, ovaries were embedded in Tissue Tek (Bayer BV, Mijdrecht,

The Netherlands) and stored at –80C. Cryosections of 5 µm were cut using a cryostat (Leica, Rijswijk, The Netherlands), mounted on Superfrost®plus glass slides (Menzel, Omnilabo International BV, Breda, The Netherlands) and stored at –80C until use.

AMH production and purification

Medium containing His6-tagged rat AMH was collected from stably transfected HEK293 cells as described previously (Durlinger, *et al.*, 2001). AMH was purified from this medium using the NiNTA superflow Ni-column (Qiagen, Westburg, Leusden, The Netherlands). Subsequently, AMH was eluted from this column using Hanks Balanced Salt Solution (HBSS) (Gibco BRL, Invitrogen, Breda, The Netherlands) containing 250 nM imidazole (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) and collected in siliconized tubes (Biozym, Landgraaf, The Netherlands) in the presence of 0.1% BSA. Next, a PD10 column (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) was used to remove the imidazole. Control medium was obtained by purifying Ni-column elution buffer over a PD10 column. Samples were stored at -20C.

Cell culture

The mouse granulosa cell line KK-1 (a kind gift of Dr. I. Huhtaniemi) was cultured in DMEM/F12 (Gibco) containing 10% FCS and penicillin (400 IU/ml) and streptomycin (0.4 mg/ml). For AMH-induced luciferase assays, KK-1 cells were seeded at 20% confluency in 24 wells plates (NUNC, VWR International BV, Amsterdam, The Netherlands) and transfected with the 3TP-luc or BRE2-Luc reporter (150 ng/well) and the indicated receptor expression vector (20 ng/well) using Fugene 6 transfection reagent (Roche Diagnostics Nederland B.V., Almere, The Netherlands). Twenty-four hours after transfection, cells were cultured for 2 hours in medium containing 0.2% FCS followed by 16 hours treatment with AMH (33 ng/ml). Luciferase was measured using the Steady-Glo® Luciferase Assay (Promega, Leiden, The Netherlands) in the TOPCOUNT (Packard, Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) luminometer. In all transfections, β-galactosidase expression plasmid (pSV40-βgal) served as internal control to normalize for transfection efficiency. Plasmids containing rat AMHRII, BMPRII, ActRIIB and TβRII and empty expression vectors were described previously (Visser, *et al.*, 2001). The BRE2-Luc plasmid was a kind gift of Dr. P. ten Dijke.

For AMH-induced gene expression experiments, KK-1 cells stably expressing the AMHRII were generated. These KK-1/AMHRII cells were cultured in DMEM/F12 in 10% FCS, penicillin (400 IU/ml) and streptomycin (0.4 mg/ml). At a cell confluency of 80%, cells were cultured for 2 hours in 0.2% FCS, followed by a 24 hour treatment with AMH (33 ng/ml). Next, cells were washed with PBS and stored at -20C in RNA-Bee (Campro Scientific, Veenendaal, The Netherlands).

Smad activation experiments and Western blot analysis

KK-1 cells were transiently transfected with Flag-tagged Smad constructs using Fugene 6 transfection reagent. Twenty-four hours after transfection, cells were starved overnight in low serum medium (0.2% FCS), followed by incubation with ligand for 1 hour. Next, cells were washed once with cold PBS and lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) containing phosphatase and protease inhibitors (Sigma). Equal amounts of protein were separated on SDS-PAGE, transferred to nitrocellulose membranes, and subjected to immunoblotting with αPS1 or

αPS2 antibodies (kind gift of Dr. A. Moustakas). Total Smad levels were detected with an α-Flag M2 monoclonal antibody (Sigma). Proteins were visualized by enhanced chemoluminescence (ECL, Amersham).

In situ hybridization

To detect expression of AMHRII and ALK2 mRNA in the ovary, *in situ* hybridizations were performed. The AMHRII and ALK2 templates to generate sense and antisense DIG-labeled transcripts for *in situ* hybridization were described previously (Visser, *et al.*, 1998, 2001). Probes were labeled using the DIG RNA labeling kit (Sp6/T7) (Roche), purified using Sephadex G-50 column (Amersham Pharmacia Biotech) and tested for labeling efficiency by Northern blotting.

Cryostat sections were air dried, fixed in 4% PFA on ice for 5 min and washed with PBS. Subsequently, sections were treated for 15 min at room temperature with proteinase K (1 μg/ml in 10 mM TrisHCl, pH 7.6) and postfixed in 4% PFA for 5 min at 4C. Slides were washed in PBS and non-specific binding was blocked by treatment of the slides with 0.1 M triethanolamine and acetic anhydride. Slides were washed with PBS and dehydrated. Probes (sense and antisense) were heated for 1 min at 85C and added to the hybridization buffer (50% formamide, 1xDenhardt's solution, 5mM EDTA, 10% dextransulphate, 500 μg/ml tRNA, 10 mM TrisHCl (pH 8.0), 300 mM NaCl, 10 mM NaH2PO4), which was also preheated at 85C. The hybridization mix was added to the slides and incubated overnight at 55C in a humidified chamber. Next, slides were washed with 4xSSC/50% formalin, 2xSCC and treated with RNAse A (20 μg/ml) at 37C for 30 min to remove none hybridized probe. Next, slides were washed with 0.2% SCC and incubated in 100 mM TrisHCl (pH 7.6), 150 mM NaCl (TN-buffer). Non-specific binding of the anti-DIG antibody was blocked using Antibody diluent (Cell Marque, ITK Diagnostics BV, Uithoorn, The Netherlands) for 30 min at room temperature. Slides were incubated overnight at 4C with an 1:1000 dilution of the anti-DIG antibody (Roche) in TN buffer. The next day, signal was detected using NBT-BCIP (Roche). The staining reaction was stopped with 2mM TrisHCl (pH=7.6), 10 mM EDTA and rinsed with water.

RNA isolation, cDNA synthesis and real-time PCR

RNA was isolated from ovaries and KK-1 cells using RNA-Bee according to the manufacturer's protocol (Campro Scientific). Additionally, 1 μg of RNase-free glycogen was added before RNA precipitation (Roche Diagnostics). Genomic DNA was removed by RNase-free DNAse (Stratagene, Amsterdam, The Netherlands) treatment for one hour at 37C in a buffer (40 mM TrisHCl, 6 mM $MgCl_2$, 2 mM $CaCl_2$) also containing the RNAse inhibitor RNAsin (Promega).

Total mRNA was measured using Ribogreen™ RNA Quantitation Kit (Molecular Probes, Leiden, The Netherlands). cDNA was synthesized using a mix of random nucleotide hexamers (Roche Diagnostics) and 200 nM oligo dT (Promega). The 25 μl RT buffer contained 8 U/μl mmLV (Promega), 0.2 mM dNTP (Amersham Pharmacia Biotech) and 1 U/μl RNAsin (Promega). All samples were checked for the presence of genomic DNA in a RT minus reaction, measuring β-actin using SYBRgreen reagent. If genomic DNA was present, a second DNAse-treatment of the RNA was performed.

Quantitative real-time PCR amplification reactions were performed using an Applied Biosystems Sequence Detector 7700. For measurements with a FAM-TAMRA probe, 40 ng of total mRNA was amplified in duplicate in the presence of 300 nM primers (Invitrogen, Breda, The Netherlands),

200 or 100 nM of FAM-TAMRA probe (Eurogentec, Maastricht, The Netherlands), 4mM MgCl2, 0.2 mM dNTPs (Amersham Pharmacia Biotech), 1.25 U AmplitaqGold in sample buffer A (Applied Biosystems) in a volume of 50 ml. Samples were heated for 2 minutes at 50C, 10 minutes at 95C and amplified for 40 cycles of 15 seconds at 95C and 60 seconds at 60C (Meijerink *et. al.*, 2001). These PCR conditions were also used to determine the expression of AMH-signaling components in KK-1 cells. Measurements using SYBRgreen (Applied Biosystems) were performed with 20 ng of total RNA in the presence of 300 nM primers (Invitrogen) in a total volume of 25 µl using the same amplification conditions. β-actin levels were used as a internal control to correct for differences in reverse transcriptase efficiency.

Primers and probes were developed using the Oligo 6.22 computer software (Molecular Biology Insights Inc., Cascade, USA) and are listed in Table 5.1. All primers and probe combination functioned at an efficiency of 100%.

Statistical analysis

Results are presented as mean ± SD or SEM. The data were analyzed for statistical differences by a dependent samples t-test or by one-way analysis of variance (ANOVA), followed by a Duncan's new multiple range test using SPSS, Inc9.0 (SPSS, Inc., Chicago, IL) computer software. Differences were considered to be significant at $P \leq 0.05$.

Table 5.1. Oligonucleotide primer sequences used to measure gene expression in KK-1/AMHRII cells and postnatal ovaries.

A. Oligonucleotide primers and FAM-TAMRA probe sequences used to amplify AMHRII, ALK2, ALK3, ALK6, Smad1, Smad5, Smad6, Smad7, Smad8 and β-actin cDNA with quantitative real-time PCR in postnatal ovaries and KK-1 cells. In addition, Smad8 primers and probes were used to measure Smad8 expression in AMH-treated KK-1/AMHRII cells and neonatal ovaries cultured with AMH.

B. Oligonucleotide primer sequences used to amplify Smad6, Smad7 and β-actin cDNA using SYBRgreen quantitative real-time PCR in KK-1/AMHRII cells treated with AMH and neonatal ovaries cultured with AMH. SYBRgreen was used because of the lack of neonatal ovary cDNA. Less cDNA is necessary to perform quantitative real-time PCR with SYBRgreen than with FAM-TAMRA probes.

A. Gene:	Primers:	Probe:	Product size (bp):
AMHRII	5′-CTC TGG CGC TAC TAC TGT G-3′ 5′-TGT CAG CCG TGC TTC T-3′	5′-AGA TCC TGA GCC GCT GTT CC-3′	257
ALK2	5′-GCC CAT TTG CAC ATA GAG-3′ 5′-AAA GGC CCA AAT ATC GAC-3′	5′-TGG TGA AGA AGA ATG GAC AGT GCT-3′	273
ALK3	5′-CAT TTC CAG CCC TAC ATC-3′ 5′-CTG TTC CAG CGG TTA GAC-3′	5′-ATG AGG ACA TGC GTG AGG TTG T-3′	206
ALK6	5′-GAC GGC CCT GAG AGT TA-3′ 5′-CAC TGG GCA GTA GGC TAA-3′	5′-ACG TCA GAT ACT TGT GGA CAG AGC A-3′	145
Smad1	5′-CGG CTG CAA ATG TAG ACT A-3′ 5′-TTG GAA ATC AAA GCC TAT GT-3′	5′-TCC TTC TGT TCG CAA ATC AAC TG-3′	302
Smad5	5′-TCT GGC TCA GTC AGT CAA C-3′ 5′-CAG AAG AAA TGG GGT TCA-3′	5′-AGT ACC ACC GAC AGG ACG TCA C-3′	218
Smad6	5′-CCC CAT CTT CGT CAA CT-3′ 5′-GCA GGA GGT GAT GAA CTG -3′	5′-CCG GGT TAC TCC ATC AAG GTG-3′	223
Smad7	5′-ACT GGA CAA CCC GGA-3′ 5′-GCA GGC TGT AGG CTT TC-3′	5′-TGT TGG TGC ACA AAG TGT TCC C-3′	95
Smad8	5′-GTA CCA AGA CAC AGC GAG TA-3′ 5′-CGG AAG TCT GAG TGT TGA TA-3′	5′-CGC CAC CTA CCC TGA CTC TTT-3′	242
β-actin	5′-CTA CGA GGG CTA TGC TCT C-3′ 5′-CTC AGG GCA TCG GAA-3′	5′-ACC GAG CGT GGC TAC AGC TT-3′	283

Table 5.1.

B. Gene:	Forward primer:	Reverse primer:	Product size (bp):
Smad6	5′-CCC CAT CTT CGT CAA CT-3′	5′-CGC TCA AAG TCG AAC AC-3′	105
Smad7	5′-CTC CAG GAC GCT GTT G-3′	5′-TGC ATG AAC TCG TGG TC-3′	108
β-actin	5′-CTA CGA GGG CTA TGC TCT-3′	5′-TCG TTG CCA ATA GTG ATG-3′	264

RESULTS

AMH induces a BMP-like response in granulosa cells

We have investigated whether AMH-signaling in the ovary is mediated through the BMP-like signaling pathway. KK-1 cells, a granulosa cell line derived from gonadal tumors from transgenic mice expressing the Simian virus 40 T-antigens driven by the mouse inhibin α-subunit promoter (Kananen, *et al.*, 1995), were transiently transfected with either the TGFβ/activin-responsive 3TP-Luc reporter or the BMP-responsive BRE2-Luc reporter. AMH treatment of KK-1 cells resulted in an activation of the BMP-responsive reporter only upon cotransfection with the AMHRII. AMH-signaling specifically requires the presence of AMHRII as none of the other type II receptors could activate the BRE2-Luc reporter in presence of AMH (Figure 5.1). In agreement with previous studies, AMH was unable to activate the TGFβ/activin-responsive reporter in KK-1 cells (Figure 5.1).

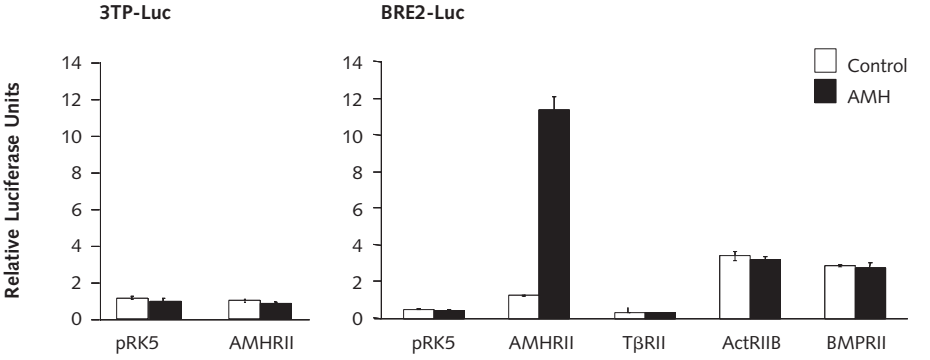


Figure 5.1: AMH signals through a BMP-like signaling pathway in KK-1 cells.
KK-1 cells were transiently transfected with a TGFβ/activin-responsive (3TP-Luc) or BMP-responsive (BRE2-Luc) reporter gene together with the indicated type II receptors or the empty expression vector pRK5. Cells were incubated overnight in the presence of control medium (control, open bars) or purified AMH (closed bars). The relative luciferase activity was measured in cell lysates and normalized to β-galactosidase activity. Data are given as mean ± SD of triplicates in one experiment and are representative for at least three independent experiments.

We next determined which Smad proteins were activated in response to AMH using phospho-specific Smad antibodies. These antibodies recognize the phosphorylated C-terminal serines of the Smad proteins but not the unphosphorylated proteins (Persson, *et al.*, 1998). KK-1 cells were transiently transfected with Smad1 or Smad2 and stimulated with AMH or TGFβ for 1 hour. As expected, TGFβ induced phosphorylation of Smad2 (Figure 5.2) but not Smad1 (results not shown). In agreement with the

AMH-induced activation of the BMP-responsive BRE2-Luc reporter, AMH treatment resulted in phosphorylation of Smad1, but not Smad2 (Figure 5.2). In conclusion, these results indicate that AMH signals through a BMP-like signaling pathway in an ovary-derived cell line.

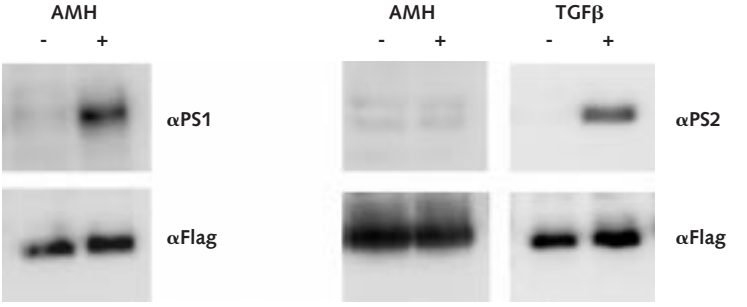


Figure 5.2: AMH induces Smad1 phosphorylation.
KK-1/AMHRII cells were transiently transfected with Flag-tagged Smad1 or Smad2 and stimulated with AMH or TGFβ. Phosphorylation of Smad proteins was detected with αPS1 or αPS2 antibodies. Total Smad proteins were detected with a α-Flag M2 antibody.

Expression of members of the AMH-signaling pathway in KK-1 cells and mouse ovaries

AMH signals through the BMP-like signaling pathway and therefore Smad1, Smad5, Smad8, the type I receptors ALK2, ALK3, ALK6 and the inhibitory Smad6 and Smad7 are candidate components of the AMH-signaling pathway. To determine which of the AMH-signaling components are present in KK-1 cells, RT-PCR analysis was performed. RNAse protection assay showed the almost complete absence of AMH and AMHRII in these cells (results not shown), which is in agreement with the lack of activation of the BMP-responsive reporter when the AMHRII was not cotransfected. All three candidate type I receptors were expressed in KK-1 cells (Figure 5.3). ALK6 was expressed at lower levels, which was supported by quantitative real-time PCR experiments (results not shown). Furthermore, KK-1 cells expressed all receptor-specific Smads (Smad1, Smad5 and Smad8) and the inhibitory Smad6 and Smad7. Thus, all candidate members of AMH-signaling pathway are expressed in the KK-1 cell line.

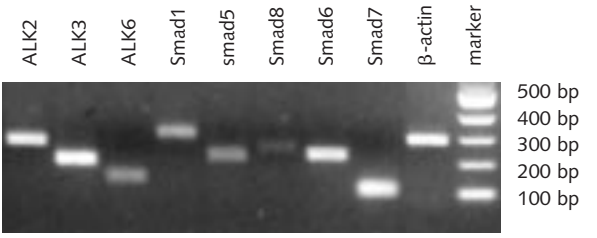


Figure 5.3: mRNA expression of members of AMH-signaling in KK-1 cells.
Expression of ALK2 (273 bp), ALK3 (206 bp), ALK6 (145 bp), Smad1 (302 bp), Smad5 (218 bp), Smad8 (242 bp), Smad6 (223 bp) and Smad7 (95 bp) was determined in KK-1 cells under quantitative real-time PCR conditions after separation on a 1.5% agarose gel.

To determine which type I receptor and Smad proteins are involved in AMH-signaling in the ovary, we measured the mRNA expression of candidate signaling components in differently aged postnatal ovaries of wild-type mice (Figure 5.4). Each time point reflects a different stage of follicle differentiation. Primordial follicles are the only follicles found in ovaries of 2-day-old mice, whereas at day 6 ovaries also contain primary follicles. In 12-day-old ovaries pre-antral follicles are present and antral follicles are first found in ovaries of 18-day-old mice. All antral and large pre-antral follicles have become atretic in ovaries of 25-day-old mice.

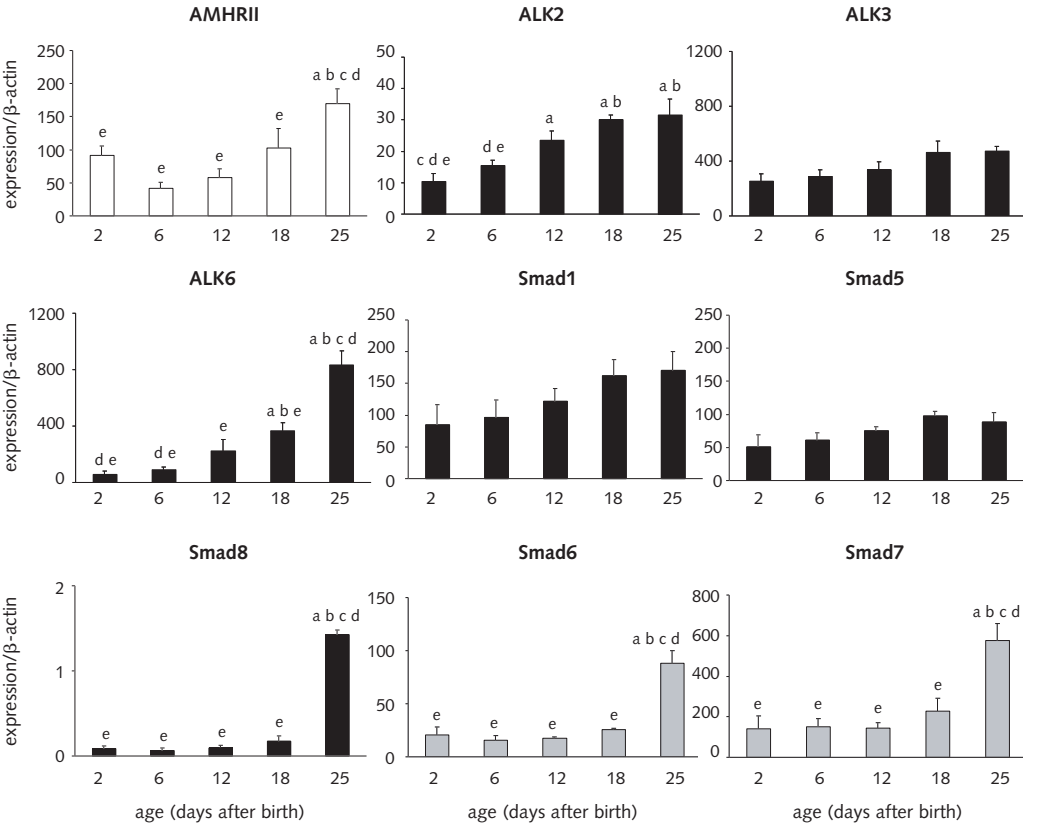


Figure 5.4. Expression levels of different candidate components of AMH-signaling in mouse ovaries.
Expression was measured in ovaries of 2-,6-, 12-, 18- and 25-day-old wild-type mice by quantitative real-time PCR with FAM-TAMRA probes. Expression of all genes has been corrected for β-actin expression. Open bars represent AMHRII, black bars represent the type I receptors, hatched bars the R-Smads and gray bars the inhibitory Smads.
Data represent means ± SEM. a, b, c, d, e indicate a significant difference from day 2, 6, 12, 18 and 25 respectively ($P \leq 0.05$). For each age 3 independent pools of ovaries were measured.
Note the difference in scale of the Y-axes of the different graphs.

AMHRII mRNA was abundantly expressed in 2-day-old ovaries, but its expression decreased from day 2 to day 6. From day 6 onward, AMHRII expression increased until day 25. All candidate type I receptors were expressed in the postnatal ovary, albeit at different levels. ALK2 was expressed at a lower level, compared to ALK3 or ALK6, but showed a 3-fold increase in expression during ovarian

development in the first 25 days. ALK3 expression levels remained constant during all timepoints studied. In contrast, ALK6 mRNA expression increased from day 2 to day 18 (6-fold) and an even stronger increase of expression was observed from day 18 to day 25.

Of the three possibly receptor-specific Smads involved, Smad1 and Smad5 were most abundantly expressed at constant levels during follicular development. In contrast, Smad8 expression could hardly be detected at any stage of ovarian development although a significant increase (8-fold) in expression was observed from day 18 to day 25. In addition, constant mRNA expression levels of the inhibitory Smads (Smad6 and Smad7) were found between day 2 and day 18. Like for Smad8, an increase in expression of Smad6 and Smad7 was found in 25-day-old ovaries (Figure 5.4).

Localization of expression of members of the AMH-signaling pathway in the ovary

To be involved in AMH-signaling, type I receptors should be colocalized with the AMHRII. Therefore, we performed *in situ* hybridizations of AMHRII and ALK2 to determine localization of expression in the ovary. The AMHRII mRNA was expressed in granulosa cells of growing follicles (Figure 5.5 A-E). Expression was first detected in granulosa cells of primary follicles (Figure 5.5C), AMHRII mRNA was highly expressed in granulosa cells of pre-antral follicles (Figure 5.5D) and disappeared in large pre-antral and antral follicles (Figure 5.5D). No expression was detected in oocytes, theca or interstitial cells (Figure 5.5A, C and D) or in the sense slides (Figure 5.5B and E).

Expression of ALK2 mRNA was detected in granulosa, theca and interstitial cells but not in the oocytes (Figure 5.5F-J). Staining levels of ALK2 mRNA were similar in the granulosa and theca cells of all follicle types and in interstitial cells (Figure 5.5F, H and I). ALK2 mRNA staining was absent in the control sections (Figure 5.5G and J).

Figure 5.5: In situ hybridization of AMHRII and ALK2 in 18-day-old mouse ovaries.
AMHRII mRNA is detected in granulosa cells of growing follicles (A, C, D) and not in the control slides (B-E). It is present in primary (PY) and small pre-antral (SPA) follicles and expression decreases in granulosa cells of large pre-antral (LPA) and antral (AF) follicles. ALK2 mRNA is detected in granulosa, theca and interstitial cells (F, H, I) and not in the control slides (G, J). No difference in expression level can be determined between granulosa and theca cells in primary, pre-antral, antral follicles and the surrounding interstitium.

