Ovarian Follicle Growth and Development:

Role of Anti-Müllerian Hormone

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Ovarian Follicle Growth and Development: Role of Anti-Müllerian Hormone

Groei en Ontwikkeling van Ovariële Follikels:
Rol van Anti-Müllerse Gang Hormoon

Proefschrift

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CONTENTS

LIST OF ABBREVIATIONS

ActRII	activin receptor type II
AHC	adrenal hypoplasia congenita
AhR	aryl hydrocarbon receptor
ALK	activin receptor-like kinase
AMH	anti-Müllerian hormone
AMHRII	AMH receptor type II
Apaf-1	apoptotic protease-activating factor-1
ArKO	aromatase knockout
ARNT	AhR nuclear translocator
bHLH	basic helix-loop-helix
BMP	bone morphogenetic protein
BrdU	5-bromodeoxyuridine
BSA	bovine serum albumin
C/EBPβ	CCAAT/enhancer-binding protein β
COX-2	cyclooxygenase 2
Dax-1	DSS-AHC critical region on the X, gene 1
DES	diethylstilbestrol
DNA	deoxyribonucleic acid
dpc	days *post coitum*
dpp	decapentaplegic
DSS	dosage-sensitive sex reversal
E#	embryonic day #
E_2	oestradiol
ER	estrogen receptor
ERE	estrogen response element
ERKO	estrogen receptor knockout
FCS	fetal calf serum
FIGα	factor in the germline α
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GDF	growth and differentiation factor
GH	growth hormone
GnRH	gonadotropin-releasing hormone
h (prefix)	human
HAS2	hyaluron synthase 2
hCG	human chorionic gonadotropin
HEK	human embryonic kidney
hpg	hypogonadal
IGF	insulin-like growth factor
kDa	kilo Dalton
KO	knockout
LH	luteinizing hormone
Lhx	Lim homeobox
LIF	leukaemia inhibitory factor
Mad	mothers against *dpp*
Mcl-1	myeloid cell leukemia-1
MH	Mad-homology domain
MIS	Müllerian inhibiting substance
MSH	MutS homologue
P	pro-oestrus
PBS	phosphate-buffered saline
PAS	Per-ARNT-Sim
PCR	polymerase chain reaction
PGC	primordial germ cell
PMDS	persistent Müllerian duct syndrome
PMSG	pregnant mare serum gonadotropin
POF	premature ovarian failure
uPA	urokinase type plasminogen activator
RT	room temperature
mRNA	messenger ribonucleic acid
S/T	serine/threonine
SEM	standard error of the mean
Sf1	steroidogenic factor
Sl	Steel
Sry	sex determining region of the Y
StAR	steroidogenic acute regulator protein
SCF	stem cell factor
TGFβ	transforming growth factor β
VIP	vasoactive intestinal polypeptide
Wt1	Wilm's tumour 1
Zfx	zinc finger transcription factor x
ZP	zona pellucida

Chapter 1

GENERAL INTRODUCTION

1.1 Introduction

The central reproductive organ of the female is the ovary. In mammalian species, the females have two ovaries, which are located in the abdomen near the kidneys. The ovary has two major functions. First of all, it produces the female gametes or oocytes, which can develop inside the ovary until they reach the developmental stage at which they can be fertilised by the male gametes. Second, the ovary produces steroid hormones, which are important for the development of female characteristics and behaviour.

In the ovary the gametes are found in special structures, the so-called ovarian follicles. Normal development of the ovary and the ovarian follicles is very important for female fertility. This chapter starts with a description of gonad formation in the mammalian female, on basis of observations on the development in female mice, and focuses on the most important factors involved in gonad formation. Furthermore, the process of ovarian follicle development is described, again on basis of data generated mainly from studies in the mouse, and also rat. Ovarian follicle development is under tight control by many hormones and growth factors, and therefore the action of only the most important regulatory factors will be mentioned. This thesis focuses on the role of anti-Müllerian hormone (AMH), one of the ovarian growth factors, in ovarian follicle development. Therefore, AMH is discussed in more detail in the last part of this Introduction.

1.2 Development of the female gonad

Normal prenatal development of the ovary, including germ cell migration, proliferation, and association with somatic cells, is the foundation for successful postnatal ovarian development, and is therefore crucial for fertility in adult females (Elvin and Matzuk, 1998).

Gonadal formation in mammals takes place early in foetal life. Although the genetic sex is determined at conception, morphologic sex differences between male and female foetuses can first be recognised at the time when their gonads become differentiated. The indifferent gonad (gonadal anlage) originates in the mesonephros (Byskov and Hoyer, 1994) (Figure 1.1A). In the mouse, massive cell proliferation occurs in the indifferent gonad at embryonic day 11.5 (E11.5), causing the sexually undifferentiated gonads to emerge from the gonadal ridges as distinct structures by E12 (Birk *et al.*, 2000) (Figure 1.1B). By that time they consist of loose mesenchymal tissue, which is covered by coelomic epithelium and is supported by the developing mesonephric tissue (Byskov and Hoyer, 1994). The coelomic epithelium has previously been termed the germinal epithelium (Gorbman *et al.*, 1983). This term is confusing, since it implies that the primordial germ cells (PGCs) are derived from this epithelial layer. However, the germ cells do not originate from this layer, but pass through this layer on their way to the inner part of the developing gonad. In the female mouse, the connection between the mesonephros and the developing gonad is obvious from the moment the gonads are formed (Byskov, 1978a; Upadhyay *et al.*, 1979). The ovarian-mesonephric connection is retained during

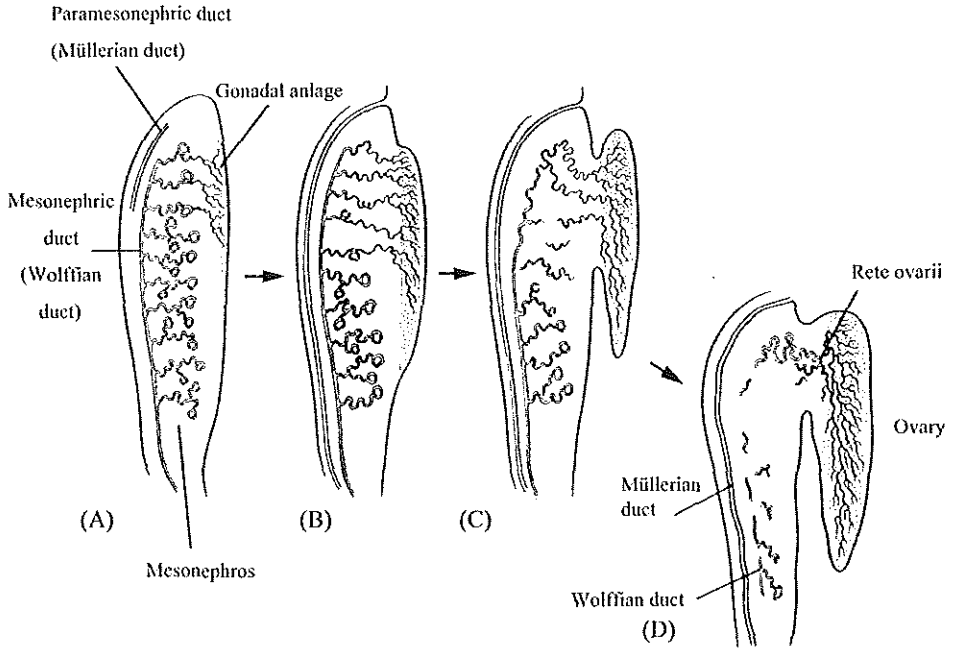

Figure 1.1
Differentiation of the ovary from the indifferent gonad and the mesonephros.
A. Indifferent gonad (gonadal anlage) is a mass of mesoderm on the dorsal body wall.
B. Indifferent gonad, supported by the mesonephric tissue, merges from the gonadal ridge.
C. Regression of the mesonephric tissue.
D. Rete ovarii, derived from the mesonephros, contributes to the formation of the ovary.
(Modified from Byskov, 1982.)

ovarian differentiation, although the mesonephric tissue gradually regresses (Figure 1.1C-D) (Byskov and Hoyer, 1994). Structures growing out of the mesonephros and connecting the mesonephric tissue and the gonads are called the rete ovarii (Figure 1.1D) (Yoshinaga *et al.*, 1988). Different experiments have shown that the mesonephros stimulates gonadal development and function and, germ cell differentiation. For example, when foetal undifferentiated mouse ovaries are stripped from mesonephric tissues and are transplanted subcutaneously into other mice, ovarian differentiation and germ cell meiosis are impaired (Byskov, 1974b).

Several autosomal genes encoding transcription factors mediate early events in the development of the indifferent gonads. In mice with disruption of the genes *Lhx1* (Lim homeobox 1), *Wt1* (Wilm's tumour 1), or *Sf1* (steroidogenic factor 1), gonadal development is arrested before sexual development can occur (Kreidberg *et al.*, 1993; Luo *et al.*, 1994; Shawlot and Behringer, 1995). Recently, it was found that in mice lacking exons 2 and 3 of the *Lhx9* gene, another member of the Lim homeo-domain gene family of tran-

Table 1.1A
Mouse models with altered gonad formation or number of germ cells

Knockout/mutant mouse model	Reproductive defect	Reference
Lhx1 knockout	Arrested gonadal development	(Shawlot and Behringer, 1995)
Wt1 knockout	Arrested gonadal development	(Kreidberg *et al.*, 1993)
Sf1 knockout	Arrested gonadal development	(Luo *et al.*, 1994)
Lhx9 knockout	Arrested gonadal development	(Birk *et al.*, 2000)
Bmp4 knockout	No formation of PGC	(Lawson *et al.*, 1999)
Bmp8b knockout	No formation of PGC	(Ying *et al.*, 2000)
Zfx knockout	Reduced PGC proliferation	(Luoh *et al.*, 1997)
At mutant	Reduced PGC numbers	(Handel and Eppig, 1979)
Gdc mutant	Reduced PGC numbers	(Pellas *et al.*, 1991)
Connexin 43 knockout	Prenatal germ cell deficiency	(Juneja *et al.*, 1999)
SCF mutant	Defect in PGC proliferation and survival	(McCoshen and McCallion, 1975)
Kit mutant	Defect in PGC proliferation and survival	(Bernex *et al.*, 1996)
Wnt4 knockout	Germ cell deficiency	(Vainio *et al.*, 1999)
Dazla knockout	Increased oocyte attrition	(Ruggiu *et al.*, 1997)
Msh5 knockout	Meiotic prophase block oocytes	(de Vries *et al.*, 1999)
Msh4 knockout	Meiotic prophase block oocytes	(Kneitz *et al.*, 2000)
AhR knockout	Impaired oocyte attrition	(Robles *et al.*, 2000)

scription factors, the gonads also fail to develop. This is due to a failure of proliferation of the mesodermal cells forming the indifferent gonad around E11.5 (Birk *et al.*, 2000). Table 1.1A shows a list of mouse models in which gonad development is impaired.

The somatic cell lineages in the indifferent gonad are derived from the mesonephros, the coelomic epithelium, or the mesenchyme, and populate the gonad together with invading PGCs. The PGCs of the mouse embryo are first detected at 7-8 days *post coitum* (dpc) (E7.5-8.5) as a small population of alkaline phosphatase-expressing cells in the extraembryonic tissue at the root of the allantois (Ginsburg *et al.*, 1990) (Figure 1.2A). Several studies suggest that the stem cells of the PGCs are of ectodermal origin (Gardner and Rossant, 1979; McMahon *et al.*, 1981). Grafting studies indicate that precursor PGCs reside in the inner cell mass of the blastocyst (Snow and Monk, 1983), and that at a later time point they are restricted to derivatives of the epiblast of the posterior primitive streak (Copp *et al.*, 1986). The precursor PGCs migrate from the extraembryonic site to the embryonic mesoderm of the primitive streak, then further to the visceral endoderm of the yolk sac, and to the developing hindgut (at 8.5 dpc) from where the PGCs migrate up through the dorsal mesoderm (9.5 dpc). Finally, they reach the gonad (Figure 1.2B) and by 12.5 dpc most PGCs are confined to the developing gonad (Tam and Snow, 1981). PGCs proliferate during their migration to the gonad. In the mouse embryo, about 10 to 100 PGCs can be identified at 8 dpc (Ozdenski, 1967; Tam and Snow, 1981). By the time