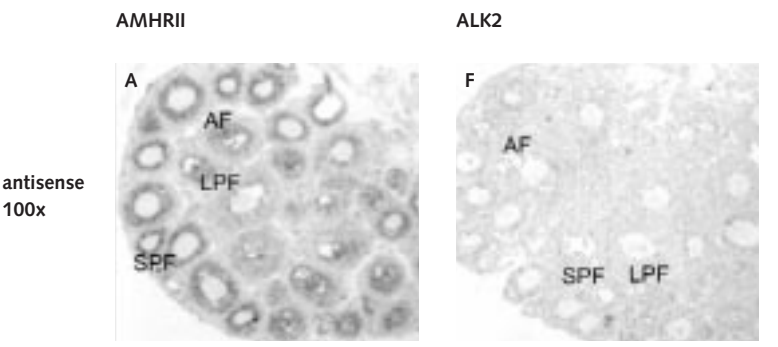
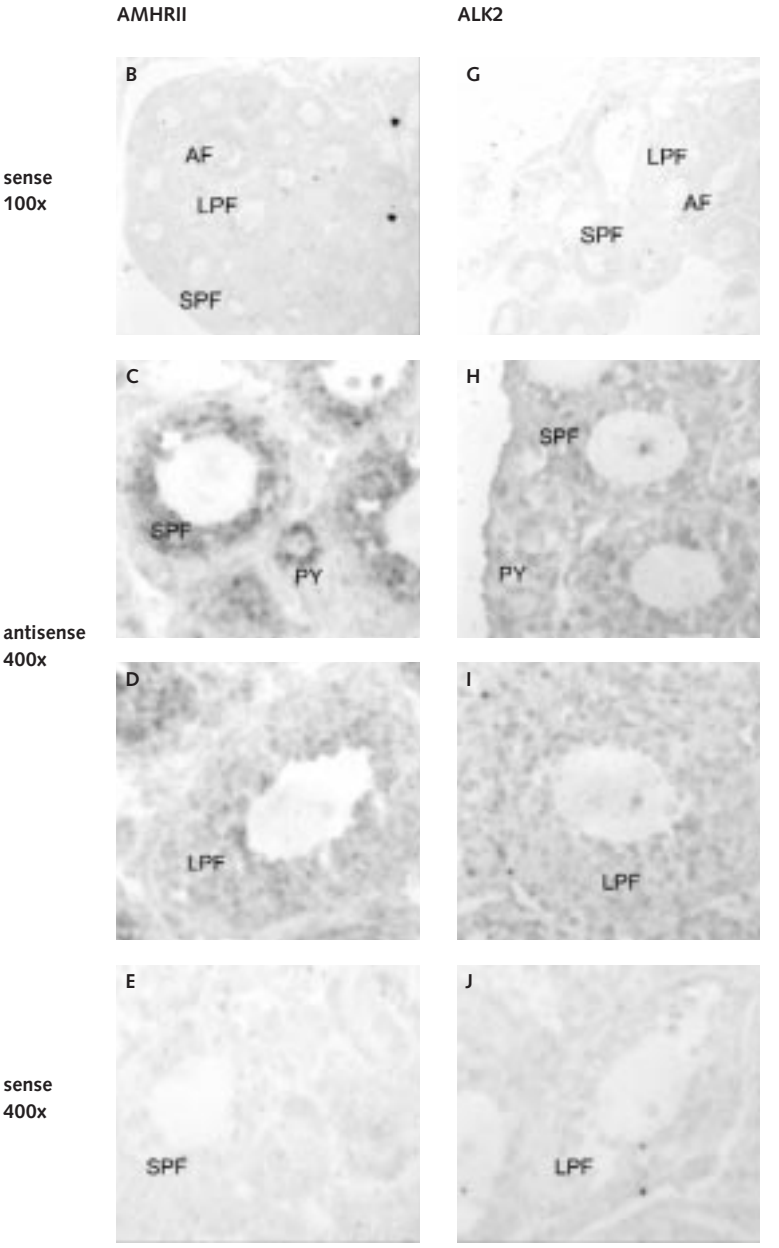


Figure 5.5:



AMH regulates its downstream signaling components

To have a further indication of AMH action in the ovary, we determined whether AMH influences the expression of the inhibitory Smads in the ovary, since several TGFβ family members stimulate the expression of these Smads. KK-1/AMHRII cells were cultured for 24 hours in the absence or presence of AMH and expression of Smad6 and Smad7 was determined using SYBRgreen reagent. AMH

strongly induced the expression of Smad6 (14-fold), the apparent stimulation of Smad7 expression (2-fold), was not significant (p=0.08) (Figure 5.6). In a timecourse experiment, we found that the upregulation of the inhibitory Smads was a rapid event, since the expression was already increased after 6 hours (results not shown). In addition, we found that AMH, similar to the results in the KK-1/AMHRII cells significantly upregulated Smad6 and Smad7 in cultured neonatal ovaries (Figure 5.6).

In a previous study (Clarke, *et al.*, 2001) it was found that Smad8 showed a sex-specific expression pattern in the Müllerian ducts, with Smad8 being expressed at a higher level in male versus female Müllerian ducts. This suggests that AMH may regulate Smad8 expression in the Müllerian ducts. Therefore, we determined whether Smad8 expression is regulated by AMH in the ovary. Indeed, AMH stimulation of KK-1/AMHRII cells and neonatal ovaries results in a strong upregulation of the hardly expressed Smad8 (10-fold respectively 37-fold) (Figure 5.6). Similar to the upregulation of Smad6 and Smad7, the upregulation of Smad8 is a rapid effect (results not shown).

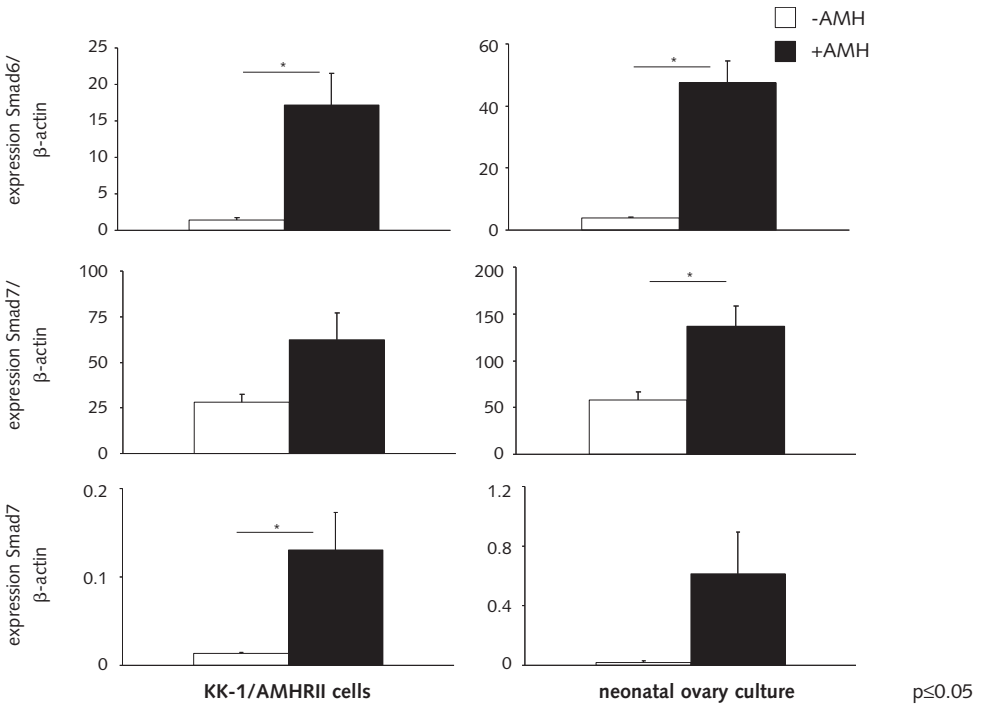


Figure 5.6: AMH regulates expression of inhibitory Smad6 and Smad7 and of the receptor-specific Smad8. Expression of Smad6, Smad7 (p=0.08) and Smad8 is upregulated by AMH in KK-1/AMHRII cells treated for 24 hours. In addition, an increase in Smad6, Smad7 and Smad8 (p=0.1) mRNA expression was also found in neonatal AMH *null* ovaries cultured for 24 hours in presence of AMH. For each treatment (control or AMH), five independent pools of KK-1/AMHRII and three independent pools of neonatal ovaries were measured. All data are presented as mean ± SEM.

DISCUSSION

The development of a primordial follicle to the pre-ovulatory follicle stage is a tightly regulated process in which intra-ovarian factors as well as systemic factors LH and FSH play an important role. AMH, a member of the TGFβ superfamily, plays an important role in this process, since AMH inhibits the recruitment of primordial follicles into the growing pool and decreases the sensitivity of large pre-antral and small antral follicles for FSH (Durlinger, *et al.*, 1999, 2001). Like all members of the TGFβ superfamily, AMH signals through serine/threonine kinase type I and type II receptors which phosphorylate the downstream Smad proteins. Based upon the Smad which is used by TGFβ superfamily members, two main signaling pathways can be distinguished. Smad2 and Smad3 are activated by TGFβs and activins through ALK4, ALK5 or ALK7 (TGFβ/activin-like signaling pathway). On the other hand, Smad1, Smad5 and Smad8 are activated by BMPs through ALK2, ALK3 and ALK6, referred to as the BMP-like signaling pathway. The type II receptor for AMH has been identified (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b) and the type I receptors ALK2, ALK3 and ALK6 have been suggested as the type I receptors for AMH. Here, we show that in the KK-1 granulosa cell line, AMH signals through the BMP-like signaling pathway since AMH in the presence of the AMHRII could activate the BMP-responsive BRE2-Luc reporter and not the TGFβ/activin-responsive 3TP-Luc reporter. In addition, AMH phosphorylates Smad1 but not Smad2 in KK-1 cells. The presence of the AMHRII is obligatory for AMH-signaling, since no other type II receptor could mediate AMH-signaling. These results are in agreement with earlier reports which showed that AMH signals through the BMP-like signaling pathway in non-ovarian fetal cell lines and Müllerian ducts (Gouédard, *et al.*, 2000; Clarke, *et al.*, 2001; Visser, *et al.*, 2001).

To investigate which candidate type I receptors and Smads can be involved in AMH-signaling in the ovary, we measured mRNA expression levels of AMHRII, ALK2, ALK3, ALK6, Smad1, Smad5 and Smad8 in ovaries of mice of various ages, which contain different follicle populations. In addition, we determined the localization of expression of AMHRII and ALK2 by *in situ* hybridization. The *in situ* hybridization experiments for ALK3 and ALK6 are ongoing. The AMHRII is like AMH (Durlinger, *et al.*, 2002 and Chapter 6 of this thesis) expressed in granulosa cells of primary and pre-antral follicles and expression decreases in large pre-antral follicles. Surprisingly, AMHRII mRNA expression in the ovary does not increase during the first 18 days of life when the granulosa cell number increases enormously. *In situ* hybridizations of fetal rat ovaries showed ubiquitous expression of AMHRII in these ovaries (Baarends, *et al.*, 1994). In 18-day-old mouse ovaries AMHRII mRNA is selectively expressed by the granulosa cells of growing follicles. Therefore, the decrease in AMHRII mRNA from 2-day-old to 6-day-old ovaries suggests a rearrangement from ubiquitous to specific expression of the AMHRII in the ovary. The mRNA expression pattern of AMHRII in postnatal mouse ovaries suggests expression of AMHRII in primordial follicles, explaining the effect of AMH on primordial follicle recruitment. Unfortunately, the histology of the *in situ* hybridization does not allow the identification of primordial follicles and therefore we were not able to determine if primordial follicles express AMHRII mRNA.

In situ hybridization revealed that ALK2 is ubiquitously expressed in the ovary. Expression was detected in granulosa, theca and interstitial cells of the ovary and quantitative real-time PCR showed that ALK2 mRNA levels increase only slightly during the first 25 days of life. These results show that in granulosa cells ALK2 mRNA expression is colocalized with AMH and AMHRII mRNA, allowing a

role for ALK2 in AMH-signaling in the ovary. Previous studies reported an overall ALK2 expression in neonatal rat ovaries with high expression in the oocytes (He, *et al.*, 1993). However, since the probes for this study contained the highly homologous kinase domain, cross-reactivity with other type I receptors could explain the discrepancy between the results.

In situ hybridization on ovaries of adult cycling rats showed ALK3 expression in oocytes, granulosa and theca cells of all follicles (Erickson and Shimasaki, 2003). These observations correlate with our results which showed a constant level of ALK3 mRNA in developing ovaries. In contrast, ALK6 mRNA levels increased from 12-day-old to 25-day-old ovaries, suggesting mRNA expression in granulosa cells of growing follicles since the number of granulosa cells strongly increases during this period. Indeed, ALK6 mRNA was strongly expressed in granulosa cells of growing follicles in adult rat ovaries (Erickson and Shimasaki, 2003). Thus, from these studies and from the present study it may be concluded that on basis of their expression pattern, each of the candidate type I receptors might be involved in AMH-signaling. Further studies, such as organ and/or cell specific knockouts, are necessary to determine exactly which type I receptor is involved in AMH-signaling in the ovary.

Levels of Smad1 and Smad5 mRNA expression did not change during the first 25 days of ovarian development, while Smad8 mRNA expression increased in 25-day-old ovaries, suggesting a role of Smad8 in atresia: the number of atretic follicles increases dramatically from 18-day-old to 25-day-old ovaries. Interestingly, Smad8 mRNA expression levels in the mesenchymal cells surrounding the Müllerian ducts is much higher in males compared to females whereas no difference in expression level could be detected for Smad1 or Smad5, suggesting regulation of Smad8 by AMH (Clarke, *et al.*, 2001). Indeed, in cultured neonatal ovaries and in KK-1/AMHRII cells AMH stimulated Smad8, but not Smad1 or Smad5 (results not shown) mRNA expression. These results suggest a role for Smad8 in AMH-signaling. However, the significance of these observations should be investigated in more detail since the expression level of Smad8 compared to that of Smad1 and Smad5 is very low.

The signaling of TGFβ family members through receptor-specific and common-mediator Smads is antagonized by the inhibitory Smad6 and Smad7. Smad7 is a general inhibitor of TGFβ/activin-like and BMP-like signaling pathways (Hayashi, *et al.*, 1997; Nakao, *et al.*, 1997; Itoh, *et al.*, 1998; Ishisaki, *et al.*, 1999; Hanyu, *et al.*, 2001), while Smad6 preferentially inhibits the BMP-like signaling pathway (Hata, *et al.*, 1998; Itoh, *et al.*, 1998; Ishisaki, *et al.*, 1999; Hanyu, *et al.*, 2001). The expression of both inhibitory Smads can be stimulated by TGFβ family members and in this way a negative feedback mechanism is formed (Afrakhte, *et al.*, 1998; Takase, *et al.*, 1998; Ishisaki, *et al.*, 1999). In addition, Clarke and colleagues found an increased Smad6 mRNA expression level in male Müllerian ducts, suggesting that AMH upregulates Smad6 mRNA expression. Indeed, in two different Leydig cell lines AMH increased expression of Smad6 and to a lesser extent Smad7 mRNA (Clarke, *et al.*, 2001). In agreement with these observations, we found stimulation of Smad6 and Smad7 mRNA expression by AMH in neonatal ovaries and in the granulosa cell line KK-1/AMHRII. No increase in Smad6 and Smad7 mRNA was detected in 2-day-old to 18-day-old ovaries *in vivo*, a time-period when AMH levels increase strongly. However, Smad6 and Smad7 expression is probably ubiquitous and *in vivo* expression of these inhibitory Smads is regulated by several members of the TGFβ superfamily which may counteract the effect of AMH.

In conclusion, AMH signals through the BMP-like signaling pathway All candidate type I receptors and Smad proteins are present in the ovary, necessitating further studies on their relative contribution to the AMH-signaling pathway. ALK6 seems to play a more important role in larger pre-antral and

antral follicles. The role of Smad8 in AMH-signaling is very interesting since its low expression is regulated by AMH, this needs further investigation. Like other members of the TGFβ superfamily, AMH upregulates expression of the inhibitory Smads, Smad6 and Smad7, forming a negative feedback loop.

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Chapter 6

GROWTH FACTORS AND PRIMORDIAL FOLLICLE RECRUITMENT

Chapter 6
GROWTH FACTORS AND PRIMORDIAL FOLLICLE RECRUITMENT

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ABSTRACT

Follicle recruitment is an important process in the regulation of the primordial follicle stock depletion in the ovary. Since the exhaustion of the follicle stock results in menopause and infertility, the understanding of the regulation of primordial follicle recruitment is of great importance. Several growth factors, such as anti-Müllerian hormone (AMH), activins, inhibins, growth and differentiation factor 9 (GDF9), bone morphogenetic protein-15 (BMP15), kit-ligand (KL), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) have been suggested to be involved in the regulation of primordial follicle recruitment. We have localized these proteins and have measured expression of their mRNAs in postnatal ovaries of mice of several ages, in which different follicle types were present. In this way, more knowledge about their role in follicle recruitment was obtained.

This study shows that AMH, activins, inhibins, GDF9, BMP15 and KL could be involved in regulation of primordial follicle recruitment, while an involvement of bFGF and NGF is less likely.

INTRODUCTION

Menopause is a major milestone in the aging process in women and starts after the last menstrual cycle. The cessation of ovarian function, which is caused by the exhaustion of the follicle stock in the ovaries, causes the almost complete absence of secretion of female sex hormones. Postmenopausal life is related with an increased risk of several diseases, like osteoporosis, cardiac failure and cognitive decline (Gibaldi, 1997; Mijatovic, *et al.*, 1999; Kesslak, 2002). Furthermore, menopause is correlated with the age at which infertility occurs, since it is preceded by a premenopausal period of 15-20 years during which fertility decreases, culminating in complete infertility approximately 10 years before menopause (te Velde, *et al.*, 1998). In view of the association of early infertility and female health problems associated with menopause, it is important to understand the processes involved in the depletion of the resting stock of primordial follicles. This limited supply of follicles is formed during fetal life and cannot be replenished at any time afterwards. Loss of the primordial follicle stock occurs through atresia and through recruitment of primordial follicles into the pool of growing follicles (Gougeon, *et al.,* 1994; Gougeon, 1996). It has been hypothesized that this recruitment is regulated by factors expressed by the growing follicles in the ovary (Hirshfield, 1994). In order to understand the process of primordial follicle recruitment it is therefore important to identify factors that are expressed by these growing follicles. Also growth factors which are involved in follicle development could be involved in regulation of primordial follicle recruitment: by influencing follicle growth and development they influence the growth factors secreted by the follicles.

Several members of the transforming growth factor β (TGFβ) superfamily have been implicated to be involved in the regulation of follicle recruitment. This is the largest known family of growth and differentiation factors and can be divided in several subfamilies, such as the TGFβs (TGFβ1-5), the activins (A, B, C, E), inhibins (A, B) and the bone morphogenetic proteins (e.g. BMP1-15, GDF1-9). In addition, anti-Müllerian hormone (AMH) is a distant member of the TGFβ superfamily (Massagué, 1998). Family members have a broad range of functions during development and in adult life, including cell cycle regulation and cell differentiation, but also in the regulation of follicular development (see also Chapter 1). Interestingly, several members of the TGFβ superfamily like AMH, inhibins, activins, growth and differentiation factor 9 (GDF9) and bone morphogenetic protein-15 (BMP15) could play an important role in the regulation of early follicle development. AMH inhibits recruitment of primordial follicles *in vivo* (Durlinger, *et al.*, 1999) and *in vitro* (Chapter 3 of this thesis). Furthermore, FSH-stimulated growth of pre-antral follicles *in vitro* is inhibited by AMH (Durlinger, *et al.*, 2001). Activin plays a role in the regulation of follicle development as has been shown in *in vitro* co-cultures of large and small pre-antral follicles. Activin, produced by larger pre-antral follicles, inhibits the growth of smaller pre-antral follicles *in vitro* (Mizunuma, *et al.*, 1999). However, the effect of activin on pre-antral follicles is controversial since in immature animals activin stimulates pre-antral follicle growth while in mature animals the opposite effect has been described (Yokota, *et al.*, 1997; Liu, *et al.*, 1998, 1999). Inhibin is known for its antagonistic actions on effects of activin. Furthermore, it was described recently that inhibin also can antagonize BMP action and therefore the action of inhibin could be more widespread than thought until recently (Wiater and Vale, 2003). The oocyte-expressed GDF9 stimulates growth of small follicles, as illustrated by a block in follicle development from primary follicles onward in GDF9 *null* mice (Dong, *et al.*, 1996).

BMP15, which is also expressed in oocytes, stimulates granulosa cell mitosis of pre-antral follicles and inhibits FSH-induced differentiation *in vitro* (Otsuka, *et al.*, 2000, 2001b; Otsuka and Shimasaki, 2002). These data indicate that TGFβ family members may play a role in the regulation of follicle growth and development. If these growth factors influence follicle growth and development they may also be involved in regulation of primordial follicle recruitment.

Besides TGFβ family members other growth factors, such as kit-ligand (KL), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF), are also involved in primordial follicle recruitment and/or early follicle development. Naturally occurring mutations in the KL gene result in impaired primordial follicle recruitment and a block in follicle growth and development after the primary follicle stage (Huang, *et al.*, 1993). *In vivo* and *in vitro* studies showed that KL is required for primordial follicle recruitment (Yoshida, *et al.*, 1997; Parrott and Skinner, 1999). A similar stimulatory effect of bFGF on primordial follicle recruitment was found (Nilsson, *et al.*, 2001). Furthermore, granulosa cell proliferation is stimulated by bFGF, showing involvement of bFGF in early follicular development (Gospodarowicz and Bialecki, 1979). Another growth factor, which might be involved in regulation of primordial follicle recruitment, is NGF. Ovaries of NGF *null* mice contain less growing follicles than their wild-type littermates (Dissen, *et al.*, 2001). In addition, follicle differentiation is stimulated by NGF through an increase in FSH receptor (FSHR) expression. The impaired growth of small follicles in NGF *null* mice can be explained by the decrease in FSHR mRNA (Romero, *et al.*, 2002). These results suggest a role for KL, bFGF and NGF in the regulation of primordial follicle recruitment.

If the growing follicle pool secretes growth factors which are involved in regulation of primordial follicle recruitment, mRNA encoding these growth factors should be expressed by growing follicles. Therefore, we measured the mRNA expression level of growth factors during ovarian development and determined the ovarian protein expression pattern.

MATERIALS AND METHODS

Animals

Mice were obtained from the animal facility (EDC) of the Erasmus MC and were maintained under standard animal housing conditions in accordance with NIH guidelines for the care and use of experimental animals. Ovaries were isolated from C57Bl6/6J wild-type mice at 1 to 10 days of age, then every other day until day 22 of age and at 25 days of age. Ovaries were fixed for 3 hours (day 1 to 12) or overnight (day 14 to 25) in Bouin's fixative, embedded in paraffin and sectioned at 5 μm. Based on the follicle population present at different ages, 2, 6, 12, 18 and 25-day-old ovaries were selected for further research. Ovaries were snap frozen in liquid nitrogen and stored at -80C, or collected and fixed for 3 hours (day 2, 6 and 12) or overnight (day 18 and 25) in Bouin's fixative for AMH, inhibin α-subunit and inhibin βA-subunit immunohistochemistry or in 3.7% formaldehyde for GDF9, bFGF, NGF and inhibin-βB subunit immunohistochemistry. Subsequently, the ovaries were embedded in paraffin and 5 μm sections were cut.

Antibodies

The AMH 7/2A antibody was raised against a synthetic peptide VPTAYAGKLLISLSEERISAHHVPN MVATECG corresponding to the C-terminus of the mature AMH protein. This human peptide sequence differs from the mouse sequence in one amino acid. In addition, the commercially available antibody MIS C-20 was used (Santa Cruz Technologies, cat# sc-6886, Santa Cruz, CA, USA). To detect expression of the inhibin α-subunit, the R1 antibody was used. This antibody recognizes free inhibin α-subunits, but also inhibin A and inhibin B (Groome, *et al.*, 1990; Arora, *et al.*, 1997). Using the E4 antibody, inhibin βA-subunit was detected. This antibody recognizes inhibin A, activin A and activin AB (Groome and Lawrence, 1991; Arora, *et al.*, 1997). Detection of the inhibin βB-subunit was performed with the C5 antibody (Groome, *et al.*, 1996). The C5 antibody recognizes inhibin βB-subunit and therefore also inhibin B, activin AB and activin B. For GDF9 two different monoclonal antibodies were used: GDF9 70 and GDF9 2/4B. GDF9 70 was raised against the synthetic peptide CVPAKYSPLSVLTIE PDGSIAYKEYEDMIATKC, corresponding to the C-terminal domain of the mature human GDF9 sequence. Between human and mouse sequences of this peptide amino acids at two positions are different. GDF9 2/4B was raised against the synthetic peptide GQETVSSELKKPLGPC, corresponding to the N-terminal domain of the mature human GDF9 sequence. This sequence differs at 7 amino acids between mouse and human GDF9. All above mentioned antibodies were used in biotinylated form. NGF protein expression was detected using the rabbit polyclonal antibody H20 (Santa Cruz Biotechnologies cat#: sc-548). Finally, the FGF-2 147 rabbit polyclonal antibody (Santa Cruz Biotechnologies cat#: sc-79) was used to detect bFGF expression. A biotinylated secondary goat anti-rabbit antibody (Dako, Glastrup, Denmark) was used to detect localization of bFGF and NGF. Immunohistochemistry for the MIS C-20 antibody was performed as described in Chapter 3. Because the AMH 7/2A, GDF9 70, GDF9 2/4B, R1, E4 and C5 antibodies were biotinylated, no use of a secondary antibody was necessary for these antibodies.

Immunohistochemistry

The localization of AMH, GDF9, NGF, bFGF and the inhibin α-, inhibin βA- and inhibin βB-subunits was studied by immunohistochemistry on ovaries of 2-, 6-, 12-, 18- and 25-day-old mouse ovaries.

Sections were mounted on slides coated with 3-aminopropyltriethoxysilane (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After deparaffinisation, sections were quenched for 20 min in 3% H_2O_2 /methanol solution to block endogenous peroxidase activity, washed with water and transferred to phosphate-buffered saline (PBS). The sections were microwaved for 3x5 min (AMH and GDF9 immunohistochemistry) or 2x10 min (inhibin α-, βA- and βB-subunits, NGF and bFGF immunohistochemistry) at 700 W in 0.01 M citric acid monohydrate buffer, pH 6.0 (Merck, Darmstadt, Germany), cooled down to room temperature (RT) and rinsed in PBS. For NGF and bFGF immunohistochemistry the sections were preincubated with normal goat serum (1:10 dilution in PBS) (Dako) for 15 min at RT. For bFGF, NGF, GDF9 and inhibin βB-subunit immunohistochemistry, endogenous biotin, biotin receptors, and avidin binding sites present in the tissues were blocked using the Avidin/biotin blocking kit (Brunschwig, Vector, Amsterdam, The Netherlands) according to the manufacturer's protocol. Sections were incubated at 4C overnight (AMH, GDF9, inhibin α- and βA-subunits, bFGF, NGF) or during 6 hours (inhibin βB-subunit) with primary antibodies against the different proteins. The antibodies were diluted in 5% BSA in PBS for AMH (MIS C-20: 0.4 ng/µl,

AMH 7/2A: 15 ng/µl), GDF9 (GDF9 70: 5.3 ng/µl, GDF 2/4B: 10 ng/µl), inhibin α-subunit (day 2-18 ovaries: 10 ng/µl, day 25 ovaries: 20 ng/µl), inhibin βA-subunit (day 2-12 ovaries: 10 ng/µl, day 18-25 ovaries: 20 ng/µl) and inhibin βB-subunit (day 2 ovaries: 1 ng/µl, day 6-25 ovaries 5 ng/µl). NGF (0.4 ng/µl) and bFGF (0.4 ng/µl) antibodies were diluted in 1.5% normal goat serum in PBS. After incubation, the sections were rinsed in PBS and the bFGF and NGF sections were subsequently treated for 30 min at RT with the biotinylated goat anti-rabbit antibody (dilution: 1:200 in 6% normal goat serum in PBS, Brunschwig, Vector). Next, sections incubated with the various antibodies were treated with streptavidin-biotin-peroxidase complex (ABC; diluted 1:200 in PBS; Dako) for 30 min at RT. They were washed three times with PBS and the peroxidase activity was developed with 0.07% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich) for seven minutes. Finally, all sections were counterstained with hematoxylin. The sections incubated with antibodies against inhibin α-, βA-, βB-subunits, bFGF and NGF were added for 4 seconds in 0.2% NH_4OH and washed with water to increase the blue color of the tissue.

Negative control sections for immunohistochemistry of AMH, GDF9 and inhibin α-, βA- and βB-subunits were incubated with 5% BSA/PBS followed by treatment with the streptavidin-biotin-peroxidase complex. NGF and bFGF control sections were incubated with peptide-primary antibody complexes, according to the manufacturer's protocol.

RNA isolation and preparation

RNA isolation, cDNA synthesis and development of primers and probes for quantitative real-time PCR were performed as described in Chapter 5. For each age group, three separately pooled groups of ovaries (n=20-40) were used. The primers and probes for the quantitative real-time PCR of AMH, KL, GDF9, BMP15, bFGF, NGF and the inhibin α-, βA- and βB-subunits are shown in Table 6.1.

Table 6.1: Oligonucleotide primers and probe sequences used to amplify AMH, GDF9, BMP15, inhibin α-, inhibin βA- and inhibin βB-subunits, NGF, kit-ligand, bFGF and β-actin mRNAs in the different aged ovaries.

Gene:	Primers:	Probe:	Product size (bp):
AMH	5'-GCT GCT GCT GCT AAA GG-3' 5'-GCG GAT TAC GGT CAG AC-3'	5'-CTC ATC CCG GAG ACC TAC CAA-3'	227
GDF9	5'-TGC TGT GGG CCT TAG A-3' 5'-TTG GTT TAT GGC AAC GA-3'	5'-CGA GTG CAG TGT CCG TAG GTG TA-3'	168
BMP15	5'-CCG GAC CAA GCA CTT AC-3' 5'-GCG AAG AAC ACT CCG TC-3'	5'-AAA GCC TTC TCC CAT GTC TAA AGC-3'	136
Inhibin α	5'-TGC ACA GGA CCT CTG AAC CA-3' 5'-TGA TAG CAC CAG AAG ATC TAG CA-3'	5'-TGA CTT CAG CCC AGC TGT GGT TCC A-3'	269
Inhibin βA	5'-ACC TCG GAG ATC ATC ACC TTT G-3' 5'-GAC TTT CAG GAA GAG CCA CAC TTC T-3'	5'-CTG ACA GGT CAC TGC CTT CCT TGG AAA TCT-3'	126
Inhibin βB	5'-ACC CAC ACA GGC GAA-3' 5'-GCA GGC CAC TCG AAG-3'	5'-AAG GCC AGC GGA TCA GTT TT-3'	306
NGF	5'-TGA AGC CCA CTG GAC TAA-3' 5'-CTG TTG AAA GGG ATT GTA CC-3'	5'-AAT AGC TGC CCG AGT GAC AGG-3'	246
Kit-ligand	5'-GAA AAG CGG TCG TGC-3' 5'-GCC AAT TTC ACA ATA TCA TTC-3'	5'-CCA ATT TGT AAA AAC GAT AAC CCG-3'	278
bFGF	5'-GCA CTT GGC CTC TCT TTA CGT GA-3' 5'-CAC AGA AGT GGC GCA TAC CTC A-3'	5'-CAT CCA AGC CTC GGC TCG GTG TCT A-3'	320
β-actin	5'-CTA CGA GGG CTA TGC TCT C-3' 5'-CTC AGG GCA TCG GAA-3'	5'-ACC GAG CGT GGC TAC AGC TT-3'	283

Statistical analysis

Results are presented as mean ± SEM. Data were analyzed for statistical differences by one-way analysis of variance (ANOVA), followed by Duncan's new multiple range test using SPSS, Inc9.0 (SPSS, Inc., Chicago, IL, USA) computer software. Differences were considered to be significant at $P \leq 0.05$.

RESULTS

Selection of ovaries

The follicle population was characterized in ovaries collected from two mice, starting the day of birth (=day 1) until day 10, then every 2 days until day 22 and day 25. Based on the age on which a new follicle class was found, five different ages were selected for detailed analysis (Figure 6.1). Ovaries of 2-day-old mice only contained primordial follicles, whereas on day 6 also primary follicles were present. In 12-day-old ovaries also pre-antral follicles were present and antral follicles were found first in ovaries of 18-day-old mice. All antral and large pre-antral follicles had become atretic in ovaries of 25-day-old mice. The criteria for atresia were the presence of picnotic nuclei in the granulosa cells and/or degeneration of the nucleus of the oocyte (Byskov, 1974; Osman, 1985). These five time points were subsequently used to determine the expression of AMH, inhibin α-, βA-, and βB-subunits, GDF9, BMP15, KL, bFGF and NGF.

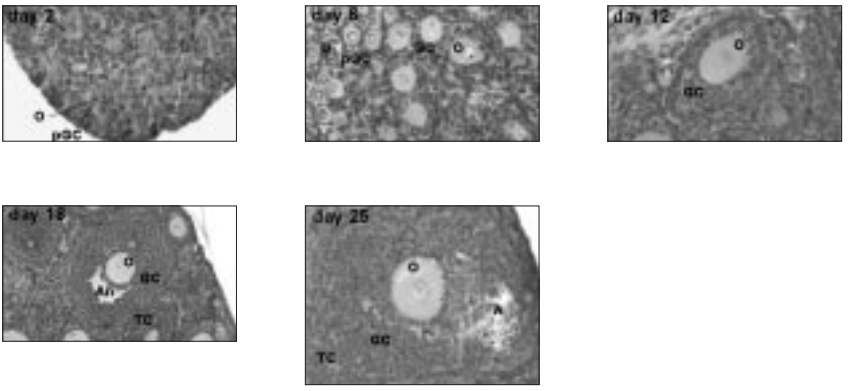
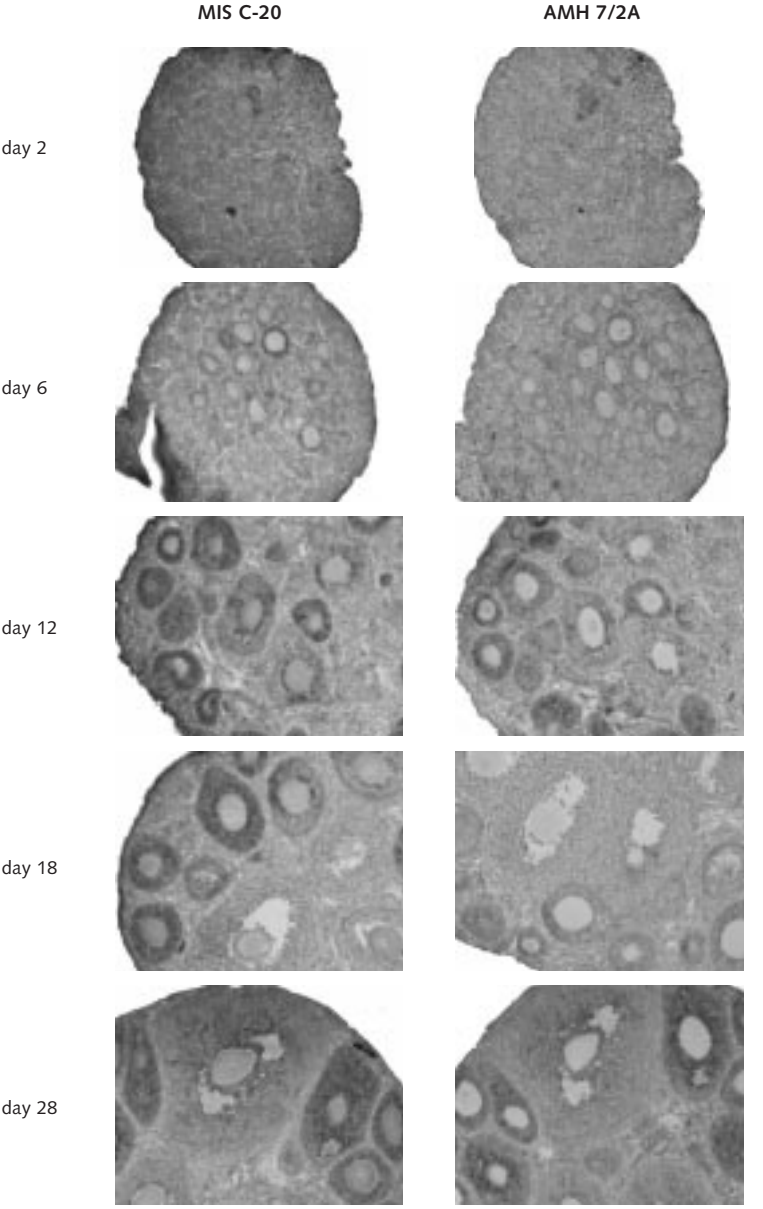


Figure 6.1: Follicle types found in postnatal ovaries.
Ovaries of 2-day-old mice only contain primordial follicles, which consist of an oocyte (O) surrounded by pre-granulosa cells (pGC). In addition to the primordial follicles, also primary follicles can be found in an ovary of a 6-day-old mouse. Oocytes of primary follicles have an increased diameter compared to primordial follicles and the granulosa cells (GC) have become cuboidal. Pre-antral follicles are first detected in 12-day-old ovaries and contain multiple layers of granulosa cells. The appearance of an antrum (An) is the characteristic of an antral follicle, which also contains granulosa (GC) and theca cells (TC). These follicles can be found in 18-day-old ovaries. Ovaries of 25-day-old mice contain atretic follicles (At) which can be recognized by several picnotic granulosa cells. Magnification x 400 for all photographs.

Figure 6.2: Immunohistochemical localization of AMH in postnatal C57Bl/6J mouse ovaries of different ages. (For a color version of this figure, see page 170.) Magnification x 100.



Immunolocalization of growth factors

Ovaries of 2-day-old mice contained only primordial follicles and immunoreactivity of TGFβ family members could be detected in these ovaries. AMH immunoreactivity could be detected first in granulosa cells of primary follicles in 6-day-old ovaries (Figure 6.2) and at later ages also in granulosa cells of

pre-antral follicles. Expression disappeared in antral or atretic follicles. Similar patterns were found with the two antibodies for detecting AMH (Figure 6.2). Inhibin α-subunit protein expression could be detected in granulosa cells of all growing and/or atretic follicles in ovaries from day 6 onward (Figure 6.3). Inhibin βB-subunit protein expression was first detected in ovaries of 12-day-old mice, while first expression of the inhibin βA-subunit protein was detected in 18-day-old ovaries. Inhibin βA- and βB-subunit proteins were detected in granulosa cells of large pre-antral, antral and atretic follicles (Figure 6.3). Expression of the GDF9 protein was first detected in the oocytes of primary follicles in 6-day-old ovaries and subsequently in all different growing and atretic follicles (Figure 6.4). No expression was detected in primordial follicles. Two GDF9 antibodies were used and some differences in expression level could be recognized. In 6-day-old ovaries, similar staining levels were found with the two GDF9 antibodies, while in 12-, 18-, and 25-day-old ovaries the staining with the GDF9 2/4B antibody was higher compared to that by the GDF9 70 antibody. Interestingly, the GDF9 70 antibody showed an increased staining in larger and atretic follicle oocytes of 25-day-old ovaries. All control slides for AMH, GDF9, inhibin α-, βA- and βB-subunits immunohistochemistry were negative (results not shown).

Figure 6.3: Immunohistochemical localization of inhibin α-, βA- and βB-subunits in postnatal C57Bl/6J mouse ovaries of different ages. (For a color version of this figure, see page 171.)
Histology of the inhibin βB-subunits differs from the inhibin a and βA-subunit immunohistochemistry due to fixation differences (formaldehyde vs. bouin). Magnification x 100.

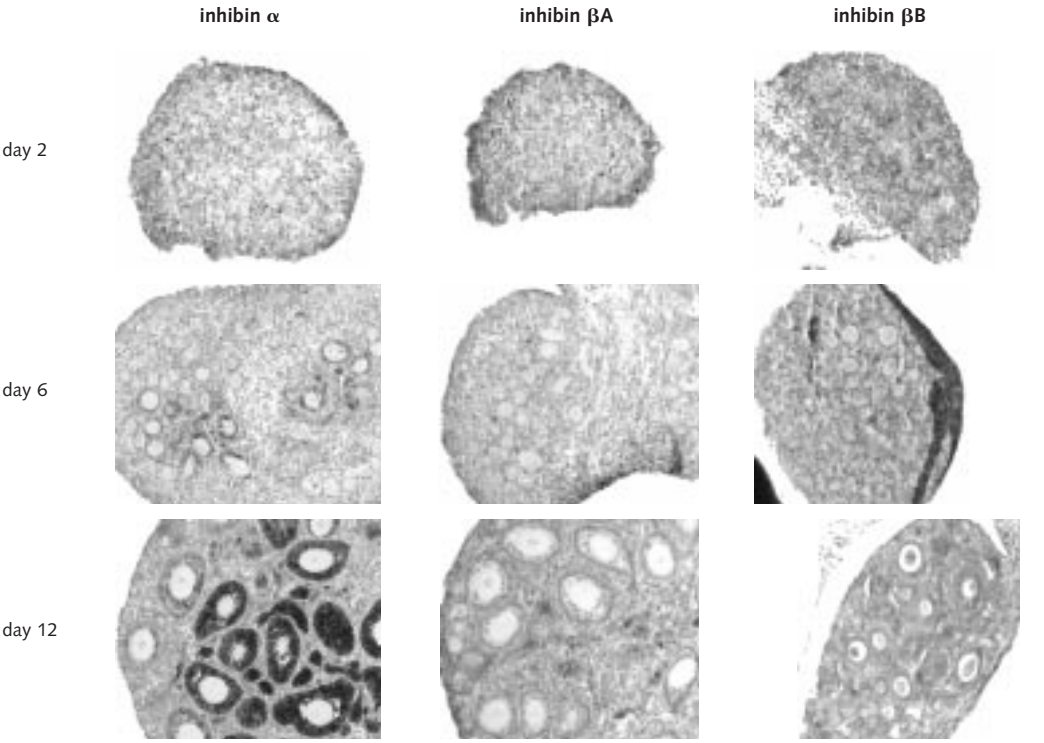


Figure 6.3

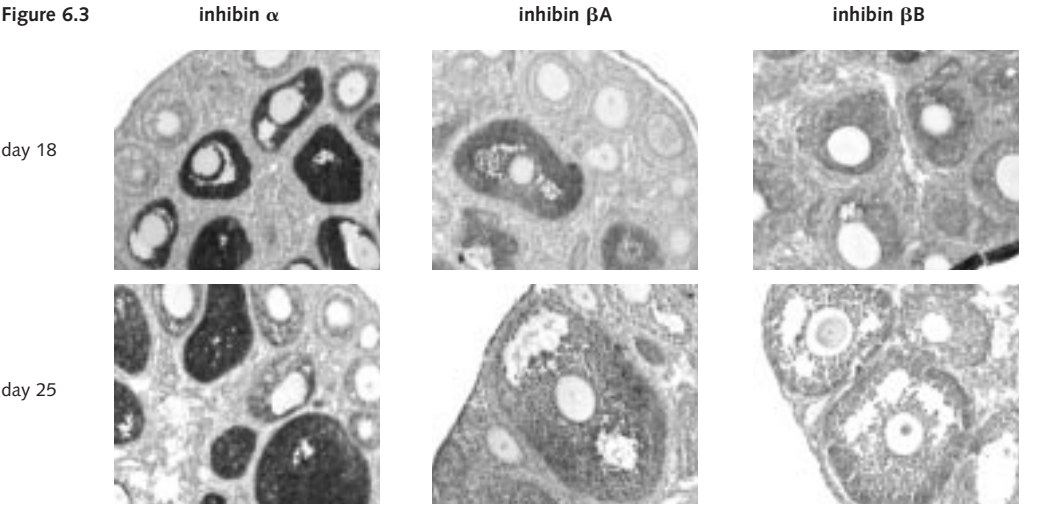


Figure 6.4: Immunohistochemical localization of GDF9 in postnatal C57Bl/6J mouse ovaries of different ages. (For a color version of this figure, see page 172.)
Magnification x 100.

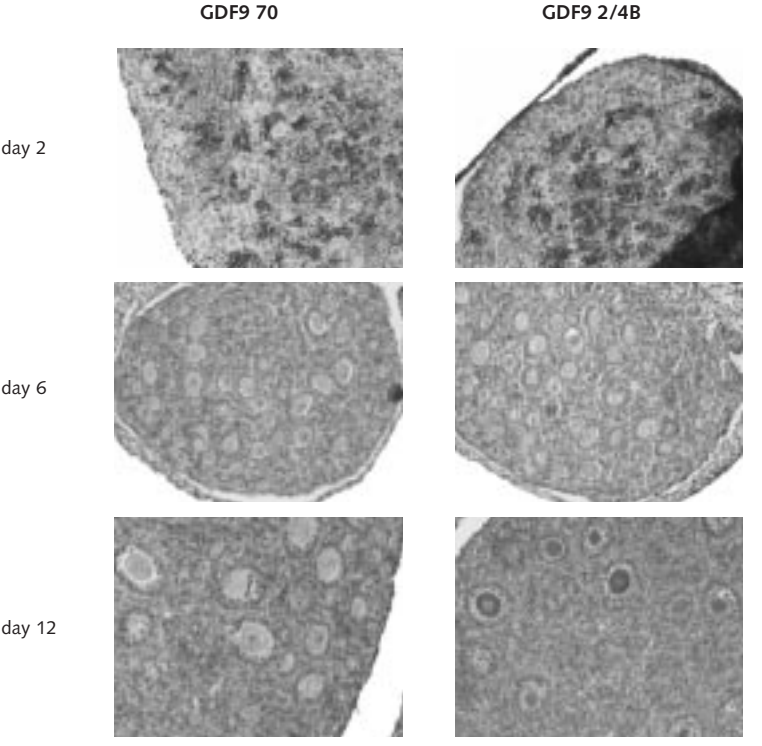
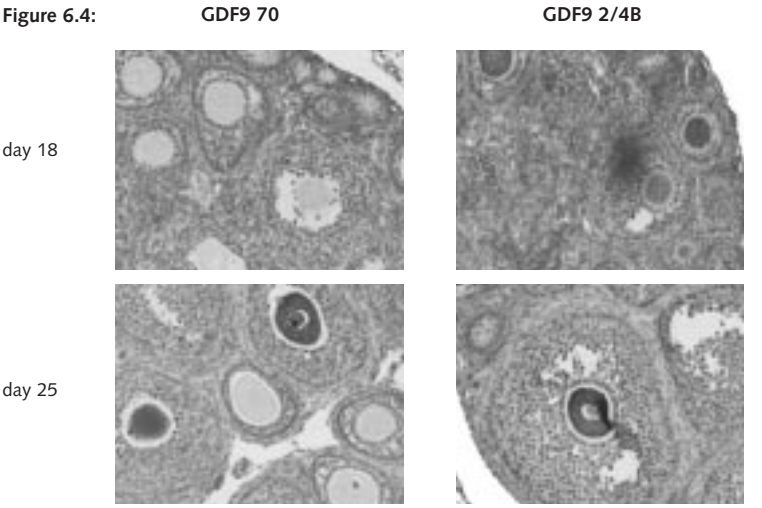


Figure 6.4:



Immunohistochemistry for NGF and bFGF revealed that these factors were not expressed by specific ovarian cell types. Expression could be found in oocytes, granulosa, theca and interstitial cells in the ovary at all ages (Figure 6.5). The expression level for bFGF in the different cell types varied during ovarian development. In 2- and 6-day-old ovaries the strongest bFGF expression was detected in oocytes and granulosa cells, while in 18- and 25-day-old ovaries interstitial cells showed the strongest bFGF staining (Figure 6.6). At every age, NGF expression was strongest in granulosa cells and oocytes of primordial to antral and atretic follicles while a lower staining was found in interstitial and theca cells. This expression was specific since the control slides, in which the peptide complex was added, no expression of bFGF or NGF could be detected (Figure 6.5).

Figure 6.5: Immunohistochemical localization of bFGF and NGF in postnatal C57Bl/6J mouse ovaries of different ages. (For a color version of this figure, see page 173.) Magnification x 100.

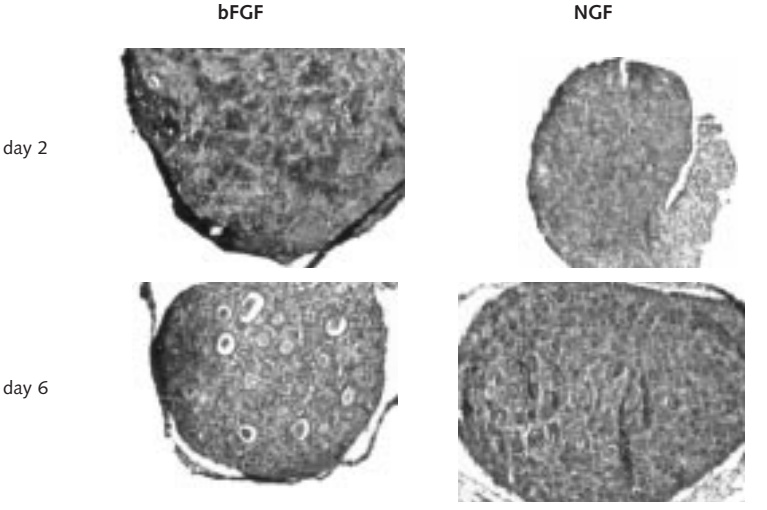


Figure 6.5

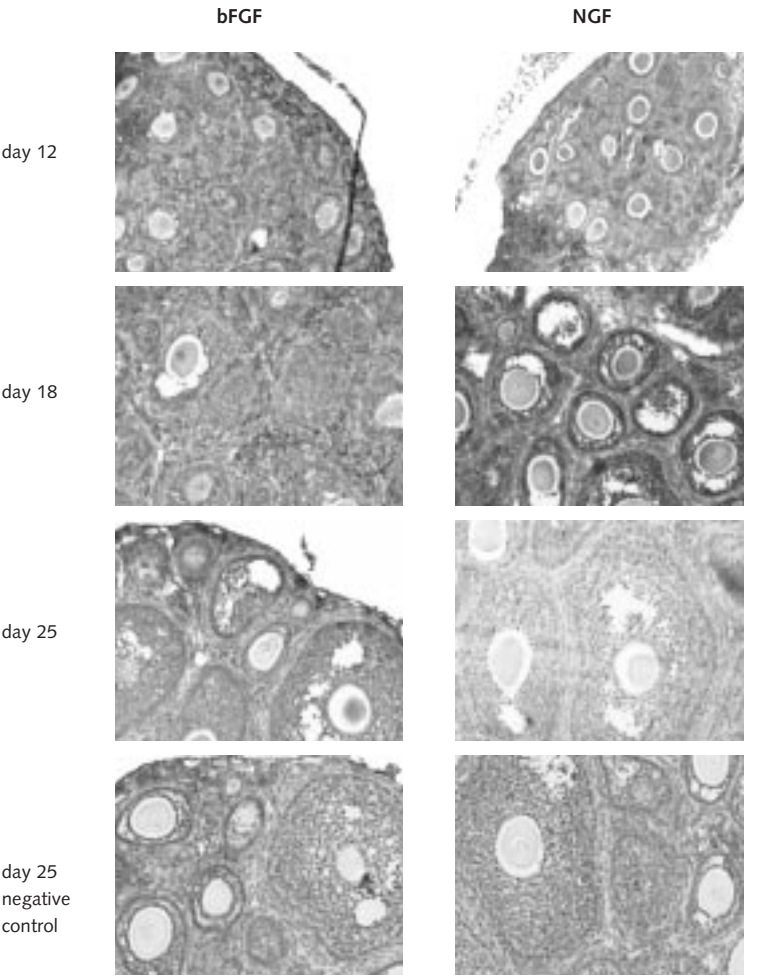
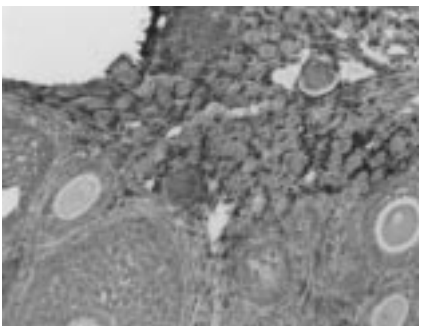


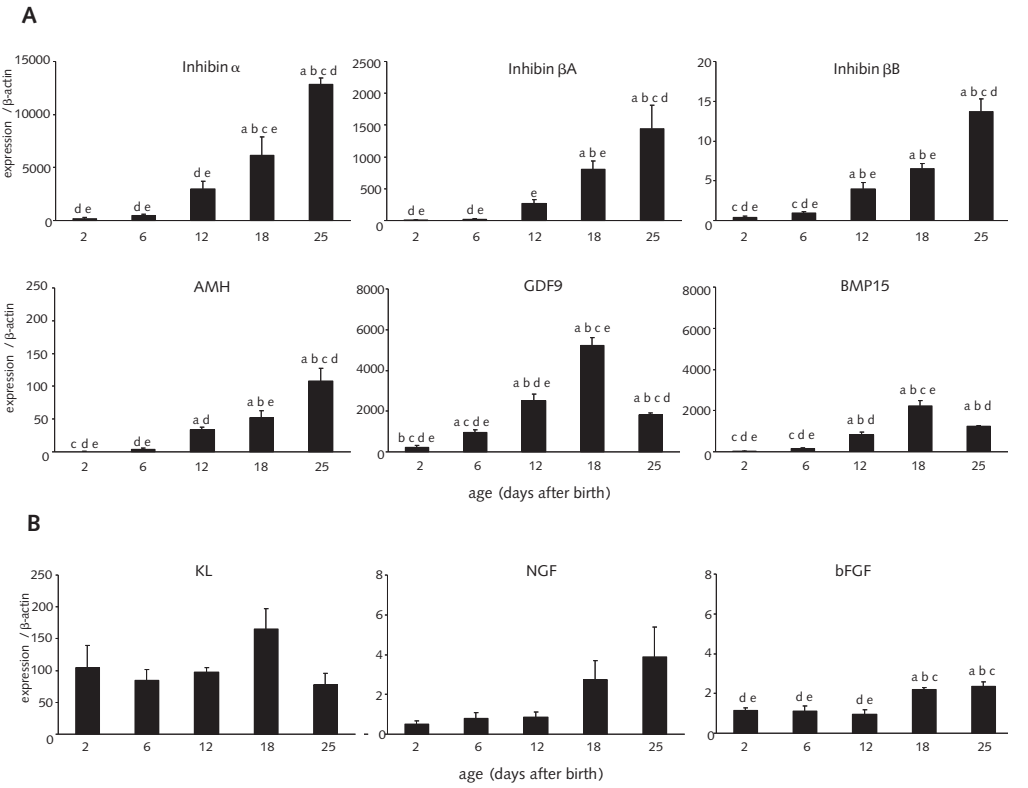
Figure 6.6: bFGF protein is expressed in the interstitial cells surrounding primordial follicles in an 18-day-old mouse ovary. (For a color version of this figure, see page 174.) Magnification x 200.



Expression of growth factors in the ovary

The mRNA expression patterns of nine growth factors in postnatal ovaries of various ages are shown in Figure 6.7. The patterns of AMH and the inhibin α-, βA- and βB-subunits were very similar during the first 25 days of life, only the level of expression was different. For all tested TGFβ family members, mRNA expression could hardly be found in 2-day-old ovaries but expression increased from day 2 to 25 (Figure 6.7A). The inhibin α-subunit was the highest expressed TGFβ superfamily member and its expression was approximately 1000 times higher than that of inhibin βB-subunit mRNA. GDF9 and BMP15 showed a similar mRNA expression pattern. Expression was low in 2-day-old ovaries and increased until day 18, where the expression level was maximal and significantly higher than at the other days. NGF and bFGF mRNA expression levels were very low compared to the expression levels of the other growth factors in the ovary (Figure 6.7B). No age-dependent change in mRNA expression levels of KL and NGF could be detected in ovaries from 2- to 25-day-old mice (Figure 6.7B). The mRNA expression pattern of bFGF was significantly increased at day 18 and 25 compared to expression in the younger animals.

Figure 6.7: Expression levels of growth factors in postnatal ovaries of different ages.
A. mRNA expression patterns of the TGFb superfamily members inhibin α-, βA-, βB-subunits, AMH, GDF9 and BMP15.
B. mRNA expression patterns of KL, NGF and bFGF. Data represent the mean ± SEM for three pools of ovaries at every age. a, b, c, d, e indicate a significant difference from day 2, 6, 12, 18 and 25 respectively (P≤0.05). Note the difference in scale of the Y-axis among the different graphs.