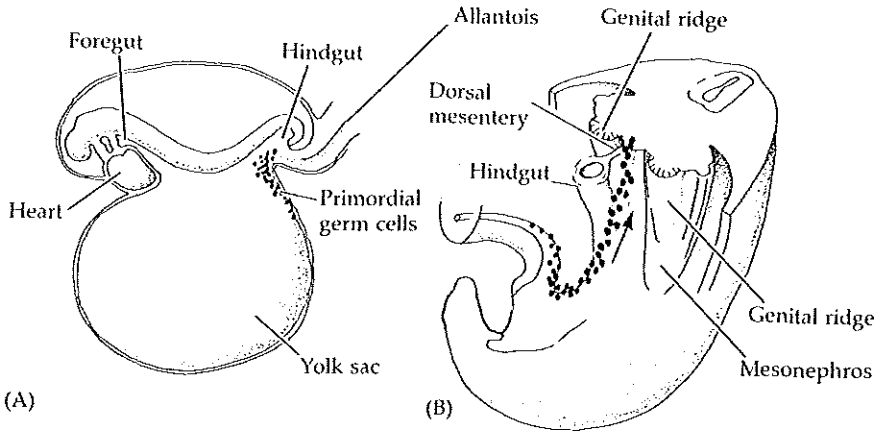

Figure 1.2
Pathway for the migration of mammalian primordial germ cells.

A. Primordial germ cells are first recognised as alkaline phosphatase-expressing cells (at E7.5-8.5 in the mouse) in the yolk sac near the junction of the hindgut and the allantois.

B. Migration of PGCs through gut and, dorsally, up the dorsal mesentery into the genital ridge.

(Modified from Gilbert, 1988.)

that they have reached the gonads their number has increased to about 4000, while by day 13.5 dpc, the gonads contain about 22,000 germ cells (Tam and Snow, 1981).

The regulation of foetal PGC development is still poorly understood. Several mouse models show that certain growth factors and transcription factors are involved in PGC development and survival. No PGCs are found in embryos in which the gene for bone morphogenetic protein 4 (BMP4), a member of the transforming growth factor β (TGFβ) superfamily of growth and differentiation factors, has been deleted. Animals heterozygous for this null mutation have fewer PGCs due to a reduction in the size of the founding population. These results show that BMP4 regulates the formation of PGC precursors and thus the size of the founding population of PGCs (Lawson *et al.*, 1999). In addition, BMP8b is also required for PGC generation. Mice with a C57Bl/6J genetic background that are homozygous for *Bmp8b* null mutation completely lack PGCs, while mice heterozygous for the *Bmp8b* null mutation have a reduced number of PGCs (Ying *et al.*, 2000). Furthermore, female mice lacking the gene *Zfx*, which is located on the X chromosome and encodes a putative zinc finger transcription factor, have reduced PGC proliferation which results in diminished fertility and shortened reproductive lifespan (Luoh *et al.*, 1997). Similarly, mutations at the atrichosis (*At*) locus (Handel and Eppig, 1979) and at the germ-cell deficient (*Gdc*) locus (Pellas *et al.*, 1991), also cause infertility, due to a significantly reduced PGC number in the developing gonad. In addition, foetal and neonatal mice homozygous for a null mutation in the gene coding for connexin 43, one of the proteins that form gap junctions, also show germ cell deficiency, which can be traced

Table 1.1B
Factors involved in proliferation and/or survival of PGCs, oogonia and oocytes *in vitro*

Factor	Germ cell type	+/- Effect on survival and/or proliferation
SCF	PGC, oogonia, oocytes	+ survival and proliferation
LIF	PGC, oogonia, oocytes	+ survival and proliferation
Interleukin-4	PGC	+ survival
Gas6	PGC	+ survival and proliferation
Neuregulin-β	PGC	+ survival and proliferation
Retinoic Acid	PGC, oogonia, oocytes	+ survival
Basic FGF	PGC	+ survival and proliferation
TNFα	PGC	+ proliferation
Activin	PGC	- proliferation
TGFβ	PGC, oogonia, oocytes	- proliferation
IGF-I	oogonia, oocytes	+ survival

back until day 11.5 of gestation (E11.5) (Juneja *et al.*, 1999). Furthermore, mutations of the genes *Steel* and *Kit*, which encode Steel factor, also named Kit ligand or stem cell factor (SCF), and its receptor Kit, respectively, result in decreased PGC numbers, due to a lack of their migration into the gonads, and increased frequency of ectopic germ cells (Bernex *et al.*, 1996; McCoshen and McCallion, 1975). These observations support several *in vitro* studies showing that SCF is a primary survival and proliferation factor for murine PGCs (Dolci *et al.*, 1991; Pesce *et al.*, 1993; Morita *et al.*, 1999). The effect of SCF on PGCs is enhanced by leukaemia inhibitory factor (LIF) (Dolci *et al.*, 1993; Morita *et al.*, 1999), while LIF alone also stimulates PGC survival and proliferation (De Felici and Dolci, 1991; Morita *et al.*, 1999). These *in vitro* culture observations are, however, not supported by genetic evidence, since knockout of LIF does not result in impaired gonadal development or fertility problems related to the ovary (Stewart *et al.*, 1992). Other *in vitro* studies show a stimulatory effect of interleukin-4 on PGC survival (Cooke *et al.*, 1996), while Gas6 and Neuregulin-β (both ligands for certain tyrosine kinase receptors) (Matsubara *et al.*, 1996; Toyoda-Ohno *et al.*, 1999) and retinoic acid (Koshimizu *et al.*, 1995) have a stimulatory effect on both PGC survival and proliferation. Basic fibroblast growth factor (Matsui *et al.*, 1992) and tumour necrosis factor-α (Kawase *et al.*, 1994) on the other hand stimulate only PGC proliferation. Activin and TGFβ1 are the only two known growth factors which have an inhibitory effect *in vitro* on PGC proliferation (Godin and Wylie, 1991; Richards *et al.*, 1999). Factors affecting PGC survival and proliferation *in vitro* are listed in Table 1.1B.