DISCUSSION

Knowledge about the regulation of primordial follicle recruitment is important for the understanding of the rate of depletion of the primordial follicle pool. An inverse correlation between the number of primordial follicles in the ovary and the rate at which they were recruited into the growing pool has been found (Hirshfield, 1994). This resulted in a constant number of antral follicles present in ovaries. This result strongly suggests a feedback mechanism between growing follicles and the resting follicle pool. Recently, several growth factors, including TGFβ superfamily members, have been identified to play a role in the regulation of primordial follicle recruitment. Here we determined the protein expression pattern and quantified mRNA expression of growth factors in mouse ovaries at different ages in order to gain more knowledge about their role in primordial follicle recruitment.

Several members of the TGFβ superfamily might play a role in follicle growth and development (see also Chapter 1). Here, we studied the expression of AMH, inhibin α-, βA- and βB-subunits, GDF9 and BMP15 mRNA expression and protein localization in postnatal ovaries. AMH and the three inhibin subunits are specifically expressed by the granulosa cells while GDF9 expression is only found in oocytes and these expression patterns are in agreement with results of other studies (Meunier, *et al.*, 1988; Tebar, *et al.*, 1997; Aaltonen, *et al.*, 1999; Elvin, *et al.*, 1999a; Hayashi, *et al.*, 1999; Jaatinen, *et al.*, 1999; Durlinger, *et al.,* 2002). No immunohistochemistry for BMP15 was performed because no antibody for BMP15 is yet available. However, *in situ* hybridization showed BMP15 mRNA expression in oocytes of growing follicles (Aaltonen, *et al.*, 1999; Jaatinen, *et al.*, 1999; Otsuka, *et al.*, 2000). This specific expression of TGFβ superfamily members by oocytes or granulosa cells of growing follicles might enable these proteins to regulate follicle development and/or primordial follicle recruitment. Interestingly, the mRNA expression levels of the oocyte-expressed factors are relatively high compared to the granulosa cell-expressed TGFβ family members, suggesting an important role of these oocyte factors in follicle development and/or primordial follicle recruitment.

Ovarian expression of mRNA of all granulosa cell-expressed TGFβ superfamily members increases during the first 25 days of life. For AMH, this result is unexpected since AMH protein was not detected in granulosa cells of antral and atretic follicles of which the number is increased in 18- and 25-day-old mice ovaries. The AMH mRNA expression pattern suggests that 25-day-old ovaries contain an increased number of small growing follicles or that AMH mRNA expression in smaller healthy follicles is increased in 25-day-old compared to 18-day-old ovaries, since the mRNA expression levels are corrected for the expression of β-actin and thus for the number of cells present in the ovary. Highest expression of inhibin α-, βA- and βB-subunit mRNA is found in 25-day-old ovaries. Inhibin α- and βA-subunit mRNA expression is stimulated by FSH in rat granulosa cell cultures (LaPolt, *et al.*, 1989, 1990) and FSH stimulates inhibin protein production in immature mouse and rat ovary culture (Yokota, *et al.*, 1997; Drummond, *et al.*, 2000). Since FSH serum levels decrease after day 18 in mice (Halpin, *et al.*, 1986) the increase in mRNA levels of inhibin α- and βA-subunits was not expected in the 25-day-old ovaries. One explanation for the discrepancy in increased inhibin subunit levels in 25-day-old ovaries might be that the ovary has become more sensitive for FSH due to an increased expression of the FSH receptor mRNA and protein. In the rat postnatal ovary, levels of FSH receptor mRNA and protein increases during postnatal ovarian development (White and Ojeda, 1981; Sokka and Huhtaniemi, 1990; Dunkel, *et al.*, 1994; Drummond, *et al.,* 1996). The present study

does not reveal if follicles produce activins or inhibins because the β-subunit antibodies used cannot distinguish between inhibins and activins containing the subunit against which the antibodies have been raised. Interestingly, inhibin α-subunit protein is expressed earlier in granulosa cells than the inhibin βA- and βB-subunits proteins. No effect of the inhibin α-subunit itself has been described, and therefore it is questionable if the inhibin a-subunit produced by the primary follicles already has an effect on follicle growth and development or primordial follicle recruitment.

In contrast to the expression pattern of TGFβ superfamily members in granulosa cells, the mRNA expression of the oocyte-expressed family members GDF9 and BMP15 is highest in 18-day-old ovaries. These mRNA expression patterns suggest that GDF9 and BMP15 mRNA are expressed strongest in large pre-antral and antral follicles. We found an increase in GDF9 protein expression in pre-antral follicles compared to primary follicles in ovaries of 12-, 18- and 25-day-old mice using two different GDF9 monoclonal antibodies, whereas we did not detect any difference in GDF9 protein staining between pre-antral or antral follicles in 18-day-old mouse ovaries. Immunohistochemistry with a polyclonal GDF9 antibody in rat ovaries showed even a decreased staining in oocytes of antral follicles (Jaatinen, *et al.*, 1999). The GDF9 mRNA expression data together with the AMH, inhibin α-, βA- and βB-subunit mRNA expression data suggest an increase in the number of growing follicles in 18-day-old ovaries. A complication is that in 25-day-old ovaries the GDF9 70 antibody, but not the GDF9 2/4B antibody, showed an increased staining in oocytes of large pre-antral and antral follicles. This increase in staining is probably not caused by an increased GDF9 protein expression but by a cross-reactivity with BMP15 protein. The peptide used to raise the GDF9 70 antibody shows a 61% homology with the BMP15 amino acid sequence, while the peptide against which the GDF9 2/4B antibody was raised shows no homology with BMP15. Furthermore, almost all follicles with an increased GDF9 70 antibody staining showed histological hallmarks of atresia. Therefore, it is possible that in atretic follicles, where proteins become degraded, the GDF9 70 antibody also recognizes degraded BMP15.

The TGFβ superfamily members studied in this chapter are specifically expressed by oocytes or granulosa cells. Since it is hypothesized (Hirshfield, 1994) that the growing follicle pool is involved in the regulation of the outgrowth of primordial follicles, these family members could be involved in regulation of this process. In contrast, bFGF and NGF are expressed by all cell types in the ovary, including the interstitial cells. Therefore, these growth factors may have a facilitating but not a regulatory role in primordial follicle recruitment. Indeed, Romero and colleagues showed a facilitating role of NGF in follicle differentiation by its stimulatory effect on FSH receptor mRNA expression (Romero, *et al.*, 2002). The protein expression of bFGF by interstitial cells surrounding primordial follicles (Figure 6.7) does not exclude a facilitating role for this growth factor in primordial follicle recruitment. Both NGF and bFGF mRNA are expressed at low levels in the ovary during the first 25 days of life. In contrast, both proteins show a general but specific staining in the ovary. The high staining of NGF in granulosa cells of growing follicles does not correlate with the absence of such an increase in mRNA expression during the first 18 days of life. A short half-life of mRNA of these proteins could explain the discrepancy between mRNA and protein levels. Therefore, studies about the mRNA and protein stability of bFGF and NGF should be performed to confirm the immunohistochemistry and real-time PCR data.

KL is required for primordial follicle recruitment since the absence of KL blocks outgrowth of follicles (Yoshida, *et al.*, 1997; Parrott and Skinner, 1999). Several reports showed an increase in expression of KL from primordial to the pre-antral stage using *in situ* hybridization and immunohistochemistry (Manova, *et al.*, 1993; Motro and Bernstein, 1993). After antrum formation, KL expression in granulosa cells surrounding the oocyte disappears and continues only in the outer granulosa cell layer of the follicle (Manova, *et al.*, 1993). This expression pattern resulted in a decrease of mRNA from the day of birth until the age of 8 days, whereafter the mRNA expression increased again (Manova, *et al.*, 1993). In contrast, we did not find a significant increase in KL mRNA expression during the first 25 days of life although the expression of KL in 18-day-old ovaries was 1.5-fold increased compared to 2-day-old ovaries. Measuring mRNA levels of KL using SYBRgreen and a different primerset showed similar results (results not shown). One explanation for not finding a significant decrease in KL mRNA levels, might be that KL mRNA is also expressed in primordial follicles since 2-day-old ovaries only contain primordial follicles and express KL mRNA. Also *in situ* hybridization on postnatal mouse ovaries suggested expression of KL in primordial follicles (Manova, *et al.*, 1993) and KL protein expression is reported in primordial follicles in monkey (Gougeon and Busso, 2000) and sheep (Tisdall, *et al.*, 1999) ovaries. Because primordial follicles form the largest group of follicles in the postnatal ovary, a small increase in KL expression in pre-antral follicles could be too small to result in a significant increase in KL mRNA expression in the total ovary. No immunohistochemistry on postnatal ovaries was performed yet, since KL mRNA and protein expression in (postnatal) ovaries of several species was already described in detail by several other groups (Manova, *et al.*, 1993; Motro and Bernstein, 1993; Tisdall, *et al.*, 1999; Gougeon and Busso, 2000). However, the results of this study show the importance of studying KL expression in postnatal mouse ovaries, focussing on the expression of KL in primordial follicles.

In conclusion, the results presented in this chapter show that AMH, GDF9, BMP15, inhibin/activin subunits and KL are specifically expressed by granulosa cells or oocytes in growing follicles and therefore may be involved in regulating the feedback signal to the primordial follicle pool. In contrast, NGF and bFGF are expressed by all cell types present in the ovary and therefore these growth factors probably may have a facilitating rather than a regulatory effect on primordial follicle recruitment.

ACKNOWLEDGEMENTS

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Chapter 7

MOLECULAR MECHANISM OF ACTION OF ANTI-MÜLLERIAN HORMONE (AMH): A SEARCH FOR AMH-REGULATED GENES

Chapter 7
MOLECULAR MECHANISM OF ACTION OF ANTI-MÜLLERIAN HORMONE (AMH): A SEARCH FOR AMH-REGULATED GENES

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ABSTRACT

Anti-Müllerian hormone (AMH) is a member of the TGFβ superfamily of growth and differentiation factors and is responsible for regression of the Müllerian ducts during male sexual development. In addition, AMH plays an important role in ovarian function since it inhibits primordial follicle recruitment and decreases the FSH-sensitivity of large pre-antral and small antral follicles. However, the molecular mechanisms by which AMH achieves these effects are largely unknown.

To understand the downstream effects of AMH we aimed to identify AMH-regulated genes. Neonatal mouse ovaries were cultured for 24 hours in the absence and presence of AMH and gene expression differences were analyzed using the Atlas™ mouse cDNA expression array. In addition, mouse KK-1/AMHRII granulosa cells were treated with AMH for 24 hours and a genome-wide search was performed using the Agilent mouse development oligo microarray.

This study identified eight upregulated and three downregulated genes, of which four genes can also be regulated by TGFβ and/or BMPs. Quantitative real-time PCR experiments should validate AMH-regulation of the identified genes. The importance of validation of differentially expressed genes obtained by Atlas array is illustrated: all identified genes turned out to be false positives. In addition, we report limitations of the Agilent mouse development oligo microarray caused by variations in dye labeling.

INTRODUCTION

Anti-Müllerian hormone (AMH) is a member of the TGFβ superfamily of growth and differentiation factors which include also inhibins, activins, bone morphogenetic proteins (BMPs) and growth and differentiation factors (GDFs) (Massagué and Chen, 2000). In 1947 it was shown by Jost that during male sex differentiation AMH produced by the testis is responsible for regression of the Müllerian ducts, the *anlagen* of female genital tract (Jost, 1947). In the testis, both Leydig and Sertoli cells are AMH target cells, since both cell types express the AMH type II receptor (AMHRII) (Baarends, *et al.*, 1994; di Clemente, *et al.*, 1994b; Racine, e*t al.*, 1998; Lee, *et al.*, 1999; Teixeira, *et al.*, 1999). Male mice, overexpressing AMH, have impaired Leydig cell function (Behringer, *et al.*, 1990; Lyet, *et al.*, 1995; Racine, *et al.*, 1998). Furthermore, AMH and AMHRII *null* mice incidentally show Leydig cell hyperplasia (Behringer, *et al.*, 1994; Mishina, *et al.*, 1996). During the spermatogenic cycle, both AMH and AMHRII are differentially expressed by Sertoli cells with the highest expression at stage VII, suggesting a role for AMH in spermatogenesis (Baarends, *et al.*, 1995a). In female mice and rats, AMH is expressed postnatally in the ovaries in granulosa cells of small growing (i.e. pre-antral and small antral) non-atretic follicles in rat and mouse (Chapter 3; Ueno, *et al.*, 1989; Münsterberg and Lovell-Badge, 1991; Hirobe, *et al.*, 1992; Baarends, *et al.*, 1995b). In the ovary, AMH inhibits outgrowth of primordial follicles (Chapter 3, Durlinger, *et al.*, 1999) and decreases the sensitivity of pre-antral and small antral follicles for FSH (Durlinger, *et al.,* 2001). Although the physiological effects of AMH in the Müllerian ducts, testis and ovary have been described, little is known about the exact molecular mechanism by which AMH establishes these effects. Few AMH-regulated genes have been identified (Table 7.1); these genes play a role in apoptosis, cell cycle, steroidogenesis, BMP/AMH-signaling pathway or in the regulation of effects of gonadotropins.

Table 7.1: AMH-regulated genes found in Müllerian ducts, ovary, testis or breast cancer tissue.

Gene:	Group:	Up (+) or down (-) regulation:	Tissue:	References:
MMP2	Apoptosis	+	Müllerian ducts	Roberts *et al.*, 2002
IEX-1S	Apoptosis/NFkB-signaling	+	Breast	Segev *et al.*, 2000, 2001
Cyp17	Steroidogenesis	-	Testis	Racine *et al.*, 1998; Teixeira *et al.*, 1999
Cyp11A	Steroidogenesis	-	Testis	Racine *et al.*, 1998
Δ5-3β-HSD	Steroidogenesis	-	Testis	Racine *et al.*, 1998
Cyp19	Steroidogenesis	-	Ovary, testis	di Clemente *et al.*, 1994; Rouiller-Fabre *et al.*, 1998
Smad6	BMP/AMH-signaling	+	Müllerian ducts, ovary, testis	Clarke *et al.*, 2001, Chapter 5 of this thesis
Smad7	BMP/AMH-signaling	+	Ovary, testis	Clarke *et al.*, 2001, Chapter 5 of this thesis
Smad8	BMP/AMH-signaling	+	Müllerian ducts, ovary	Clarke *et al.*, 2001, Chapter 5 of this thesis
LHR	Gonadotrophin receptor	-	Ovary	di Clemente *et al.*, 1994; Racine *et al.*, 1998
p16	Cell cycle	+	Ovary	Ha *et al.*, 2000

Two AMH-regulated genes are involved in apoptosis: matrix metalloproteinase 2 (MMP2) and IEX-IS. During Müllerian duct regression, epithelial cells become apoptotic after they become detached from the basement membrane (Roberts, *et al.*, 1999; Allard, *et al.*, 2000). This suggests a role for MMPs since these enzymes are involved in degradation of extracellular matrix and cell surface proteins (Vu and Werb, 2000).

Indeed, MMP2 is necessary for Müllerian duct regression and its expression is under control of AMH (Roberts, *et al.*, 2002). The second AMH-regulated gene involved in regulation of apoptosis is the NFκB-regulated gene IEX-IS. In the breast tumor cell line T47D, AMH induces growth inhibition and apoptosis and it was suggested that this is mediated through the NFκB-signaling cascade (Segev, *et al.*, 2000) since the mRNA levels of the IEX-IS gene are upregulated after 1 hour of AMH stimulation (Segev, *et al.*, 2000, 2001). Involvement of the IEX-1S in apoptosis is supported by the fact that expression of this gene by itself induces apoptosis in T47D cells (Segev, *et al.*, 2000). However, if AMH signals through the NFκB-signaling pathway still needs to be proven.

Four steroidogenic enzymes are known to be regulated by AMH: P450 17α-hydroxylase/C17-20 lyase (Cyp17), cytochrome P450 cholesterol side-chain cleavage ($P450_{SCC}$, Cyp11A), Δ5-3β-hydroxysteroid dehydrogenase (Δ5-3β-HSD) and aromatase (Cyp19). AMH suppresses testosterone serum levels by inhibiting the expression of several steroidogenic enzymes. This is mainly caused by a decreased expression of Cyp17, the enzyme that catalyzes the conversion of progesterone to androstenedione (Racine, *et al.*, 1998; Teixeira, *et al.*, 1999). In addition, also mRNA expression of Cyp11A and Δ5-3β-HSD is decreased (Racine, *et al.*, 1998). Cyp19 is another steroidogenic enzyme of which expression is inhibited by AMH. A decreased FSH-stimulated Cyp19 mRNA expression was found in cultured immature rat granulosa cells treated with AMH (di Clemente, *et al.*, 1994a). Furthermore, AMH inhibits Cyp19 expression in cultured Sertoli cells (Rouiller-Fabre, *et al.*, 1998).

The candidate downstream mediators of AMH-signaling (Smad1, -5 and -8) are all expressed in the mesenchyme cells surrounding the Müllerian ducts. Interestingly, the Smad8 mRNA expression level is much higher in males compared to females while no difference in expression level could be detected for Smad1 or Smad5 (Clarke, *et al.*, 2001). Indeed, AMH stimulates the expression of Smad8 in cultured neonatal ovaries and in the mouse granulosa cell line KK-1/AMHRII (Chapter 5). In addition, Smad6 mRNA expression is also upregulated in mesenchymal cells of the male Müllerian duct. Expression of Smad6 and also Smad7, another inhibitory Smad, is regulated by AMH in neonatal ovaries, the KK-1/AMHRII cell line (Chapter 5) and in Leydig cell lines (Clarke, *et al.*, 2001).

Another AMH-regulated gene is the LH receptor (LHR). AMH inhibits the FSH-induced expression of the LHR in cultured rat and porcine granulosa cells (di Clemente, *et al.*, 1994a). However, this inhibition is only achieved *in vitro* and not *in vivo*, since LHR expression is normal in mice overexpressing AMH (Racine, *et al.,* 1998). AMH inhibits cell cycle progression in the human ovarian cancer cell line OVCAR8 by upregulating p16 protein expression which inhibits cell cycle progression by binding to cyclin dependent kinase 4 (cdk4) and cdk6 (Sherr, 1995; Ha, *et al.*, 2000). This upregulation of p16 protein is probably achieved by an increase in translation of p16 mRNA, since no change in p16 mRNA level was found (Ha, *et al.*, 2000).

In conclusion, several AMH-regulated genes in different AMH target tissues have been described. However, it is not clear if these genes are responsible for the effects of AMH on primordial follicle recruitment and/or inhibition of FSH-sensitivity of large pre-antral and small antral follicles. To identify AMH-regulated genes, which are involved in these processes in the ovary, we cultured neonatal ovaries and KK-1/AMHRII mouse granulosa cells in the presence and absence of AMH. Differences in gene expression were studied using the Atlas™ mouse cDNA expression array and the Agilent mouse development oligo microarray.

MATERIALS AND METHODS

Animals

Mice were obtained from the animal facility (EDC) of the Erasmus MC. The mice were maintained under standard animal housing conditions in accordance with NIH guidelines for the care and use of experimental animals. The AMH *null* mouse genotype was determined as described by Durlinger *et al.* (1999).

Ovary and cell culture

Ovaries of 2-day-old wild-type and AMH *null* mice were cultured for 24 hours in the presence of purified AMH (267 ng/ml) or control medium. Culture conditions were as described previously (Chapter 3). Rat His6-tagged AMH was purified from conditioned medium of stably transfected cells as described previously (Durlinger, *et al.*, 2001, Chapter 5 of this thesis). After culture ovaries were snap-frozen in liquid nitrogen and stored at –80C.

The KK-1/AMHRII cell line (Chapter 5 of this thesis) was cultured in DMEM/F12 with 10% FCS, penicillin (400 IU/ml) and streptomycin (0.4 mg/ml). At a cell confluency of 80%, cells were further cultured for 2 hours in 2% FCS, followed by a 24 hour treatment with AMH (33 ng/ml). Next, cells were washed with PBS and stored at -20C in RNA-Bee (Campro Scientific, Veenendaal, The Netherlands).

RNA isolation of cultured ovaries and of KK-1/AMHRII cells using RNA-Bee was performed as described in Chapter 5. Gene expression differences were analyzed using the Agilent mouse development oligo microarray.

Hybridization on Atlas™ mouse cDNA expression array filters

Neonatal ovary cultures were used to search for AMH-regulated genes using the Atlas™ mouse cDNA expression array filters. After RNA isolation, the presence/absence of genomic DNA in the RNA samples was tested using a 40 cycle PCR on the mouse L1-repeat gene (forward primer: 5'-GAG ATT ACC AGA TGG CGA AAG-3' and reverse primer 5'-GCC GAT GTT CTC TAT GGA ATC-3'). For the 50 μl PCR reaction, 0.5 pmol/μl primers were used in a PCR buffer containing 1.2 mM DTT, 50 mM KCl, 10 mM TrisHCl, 0.25 mM dNTP (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 1.5 mM $MgCl_2$ and 0.05 units/μl Supertaq (Sphearo-Q, Leiden, The Netherlands). The PCR reaction mixtures were preheated at 95C for 5 minutes followed by 40 PCR cycles (30 seconds denaturation at 94C, 45 seconds annealing at 60C, 30 seconds extension at 72C) and a final extension step of 10 minutes at 72C. The products were separated by electrophoresis on a 1.5% agarose gel. If a product was found, the DNAse treatment was repeated.

cDNA was made from total RNA using a mixture of array gene-specific primers and was labeled with ^{32}P-dATP. Probe synthesis, labeling, purification and hybridization to the filters was performed following the instructions of the manufacturer (Clontech, USA). The filters were exposed to a phosphorimaging screen at room temperature and scanned using the PhosphorImager (Molecular Dynamics, Sunnyvale, USA). The results were analyzed using BD AtlasImage™ Software 2.7.

Hybridization on Agilent mouse development oligo microarray

The response of the KK-1/AMHRII cells to AMH was checked by determining the expression of Smad6 (forward primer 5'-CCC CAT CTT CGT CAA CT-3' and reverse primer 5'-CGC TCA AAG TCG AAC AC-3') and Smad7 (forward primer 5'-CTC CAG GAC GCT GTT G-3' and reverse primer 5'-TGC ATG AAC TCG TGG TC-3') using quantitative real-time PCR (as described in Chapter 5).

Genomic DNA present in the RNA samples used to hybridize the Agilent mouse development oligo microarray was removed using the SV Total RNA Isolation system (Promega, Leiden, The Netherlands). In addition, the quality of RNA was tested using the Lab-on-a-chip 2100 Bioanalyzer (Agilent) performed according to the protocol of the manufacturer by ServiceXS (Leiden, The Netherlands).

The Cy5/Cy3 labeling of RNA and the hybridization on the Agilent mouse development oligo microarray were performed by Service XS, according to the protocols of the manufacturer of Agilent Fluorescent Direct label kit and Agilent Oligonucleotide microarray hybridization (Agilent Technologies, USA). Hybridization was performed in duplicate with a reverse dye labeling (dye-swap) to check for preferential dye incorporation. The data were analyzed using the Agilent Feature Extraction software. Genes were considered to be significantly up- or downregulated if in one array the Cy3/Cy5 and in the other array the Cy5/Cy3 ratio was equal to or larger than 1.5, if the signal level was above background (=average signal of negative controls + 2xSD) (Wang, et al., 2001) and when the p-value was lower than 10^{-3}.

cDNA synthesis and quantitative real-time PCR

AMH-regulated genes identified by the Atlas™ mouse cDNA expression array filters were re-examined using quantitative real-time PCR. RNA samples were first tested for the presence of genomic DNA in a RT minus reaction, measuring β-actin (primers see Table 7.2) using SYBRgreen reagent as described in Chapter 5. Subsequently, cDNA synthesis and quantitative real-time PCR using SYBRgreen were performed as described in Chapter 5. Primers used are shown in Table 7.2.

Table 7.2: Oligonucleotide primer sequences used to validate gene expression differences found with the Atlas™ mouse cDNA expression array filters.

Gene:	Forward primer:	Reverse primer:	Product size (bp):
RelA	5'-AAG CCT TCC CGA AGT G-3'	5'-CCT CCG AAA GCG AGA T-3'	112
Frizzled 3	5'-AAA CAG AGT TCG GAT TGA GA-3'	5'-AAG AGT GGC ACA AGG TAG AG-3'	109
Cathepsin L	5'-GTG GGC TAT GGC TAT GAA-3'	5'-TCA TTA CCG CTA CCC ATC-3'	181
Integrin α6	5'-GAA GCC GAT TCG TCT AAA-3'	5'-TTC CCA GCG ATT GAA TAG-3'	225
β-actin	5'-CTA CGA GGG CTA TGC TCT-3'	5'-TCG TTG CCA ATA GTG ATG-3'	264

Table 7.3: AMH-regulated genes obtained using the Atlas™ mouse cDNA expression array.
Wild-type neonatal mouse ovaries were cultured for 24 hours in the absence or presence of AMH.

Gene:	Group:	ratio (+AMH/-AMH):
Glutathione S-transferase mu 2 (GSTM2); GST5-5	Detoxification	0.3
Integrin alpha 6 (ITGA6)	Extracellular matrix	0.4
Frizzled homolog 3 (FZD3; FZ3)	Wnt-signaling	0.5
Cathepsin L (CTSL); major excreted protein (MEP)	Protease	1.7
Transcription termination factor 1 (TTF1)	Transcription factor	2.1
RelA proto-oncogene; NF-kappa-B transcription factor p65 subunit (NF-κB p65)	NFκB-signaling	2.3
Special AT-rich sequence-binding protein 1 (SATB1)	Transcription factor	2.5

RESULTS

Identification of AMH-regulated genes using the Atlas™ mouse cDNA expression array filters

Previously, we have shown that neonatal ovaries are sensitive to AMH (Chapter 3). Therefore, to screen for AMH-regulated genes in the ovary, neonatal ovaries were cultured for 24 hours in the absence or presence of AMH. Using the Atlas Array expression system, which contains 588 different mouse genes, AMH-induced differences in gene expression were studied (Figure 7.1). Using the BD AtlasImage™ Software 2.7 four up- and three downregulated genes were identified (Table 7.3).

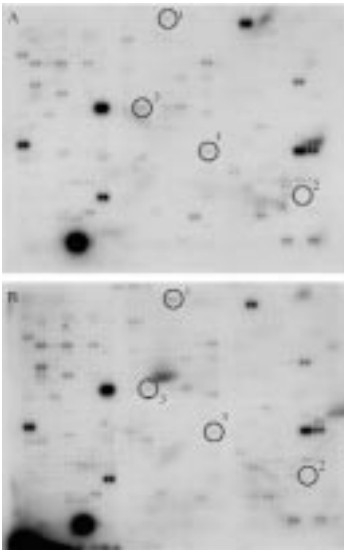

Figure 7.1: cDNA expression profile of 2-day-old neonatal ovary culture of wild-type mice cultured for 24 hours in the absence or presence of AMH.
Membranes were hybridized under identical conditions with 5 μg total ^{32}P-labeled cDNA. Differentially expressed genes were identified using BD AtlasImage™ Software 2.7 and are marked by numbers: 1=RelA, 2=cathepsin L, 3=frizzled 3 and 4=integrin a6.

We focussed on RelA, cathepsin L, integrin a6 and frizzled3 since in the literature a connection of these genes with AMH can be found and/or a role in follicular development has been described. Quantitative real-time PCR was performed to confirm the AMH-induced differential expression of these genes. However, none of these four genes was found to be differentially regulated using this technique neither in neonatal ovaries nor in KK-1/AMHRII cells (Figure 7.2).

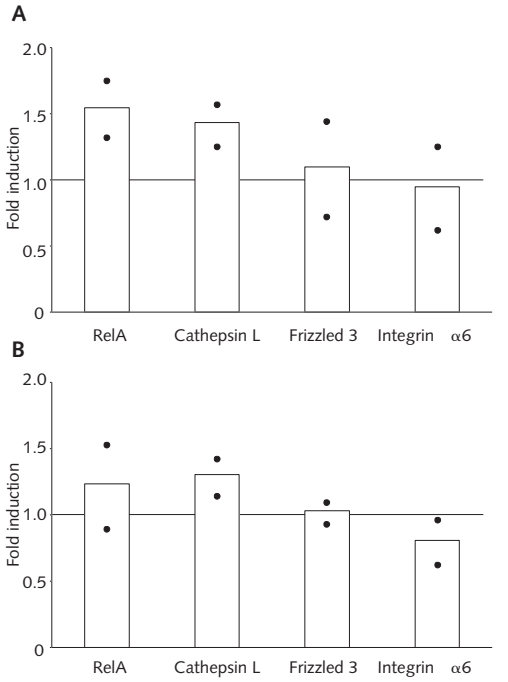

Figure 7.2: Validation of AMH-regulated gene expression in 2-day-old neonatal AMH null ovaries (A) and in KK-1/AMHRII cells (B).
Gene expression of RelA, cathepsin L, frizzled3 and integrin α6 was measured by quantitative real-time PCR using SYBRgreen. Gene expression was corrected for β-actin and fold induction was determined as ratio of gene expression +AMH/-AMH. The bars represent the mean fold induction and the spots represent the fold induction of the two independent experiments.

Identification of AMH-regulated genes using the Agilent mouse oligo microarray

Because the Atlas array did not yield any AMH-regulated genes, we decided to perform a genome-wide screen using the Agilent developmental oligo array. This glass-slide based array contains over 20,000 mouse genes and ESTs. For this expression analysis, KK-1/AMHRII cells were stimulated with AMH for 24 hours. As described in Chapter 5, we found that Smad6 and Smad7 gene expression is upregulated in KK-1/AMHRII cells after 24 hours of culture in the presence of AMH. To test the quality of the response of the KK-1/AMHRII cells to AMH, the expression of Smad6 and Smad7 was measured before using this RNA to hybridize to the Agilent array. Indeed, expression of Smad6 and Smad7 was stimulated about 18- and 4-fold respectively by AMH and the RNA samples were subsequently used.

Of the 20,000 genes present on the Agilent array, the Cy3 and Cy5 signal levels of almost 89% (about 18,000 genes) were above background levels on both arrays (=average signal of negative controls + 2xSD) and had a p-value smaller than 10^{-3}. However, only eight upregulated and three downregulated genes were identified (Table 7.4) and they are in the process of being validated using quantitative real-time PCR.

Table 7.4: Candidate AMH-regulated genes obtained using the Agilent mouse development oligo microarray. KK-1/AMHRII cells were cultured for 24 hours in the absence or presence of AMH. The p-value respresents the significance of the log(Cy5signal/Cy3signal) per feature after dye normalization (Agilent Technologies, USA).

Gene:	Abbreviation:	Group:	Ratio (+AMH/-AMH)		p-value	
			array 1:	array 2:	array 1:	array 2:
Ribosomal protein L24	Rpl24	Ribosomal protein	0.6	0.6	$1.1*10^{-4}$	$7.6*10^{-7}$
Neuronal precursor cell expressed, developmentally down-regulated gene 9	Nedd9	Ubiquitin-proteosome pathway	0.6	0.7	$3.5*10^{-4}$	$1.3*10^{-5}$
Transferrin receptor	Trfr	Iron uptake	0.6	0.6	$1.2*10^{-4}$	$2.0*10^{-4}$
Tumor necrosis factor, alpha-induced protein 3	Tnfaip3	NFkB-signaling	1.6	1.6	$4.9*10^{-5}$	$8.0*10^{-5}$
Crystallin alpha B	Cryab	Heat shock protein	1.6	1.9	$2.0*10^{-7}$	$3.4*10^{-5}$
CD24a antigen/heat stable antigen		Differentiation marker	1.7	1.6	$1.2*10^{-4}$	$1.7*10^{-5}$
Procollagen C-endopeptidase enhancer 2	Pcolce2	Protease	1.7	1.7	$5.0*10^{-6}$	$7.1*10^{-6}$
Unknown EST, similar to Homo sapiens clone RP11-349E11 from 7p14-15		EST	2.0	1.7	$3.4*10^{-5}$	$1.7*10^{-7}$
Unknown EST, similar to Homo sapiens clone RP11-337C18 on chromosome 1,		EST	2.2	2.5	$3.1*10^{-10}$	$1.1*10^{-8}$
Latexin	Lxn	Enzyme inhibitor	3.5	1.9	$9.1*10^{-8}$	$1.3*10^{-17}$
Inhibitor of DNA binding 2	Id2	Transcription factor	5.5	2.7	$1.9*10^{-13}$	$9.3*10^{-24}$

DISCUSSION

Two different types of arrays were used in the search for AMH-regulated genes. Using the Atlas mouse cDNA expression array, four upregulated and three downregulated genes were identified in wild-type neonatal ovaries stimulated with AMH *in vitro* (Table 7.3). Based on the literature, four of these genes were selected for validation. RelA (NFκB) has been shown to be involved in the AMH-induced growth inhibition in two breast tumor cell lines and in a mammary epithelial cell line (Segev, *et al.*, 2000, 2001). This growth inhibition was associated with an increase in RelA activity (Segev, *et al.*, 2000, 2001). Cathepsin L, a lysosomal cysteine protease member of the papain family (Kirschke, *et al.*, 1998), has been suggested to be important for tissue remodeling and cell migration (Ishidoh and Kominami, 1998). Interestingly, cathepsin L has been implicated in folliculogenesis, since cathepsin L expression in granulosa cells of pre-ovulatory follicles is important for ovulation (Robker, *et al.*, 2000). However, to ensure that cathepsin L is an AMH-regulated gene *in vivo* further research is needed, since AMH is not expressed in pre-ovulatory follicles where cathepsin L is expressed. In the testis, cathepsin L is differentially expressed in Sertoli cells during the various stages of spermatogenesis. Interestingly, its expression is highest during stage VI and VII of the seminiferous epithelium, when also AMHRII expression is highest in Sertoli cells (Zabludoff, *et al.*, 2001). Laminin degradation is associated with cathepsin L expression and laminin α5 expression in basement membrane of embryonic testicular chords disappears when Sertoli cells start expressing AMH (Frojdman, *et al.*, 1999; Zabludoff, *et al.*, 2001).

The receptor for laminin is the integrin receptor α6β1 or α6β4 (Burgeson, *et al.*, 1994). This receptor is involved in adhesion of cells to the extracellular matrix and is a modulator of growth, development and differentiation. Integrin α6 is also involved in development and sex differentiation of the mouse ovary (Frojdman and Pelliniemi, 1995) and in luteinization of human granulosa cells during the periovulatory phase (Fujiwara, *et al.*, 1998). Finally, frizzled3 is a member of the frizzled receptor family that mediates Wnt-signaling. Wnt family members are involved in many processes including sex differentiation. For example Wnt-4 is involved in embryonic ovarian development and Wnt-7a is necessary for AMHRII expression in the Müllerian ducts (Parr and McMahon, 1998; Vainio, *et al.*, 1999). Wnt binding to frizzled leads to accumulation of β-catenin. Accumulation of β-catenin is found in Müllerian ducts upon AMH exposure (Allard, *et al.*, 2000). However, none of these four genes was differentially expressed in AMH *null* neonatal ovary culture or KK-1/AMHRII cells in the validation experiment using quantitative real-time PCR, underlining the necessity to validate candidate regulated genes by an alternative quantitative method. The failure to identify AMH-regulated genes was caused by technical problems (high and varying background levels) and due to the limited number of genes present on these filter arrays.

Thus a more extensive study was necessary to identify AMH-regulated genes, which are potentially involved in the regulation of recruitment of primordial follicles and in regulation of FSH-sensitivity of pre-antral and antral follicles. KK-1/AMHRII cells were stimulated with AMH for 24 hours and a genome wide screen was performed using the Agilent mouse oligo microarray representing over 20,000 mouse genes. The low number of identified AMH-regulated genes, eight upregulated and three downregulated genes, is not caused by a lack of response of the cell culture since in the same samples Smad6 and Smad7 were found to be stimulated by AMH. Unfortunately, Smad6 and Smad7 were not present on the Agilent array and could not be used to check the quality of the procedure. A major problem of the Agilent array seemed to be the variation of dye-incorporation. The Agilent array was performed in duplicate, using the same batch of RNA, where in one experiment RNA from cells treated with AMH was labeled with Cy3 and in the other experiment with Cy5 and the untreated cells were labeled in reverse. When the ratios of Cy3/Cy5 from the genes with a signal above background are plotted in a graph, a negative slope would be expected since genes should have an inversed Cy3/Cy5 ratio in array 1 compared to array 2 if the dye labeling would be similar. However, a positive slope was found when the ratios of genes expressed above background in both arrays were plotted (Figure 7.3). Quadrant I contains the genes which have incorporated more Cy3 than Cy5 while quadrant III contains genes which have incorporated more Cy5 than Cy3. AMH-induced genes are highlighted by triangles in quadrant IV, while AMH-downregulated genes are highlighted by triangles in quadrant II. The Cy3/Cy5 ratios of these genes are inversed between both arrays and this Cy3/Cy5 or Cy5/Cy3 ratio is equal to or larger than 1.5-fold. Differences in labeling between the two array experiments might explain the small number of identified genes (Figure 7.3). The large number of genes in quadrant I and III suggest that many genes preferentially incorporate the Cy3 or the Cy5 dye. Indeed, two Agilent arrays hybridized with the same RNA sample (self-self hybridization experiments) showed a preferential dye labeling in about 10% of the expressed genes (data kindly provided by Dr. J. T. den Dunnen, Leiden University Medical Centre). However, only a small overlap (0.4%) of the genes which preferentially incorporated the Cy3 or the Cy5 dye in both arrays was found, indicating variation in dye labeling between experiments. The variation in dye labeling can mask

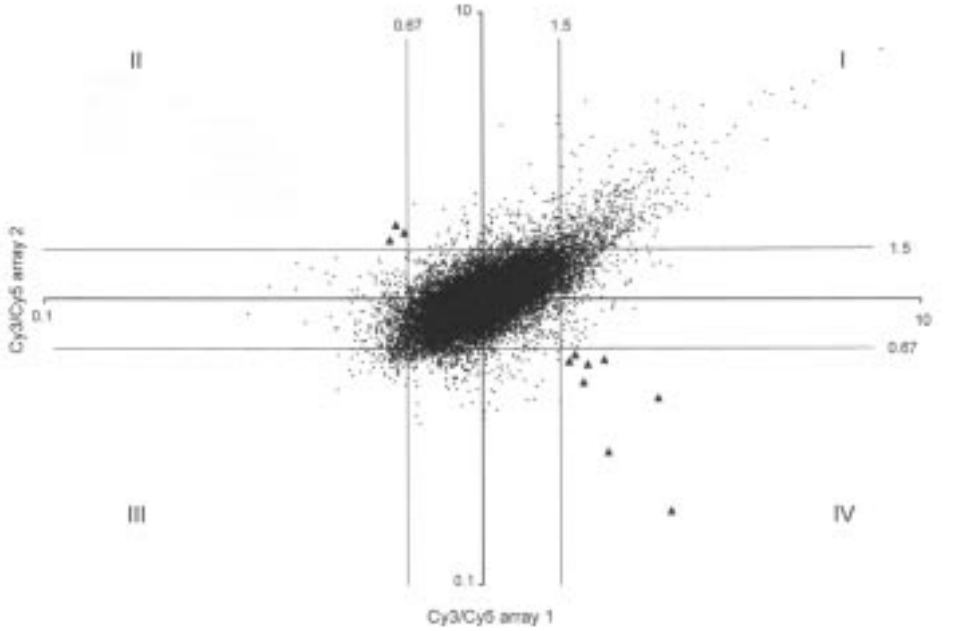

Figure 7.3: Logarithmic plot of the Cy3/Cy5 ratio of reverse dye labeling of two arrays.
In array 1, cDNA of KK-1/AMHRII cells treated with AMH was labeled Cy3, while in array 2 this cDNA was labeled with Cy5. Preferentially incorporated genes can be found in quadrant I and III, causing a positive correlation between the ratios of the two arrays. AMH-upregulated genes are highlighted by triangles in quadrant I and have a Cy3/Cy5 ratio above 1.5 in array 1 and below 0.67 in array 2. AMH-downregulated genes are highlighted by triangles in quadrant II and have a Cy3/Cy5 ratio below 0.67 in array 1 and above 1.5 in array 2. Only genes with signals above background are plotted in this graph.

genes which are differentially expressed by AMH. Thus, the small number of apparently AMH-regulated genes found in the KK-1/AMHRII cell line using the Agilent array might be caused by the variation in labeling. Another explanation for the small number of identified genes could be the use of the KK-1/AMHRII cell line. AMH is involved in follicle development in primary and pre-antral follicles. The KK-1/AMHRII cells are derived from granulosa cell tumors and may have lost the granulosa cells characteristics of pre-antral follicles.

Some of the identified candidate genes are members of families of proteins or have related functions: iron uptake (Trfr), ubiquination (Nedd9), members of NFκB-signaling (Tnfaip3), transcription factors (Id2), heat shock proteins (Cryab), differentiation markers (CD24a antigen), enzyme inhibitors (Lxn), proteases (Pcolce2) and ribosomal proteins (Rpl24) (see also Table 7.4). Also two ESTs were identified. The transferrin receptor (Trfr) is a very interesting candidate AMH-regulated gene since this receptor is necessary in the cell cycle because of its role in iron uptake (Neckers and Cossman, 1983), suggesting that downregulation of this receptor by AMH could inhibit granulosa cell proliferation. In addition, an inhibitory effect of transferrin is reported on differentiation of *in vitro* cultured granulosa cells by inhibiting FSH-induced aromatase activity (Li, *et al.*, 1991) and inhibin and progesterone production (Yu and Findlay, 1991; Kawano, *et al.*, 1995). AMH is known to inhibit aromatase activity

and FSH-sensitivity of large pre-antral and antral follicles and therefore it is unexpected that a receptor which also could achieve these effects is downregulated by AMH.

Nedd proteins are ubiquitin-like proteins (Yeh, *et al.*, 2000) and Nedd9 is expressed in KK-1/AMHRII granulosa cells and oocytes (EST K0232A09-3 from the NIA mouse unfertilized egg cDNA library). Downregulation of Nedd9 by AMH could be important since a family member Nedd8 is involved in ubiquination of steroid receptors (Fan, *et al.*, 2002) and the inhibitor of NFκB-signaling IkBα (Tanaka, *et al.*, 2001). Interestingly, AMH induces expression of the NFkB regulated gene IEX-1S in breast tumor cell lines (Segev, *et al.*, 2000, 2001). However, whether also Nedd9 is involved in ubiquitination of NκBα should be determined. In addition, also the AMH-upregulated gene tumor necrosis factor alpha-induced protein 3 (Tnfaip3) is involved in the NFκB-signaling pathway (Baltathakis, *et al.*, 2001). If regulation of Tnfaip3 by AMH can be confirmed by quantitative real-time PCR, a role of the NFκB-signaling downstream of AMH-signaling would be probable.

Inhibitors of DNA binding or differentiation, referred to as Id proteins, antagonize the effect of helix-loop-helix transcription factors which play a role in cell growth, differentiation and tumorigenesis (Norton, *et al.*, 1998). Id2 expression is associated with dedifferentiation of cells (Coppe, *et al.*, 2003) and Id2 is therefore interesting as an AMH-upregulated gene, since inhibition of FSH-sensitivity of pre-antral follicles by AMH is a measure of inhibition of differentiation of granulosa cells. Another protein that could inhibit differentiation is the AMH-upregulated gene Cd24a. This gene encodes the murine differentiation marker heat stable antigen (HSA) in hematopoietic cell lineages, which is generally highly expressed in immature precursor cells and low in differentiated cells (Bruce, *et al.*, 1981; Linton, *et al.*, 1989). However, little is known about the function of Cd24a and it is unknown what the role of this gene in the ovary is. Also nothing is known about the role in the ovary of the candidate AMH-regulated gene Cryab, a heat shock protein expressed by lenticular tissues of the eye, but also heart, brain kidney, placenta, lung and striated muscle (Iwaki, *et al.*, 1990; Kato, *et al.*, 1991; Flugel, *et al.*, 1993).

Procollagen C-endopeptidase enhancer (Pcolce2) specifically binds to procollagen C-propeptides, thereby facilitating cleavage by procollagen C-proteinases into collagen (Ricard-Blum, *et al.*, 2002). Collagen is the most important extracellular matrix component and could be important in maintaining the follicle structure. In addition, Pcolce2 functions as a tissue inhibitor of metalloproteinases (TIMPs) when the netrin-like domain of this protein is released after proteolysis (Kessler, *et al.*, 1990; Banyai and Patthy, 1999; Mott, *et al.*, 2000). BMP-signaling can be stimulated by Pcolce2 since the CUB domains bind to BMP1 thereby enhancing its activity and stimulating the release of BMPs bound to chordin (Hulmes *et al.*, 1997). Another candidate AMH-regulated gene is latexin, a carboxy peptidase A inhibitor (Balint, *et al.*, 2003). A function for this protein in the ovary is unknown.

AMH signals through the BMP-like signaling pathway (Gouédard, *et al.*, 2000; Clarke, *et al.*, 2001; Visser, et al., 2001, Chapter 5 of this thesis). Like the AMH-regulated genes identified using the Agilent array, BMP-regulation has been described for factors involved in cell cycle, transcription factors, heat shock proteins, factors involved in extracellular matrix and Id-proteins (Balint, *et al.*, 2003) and the candidate AMH-regulated genes Id2 (Hollnagel, *et al.*, 1999; Balint, *et al.*, 2003) and latexin (Balint, *et al.*, 2003) have been described to be regulated by BMPs. In addition to regulation of expression by BMPs, Id2 mRNA expression is also under control of TGFβ (Hacker, *et al.*, 2003; Sugai, *et al.*, 2003). Furthermore, TGFβ inhibits expression of the transferrin receptor (Smeland, *et al.*,

1987; Cross and Cambier, 1990; Ruegemer, *et al.*, 1990) and stimulates expression of Cryab (Welge-Lussen, *et al.*, 2000). Regulation of gene expression by the both TGFβ/activin-like and BMP-like signaling pathways suggests that Smad4 or other signaling pathways (like MAPK) are involved.

In conclusion, using the Agilent array we identified 11 candidate AMH-regulated genes. This low number of AMH-regulated genes may be caused by the preferential dye-incorporation of many genes. The identified AMH-regulated genes should be validated by quantitative real-time PCR. Confirmation of identified genes using an array is important as shown by the Atlas™ mouse cDNA expression array where all identified AMH-regulated genes were false positives.

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Chapter 8

GENERAL DISCUSSION

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8.1 INTRODUCTION

Female reproduction is dependent on successful development of a primordial follicle into a pre-ovulatory follicle. In women, the limited primordial follicle pool is formed during fetal life, whereafter this pool is gradually depleted since no new primordial follicles can be formed (for review see Chapter 1). Depletion of this follicle pool is associated with decreased fertility, followed by infertility and ends in menopause, which is associated with increased risk for several health problems (osteoporosis, cardiovascular disease). Recruitment of primordial follicles into the growing pool is an important regulatory checkpoint in primordial follicle stock exhaustion. The rate of primordial follicle recruitment is determined by growth factors, including members of the TGFβ superfamily, which are produced by the growing follicles. These growing follicles can develop into a pre-ovulatory follicle or undergo atresia. Thus, for understanding the regulation of primordial stock depletion, follicular development and atresia are important processes to investigate.

Anti-Müllerian hormone (AMH), a member of the TGFβ superfamily, is produced by the granulosa cells of small growing follicles in the ovary and inhibits recruitment of primordial follicles. The major objective of this thesis was to obtain more insight in the role of AMH and the molecular mechanisms by which AMH achieves this effect. We studied the effect of AMH on primordial follicle recruitment in neonatal ovaries, investigated the AMH-signaling pathway and searched for AMH-regulated genes. In this Chapter, the mechanisms by which AMH could achieve its specific effects is discussed. In addition, the role of AMH in follicle development compared to the role of the systemic factors LH and FSH and the clinical relevance of AMH are discussed.

8.2 MODULATION OF TGFβ SUPERFAMILY SIGNALING

The TGFβ superfamily is the largest known family of growth- and differentiation factors and presently consists of 35 family members (Chang, *et al.*, 2002). All members of the TGFβ family signal through serine/threonine (S/T) kinase type II and type I receptors (for a review see Chapter 2). The signaling mechanisms of the TGFβ superfamily members has intriguing properties since only five type II and seven type I receptors have sofar been identified in mammals. Combinations of a ligand with different type II and type I receptors can generate ligand- and cell-specific effects. After activation of the receptors, receptor-specific Smads are activated. However, different ligands may activate the same downstream Smads. In addition, one ligand can mediate different effects in different cell types. Therefore, the question arises how so many different ligands can generate so many cell-specific signals through a relatively limited system of transduction molecules. This section discusses the mechanisms of modulation of signaling of TGFβ family members at the level of the ligand, of the receptor and further downstream which are involved in transducing the specific signal for the TGFβ family members and which also could be involved in achieving specific effects of AMH.

8.2.1 Ligand

Signaling of TGFβ superfamily members can be regulated, both positively and negatively, at the level of the ligand. Formation of homo- or heterodimers determines the presence of a ligand with a certain biological effect and/or potency. The availability of the ligand for binding to its receptor can be regulated by docking proteins which can bind to the ligand, other extracellular proteins, antagonists and accessory receptors present on the membrane. This section will discuss these sites of modulation of TGFβ superfamily signaling at the level of the ligand (Figure 8.1).

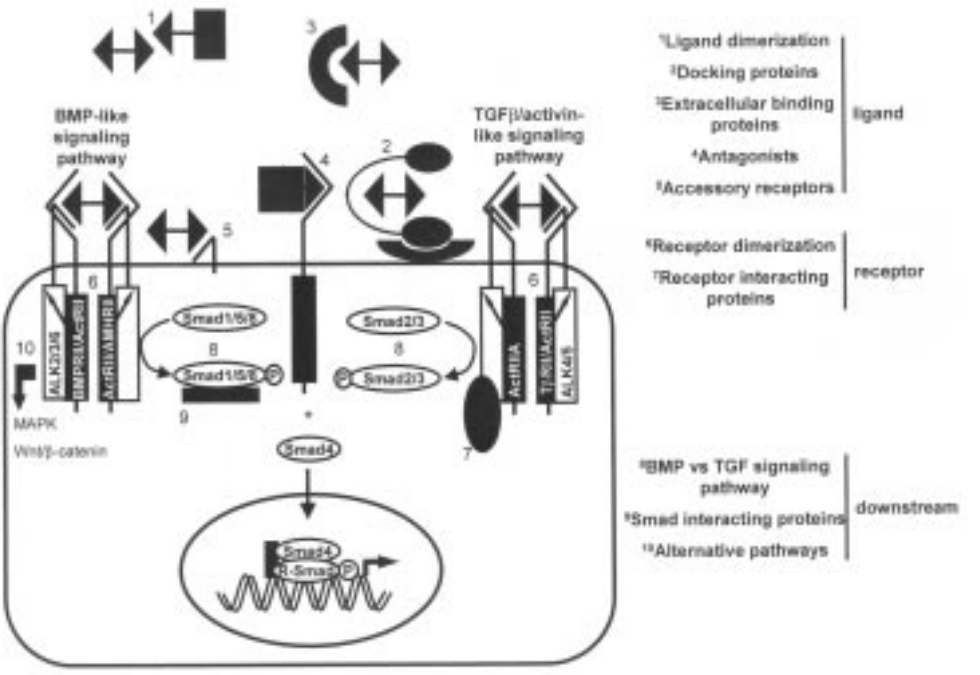

Figure 8.1: Sites of modulation of signaling of TGFβ superfamily members.

TGFβ superfamily members are synthesized as precursor proteins which are able to form homo- or heterodimers (for review see Chapter 2). Although most members of the TGFβ superfamily form homodimers, formation of heterodimers has also been described. The regulation of homo/heterodimer formation influences the biological effects of the ligands since heterodimers can have different biological effects and/or biological potency compared to homodimers. The heterodimer TGFβ1.2, a combination of TGFβ1 and TGFβ2, binds more efficiently to the type II and I receptors than TGFβ2 but less efficiently than TGFβ1 (Cheifetz, e*t al.*, 1988). In addition, the biological potency of the TGFβ1.2 heterodimer as measured by cell proliferation lies in between the potencies of TGFβ1 and TGFβ2 homodimers. Thus, the TGFβ heterodimer has a similar biological effect compared to the homodimers, but its potency is different. Several BMP heterodimers has also been described, but in contrast to TGFβ the biological effects of the BMP heterodimers are different from those of the homodimers. The BMP4/7 and BMP2/7 heterodimers induce ventral mesoderm in Xenopus animal caps, whereas BMP2, BMP4 and BMP7 do not (Suzuki, *et al.,* 1997; Nishimatsu and Thomsen, 1998).

In addition, BMP2/7 and BMP2/6 heterodimers are more potent in the alkaline phosphatase induction assay as well as in inducing cartilage and bone in an *in vivo* assay compared to their homodimers (Israel, *et al.*, 1996). Recently, also the heteromeric protein BMP15/GDF9 has been identified by immunoprecipitation and immunoblotting. However the biological effect of this heterodimer is unknown (Liao, *et al.*, 2003). The best known heterodimers are inhibin A, inhibin B and activin AB, which are formed by a combination of inhibin α- and inhibin βA-subunits, inhibin α- and inhibin βB-subunits and inhibin βA- and inhibin βB-subunits respectively. Inhibins antagonize the biological effects of activins and BMPs. Thus, the effects of TGFβ superfamily members can be regulated by the secretion of homo- and heterodimers, since differences in biological effects and potency are described between the homo- and heterodimers.

For AMH, the formation of heterodimers has not been reported yet, but it cannot be excluded. In principle, all TGFβ family members, expressed in the same cell types (granulosa cells of primary or pre-antral follicles) and at the same time as AMH, are candidates for heteromerization with AMH. One possible candidate for AMH heteromerization is the inhibin α-subunit, since both AMH and inhibin α-subunit are expressed in granulosa cells of primary and pre-antral follicles (Chapter 6). Furthermore, inhibin βA- and inhibin βB-subunits, which form heteromeric complexes with the inhibin α-subunit, are not expressed in these cells until the follicle has developed into a large pre-antral follicle. Thus, the inhibin α-subunit is expressed without being able to form a known dimer (Chapter 6). Therefore, it is possible that the inhibin α-subunit forms a dimer with another TGFβ superfamily member, for example AMH. Since antibodies against AMH and inhibin α-subunit are available, it would be interesting to investigate the existence of this dimer in follicle fluid and serum.