While the primordial germ cells undergo mitosis, the somatic cells of the gonads in both sexes proliferate as well. As the cells proliferate, they orient themselves into morphological distinct, but transitory embryonic tissues. The somatic cells intersperse themselves in between the germ cells, which at first are found in clusters, but are gradually separated from one another by intervening somatic cells and form the primary sex cords (Hirshfield,

1991). The origin of the germ cell supporting cell is still not completely clear. A dual origin of these cells was proposed (Byskov, 1978a; Wartenberg, 1978), based on the observation that in the developing gonad cords or clumps of germ cells are not only connected to the mesonephros at the basal part of the gonad (Byskov, 1975), but also to the surface epithelium (Merchant, 1975; Paranko, 1987). These observations lend support to the idea that the surface epithelium proliferates downward and/or mesonephric cells move up in between the germ cells, both giving rise to the germ cell supporting cell lineages (Byskov and Hoyer, 1994).

Thus, initial gonad formation is characterised by several, carefully timed events. First there is the migration of primordial germ cells through the coelomic epithelium and into the underlying mesenchymal tissue. Subsequently, mesonephric cells and other cell types are released and migrate into the same area. Finally, the cell lineages that support the germ cells are established (Byskov and Hoyer, 1994).

1.3 Oogenesis

Around the time that the sex of the gonads is morphological recognisable, which occurs around E12.5 (Byskov and Grinsted, 1981), the PGCs in the ovary are called oogonia (Byskov and Hoyer, 1994). The oogonia continue to divide mitotically in the developing female gonad until they enter the first stages of meiosis I. Often groups of germ cells divide synchronously during mitotic division, and also exhibit synchrony while passing through the transitory stages of the meiotic prophase (Borum, 1967). Germ cells of such groups are often connected by intercellular bridges (Gondos, 1978; Pepling and Spradling, 1998), which are involved in the transfer of different substances (*e.g.* gene products) between germ cells (Gondos, 1973; Gondos, 1978). These intercellular bridges are found in the female mouse from 11.5 to 17.5 dpc (Pepling and Spradling, 1998). Oogonia are called oocytes from the moment they enter the first stages of meiosis I. In the mouse and some other mammalian species the conversion of oogonia into primary oocytes depends on the contact of the oogonia with cells derived from the rete ovarii. It has been postulated that these cells, which are of mesonephric origin, produce a meiosis-inducing substance. Evidence for the existence of such a meiosis-inducing factor comes from studies using cocultures between foetal mouse ovaries and testes, showing that ovaries containing germ cells in early stages of meiosis induce meiosis in male germ cells of cocultured foetal testes, which normally do not enter meiosis prenatally (Byskov and Saxen, 1976). However, it seems that in these experiments male germ cells enter meiosis because the production and/or local accumulation of a meiosis-inhibiting substance is disrupted as a consequence of damage to the testicular tissue during the preparation of the foetal testis (McLaren and Southee, 1997). By E17.5 all germ cells have entered meiosis in the mouse (Evans *et al.*, 1982) and the oocytes progress through the stages of meiotic prophase: leptotene, zygotene, pachytene and diplotene (Franchi and Baker, 1973). However, the meiotic process is not completed and the oocyte becomes arrested in the diplotene stage (dictyate) of the first meiotic division (Bacharova, 1985).

Coincident with their entry into meiosis, vast numbers of germ cells undergo attrition, since germ cells passing through the transitory stages of meiotic prophase are very vulnerable (Beaumont and Mandl, 1962). It has been proposed that germ cells which during the pachytene stage have unpaired or incompletely paired chromosomes, are selectively destroyed, or later show aberrant meiotic disjunction (Burgoyne, 1984). The presence of two X chromosomes at the beginning of meiosis may promote the survival and function of oocytes, although conflicting results have been found in mice and humans. Germ cells of human 45,XO (Turner's syndrome) ovaries start to degenerate at about the third month of foetal life, which is coincident with the start of meiosis, and these women are infertile (Singh and Carr, 1966). In contrast, XO mice are fertile, although many oocytes degenerate during meiosis (Burgoyne and Baker, 1985). Oocyte loss in XO mice and women may be due to X dosage deficiency, since in normal XX oocytes both X chromosomes are transcriptionally active from the onset of meiosis (Burgoyne, 1981). It has also been suggested that the crucial factor determining survival of the germ cells may be the enclosure within follicles (Byskov, 1986). As soon as the oocytes become enclosed by pregranulosa cells they became arrested in diplotene. Those that are not enclosed and thus are not arrested in diplotene continue on through diakinesis and degenerate.

Several mouse models have shown the involvement of certain gene products in determining the number of oocytes. Wnt4, a signalling molecule necessary for the formation of the Müllerian ducts, also seems to be directly involved in maintaining development of the female germ line since the ovaries of Wnt4-deficient mice contain at birth less then 10% of the oocytes found in wild type females (Vainio *et al.*, 1999). Another knockout mouse model, the Dazla-deficient mouse model, revealed that the *Dazla* gene product is involved in gametogenesis. *Dazla* encodes a protein containing RNA-binding elements and is found in the cytoplasm of oogonia and oocytes (Brekhman *et al.*, 2000; Seligman and Page, 1998). Dazla-deficient males and females are infertile, and examination of postnatal ovaries showed that these ovaries are devoid of oocytes and therefore also of follicles. This defect was caused by a marked deficiency of oocytes at E18.5, while many of the oocytes remaining were undergoing attrition (Ruggiu *et al.*, 1997). Also the ovaries of Msh5-deficient females are completely devoid of oocytes. Msh5 is a member of the family of MutS-like proteins, which are involved in DNA mismatch repair (Pochart *et al.*, 1997). Msh5, however, appears not to be involved in DNA mismatch repair, but rather in facilitating crossing-over between homologs during meiosis. A close examination of the oocytes which are still present at E15 until E18, showed that oocytes of Msh5-deficient females remain in the zygotene stage of the first meiotic prophase instead of going on to pachytene and diplotene stage (de Vries *et al.*, 1999). The same effect was also found in Msh4-deficient females (Kneitz *et al.*, 2000). The aryl hydrocarbon receptor (AhR), a basic helix-loop-helix (bHLH) transcription factor, is involved in oocyte destruction. AhR is abundantly and exclusively expressed in oocytes and granulosa cells of follicles at all stages of development. AhR seems to increase oocyte destruction, since AhR-deficient females revealed a two-fold higher number of primordial follicles than wild type females at day 4 postpartum. An *in vivo* experiment supported this finding, since AhR-deficiency attenuated the magnitude of oocyte apoptosis in foetal ovaries cultured without hormo-

nal support for 72 hours. It could imply that one function of AhR is to mediate programmed cell death signalling in the female germ line (Robles *et al.*, 2000).

In vitro studies have also shown the involvement of several growth factors and other receptor ligands in the survival of oocytes and/or the survival or proliferation of oogonia, just as with the PGCs. For both insulin-like growth factor-I (IGF-I) (Morita *et al.*, 1999) and retinoic acid (Morita and Tilly, 1999), it has been shown that they stimulate the survival of oogonia and oocytes. In addition, a combination of SCF and LIF has a positive effect on the survival of both oogonia and oocytes (Morita *et al.*, 1999). Cotreatment with IGF-I or SCF/LIF and TGFβ partially reversed the survival actions of IGF-I or SCF/LIF (Morita *et al.*, 1999), indicating the inhibitory action of TGFβ on the survival of oocytes and oogonia. Table 1.1A shows a list of mouse models with altered gonad formation and/or altered number of germ cells, while Table 1.1B gives an overview of factors involved in germ cell proliferation and/or survival *in vitro*.

Several laboratories have independently confirmed the involvement of apoptosis, or programmed cell death, in the attrition of ovarian germ cells including PGCs (Coucouvanis *et al.*, 1993; Morita *et al.*, 1999; Pesce and De Felici, 1994; Ratts *et al.*, 1995). One of the factors involved in control of apoptosis, Bcl2 (B-cell leukemia/lymphoma-2), is involved also in the survival of ovarian germ cells, since in *Bcl2* knockout females numerous aberrantly formed primordial follicle-like structures containing a single layer of granulosa cells without an oocyte are found (Ratts *et al.*, 1995).

Many oogonia and oocytes degenerate during the embryonic period in mammals. The population of germ cells mainly increases due to oogonial proliferation. Peak numbers of germ cells in both mice and rats are about 70,000-80,000. A dramatic fall in these numbers largely due to atresia occurs mainly at the pachytene stage (Burgoyne and Baker, 1985). It has been estimated that eventually over two-thirds of the initial female germ cell pool is lost by the time of birth in mice, resulting from attrition from both oogonia and oocytes (Borum, 1967). Thus, the number of oocytes present in the ovaries at birth is a function of germ cell proliferation and the extent of attrition.

1.4 Folliculogenesis

Throughout reproductive life, the ovaries of mice and other mammals contain a mixed population of follicles in different stages of development. All follicles start as a primordial follicle, of which some will eventually reach the preovulatory stage. Between these two stages of follicular development the follicle undergoes many changes, which are under the influence of many paracrine and endocrine factors.

In the following paragraphs, the process of follicle development is described from the moment that primordial follicle growth is initiated until the formation of the ultimate follicle stage, the preovulatory follicle, while also the most important regulators of follicular development are mentioned. Furthermore, follicular development is divided into two stages: early and late follicular development. This division is based on differences in both morphological and functional aspects of the follicles, which will be described below.

1.4.1 Initiation of folliculogenesis

During the meiotic prophase, in the fetal ovary, a specialised population of somatic (pregranulosa) cells encloses the oocyte that then reaches the diplotene arrest, and together they form a primordial follicle. The time of appearance of the first primordial follicles varies between species and depends on when the oocytes reach the diplotene stage of the first meiotic division (Byskov, 1986). In mice, the first primordial follicles were reported on day 2 postpartum (Peters, 1978), while they are found 4.5 months postfertilization in humans (Byskov, 1986). In mice, follicles begin to form in the inner part of the ovary (medulla) where the oocytes first reach the diplotene stage of meiosis. Gradually the more peripherally situated follicles (situated in the cortex) begin to form (Peters, 1969b). This cortex-medulla differentiation pattern is organised around E13, the time when meiosis begins in the foetal mouse ovary and is fully activated after E16, when the last germ cells have entered meiosis (Byskov *et al.*, 1997). In many species, the granulosa cells of the developing follicles are connected with the rete ovarii. Some cells derived from the rete ovarii contribute to the pregranulosa cell layer (Byskov *et al.*, 1977), whereas other cells from the rete ovarii appear to differentiate to the first interstitial cells (Hoyer and Byskov, 1981). The oocytes of primordial follicles remain arrested in the prophase of the first meiotic division, and meiosis I will not be completed until around ovulation.

Follicular formation needs the presence of an oocyte. Absence of oocytes prevents morphogenesis of the ovarian follicles. Exposure of female foetuses to busulphan, which causes the destruction of all germ cells, results in a cordlike appearance of the ovarian stromal tissue (Kasuga and Takahashi, 1986). Thus, germ cells are required for progression from the ovarian primary sex cord stage to the formation of follicles (Merchant, 1975). Ectopic germ cells that have ended up in the adrenal gland instead of in the developing ovary induce the surrounding adrenal tissue to assume an orientation characteristic of granulosa cells in primordial follicles, which also clearly illustrates the organising power of the oocyte (Upadhyay and Zamboni, 1982). One factor of which it has been shown that is closely involved in primordial follicle formation is Factor In the Germline alpha (FIGα). FIGα is a germline bHLH transcription factor that is first detected in oocytes at E13.5 Female mice lacking FIGα have normal gonadogenesis during gestation but are unable to form primordial follicles postnatally, which results in massive depletion of oocytes (Dean, 2000).