TGFβ superfamily members are synthesized as precursors and cleavage of these proteins is necessary to obtain biological activity of the mature growth factor (for a review see Chapter 2)(Gentry, *et al.*, 1988); this is also true for AMH (Pepinsky, *et al.*, 1988; Kurian, *et al.*, 1995). After cleavage, the N-terminal peptide and the mature protein dissociate. This is necessary for full bio-activity of the mature protein (Gentry and Nash, 1990) with the exception of AMH which is the only known TGFβ family member where continuous association between the mature and the N-terminal domain is necessary for full biological activity (Wilson, *et al.*, 1993). The precise function of this N-terminal domain in AMH-signaling is unknown. However, a role as a docking protein can be suggested, based on the function of the N-terminal domain of TGFβ. The N-terminal domain of TGFβ, named latency associate peptide (LAP), can bind to the latency binding protein (LTBP) which localizes TGFβ to the extracellular matrix, generating a reservoir of TGFβ at the cell membrane of certain cells (reviewed by Khalil, 1999). TGFβ is released from this complex by the proteolytic enzyme plasmin, which removes LAP from the complex, thereby releasing TGFβ. Sofar, no AMH-docking proteins have been described.

Another mechanism for cell-specific signaling of TGFβ superfamily members is modulation of the availability of TGFβ superfamily members by extracellular proteins, which prevent binding of the ligand to their receptors (Table 8.1 and Figure 8.1), antagonists (Figure 8.1) or by accessory receptors which inhibit or facilitate ligand binding to their receptors (Figure 8.1). Several ligand-binding proteins of the TGFβ superfamily have been identified. BMP action is inhibited by sclerostin (Winkler, *et al.*, 2003), noggin (Zimmerman, *et al.*, 1996), chordin (Blader, *et al.*, 1997), and the DAN family members Dan (Stanley, *et al.*, 1998), gremlin (Hsu, *et al.*, 1998), and cerebrus (Bouwmeester, *et al.*, 1996; Piccolo, *et al.*, 1996, 1999; Yokouchi, *et al.,* 1999).

In addition, follistatin (de Winter, *et al.*, 1996; Fainsod, *et al.*, 1997; Iemura, *et al.*, 1998) and follistatin-like protein (Tsuchida, *et al.*, 2000) inhibit the actions of BMPs and activins. That these extracellular binding proteins can have an important role in regulating normal signaling of their ligands is shown by sclerostin. Sclerosteosis is a human disease typified by high bone mass due to the loss of sclerostin expression (Winkler, *et al.*, 2003). Furthermore, overexpression of sclerostin by transgenic mice, resulted in low bone mass and decreased bone strength through reduction of osteoblast activity caused by decreased BMP-signaling (Winkler, *et al.*, 2003). Sofar, no effect of any of these extracellular ligand-binding proteins on AMH-signaling have been investigated. However, follistatin might be a possible candidate, since this protein is expressed in the ovary by granulosa cells of growing follicles and plays a role in follicle development. Mice overexpressing follistatin show a block in folliculogenesis, caused by blocking the activity of activin and possibly also GDF9 (Guo, *et al.*, 1998). However, whether recruitment of primordial follicles is influenced by follistatin, and thus a role of follistatin in AMH-signaling, is unknown and needs further research.

Table 8.1: Extracellular binding proteins for TGFβ superfamily members.

Extracellular protein:	Binds:	Reference:
Sclerotin	BMPs	Winkler *et al.*, 2003
Noggin	BMPs	Zimmerman *et al.*, 1996
Chordin	BMPs	Blader *et al.*, 1997
Dan	BMPs	Stanley *et al.*, 1998
Gremlin	BMPs	Hsu *et al.*, 1998
Cerebrus	BMPs, activins	Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1996, 1999; Hsu *et al.*, 1998; Yokouchi *et al.*, 1999
Follistatin	activins, BMPs	de Winter *et al.*, 1996; Fainsod *et al.*, 1997; Iemura *et al.*, 1998
Follistatin-like protein	activins, BMPs	Tsuchida *et al.*, 2000

Alternatively, ligand binding to the receptor can be inhibited by competitive binding of antagonists to the receptor. Inhibins are known for their antagonizing effect on activin signaling by direct binding of inhibin to ActRII and ActRIIB, thereby preventing activin binding (Attisano, *et al.*, 1992; Martens, *et al.*, 1997). No reports have been published in which inhibin binding to the AMHRII is described. However, it can be suggested that inhibin may inhibit AMH signaling by binding to betaglycan (see next section).

As described above, binding of TGFβ superfamily members to their receptors can be modulated by extracellularly localized antagonists. In addition, ligand binding can also be positively or negatively modulated by accessory receptors expressed at the membrane. In this section, this will be illustrated by the role of betaglycan and BAMBI in TGFβ superfamily members signaling. Betaglycan stimulates TGFβ signaling by binding of TGFβ and thereby facilitating TGFβ binding to the type II receptor. Although all three TGFβ forms are bound by betaglycan, the effect on signaling is most evident for TGFβ2 since this TGFβ shows the lowest affinity for the TβRII (reviewed by Massagué, 1998). Besides binding of TGFβ, betaglycan also binds inhibin (Lewis, *et al.*, 2000). Binding of inhibin to betaglycan increases the affinity of inhibin for ActRII and ActRIIB, resulting in an inhibition of activin signaling through these receptors (Lewis, *et al.*, 2000; Chapman, *et al.*, 2002). Recently, it was reported that inhibin by binding to betaglycan also can form a complex with BMPRII and antagonize BMP-signaling (Wiater and Vale, 2003). Thus, betaglycan facilitates TGFβ-signaling, but inhibits, by facilitation of inhibins action, activin and BMP-signaling.

Another protein which inhibits downstream signaling is BMP and activin membrane-bound inhibitor (BAMBI). This protein has a high homology with the TGFβ superfamily type I receptors but lacks the intracellular kinase domain, which is necessary for type I receptor activation. BAMBI can interacts with all type I receptors (except ALK2) and the presence of BAMBI in a receptor complex with type II receptors prevents downstream signaling for TGFβ, activins and BMPs (Onichtchouk, *et al.*, 1999).

No data about a role of the accessory receptors betaglycan or BAMBI in AMH-signaling are available. However, it would be interesting to investigate the role of these accessory proteins in AMH-signaling. Inhibin and betaglycan are expressed in the ovary (Findlay, *et al.,* 2001) and it might be possible that betaglycan together with inhibin can antagonize AMH-signaling by preventing binding of AMH to the AMHRII, similar to the inhibition of BMP-signaling by this proteins. In addition, a modulating role of BAMBI in AMH-signaling is possible since AMH signals through ALK3 (Jamin, *et al.*, 2002). In conclusion, signaling of TGFβ superfamily members can be modulated at ligand level by heteromerization, antagonists and accessory proteins and this could contribute to ligand specific effects. Thus, although not much is known yet about modulation of AMH-signaling at the level of the ligand, many sites of modulation are possible.

8.2.2 Receptor

TGFβ superfamily members signal through binding to serine/threonine (S/T) kinase type II and type I receptors. Five type II (TβRII, ActRII, ActRIIB, BMPRII, AMHRII) and seven type I receptors (ALK1-7) have been identified in mammals (for a review see Chapter 2). Binding of TGFβ family members to the type II receptor induces the formation of a hetero-tetrameric complex containing two type II and two type I receptors (Wrana, *et al.*, 1994; Heldin, *et al.*, 1997; Kirsch, *et al.*, 2000). The combination of the ligand with type II and type I receptors is responsible for a ligand-specific signal into the cell. In addition, some receptor-interacting proteins have been described which might influence type I receptor activity. This section describes the modulation of signaling at the level of the receptor by which AMH-signaling might be influenced.

Five type II receptors are known and most of these receptors signal for more than one ligand. So can several BMPs bind to the BMPRII or ActRII receptors (ten Dijke, *et al.*, 1994; Rosenzweig, *et al.*, 1995; Yamashita, *et al.*, 1995). In contrast, AMH is the only described ligand for the AMHRII, and mice lacking AMH (Behringer, *et al.*, 1994) or the AMHRII (Mishina, *et al.,* 1996; Arango, *et al.*, 1999) show absence of Müllerian duct regression. Furthermore, the phenotypes of the testis in AMH *null* and AMHRII *null* mice are similar, showing the production of functional sperm and hyperplasia of Leydig cells (Behringer, *et al.*, 1994; Mishina, *et al.*, 1996). In contrast, the ovarian phenotype of the AMH null and MRKI(-/-) mice are not completely identical (Chapter 4), since a larger decrease of the number of primordial follicles is found in ovaries of MRKI(-/-) mice than in AMH(-/-) mice. This difference in ovarian phenotype might be explained by ligand-independent AMHRII-signaling. Furthermore, it is likely that AMH only signals through the AMHRII and not through other type II receptors. This is shown by transfection experiments, where AMH only signals through AMHRII and not through TβRII, ActRIIB or BMPRII (Chapter 5).

Ligands can signal through more than one of the seven type I receptors as is shown for BMPs (ten Dijke, *et al.,* 1994; Ebisawa, *et al.*, 1999), activins (Attisano, *et al.*, 1993) and TGFβs (Franzen, *et al.*, 1993; Oh, *et al.*, 2000).

The affinity for the type I receptor can be dependent on the cell line, shown by BMP6, which binds strongly to ALK2 but weakly to ALK3 in mouse undifferentiated mesenchymal cells (C2C12), while a strong binding to ALK6 and a weak binding to ALK2 and ALK3 were found in rat osteoprogenitor cells (ROB-C26 cells) (Ebisawa, *et al.*, 1999). Furthermore, signaling of a ligand through two different type I receptors can result in a synergistic effect. The alkaline phosphatase activity and the type I receptor-induced transcriptional activity is synergistically induced by a combination of signaling through ALK2 and ALK3 or ALK2 and ALK6 (Aoki, *et al.*, 2001). The combination of signaling through two different type I receptors might activate the downstream signaling pathways more efficiently than signaling through one type I receptor. These results show that cell-specific effects may be achieved through expression of different type I receptors in different cell types.

The receptor complex through which TGFβ superfamily members signal contains two type II and two type I receptors. In endothelial cells, TGFβ can mediate its signal through two receptor complexes containing TβRII/ALK5 or TβRII/ALK1/ALK5 (Goumans, *et al.*, 2002, 2003). Signaling through the TβRII/ALK5 receptor complex results in inhibition of migration and proliferation of the endothelial cells, while signaling through the TβRII/ALK1/ALK5 receptor complex stimulates these processes. In addition, different genes are induced by the two receptor complexes (Goumans, *et al.*, 2002). It appeared that ALK1 is a negative mediator of TβRII/ALK5-signaling and that endothelial cells can regulate TGFβ-induced responses by expression of ALK1 (Goumans, *et al.*, 2003). In addition, heteromeric ALK3/ALK6 complexes are found and formation of these complexes can be induced by BMP2 (Gilboa, *et al.*, 2000). However, whether these ALK3/ALK6 complexes are functional needs to be determined.

Candidate type I receptors for AMH-signaling are ALK2, ALK3 and ALK6 (Gouédard, *et al.*, 2000; Clarke, *et al.*, 2001; Visser, *et al.*, 2001) and all three type I receptors are expressed in the ovary (Chapter 5, Erickson and Shimasaki, 2003). Dominant-negative and antisense approaches in cell lines showed that ALK2 is necessary for AMH-signaling (Clarke, *et al.,* 2001; Visser, *et al.*, 2001). In addition, Müllerian duct regression is inhibited by antisense ALK2 (Visser, *et al.*, 2001). ALK3 also plays a role in AMH-signaling, as indicated by the presence of persistent Müllerian ducts in conditional ALK3 *null* mice (Jamin, *et al.*, 2002). Furthermore, the involvement of ALK6 in AMH-signaling was suggested, based on the observation that AMH-induced a ligand-dependent AMHRII/ALK6 receptor complex (Gouédard, *et al.*, 2000). However, the ALK6 *null* mouse model (Clarke, *et al.*, 2001), the expression pattern of ALK6 in the Müllerian ducts (Dewulf, *et al.*, 1995; Clarke, *et al.*, 2001) and *in vitro* experiments in which the role of ALK6 in AMH-signaling was investigated (Clarke, *et al.*, 2001; Visser, *et al.*, 2001) suggest that ALK6 is not involved in AMH-signaling in Müllerian duct regression, whereas a role for ALK6 in AMH-signaling in the ovary cannot be ruled out yet.

AMH has several effects on follicles in the ovary: it inhibits primordial follicle recruitment (Chapter 3, Durlinger, *et al.*, 1999) and decreases the sensitivity for FSH in large pre-antral and small antral follicles (Durlinger, *et al.*, 2001). It can be suggested that AMH achieves these different effects by signaling through different receptor complexes. An indication for this hypothesis is that AMH stimulates MMP2 expression in the Müllerian ducts (Roberts, *et al.*, 2002) but not in KK-1 cells (our unpublished observations). However, this difference may also be explained by varying expression of transcription factors and/or signaling through different signaling pathways. In addition, AMH-signaling might be mediated by a receptor complex containing two different type I receptors. The strongest indication for this hypothesis is given by the ALK3 conditional mutant mice, which do not show

Müllerian duct regression (Jamin, *et al.*, 2002). However, crossbreeding of the ALK3 conditional mutant mice with transgene mice that overexpress AMH rescues the regression of the Müllerian ducts (Jamin, *et al.*, 2003). These results indicate involvement of ALK3 in AMH-signaling in the Müllerian ducts, which can be mediated by another type I receptor if AMH is highly expressed. This other type I receptor is probably ALK2 since the absence of ALK2 expression prevents Müllerian duct regression (Visser, *et al.*, 2001). It can be suggested that ALK2 and ALK3 form heteromeric complexes since both receptors are necessary for Müllerian duct regression.

To test which type I receptors are involved in AMH-signaling in the ovary, several approaches are applicable. We have transfected plasmids containing dominant-negative type I receptors into KK-1 cells. However this approach was not successful since too large amounts of plasmid had to be used causing non-specific side-effects. To avoid transfection problems, silencing of genes with morpholinos or small interfering RNA might be a more successful approach. The use of antisense oligos (morpholinos) has proven its value in the Müllerian duct system (Visser, *et al.*, 2001) and might be successful in ovarian cell lines. Another approach for the identification of type I receptors involved in AMH-signaling is the generation of mice lacking the expression of a type I receptor. This approach is complicated since ALK2 *null* (Mishina, *et al.*, 1999) and ALK3 *null* (Mishina, *et al.*, 1995) mice die before birth. The ALK6 *null* mouse is viable but does not show Müllerian duct regression, suggesting that ALK6 is not necessary for this AMH-induced event (Yi, *et al.*, 2000; Clarke, *et al.*, 2001). The ovarian function in these ALK6 *null* mice is aberrant, as shown by irregular estrus cycles and cumulus expansion resulting in the absence of fertilization *in vivo* (Yi, *et al.*, 2001). This ovarian phenotype shows that the interpretation of type I receptor knockout and also conditional knockout mouse models like for ALK3 (Jamin, *et al.*, 2002) is complicated since these receptors are involved in signaling pathways of several ligands. Therefore, it will be difficult to link the observed phenotypes to the absence of AMH-signaling. Identification of type I receptors involved in AMH-signaling could be achieved using immunoprecipitation of AMHRII and type I receptors. However, this technique was not very successful since it AMHRII in overexpressing systems bind to all type I receptors (unpublished results J.A. Visser). Fluorescence resonance energy transfer (FRET) analysis might be a useful technique in the search for AMH-induced receptor interactions. Through labeling of AMHRII with a donor fluorophore and the different type I receptors with an acceptor fluorophore, AMH-induced receptor type II and type I association may be shown since the energy of the excited donor fluorophore is transmitted to the acceptor fluorophore when these fluorophores are in near proximity (reviewed by Sekar and Periasamy, 2003). FRET analysis would be a new approach for the study of combinations of TGFβ superfamily receptors.

Receptor signaling can be modulated within the cell by proteins which bind to these receptors (Table 8.2). Smad6 and Smad7 antagonize signaling by TGFβ superfamily members by binding to the type I receptors, thereby preventing binding of receptor-specific Smads (Hayashi, *et al.*, 1997; Imamura, *et al.*, 1997; Nakao, *et al.*, 1997; Souchelnytskyi, *et al.*, 1998; Lebrun, *et al.*, 1999; Hanyu, *et al.*, 2001). Less well described receptor binding proteins are BMP receptor associated molecule (BRAM) which binds to ALK3 (Kurozumi, *et al.*, 1998) and the Smad1 antagonistic effector (SANE) which binds to ALK3 and ALK6, thereby preventing Smad1 phosphorylation (Raju, *et al.*, 2003). Since these receptor binding proteins can bind to ALK3 or ALK6, they may be involved in modulating AMH-signaling. However, more research is necessary at the effects of these interactions.

Table 8.2: Receptor binding proteins involved in BMP-signaling.

Protein:	Interacts with:	Effect:	Reference:
Smad6	ALK2, ALK3, ALK6	Inhibitor, antagonizes BMP-like signaling, by inhibiting receptor-specific Smad activation	Imamura *et al.*, 1997; Ishisaki *et al.*, 1999; Lebrun *et al.*, 1999
Smad7	All type I receptors	Inhibitor, antagonizes BMP-like and TGFβ/activin-like signaling, by inhibiting receptor-specific Smad activation	Hayashi *et al.*, 1997; Nakao *et al.*, 1997; Souchelnytskyi *et al.*, 1998; Ishisaki *et al.*, 1999; Lebrun *et al.*, 1999; Hanyu *et al.*, 2001
BRAM	ALK3	Unknown	Kurozumi *et al.*, 1998
SANE	ALK3, ALK6	Antagonizes BMP signaling by binding to receptor and preventing Smad1 activation	Raju *et al.*, 2003

In conclusion, specificity of AMH-signaling can be obtained by the type II receptor and the type I receptors used. AMH signals through a specific type II receptor and uses probably three different type I receptors. Furthermore, receptor binding proteins can influence type I receptor activity.

8.2.3 Downstream signaling

After binding to the receptor complex, TGFβ superfamily members activate downstream signaling pathways. Two main Smad-signaling pathways are activated by TGFβ superfamily members: the TGFβ/activin-like or the BMP-like signaling pathway (Figure 8.1). Smad2 and Smad3 are activated by TGFβs and activins through the type I receptors ALK4, ALK5 and ALK7, which form a receptor complex with TβRII, ActRII or ActRIIB (TGFβ/activin-like signal pathway). On the other hand, Smad1, Smad5 and Smad8 are activated by BMPs through the type I receptors ALK2, ALK3 and ALK6, which form a complex with BMPRII, ActRII or ActRIIB (BMP-like signaling pathway). Crossovers between these two main signaling pathways have also been described. TGFβ signals in most cells through TβRII and ALK5 resulting in phosphorylation of Smad2 and Smad3. In endothelial cells, also signaling through TβRII and a ALK1/ALK5 receptor complex has been described which results in phosphorylation of Smad1 and Smad5 (Goumans, *et al.*, 2002, 2003). In addition, GDF9 binds to the BMPRII and an unidentified type I receptor. Unexpectedly, not Smad1 but Smad2 is phosphorylated after GDF9 binding (Kaivo-Oja, *et al.*, 2003; Roh, *et al.*, 2003), suggesting that ALK4 or ALK5 are involved in the GDF9/BMPRII complex.

AMH signals through the BMP-like signaling pathway in non-ovarian tissues (Gouédard, *et al.*, 2000; Clarke, *et al.*, 2001; Visser, *et al.*, 2001). In this thesis, we show that also in the ovary AMH signals through the BMP-like signaling pathway, since in the granulosa cell line KK-1 only the BMP-responsive and not the TGFβ-responsive promoter can be activated (Chapter 5). In agreement with this result, AMH treatment of KK-1 cells causes Smad1 but not Smad2 phosphorylation (Chapter 5). Therefore, in this section the focus is on modulation of the BMP-like signaling pathway. Binding of BMPs or AMH to their receptors results in activation of Smad1, Smad5 or Smad8. An important question is how the ligands can generate their specific signal. Although this question cannot be completely answered, downstream signaling can be modulated by Smad-interacting proteins or by alternative pathways.

After BMPs have been bound to their receptors and have activated the Smad proteins, their signaling can be modulated by several interacting repressors and/or activators that are present in the cytoplasm or nucleus and can bind to the receptor-specific Smads, the common Smad4 and the inhibitory Smads. For example, the receptor-specific Smad–Smad4 complex formation can be inhibited

by Smad6 (Hata, *et al.*, 1998) and the inhibitory action of Smad6 can be prevented by the SH3 domain of STAM (AMSH) (Itoh, *et al.*, 2001) or enhanced by Tob (Yoshida, *et al.*, 2003). In addition, several transcription factors have been described to form a complex with the receptor-specific Smad-Smad4 complex, thereby enhancing or inhibiting gene transcription (For review see Zwijsen, *et al.*, 2003). Thus, downstream BMP-signaling can be modulated by several proteins. Since these proteins bind Smad1, 5 and/or 8, they could also be involved in modulating AMH-signaling, although more research is necessary to obtain data which show involvement of these proteins.

Not only the Smad-signaling pathway will mediate BMP signals but also the MAPK and possibly the Wnt/β-catenin signaling pathways are involved in BMP and AMH-signaling. The differential activation of these signaling pathways by AMH and the different BMPs may result in ligand-specific effects in the cells. The MAPK-signaling pathway plays an important role in mediating BMP-signaling. Three MAPK cascades have been identified: the extracellular signal regulated kinases (ERKs), p38-MAPK, and the c-Jun N-terminal kinases (JNKs) and all three signaling pathways might be involved in BMP-signaling. A mutation in the BMPRII showed that MAPK-signaling is induced by another part of the receptor than the Smad-signaling pathway showing the activation of the MAPK-pathway is independent of the Smad-signaling pathway (Rudarakanchana, *et al.*, 2002). Erk1 and Erk2 are involved in BMP15-induced proliferation of granulosa cells, since an inhibitor of these MAPK inhibits this BMP15-induced effect (Moore, *et al.*, 2002). MAPK-signaling by BMP is cell type dependent since BMP4 inhibits neuronal induction in Xenopus ectoderm by inhibition of Erk2 and p38 signaling (Uzgare, *et al.*, 1998; Goswami, *et al.*, 2001), whereas it stimulates phosphorylation of Erk1/2 and p38-MAPK in an osteoblast-like cell line (Kozawa, *et al.*, 2002). Likewise, BMP2 induces Erk- (Gallea, *et al.*, 2001) and p38- (Gallea, *et al.*, 2001; Lee, *et al.*, 2002) signaling in osteoblast differentiation. Recently, it was shown that also JNK can be activated by BMP2 in an osteoblast cell line through induction of protein kinase D (Lemonnier, *et al.*, 2003) and BMP7 inhibits IL1β-induced JNK activation (Lee, *et al.*, 2003). Thus, all three MAPK-signaling pathways can be involved in mediating BMP- and therefore probably also AMH-signaling.

A role for the MAPK-signaling pathway in AMH-signaling was suggested by studies on Müllerian duct regression. Apoptotic cells in the Müllerian ducts show a decreased expression of JNK and p38 proteins. When the expression of JNK and p38 was restored by treating the cells with diethylstilbestrol (DES), apoptosis in the Müllerian ducts was inhibited, suggesting a role of these signaling pathways in this AMH-regulated process (Teng, 2001).

Recently, a connection between the Wnt/β-catenin and the BMP-signaling pathway was reported (Hussein, *et al.*, 2003; Soshnikova, *et al.*, 2003). Wnt members are involved in developmental processes, cell proliferation, differentiation and survival (Wodarz and Nusse, 1998; Eastman and Grosscheldl, 1999; Huelsken and Birchmeier, 2001). After binding of Wnt molecules to their receptors, β-catenin is stabilized, which interacts with Lef transcription factors and regulates gene expression (Behrens, *et al.*, 1996; Eastman and Grosscheldl, 1999; van de Wetering, *et al.*, 2002). In apical ectodermal ridge formation, β-catenin action is influenced by ALK3, showing involvement of the BMP-signaling pathway (Soshnikova, *et al.*, 2003). Also in the Müllerian ducts it has been suggested that AMH signals through or interacts with the Wnt/β-catenin pathway since AMH-signaling results in an accumulation of β-catenin and Lef1 (Allard, *et al.*, 2000). However, whether AMH signals through the Wnt/β-catenin pathway or influences this pathway by for example downregulated genes, needs to be determined.

8.3 TGFβ SUPERFAMILY MEMBERS VERSUS FSH/LH IN FOLLICULOGENESIS

During follicle development, two important sites of regulation can be recognized: recruitment and selection (see Figure 1.1). At recruitment, primordial follicles enter the growth phase from the dormant follicle pool. The oocyte diameter starts to increase, the granulosa cells become cuboidal and start to proliferate and the primordial follicle develops into a primary follicle. When more than one layer of granulosa cells have been formed the follicle is called pre-antral (for review see Chapter 1). After formation of the antrum, the follicle is named antral follicle. At selection, large pre-antral follicles in mice and rats and antral follicles in human are selected for growth until the pre-ovulatory stage. Only those follicles that have become sensitive enough for FSH will be rescued from atresia (McGee and Hsueh, 2000).

8.3.1 Follicle development

Follicle development is regulated by the gonadotropins LH and FSH, which are produced by the pituitary. In the literature, these two hormones are described as the most important for follicle development. However, the ovary produces several growth factors belonging to the TGFβ superfamily, like BMP2, BMP3, BMP4, BMP6, BMP7, BMP15, GDF9, AMH, TGFβ, activins and inhibins which can also be very important for follicle development (for review see Chapter 1, Figure 1.1). That ovarian factors can have a dominant role in follicle development is shown by the FSHβ/AMH deficient mice in which the ovaries, like the ovaries of AMH *null* mice, contain more growing and less primordial follicles compared to wild-type mice ovaries (Durlinger, *et al.*, 2001). In contrast, the ovaries of FSHβ *null* mice show similar numbers of primordial and growing follicles compared to ovaries of wild-type mice. Although both gonadotropins and TGFβ superfamily members are important for follicle development, their roles are distinct. In this section, the effects of the gonadotropins and several members of the TGFβ superfamily on follicle development and the interaction between these two groups of hormones and growth factors are discussed.

TGFβ superfamily members are involved in follicle development from recruitment of the primordial follicles until ovulation of the oocyte. AMH inhibits recruitment of primordial follicles into the growing pool as shown by the AMH *null* mice (Durlinger, *et al.*, 1999) and neonatal ovary cultures (Chapter 3), while BMP4 stimulates primordial follicle recruitment in neonatal ovary cultures (Nilsson and Skinner, 2003). Gonadotropins are not obligatory for primordial follicle recruitment, since growing follicles are found in FSHβ *null*, FSHR *null* and LHR *null* mice (Kumar, *et al.*, 1997; Dierich, *et al.*, 1998; Zhang, *et al.*, 2001). In addition, primordial follicle recruitment cannot be directly stimulated by FSH or LH since these primordial follicles do not express the receptors for these gonadotropins (for review see Chapter 1)(Rannikki, *et al.*, 1995; Teerds and Dorrington, 1995; O'Shaughnessy, *et al.*, 1996, 1997; Oktay, *et al.*, 1997).