Some primordial follicles begin to grow as soon as they are formed, but most spend months (mice) or years (humans) in the quiescent state. Thus, the primordial follicles constitute the resting stock of nongrowing follicles, which is progressively depleted during reproductive lifespan, when primordial follicles continuously leave the nongrowing pool through conversion into primary follicles. The process in which primordial follicle growth is initiated is called initial recruitment (McGee and Hsueh, 2000). At first, the reawakened follicle is difficult to distinguish from its dormant neighbours. The first sign of growth is the resumption of cell proliferation by the squamous pregranulosa cells, which can be demonstrated by autoradiography following [^{3}H]-thymidine incorporation (Hirshfield, 1991). Later stages of growth can be recognised by an increase in size of the oocyte and

a change in shape of the granulosa cells, which assume a cuboidal shape (Lintern-Moore and Moore, 1979). Initiation of follicle growth is not restricted to sexual maturity, but in fact begins within the first week of life in the female mouse and during foetal development in the human.

The pool of primordial follicles is not a homogeneous population. Several studies have shown that primordial follicles differ with respect to the cell lineages of their pregranulosa cell population, and with respect to the time during embryonic development at which their oocytes entered meiosis (Henderson and Edwards, 1968; Hirshfield, 1992). It has been suggested that these differences between the primordial follicles cause some primordial follicles to initiate growth earlier than others. The production-line hypothesis suggests that follicles containing oocytes that entered meiotic arrest early during development, also re-initiate growth relatively early during life, while follicles containing oocytes that enter meiotic arrest later during development start growth only later in life (Henderson and Edwards, 1968). This hypothesis is supported by the fact that the ovarian cortex-medulla growth pattern seems to arise concurrently with the beginning of meiosis, suggesting that the organisation of the characteristic cortex-medulla growth pattern within the ovary is related to the meiotic process (Byskov *et al.*, 1997). Although other studies have confirmed this model (Polani and Crolla, 1991; Hirshfield, 1992), the production-line hypothesis has been challenged by others (Meredith and Doolin, 1997). The observation that the pregranulosa cells surrounding a primordial follicle are derived from two different cell populations has led to the hypothesis that the overall response threshold of each primordial follicle to an activation stimulus would be a function of the proportion of cells from each of the two populations of granulosa cells (Hirshfield, 1992). This latter hypothesis, however, has not been extensively examined.

The factors that initiate primordial follicle growth are largely unknown. Some investigators have proposed that the emergence of follicles from the stock of primordial follicles is independent of extra-follicular influences and is a pre-programmed feature of the follicle. This means that the number of primordial follicles that begins to grow within any given time period is a fixed percentage of the stock of primordial follicles, similar to the process of radioactive isotopic decay. This implies that when the stockpile of primordial follicles is reduced, less primordial follicles will enter the pool of growing follicles (Edwards *et al.*, 1977). This model, however, does not appear to hold, since the onset of follicle growth has been shown to be subject to external modulation (see below). The emergence of primordial follicles from the stock may also be regulated by systemic or local factors, and a reduction in the stockpile of primordial follicles may lead to a compensatory increase in the proportion of primordial follicles that start to grow (Jones and Krohn, 1961).

Hypothetical factors from atretic (Tsafriri and Braw, 1984) or growing follicles (Cran and Moor, 1980) have been proposed to affect the onset of primordial follicle growth. However, despite many extensive investigations, the factors triggering initial follicle growth remain largely unknown. Two candidate factors, the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are the main regulators of function of large ovarian follicles, have been shown to affect the size of the pool

of primordial follicles. Eliminating LH and FSH from the circulation in adult mice by hypophysectomy results in a larger pool of primordial follicles than in non-hypophysectomized mice (Wang and Greenwald, 1993). In addition, an increase in the FSH level as a result of unilateral ovariectomy in aged rats results in increased primordial follicle loss (Meredith *et al.*, 1992). Indeed, the increased levels of LH and FSH in ageing rats compared to young rats are accompanied by increased primordial follicle loss (Marcus *et al.*, 1993; Richardson *et al.*, 1987). Furthermore, high tonic LH levels reduce the number of resting primordial follicles, since in transgenic mice overexpressing a long acting form of LH, primordial follicles are lost from the resting pool more rapidly than in controls (Flaws *et al.*, 1997). However, FSH and LH are unlikely to exert *direct* actions on the primordial follicles, because full-length LH and FSH receptor mRNA expression could not be detected in primordial follicles (Rannikki *et al.*, 1995; Teerds and Dorrington, 1995; O'Shaughnessy *et al.*, 1996; Oktay *et al.*, 1997; O'Shaughnessy *et al.*, 1997). Although gonadotropins appear to affect initial recruitment, they are not essential for the initiation of primordial follicle growth. In FSHβ deficient female mice, follicle growth occurs up to the early antral stage (Kumar *et al.*, 1997). Furthermore, primordial follicle growth commences in cultured new-born mouse ovaries without the addition of gonadotropins to the culture medium (Eppig and O'Brien, 1996) (Chapter 3 of this thesis).

Another factor that might be involved in the initiation of follicle development is FIGα, which mRNA expression is mainly found in the oocytes of primordial follicles, although a very low expression level was also found in oocytes of growing follicles. FIGα can form a heterodimer with E12, another bHLH protein, and binds to a conserved E-box upstream of the transcription start site of the three mouse zona pellucida genes *Zp1*, *Zp2* and *Zp3* (Liang *et al.*, 1997). It is suggested that FIGα might play an important role, presumably in conjunction with other factors, in co-ordinating the oocyte-specific expression of mouse *Zp1*, *Zp2* and *Zp3* (Liang *et al.*, 1997). Based on this regulatory effect on transcription of zona pellucida genes and on its expression in oocytes of primordial follicles it is suggested that FIGα also might regulate the transcription of many other oocyte genes in the primordial follicle, potentially including a growth factor that signals the pregranulosa cells to initiate replication (Elvin *et al.*, 1999b).