After recruitment, primary follicle development requires GDF9 since follicle development is blocked at the primary follicle stage in GDF9 *null* mice (Dong, *et al.*, 1996). First effects of LH and FSH on follicle growth and development have been described on pre-antral follicles, which growth is stimulated by LH (for review see Chapter 1)(Cortvrindt, *et al.*, 1998; Wu, *et al.*, 2000) and FSH (for review see Chapter 1)(Yokota, *et al.*, 1997; Mizunuma, *et al.*, 1999; Gutierrez, *et al.*, 2000).

Also the TGFβ superfamily members GDF9 (Hayashi, *et al.*, 1999), activin A (only follicles from immature but not from adult mice) (Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999) and TGFβ (follicles of adult but not immature mice) (Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999) have been described to stimulate pre-antral follicle growth.

Markers for increase in follicle development are estrogen and inhibin production by the follicles. LH and FSH together are involved in stimulation of the estrogen production, since LH stimulates the production of androgens by theca cells while FSH stimulates the conversion of androgens into estrogens by the granulosa cells. Also TGFβ superfamily members can stimulate pre-antral follicle development. Activin A stimulates the development of cultured pre-antral follicles from immature but not from adult mice as shown by an increase in inhibin and estrogen production (Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999). TGFβ stimulates inhibin and estrogen production in adult but not immature follicles (Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999). In addition, a stimulatory effect on pre-antral follicle development of GDF9 has been found, since an increase in inhibin α-subunit mRNA expression was detected after treatment with this oocyte factor (Hayashi, *et al.*, 1999). Besides stimulation of growth and development of immature pre-antral follicles, activin is involved in regulation of pre-antral follicle growth of adult mice since activin production by large pre-antral follicles keep small pre-antral follicles dormant (Mizunuma, *et al.*, 1999). In conclusion, follicle development until the point of selection is regulated by several TGFβ superfamily members, while the gonadotropins LH and FSH have a subordinate role.

In rodents, large pre-antral follicles become selected for further growth towards the pre-ovulatory stage by FSH. Follicle growth and development from the point of selection is dominantly regulated by FSH and LH, while the TGFβ superfamily members play a less important role. The importance of FSH in this process is clearly shown by the FSHβ *null* (Kumar, *et al.*, 1997) and FSHR *null* (Dierich, *et al.*, 1998) mice of which the ovaries do not contain any antral follicle. The sensitivity of follicles to FSH is inhibited by AMH, thereby decreasing the number of follicles selected for pre-ovulatory growth (Durlinger, *et al.*, 2001). Follicle development becomes dependent on LH after formation of the antrum as shown in the LHR *null* mice of which the ovaries do not contain pre-ovulatory follicles (Zhang, *et al.*, 2001). FSH stimulates antral follicle growth by stimulating granulosa cell proliferation. In addition, antrum formation is induced by FSH, a process which is enhanced by LH. During the last phase of follicle development, LH is involved in successful maturation of the follicles and is essential for ovulation.

8.3.2 Modulation of FSH action by TGFβ superfamily members

As described above, both FSH and TGFβ superfamily members are involved in follicle development. Interestingly, FSH action is modulated by several TGFβ superfamily members (Table 8.3). The FSH-induced growth of pre-antral follicles is e.g. inhibited by AMH (Durlinger, *et al.*, 2001) and activin (Yokota, *et al.*, 1997; Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999), but enhanced by GDF9 (Hayashi, *et al.*, 1999) and TGFβ (both in adult and immature mice pre-antral follicles) (Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999). In addition, the FSH-stimulated differentiation of follicles, measured by increasing inhibin α mRNA expression is enhanced by GDF9 (Hayashi, *et al.*, 1999) which suggests that GDF9 in addition to FSH stimulates follicle development.

FSH stimulated estrogen production in pre-antral follicles of adult mice is inhibited by activin (Yokota, *et al.*, 1997; Liu, *et al.*, 1999; Mizunuma, *et al.*, 1999). In antral and pre-ovulatory follicles, FSH induces differentiation as shown by increased LHR expression and estrogen and progesterone production and these effects are all inhibited by GDF9 (Vitt, *et al.*, 2000a). In addition, FSH-induced antral follicle growth is inhibited by AMH (Durlinger, *et al.*, 2001). Thus, these experiments show a differentiating effect of FSH on follicle development while TGFb superfamily members mainly have an FSH-inhibitory effect.

Table 8.3: Modulation of effects of FSH action by TGFβ family members during follicle development.

Follicle type:	Effect on:	FSH effect:	FSH effect positively (+) or negatively (-) modulated by TGFβ family member:
Pre-antral	Growth	+	GDF9 +
			Activin (immature mice) +
			AMH -
	Inhibin production	+	GDF9 +
	Estrogen production	+	Activin (mature mice) -
Antral	Growth	+	AMH -
Pre-ovulatory	Estrogen production	+	GDF9 -
	Progesterone production	+	GDF9 -
	LHR expression	+	GDF9 -

Many experiments have been performed to study the effects of TGFβ superfamily members on FSH regulation of granulosa cell cultures of immature rats (Table 8.4). Since granulosa cells luteinize when cultured in the presence of FSH (Thanki and Channing, 1976), the exact developmental stage of cultured granulosa cells is unclear and therefore discussed separately from the follicle culture experiments. FSH-induced estrogen production is stimulated by BMP4, BMP7 and activin (Xiao, *et al.*, 1992; Shimasaki, *et al.*, 1999). The inhibin production, a measurement for follicle differentiation (also measured by inhibin α-, βA- or βB-subunit mRNA transcription) is inhibited by several TGFβ superfamily members (Xiao, *et al.*, 1992; Otsuka, *et al.*, 2001a, 2001b). Progesterone production and LHR expression are signs of luteinization of the granulosa cells. Both LHR expression and progesterone production are stimulated by FSH and inhibited by TGFβ superfamily members, causing the inhibition of luteinization by these TGFβ superfamily members (Xiao, *et al.*, 1992; di Clemente, *et al.*, 1994a; Elvin, *et al.*, 1999a; Shimasaki, *et al.*, 1999; Otsuka, *et al.*, 2001a). A discrepancy is found concerning the regulation of FSHR mRNA expression by FSH, since stimulation and inhibition of FSHR mRNA by FSH has been described. In both experiments, the FSH effect was inhibited by TGFβ superfamily members (Xiao, *et al.*, 1992; Otsuka, *et al.*, 2001a, 2001b).

Together these results show that FSH and LH are the driving forces of follicle development from selection to ovulation while TGFβ superfamily members have a dominant role in follicle development until selection but also are involved in follicle development after this regulatory point. In addition, ovarian factors modulate actions of FSH on follicle development: in general they inhibit FSH-induced differentiation.

Table 8.4: FSH effects modulated by members of the TGFβ superfamily in granulosa cell culture.

FSH effect:	TGFβ family member modulation:	References:
Stimulates estrogen synthesis	Stimulated by BMP4, BMP7, activin (granulosa cells of follicles of immature mice)	Xiao *et al.*, 1992; Shimasaki *et al.*, 1999
Stimulates inhibin production	Stimulated by activin (granulosa cells of follicles of immature mice)	Xiao *et al.*, 1992
Stimulates inhibin α mRNA expression	Inhibited by BMP6, BMP15	Otsuka *et al.*, 2001a, 2001b
Stimulates inhibin βA mRNA	Inhibited by BMP6, BMP15	Otsuka *et al.*, 2001a, 2001b
Stimulates inhibin βB mRNA	Inhibited by BMP6, BMP15	Otsuka *et al.*, 2001a, 2001b
Stimulates progesterone production	Inhibited by GDF9, BMP15, BMP6, BMP4, BMP7	Elvin *et al.*, 1999; Shimasaki *et al.*, 1999; Otsuka *et al.*, 2001a
	Stimulated by activin (granulosa cells of follicles of immature mice)	Xiao *et al.*, 1992; Shimasaki *et al.*, 1999
Stimulates LHR expression	Inhibited by GDF9, AMH, BMP6	di Clemente *et al.*, 1994; Elvin e*et al.*, 1999; Otsuka *et al.*, 2001a
Stimulates FSHR expression	Inhibited by BMP6, BMP15	Otsuka *et al.*, 2001a, 2001b
Inhibits FSHR expression	Inhibited by activin (granulosa cells of follicles of immature mice)	Xiao *et al.*, 1992

8.4 AMH AND THE CLINIC

Interestingly, in ovaries of the mouse (Chapter 3, Münsterberg and Lovell-Badge, 1991; Taketo, *et al.*, 1993; Durlinger, *et al.*, 2002), the rat (Hirobe, *et al.*, 1994; Baarends, *et al.*, 1995b) as well as the human (Weenen, *et al.*, 2004), expression of AMH is found in granulosa cells of follicles immediately after primordial follicle recruitment and expression is continued during follicle growth until the point of selection. Thus, AMH is expressed in follicles that grow independently of FSH stimulation (for review see Chapter 1). This is reflected by the absence of large AMH serum fluctuations in female serum levels during the menstrual cycle (Cook, *et al.*, 2000). Many studies have been performed to investigate the correlation of AMH serum levels with ovarian function and in this section these studies are discussed.

Throughout life, the primordial stock decreases and when a women has reached the menopause, the primordial follicle pool is almost depleted (for review see Chapter 1)(Baker, 1963). The decrease of the primordial follicle stock in the ovary is called ovarian aging. However, ovarian aging is difficult to measure since the size of the primordial follicle stock in women is difficult to determine. However, it appears that the number of growing follicles is correlated to the size of the primordial follicle stock from which they are recruited (Gougeon, *et al.*, 1994; Scheffer, *et al.*, 1999). Since AMH is produced by the growing follicles, the hypothesis that AMH might be a candidate marker of ovarian reserve was tested. Indeed, a negative correlation was found between age and AMH levels (de Vet, *et al.*, 2002). In addition, AMH serum levels showed a strong correlation with the number of growing antral follicles. This correlation was stronger than the correlation of the number of growing antral follicles with FSH or inhibin B serum levels (de Vet, *et al.*, 2002; Fanchin, *et al.*, 2003). In line with these results, AMH serum levels are undetectable after menopause (Lee, *et al.*, 1996; de Vet, *et al.*, 2002). Thus, AMH correlates with the number of growing follicles present in the ovary and ovarian aging.

Fertility in women is determined by the ovarian reserve, which represents both the quantity and quality of the ovarian follicle pool. Besides aspects such as oocyte quality, the number of primordial follicles that are left in the ovary is also an important parameter for ovarian reserve (te Velde and Pearson, 2002). Therefore, a marker that reflects all follicles that have made the transition from the primordial follicle pool to the growing pool may be a good indirect marker of the quantitative aspects of the ovarian reserve. Indeed, low AMH serum levels were associated with a decreased response to ovarian stimulation for IVF (Seifer, *et al.*, 2002; van Rooij, *et al.*, 2002). Although AMH serum levels may not be the only marker of ovarian reserve, the relatively small fluctuations in AMH serum level during the menstrual cycle (Cook, *et al.*, 2000) indicate that a predictive blood sample may be drawn at any moment during the menstrual cycle, which further supports the advantage of the use of AMH as a marker of ovarian reserve over antral follicle count using ultrasound. An interesting new field of research is the role of AMH during follicle development of an individual follicle. The immature female gametes in the ovarian cortex can be cryopreserved (Gosden, 2000), for example for females undergoing cancer treatment. At this moment, it is still impossible to mature these primordial follicles to a pre-ovulatory follicle *in vitro*. However, the *in vitro* culture of follicles is still under development. AMH is expressed by granulosa cells of growing follicles until selection and AMH production by these follicles might give an indication of developmental stage of the follicle. During the *in vitro* maturation of follicles different concentrations of FSH, LH and growth factors might be needed, and AMH may be an excellent marker of follicle developmental stage to indicate successful maturation of follicles.

AMH serum levels may also give an indication of abnormal ovarian function. In patients with polycystic ovary syndrome (PCOS), much higher AMH serum levels were found than in normo-ovulatory women (Fallat, *et al.*, 1997; Cook, *et al.*, 2002; Laven, *et al.*, 2004). In these PCOS patients a higher number of immature oocytes was found after ovarian stimulation for IVF treatment (Fallat, *et al.*, 1997). Furthermore, increased serum levels of AMH are found in most women with granulosa cell tumors (Gustafson, *et al.*, 1992; Lane, *et al.*, 1999; Long, *et al.*, 2000; Rey, *et al.*, 2000).

In conclusion, AMH is a good marker for ovarian function. Decreasing AMH serum levels show a decrease in ovarian reserve, with poor prognosis for good results in IVF. In addition, an increased AMH serum level is an indication for PCOS and can be used to detect the presence of granulosa cell tumors.

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Summary

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SUMMARY

Menopause, the end of menstrual cycling, is a major milestone in the aging process in women. The cessation of ovarian function is accompanied by the almost complete absence of female sex steroid hormone secretion by the ovaries in postmenopausal life. Consequently, before and after the menopausal transition very different physiological hormonal regimens prevail, which have a major impact on healthy aging of women and their quality of life. After menopause, due to the loss of exposure to protective female sex steroid hormones, women have an increased risk for different health problems (such as osteoporosis, cardiovascular diseases and a decreased cognitive function). In addition, the age at menopause is associated with the age at which infertility starts. Approximately, 20 years before menopause female fertility starts to decrease, culminating through sub-fertility into complete infertility 10 years before menopause. Since the age at menopause affects the length of the postmenopausal period and the age of infertility, it is of utmost importance to understand the process leading to menopause. Menopause is caused by the exhaustion of the follicle stock in the ovaries. The limited supply of primordial follicles is formed during fetal life and cannot be replenished afterwards. The number of primordial follicles decreases by outgrowth of follicles or by atresia. Studies on the anti-Müllerian hormone (AMH) *null* mice showed that AMH inhibits the outgrowth of primordial follicles and thereby inhibits the exhaustion of follicles in the ovary.

This thesis focuses on the role of AMH in the ovary and investigates through which molecular mechanisms these effects are achieved.

In the General Introduction (Chapter 1), the process of follicle development from primordial to pre-ovulatory follicle is described. This process is regulated by follicle-stimulating hormone (FSH) and luteinizing hormone (LH), produced by the pituitary, but also by several intra-ovarian factors. One important family of growth- and differentiation factors which plays a role in follicle development is the transforming growth factor β (TGFβ) superfamily, to which also AMH belongs. The involvement of several TGFβ family members in follicle development is described and the emphasis is on the known effects of AMH on follicle development and the clinical relevance of these findings.

AMH is a member of the TGFβ superfamily and family members signal through a receptor complex consisting of a type II and a type I transmembrane serine/threonine kinase receptor. The production of TGFβ superfamily members, the mechanism of receptor activation and downstream signaling are described in Chapter 2.

The inhibitory effect of AMH on primordial follicle recruitment was investigated in an *in vitro* study in which 2-day-old neonatal wild-type ovaries were cultured in the absence or presence of AMH (Chapter 3). The ovaries of 2-day-old mice contain only primordial and no growing follicles, and therefore provide a good model for studying the effect of AMH on primordial follicle recruitment. After 2 or 4 days of culture of the neonatal ovaries, less growing follicles were found in the ovaries cultured in the presence of AMH compared to ovaries cultured in the absence of AMH. Furthermore, the mRNA levels of the inhibin α-subunit, expressed by early growing follicles detected by immunohistochemistry, was lower in the ovaries cultured in the presence of AMH. No effect on mRNA levels of the AMH type II receptor (AMHRII) or the oocyte markers zona pellucida protein 3 (ZP3) and growth and differentiation factor 9 (GDF9) was detected after culturing ovaries in the presence of AMH.

AMH signals through a receptor complex containing type II and type I serine/threonine kinase receptors. The AMH type II receptor (AMHRII) is responsible for AMH-signaling in the Müllerian ducts, since the absence of the receptor results in the absence of Müllerian duct regression. To investigate if this AMHRII is also responsible for AMH-signaling in the ovary, we studied the follicle population of ovaries of 4-month-old mice lacking expression of the AMHRII, the MIS receptor knock-in (MRKI) mice (Chapter 4). Similar to the results found in the AMH *null* mice ovaries, ovaries of MRKI(-/-) mice contained more growing and less primordial follicles than their wild-type littermates. The increased number of growing follicles was accompanied by increased inhibin A and inhibin B and decreased FSH serum levels, albeit that these differences were not significant due to the small number of wild-type mice investigated. The number of total and primordial follicles in the MRKI(-/-) mice ovaries was smaller compared to the AMH(-/-) ovaries, suggesting an increased recruitment of primordial follicles. This increased recruitment is probably caused by the absence of ligand-independent activity of the AMHRII in the MRKI(-/-) mice ovaries. These experiments show that AMH signals through the AMHRII in the ovary.

Two main signaling pathways can be distinguished for TGFβ superfamily members: the BMP-like and TGFβ/activin-like signaling pathway. In Chapter 5, we investigated through which signaling pathway AMH transduces its signal in the ovary. In addition, we studied if AMH can regulate its own signaling by changing gene expression of its receptors or downstream signaling mediators. Using the mouse granulosa cell line KK-1, we showed that AMH signals through the BMP-like signaling pathway. The different candidate type I receptors and receptor-specific and inhibitory Smads are expressed by ovaries of postnatal ovaries, showing possible involvement in primordial follicle recruitment. The AMHRII and one of the candidate type I receptors, ALK2, are expressed by granulosa cells of pre-antral and antral follicles, shown by *in situ* hybridization. In addition, we showed by quantitative real-time PCR that AMH upregulates the mRNA expression of the inhibitory Smad6 and Smad7, showing an intracellular negative feedback mechanism. Furthermore, AMH upregulates the expression of the receptor-specific Smad8. However, the importance of this finding needs further research since the expression of Smad8 is very low.

Primordial follicle recruitment is under control of the growing follicle pool. To identify the importance of several intra-ovarian growth factors in this process, we studied the mRNA expression and protein localization in postnatal ovaries. The age of the ovaries was selected on basis of the presence of different follicle types. Ovaries of 2-day-old mice only contained primordial follicles, whereas on day 6 also primary follicles were present. In 12-day-old ovaries pre-antral follicles first appeared and antral follicles were found first in ovaries of 18-day-old mice. All antral and large pre-antral follicles had become atretic in ovaries of 25-day-old mice. At these five different ovarian ages the protein localization and mRNA expression levels were determined of growth factors which are or could be involved in primordial follicle recruitment. Granulosa cells of small growing follicles expressed AMH, activins, inhibins and kit-ligand, while growth and differentiating factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) were expressed by the oocytes of small growing follicles. Therefore, these growth factors could be involved in primordial follicle recruitment. However, the intra-ovarian growth factors basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) were expressed by oocytes, granulosa, theca and interstitial cells. Since these growth factors are so generally expressed, it is unlikely that bFGF and NGF play a regulatory role in primordial follicle recruitment.

AMH inhibits recruitment of primordial follicles and decreases the sensitivity of large pre-antral and small antral follicles for FSH. However, the molecular mechanisms by which AMH achieves these effects are largely unknown. To understand the downstream effects of AMH we aimed to identify AMH-regulated genes (Chapter 7). We cultured neonatal mouse ovaries and the mouse granulosa cell line KK-1/AMHRII for 24 hours in the absence or presence of AMH. To identify gene expression differences in neonatal ovaries we used the Atlas™ mouse cDNA expression array and to identify gene expression differences in KK-1/AMHRII cells we used the Agilent mouse development oligo microarray. Differences in gene expression found on the arrays were validated using quantitative real-time PCR. The genes identified from neonatal ovary culture and the Atlas™ mouse cDNA expression array could not be confirmed by quantitative real-time PCR. Validation of the genes identified from the KK-1/AMHRII and the Agilent mouse development oligo microarray is ongoing.

The General Discussion (Chapter 8) summarizes the mechanisms by which AMH could achieve its specific effects. In addition, the role of AMH in follicle development compared to the role of the systemic factors LH and FSH and the clinical relevance of AMH are discussed.

Samenvatting

Samenvatting

SAMENVATTING

Menopauze is een mijlpaal in het verouderingsproces van vrouwen en de postmenopausale periode begint na de laatste menstruele cyclus. De achteruitgang van de ovariële functie veroorzaakt de bijna complete afwezigheid van afgifte van vrouwelijke geslachtshormonen door de ovaria. Na de menopauze hebben vrouwen een verhoogde kans op verscheidene gezondheidsproblemen zoals osteoporose, veranderingen in cognitieve functies (bijv. de ziekte van Alzheimer) en cardiovasculaire ziekten. Aan de andere kant is er een verband tussen de leeftijd waarop een vrouw in de menopauze komt en de leeftijd waarop zij onvruchtbaar wordt. Circa twintig jaar voor de menopauze begint de vruchtbaarheid af te nemen en 10 jaar voor de menopauze is de vrouw volledig onvruchtbaar. In het licht van mogelijk vroege onvruchtbaarheid en de gezondheidsproblemen geassocieerd met de menopauze is het van groot belang om de processen die een rol spelen bij het ontstaan van de menopauze te begrijpen.

De menopauze wordt veroorzaakt door de uitputting van de follikelvoorraad in de ovaria. De gelimiteerde voorraad primordiale follikels wordt gevormd tijdens de foetale ontwikkeling en kan later niet meer aangevuld worden. Na de vorming van de primordiale follikelvoorraad, neemt het aantal primordiale follikels af door uitgroei of door sterfte van deze follikels. Studies aan muizen die geen anti-Müllerse gang hormoon (AMH) eiwit kunnen maken, hebben laten zien dat AMH de uitgroei van de primordiale follikels remt en daarmee de uitputting van de voorraad follikels in de ovaria tegengaat.

Dit proefschrift richt zich op de functie van AMH in het ovarium en onderzoekt door welke moleculaire werkingsmechanismen de effecten van AMH worden bereikt.

In de Algemene Introductie (Hoofdstuk 1) wordt het ontwikkelingsproces van een primordiale follikel tot een pre-ovulatoire follikel beschreven. Dit proces wordt gereguleerd door follikelstimulerend hormoon (FSH) en luteiniserend hormoon (LH), welke door de hypofyse geproduceerd worden. Verder wordt de ontwikkeling van een follikel gereguleerd door groeifactoren die in het ovarium geproduceerd worden. Een zeer belangrijke familie van groei- en ontwikkelingsfactoren is de *transforming growth factor* β (TGFβ) superfamilie. Hoofdstuk 1 geeft een overzicht van de betrokkenheid van meerdere TGFβ familieleden bij follikelontwikkeling. De nadruk van dit hoofdstuk ligt op de reeds bekende effecten van AMH op follikelontwikkeling en de klinische relevantie van deze bevindingen. AMH is lid van de TGFβ superfamilie van groei- en ontwikkelingsfactoren. Groeifactoren die bij de TGFβ familie horen geven hun signaal in de cel door via een receptorcomplex bestaande uit type II en type I transmembraan serine/threonine kinase receptoren. Hoofdstuk 2 beschrijft de productie van TGFβ familieleden in de cel, het mechanisme van de activatie van hun receptor en het doorgeven van hun receptor signaal in de cel.

Het remmend effect van AMH op de uitgroei primordiale follikels werd onderzocht in een kweeksysteem, waarbij ovaria van 2 dagen oude muizen werden gekweekt in de af- en aanwezigheid van AMH (Hoofdstuk 3). De ovaria van 2 dagen oude muizen bevatten alleen primordiale follikels en zijn om die reden een zeer goed studieobject voor het meten van de effecten van AMH op de primordiale follikel uitgroei. Na 2 of 4 dagen worden minder groeiende follikels gevonden in ovaria die gekweekt zijn in aanwezigheid van AMH vergeleken met de ovaria die in afwezigheid van AMH zijn gekweekt. Verder toonden we met behulp van immunohistochemische technieken aan dat de inhibine α-subunit wordt geproduceerd door kleine groeiende follikels. Dat AMH de uitgroei van primordiale follikels in de gekweekte ovaria remt werd bevestigd door metingen van inhibine α-subunit mRNA.

De ovaria die gekweekt werden in afwezigheid van AMH bevatten meer inhibine α-subunit mRNA dan de ovaria gekweekt in aanwezigheid van AMH. AMH had geen effect op de mRNA expressie van de AMH type II receptor (AMHRII) of de door oocyten geproduceerde eiwitten *zona pellucida 3* (ZP3) en *growth differentiation factor 9* (GDF9).

Het signaal van AMH wordt doorgegeven d.m.v. een receptorcomplex dat een type II en type I receptoren bevat. De AMH type II receptor (AMHRII) is verantwoordelijk voor het doorgeven van het signaal van AMH in de Müllerse gangen tijdens de embryonale periode, resulterend in regressie van de Müllerse gangen in mannen. Vrouwen maken geen AMH tijdens de embryonale periode en daardoor gaan de Müllerse gangen niet in regressie en ontwikkelen zich in de eileiders, baarmoeder en het bovenste deel van de vagina. Wij onderzochten of dezelfde AMHRII ook verantwoordelijk is voor het doorgeven van het signaal van AMH in de ovaria. Hiervoor bestudeerden we de follikel populatie in ovaria van 4 maanden oude (MRKI(-/-)) muizen die geen AMHRII tot expressie brengen (Hoofdstuk 4). De ovaria van MRKI(-/-) muizen bevatten meer groeiende en minder primordiale follikels dan in de controle dieren en deze resultaten zijn vergelijkbaar met de resultaten gevonden in de ovaria van muizen die geen AMH (AMH(-/-)) tot expressie brengen. Het toegenomen aantal groeiende follikels ging vergezeld met toegenomen serumconcentraties van inhibine A en inhibine B en afgenomen serumconcentratie van FSH. Deze verschillen waren niet significant, waarschijnlijk als gevolg van het kleine aantal controle dieren dat tot nu toe in de studie was opgenomen. Ovaria van MRKI(-/-) muizen bevatten minder follikels in totaal en minder primordiale follikels dan gevonden werden in AMH(-/-) dieren. Het fenotype van de MRKI(-/-) muizen is sterker dan de AMH(-/-) muizen. Dit resultaat zou kunnen wijzen op basale activiteit van de AMHRII. Dus, de experimenten in de MRKI muizen hebben laten zien dat AMH in de ovaria signaleert door middel van de AMHRII.

Twee algemene signaaltransductie routes voor de TGFβ superfamilie leden kunnen worden onderscheiden: de BMP- en de TGFβ/activine-signaaltransductie route. In Hoofdstuk 5 onderzochten we via welke signaaltransductie route het signaal van AMH wordt doorgegeven in het ovarium. Vervolgens hebben we onderzocht of AMH de sterkte van zijn eigen signaal in de cel kan reguleren door de expressie van zijn type I en type II receptoren en downstream eiwitten te beïnvloeden. Voor dit onderzoek gebruikten we de muizen granulosa cellijn KK-1. We ontdekten dat AMH zijn signaal in de cel doorgeeft via de BMP-signaaltransductie route. De verschillende kandidaat type I receptoren, receptor-specifieke en remmende Smads komen tot expressie in de ovaria van postnatale muizen, en kunnen dus allemaal betrokken zijn bij het doorgeven van het AMH signaal en dus ook bij primordiale follikel uitgroei. Met *in situ* hybridisatie hebben we aangetoond dat de AMHRII en één van de kandidaat type I receptors, ALK2, tot expressie komen in granulosa cellen van pre-antrale en antrale follikels. Ook hebben we door middel van *quantitative real-time PCR* laten zien dat AMH de genexpressie van de remmende Smads (Smad6 en Smad7) stimuleert en op deze manier wordt een intracellulair negatief terugkoppelingsmechanisme gevormd. De genexpressie van de receptor-specifieke Smad8 wordt ook gestimuleerd door AMH. Het belang van deze bevinding moet echter verder onderzocht worden omdat het expressieniveau van Smad8 erg laag is.