Recently, one factor has been identified that is intimately involved in primordial follicle recruitment. Stem cell factor (SCF) has a direct stimulatory effect on the initiation of primordial follicle growth in 4-day-old rat ovaries in culture. The effect of SCF was completely inhibited by an antibody that blocks SCF binding to the extracellular domain of the stem cell factor receptor (Kit) (Parrott and Skinner, 1999).

Not all primordial follicles leave the resting pool by initiating growth. Some primordial follicles are lost by migration through the ovarian surface epithelium. This is especially obvious in new-born mice, in which the number of primordial follicles that move to the peri-ovarian sac reaches a peak in the first week of life and continues until about day 28 (Wordinger *et al.*, 1990). Other primordial follicles are lost from the stock by attrition. The extent of this phenomenon, however, is difficult to determine since adequate morphological markers of degeneration of primordial follicles are not available. Furthermore, degenerating primordial follicles may be eliminated from the ovary very rapidly,

escaping accurate counting (Hirshfield, 1994). One factor, which is clearly involved in primordial follicle survival is Bax, encoded by the *bcl2-associated-x* gene and a member of the *Bcl2* gene family. In Bax-deficient females, the number of primordial follicles at 4 days of age is similar to the number found in wild type females of the same age. However, at day 42 of age about three times more primordial follicles are found in the Bax-deficient females, which is the result of a lower incidence of atresia of primordial follicles in these females (Ratts *et al.*, 1995).

1.4.2 Early follicular development

Follicle development from the primary follicle stage to the small antral stage involves several processes (Hirshfield, 1991). First of all, the squamous pregranulosa cells surrounding the oocyte become cuboidal, and from this moment onward the follicle is called a primary follicle. Coincident with this transition in granulosa cell morphology, the oocyte starts to grow, and reaches full size early in the developmental process. During the growth of mammalian oocytes, there is continuous coupling with the surrounding granulosa cells via gap junctions (Eppig, 1991). According to morphological evidence, this coupling begins with primordial follicle development, and expands during folliculogenesis (Mitchell and Burghardt, 1986). Via these gap junctions communication between the oocyte and the granulosa cells by different molecules and growth factors can take place (Eppig, 1991; Dong *et al.*, 1996). Also during oocyte growth the zona pellucida is formed (Figure 1.3). The zona pellucida, which is an extracellular glycoprotein matrix between the oocyte and the surrounding granulosa cells, consists of several proteins which are secreted by

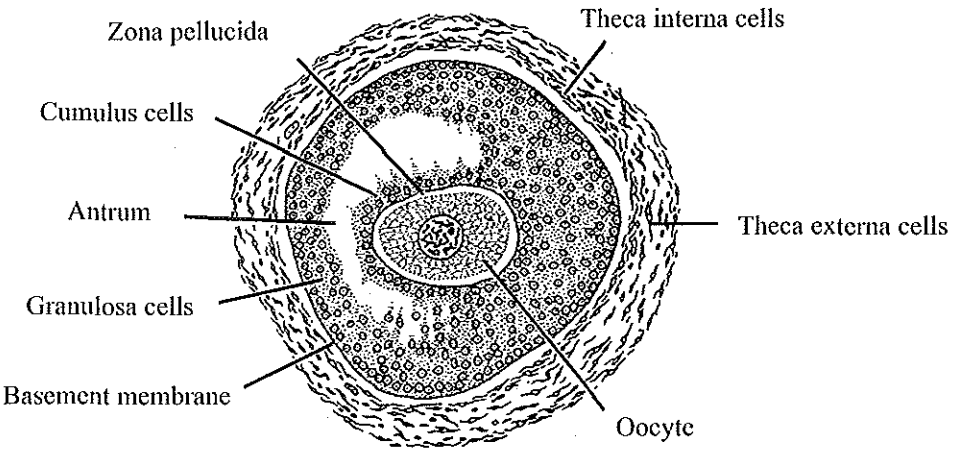

Figure 1.3
Structural organisation of an antral ovarian follicle.
(modified from Gilbert, 1988)

the oocyte. The surrounding granulosa cells probably do not contribute to the formation of the zona pellucida (Zamboni and Upadhyay, 1983). The zona pellucida is critical for species-specific fertilisation, the subsequent block to polyspermy, and passage of the early embryo through the oviduct prior to implantation (Yanagimachi, 1994). The basement membrane, which consists of collagen, laminin and fibronectin, is formed around the layer of granulosa cells as soon as the primordial follicle arises (Figure 1.3). The granulosa cells proliferate extensively, and during preantral follicle growth several layers of granulosa cells are formed. From the moment that two distinct layers of granulosa cells are apparent around the oocyte the follicle is called a secondary follicle. The formation of a distinct theca layer outside the basement membrane occurs when follicular growth is well underway (Figure 1.3). The theca cells are derived from ovarian stroma cells (Hoage and Cameron, 1976). In mice, the first recognisable theca cells are found when the oocyte is fully grown and the follicle has acquired 2-3 layers of granulosa cells (Peters, 1969a). As the follicle grows, theca cells proliferate along with the granulosa cells, which results in several layers of theca cells. During follicle growth, the basement membrane, which separates the granulosa cells from the theca cells, expands or is remodelled to accommodate the increasing contour of the follicle. The mechanism by which the expansion occurs while maintaining the integrity of the basement membrane remains to be elucidated (Hirshfield, 1991).