Primordiale follikeluitgroei wordt deels gereguleerd door de groeiende follikels. Om het belang van verschillende intra-ovariële groeifactoren in dit proces te bestuderen, hebben we de eiwit lokalisatie en mRNA expressie van een aantal belangrijke groeifactoren bestudeerd in postnatale ovaria. De leeftijd van de ovaria is gekozen op basis van aanwezigheid van verschillende type follikels. Ovaria van 2 dagen

oude muizen bevatten alleen primordiale follikels. De eerste primaire follikels worden gevonden in ovaria van 6 dagen oude muizen. Ovaria van 12 dagen oude muizen bevatten pre-antrale follikels en antrale follikels zijn aanwezig in 18 dagen oude muizen. Alle grote pre-antrale en antrale follikels zijn atretisch in ovaria van 25 dagen oude muizen. In ovaria van muizen met deze 5 verschillende leeftijden hebben we de localizatie van eiwit en de hoogte van expressie van het mRNA van een aantal groeifactoren bepaald. Granulosa cellen van kleine groeiende follikels bevatten AMH, activines, inhibines en kit-ligand, terwijl de GDF9 en *bone morphogenetic protein 15* (BMP15) tot expressie komen in de oocyten van deze kleine groeiende follikels. Deze groeifactoren kunnen dus betrokken zijn bij primordiale follikel uitgroei. De intra-ovariële groeifactoren *basic fibroblast growth factor* (bFGF) en *nerve growth factor* (NGF) komen tot expressie in de oocyten en in granulosa, theca en interstitiële cellen. Omdat deze groeifactoren zo algemeen tot expressie komen is het onwaarschijnlijk dat bFGF en NGF een specifiek regulerende rol spelen bij primordiale follikel uitgroei.

AMH remt de uitgroei van primordiale follikels en remt de gevoeligheid van grote pre-antrale en antrale follikels voor FSH. De moleculaire mechanismen waardoor AMH dit effect bereikt zijn onbekend. Om de effecten van AMH te ontrafelen wilden we AMH-gereguleerde genen opsporen (Hoofdstuk 7). We kweekten neonatale ovaria en de muizen granulosa cellijn KK-1/AMHRII voor 24 uur in af- of aanwezigheid van AMH. Genexpressieverschillen in de neonatale ovaria werden bestudeerd met de Atlas™ mouse cDNA expressie array en de genexpressie verschillen in de KK-1/AMHRII cellen werden bestudeerd met de Agilent mouse development oligo microarray. Verschillen in genexpressie gevonden met de arrays werden gecontroleerd met quantitatieve real-time PCR. De AMH-gereguleerde genen gevonden in de neonatale muizen ovaria konden niet worden bevestigd met de quantitatieve real-time PCR. De genen welke zijn geïdentificeerd in de KK-1/AMHRII cellen worden op dit moment gecontroleerd.

De Algemene Discussie (Hoofdstuk 8) geeft tenslotte een overzicht van de mechanismen waardoor AMH zijn effecten kan bereiken. Ook de rol van AMH in follikelontwikkeling wordt vergeleken met de rol van LH en FSH. Als laatste wordt de klinische relevantie van het meten van AMH concentraties in het bloed besproken.

Publications

Publications

LIST OF PUBLICATIONS

Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JTJ, Grootegoed JA and Themmen APN. 2001. Anti-Müllerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. Endocrinology 142: 4891-4899.

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Curriculum Vitae

Curriculum Vitae

CURRICULUM VITAE

Maria Johanna Godefrida Gruijters was born on October 22th 1975 in Geldrop. Her secondary education was taken at the Scholengemeenschap Jan van Brabant (VWO) in Helmond. In 1994, she started the study Biology at the University of Utrecht, which was continued with the study Fundamental Biomedical Sciences a year later. Her first 7.5 months doctoral training was followed at the Department of Biology Education at the University of Utrecht where she studied Health Education under supervision of Dr. Aart-Jan Waarlo. The theoretical part (3 months) was followed by a 4.5 months research period for the Diabetesvereniging Nederland (DVN). During these 4.5 months she, together with Martijn Planken, studied the role which the DVN should play in supporting and educating young people between the age of 12 and 21 years suffering from diabetes. She followed her second doctoral training of 7.5 months at the Group for Comparative Endocrinology of the Department of Zoology at the University of Utrecht. Under supervision of Dr. Jan Bogerd and Prof. dr. Henk J. Th. Goos, she cloned and partly characterized four genes from a genomic library of the African catfish. The study Fundamental Biomedical Sciences was successfully (cum laude) completed in May 1999.

From May 1999 until October 2003, the PhD research, described in this thesis, was performed first at the Department of Endocrinology & Reproduction and from January 2002 at the Department of Internal Medicine at the Erasmus MC (Rotterdam) under supervision of Dr. ir. Axel P.N. Themmen and Prof. dr. Frank H. de Jong.

As of March 2004 she is working as a data manager at the HOVON (Hemato-Oncology for Adults in The Netherlands) data center, Department of Trials & Statistics at the Erasmus MC, Rotterdam.

Dankwoord

Dankwoord

Ongeveer 5 jaar heb ik als aio gewerkt, eerst bij de vakgroep Endocrinologie & Voortplanting, daarna bij de vakgroep Inwendige Geneeskunde. Deze jaren waren drukke, leerzame maar vooral ook hele leuke jaren. Promoveren doe je niet alleen. Daarom wil ik op deze plek die mensen bedanken die op de een of andere manier behulpzaam zijn geweest bij mijn onderzoek of die deze jaren hebben gemaakt tot jaren waar ik met heel veel plezier aan terugdenk.

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Figure 3.3: Immunohistochemical localization of AMH and inhibin α-subunit in freshly isolated (control) and cultured neonatal C57Bl/6J mouse ovaries.

A-B. Section of a 2-day-old control mouse ovary. No AMH (A) and inhibin α-subunit (B) protein expression is found in naked oocytes (no), primordial follicles (pr) and interstitial tissue (I). Scale bar 20 µm.

C-D. Section of a 4-day-old control mouse ovary. AMH protein expression (C) and inhibin α-subunit protein expression (D) are found in several granulosa cells of early primary follicles. Scale bar 20 µm.

E-F. Section of a 6-day-old control mouse ovary. Abundant AMH protein expression (E) and inhibin α-subunit protein expression (F) are found in granulosa cells of primary follicles (P) and early secondary follicles (S). Scale bar 20 µm.

G-H. Section of a 2-day-old mouse ovary cultured for 2 days in the absence of added AMH. High AMH protein expression (G) and inhibin α-subunit protein expression (H) are found in granulosa cells of primary follicles (P). Scale bar 20 µm.

I-J. Section of a 4-day-old control mouse ovary. For AMH some background staining is found in the interstitium (I) and the oocytes, while for inhibin α-subunit very low background staining is only found in the oocytes (J). Scale bar 20 µm.

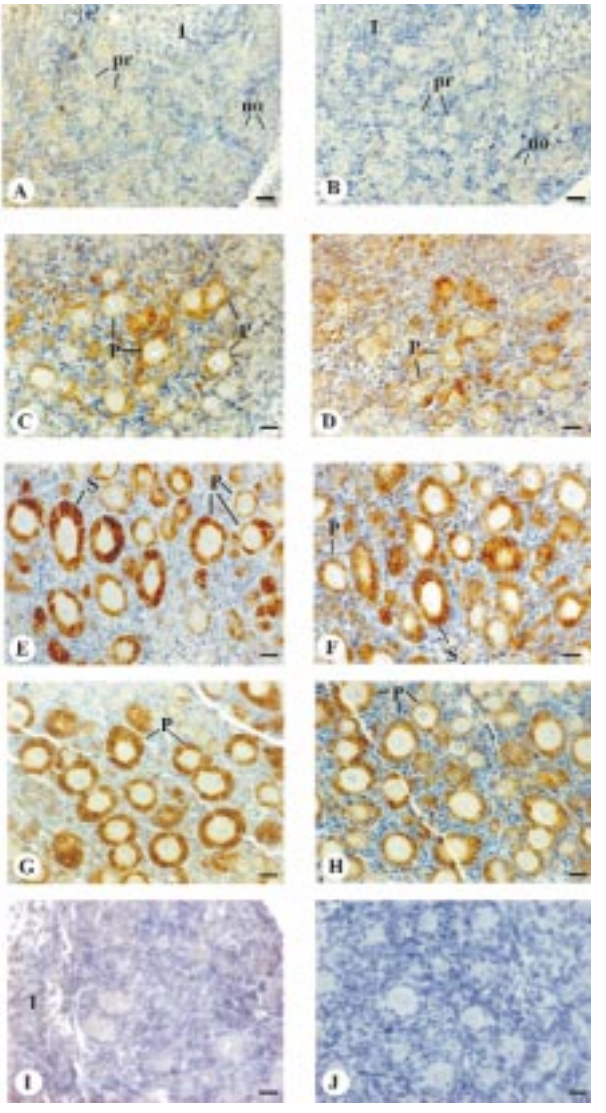


Figure 6.2: Immunohistochemical localization of AMH in postnatal C57Bl/6J mouse ovaries of different ages. Magnification x 100.

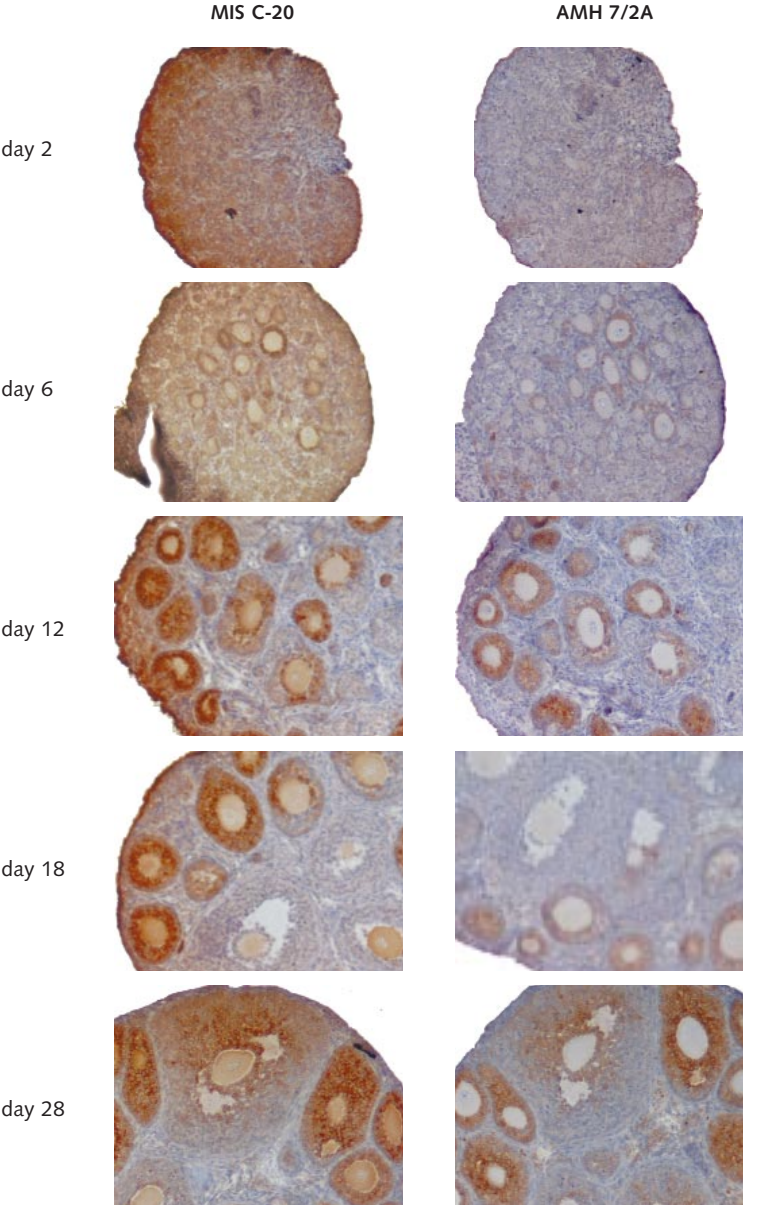


Figure 6.5: Immunohistochemical localization of bFGF and NGF in postnatal C57Bl/6J mouse ovaries of different ages. Magnification x 100.

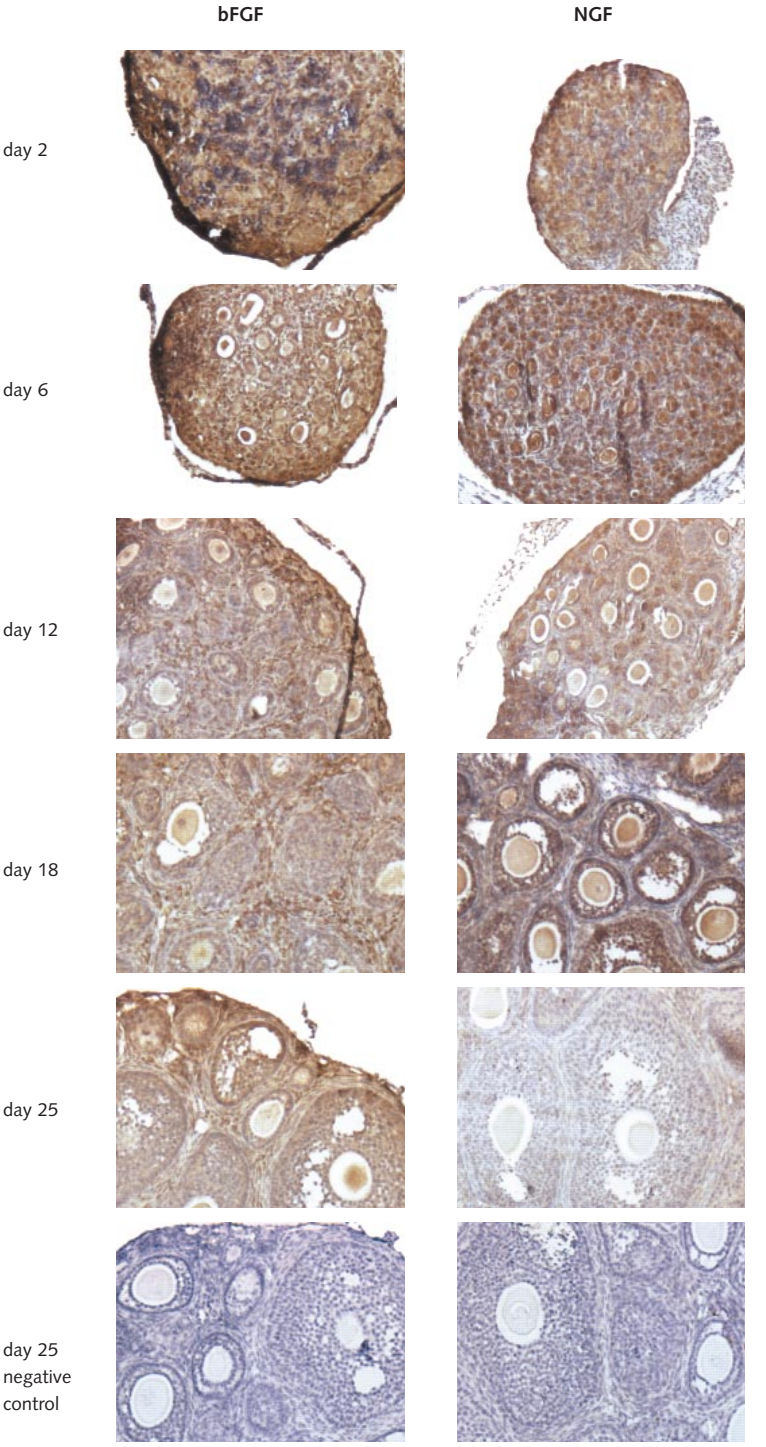


Figure 6.4: Immunohistochemical localization of GDF9 in postnatal C57Bl/6J mouse ovaries of different ages. Magnification x 100.

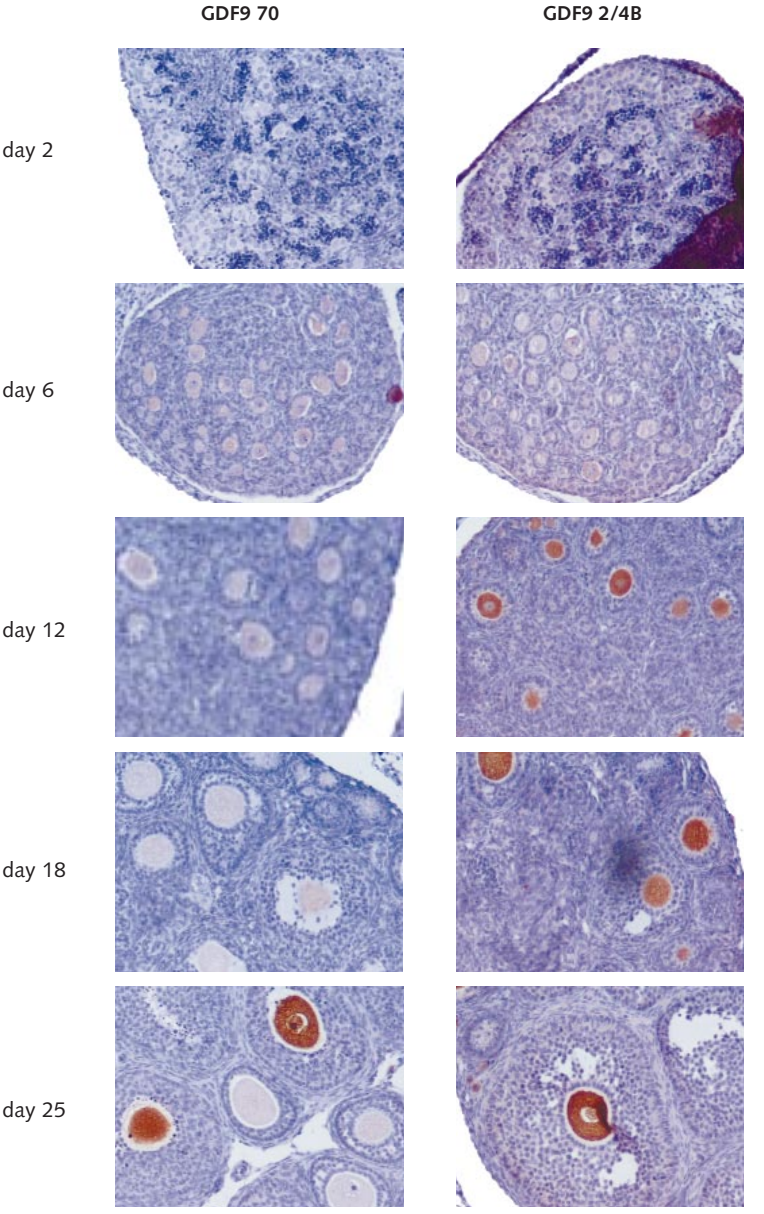


Figure 6.3: Immunohistochemical localization of inhibin α-, βA- and βB-subunits in postnatal C57Bl/6J mouse ovaries of different ages.
Histology of the inhibin βB-subunits differs from the inhibin a and βA-subunit immunohistochemistry due to fixation differences (formaldehyde vs. bouin). Magnification x 100.

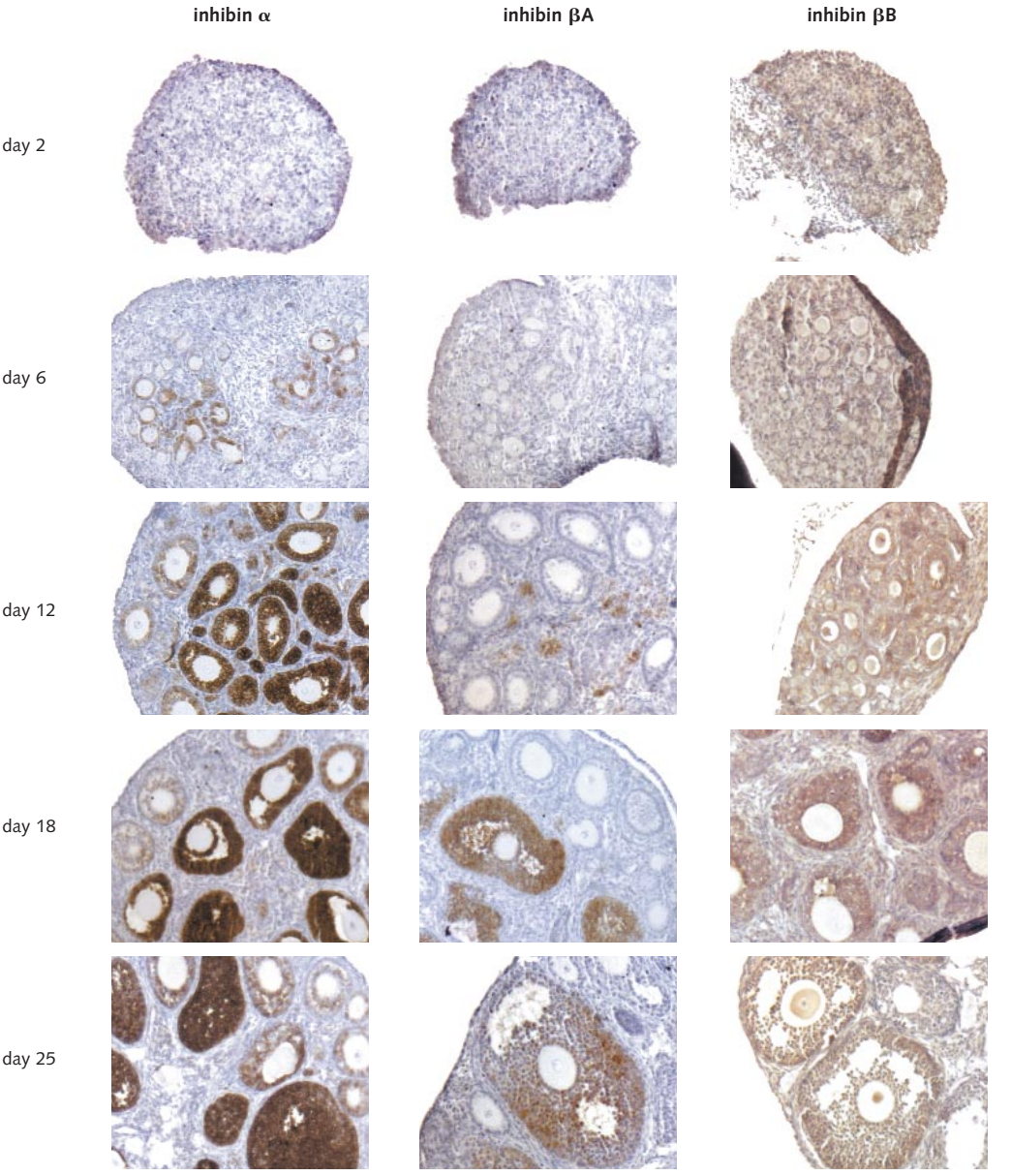
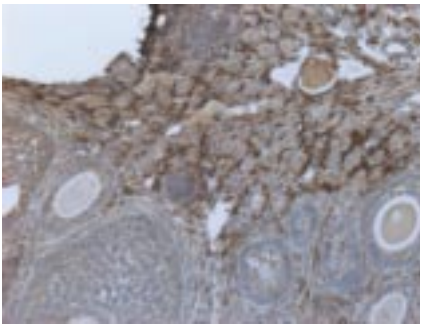


Figure 6.6: bFGF protein is expressed in the interstitial cells surrounding primordial follicles in an 18-day-old mouse ovary. Magnification x 200.



Stellingen behorende bij het proefschrift

Anti-Müllerian Hormone: Function and Molecular Mechanism of Action in the ovary
Anti-Müllerse gang hormoon: Functie en moleculair werkingsmechanisme in het ovarium

19 mei 2004
Erasmus Universiteit Rotterdam
Marja Gruijters

1 AMH en BMP4 geven beide hun signaal door via de BMP signaal transductieroute maar hebben een tegengesteld effect op de primordiale follikeluitgroei. Dit gegeven suggereert een belangrijke rol voor de type II receptor in het doorgeven van het signaal.
dit proefschrift
Nilsson *et al.* (2003) Biol Reprod 69: 1265-72

2 Validatie van met een array geïdentificeerde hormoon gereguleerde genen met behulp van een onafhankelijke techniek is noodzakelijk.
dit proefschrift

3 ALK2 en ALK3 zijn de enige type I receptoren die het het signaal van AMH doorgeven.
dit proefschrift
Visser *et al.* (2001) Mol Endocrinol 15:936-45
Clark *et al.* (2001) Mol Endocrinol 15:946-59
Jamin *et al.* (2002) Nat Genet 32:408-410

4 Beschrijvend onderzoek kan, net zoals experimenteel onderzoek, nieuw licht werpen op bestaande hypothesen over effecten van hormonen.
dit proefschrift

5 De overeenkomsten in de fenotypes van de AMH *null* muizen en de AMHRII *null*, de AMHRII knock-in en de AMH overexpressor/AMHRII *null* muizen bewijzen dat AMH het enige ligand is voor de AMHRII.
dit proefschrift
Behringer *et al.* (1994) Cell 79:415-25
Durlinger *et al.* (1999) Endocrinology 140:5789-96
Mishina *et al.* (1996) Genes Dev 10:2577-87
Jamin *et al.* (2003) Mol Cell Endocrinol 211:15-19

6 Als onderzoekers *"quantitative real-time PCR"* gebruiken, wil dat niet zeggen dat zij deze techniek voldoende begrijpen om de resultaten van de experimenten op een correcte manier te interpreteren.
Drummond *et al.* (2002) J Endocrinology 143:1423-33

7 Het gegeven dat GDF9 bindt aan de BMPRII en toch Smad2 fosforyleert werpt de dogma's over de methode van signalering van TGFβ familieleden omver.
Roh *et al.* (2003) Endocrinology 144:172-8
Kaivo-Oja *et al.* (2003) J Clin Endocrinol Metab 88:755-62

8 AMH is een goede additionele marker voor ovariële functie.
Cook *et al.* (2002) Fertil Steril 77:141-6
de Vet *et al.* (2002) Fertil Steril 77:357-362
van Rooij *et al.* (2002) Hum Reprod 17:3065-71
Laven *et al.* (2004) J Clin Endocrinol Metab 89:318-23

9 Het belang dat de Nederlandse regering zegt te hechten aan het ontwikkelen van de kenniseconomie, geïllustreerd door het oprichten van een platform met de minister-president als voorzitter, staat in schril contrast met de relatief lage overheidsuitgaven voor onderzoek en onderwijs in Nederland in vergelijking met die in andere westerse landen.
Rapport OESO: Education at a glance 2003

10 Onderzoekers zouden alleen als auteur moeten optreden van een wetenschappelijk artikel als zij het volledig eens zijn met de inhoud.
Argos, 14 november 2003
The Oral Contraceptive and Hemostasis Study Group (2003) Contraception 67:173-185

11 Elvis leeft!