Compared to the initiation of primordial follicle growth, much more is known about the regulation of subsequent follicle development. Several hormones and growth factors are known to influence early follicular development. FSH is not essential for follicle growth from the primordial to the large preantral stage. In hypophysectomized mice (Wang and Greenwald, 1993) and rats (Lane and Greep, 1935; Paesi, 1949), for example, follicles develop to the early antral stage. Also the FSHβ deficient mouse model (Kumar *et al.*, 1997) or the hypogonadal mouse model (*hpg* mouse) (Halpin and Charlton, 1988), in which a naturally occurring mutation in the gene encoding gonadotropin-releasing hormone (GnRH) markedly reduces the synthesis of FSH and LH from the pituitary (Cattanach *et al.*, 1977), show that follicles progress to the early antral stage. However, several studies have shown that FSH does influence early follicle growth. In immature prepubertal mice (Pedersen, 1969) and rats (Hage *et al.*, 1978), the relatively high FSH levels between day 10 and 20 after birth (Dullaart *et al.*, 1975; Uilenbroek *et al.*, 1976) cause a higher number of growing follicles compared to adult animals. Furthermore, treatment of immature rats with GnRH-antagonist severely suppressed the gonadotropin serum level and decreased the number of growing follicles during the prepubertal period (Van Cappellen *et al.*, 1989; McGee *et al.*, 1997). In contrast, treatment with FSH of intact, hypophysectomized, or GnRH-antagonist-treated juvenile rats, increased preantral follicle development (McGee *et al.*, 1997). Another factor involved in early follicle growth, of which the effects are closely related to FSH action, is vasoactive intestinal polypeptide (VIP). This neurotransmitter is found in ovarian nerves, and exposure of 2-day-old rat ovaries containing many primary follicles to VIP, resulted in the activation of *FSH receptor (FSHR)* gene expression in granulosa cells of these follicles. The encoded FSHR appeared to be biologically active, since FSH was able to induce cAMP formation and to stimulate

follicle growth in ovaries pretreated with VIP (Mayerhofer *et al.*, 1997). *In vivo* VIP expression precedes the appearance of the full-length FSHR (Malamed *et al.*, 1992), which is present from postnatal day 3 in the rat ovary (Rannikki *et al.*, 1995).

Also several growth factors are necessary for preantral follicle development. Three variants of the *Steel* locus, *Steel t (Slt)*, *Steel panda* (Sl^{panda}), and *Steel contrast* (Sl^{con}) encode different forms of SCF, the ligand for the receptor Kit. These mutants permit prenatal ovary development, but cause growth arrest of ovarian follicles after the initiation of primordial follicle growth but before the development of the secondary follicle stage (Kuroda *et al.*, 1988; Huang *et al.*, 1993; Bedell *et al.*, 1995). The receptor for SCF, Kit, is expressed in oocytes of primordial and growing follicles, differentiated theca cells and undifferentiated stroma cells (Manova *et al.*, 1990; Manova *et al.*, 1993; Motro and Bernstein, 1993), while SCF is only found in the granulosa cells of growing follicles (Manova *et al.*, 1993; Motro and Bernstein, 1993). *In vitro* studies have also shown that SCF not only has a stimulatory effect on initial recruitment, but also has several effects on isolated oocytes including promotion of growth and maintenance of meiotic arrest (Packer *et al.*, 1994; Ismail *et al.*, 1996). In addition, SCF directly stimulates theca cell proliferation and steroid production during follicular growth (Parrott and Skinner, 1997), and induces proliferation and organisation of ovarian stromal-interstitial cells around small follicles, eventually leading to the formation of theca cell layers (Parrott and Skinner, 2000). SCF mRNA expression is highly elevated in follicles of mice lacking growth and differentiation factor 9 (GDF9), a member of the TGFβ family, suggesting that GDF9 is one of the oocyte-secreted paracrine factors that negatively regulates SCF expression (Elvin *et al.*, 1999b).

GDF9 itself also has an effect on early follicular development. GDF9 is first expressed in oocytes of primary follicles, and its expression persists in the oocyte throughout follicular development until ovulation (McGrath *et al.*, 1995). GDF9-deficient females are infertile and show a block in development at the early primary follicle stage. Follicles beyond the one-layer follicle stage are abnormal, and contain an asymmetrical arrangement of atypical granulosa cells, which exhibit signs of steroidogenic activity. The oocytes in these follicle-like structures degenerate, while the zona pellucida collapses. Oocytes from GDF9-deficient mice are slightly larger than oocytes from wild type mice, and their acquisition of meiotic competence is impaired. Furthermore, the theca layer fails to develop and no atretic follicles are found (Dong *et al.*, 1996). *In vitro* studies in which preantral follicles or ovarian explants from 6-day-old rats were cultured in the presence or absence of recombinant rat GDF9, show that GDF9 enhances growth and differentiation of preantral follicles (Hayashi *et al.*, 1999). GDF9 also has an effect at later stages of follicular development (see Paragraph 1.4.3). GDF9 is not the only member of the TGFβ family that is expressed in the oocyte. GDF9B, a factor closely related to GDF9 and also known as BMP15, and BMP6 are also found in the oocyte. BMP6 expression is also at lower levels found in granulosa cells. In contrast to the dramatic ovarian phenotype of the GDF9 knockout mouse, BMP6 deficient females are fertile and produce normal litters (Solloway *et al.*, 1998), indicating that BMP6 has either no function in the ovary or that there is functional redundancy with other ovarian TGFβ family members (Elvin *et al.*, 2000). In a certain strain of sheep (Inverdale) a naturally occurring null mutation in *GDF9B* is found.

In female sheep homozygous for this mutation ovarian follicles do not grow beyond the primary stage of development. The oocytes of these follicles, however, continue to grow until they are unable to be supported by the surrounding granulosa cells, whereupon the oocytes degenerate. Inactivation of only one copy of *GDF9B*, however, increases ovulation rate (see also Paragraph 1.4.3). Other BMPs are also found in the ovary. *In situ* hybridisation identified strong mRNA labelling for BMP4 and BMP7 in the theca cells, while BMP receptor types IA, IB and II are expressed in the granulosa cells and oocytes of most follicles in ovaries of normal cycling rats (Shimasaki *et al.*, 1999). Both BMP4 and BMP7 have an effect on FSH-induced steroidogenesis of granulosa cells (see Paragraph 1.4.3).

Activins and inhibins, also members of the TGFβ family of growth and differentiation factors, affect preantral follicle growth and antral follicle development. Activins and inhibins are produced, among other cell types, by ovarian granulosa cells, and these factors stimulate and inhibit, respectively, pituitary FSH synthesis and secretion (Vale *et al.*, 1990). *In vitro* culture experiments of preantral follicles from prepubertal (7-day-old) mice and adult mice showed age-dependent effects of activin on follicle diameter, and estradiol (E_2) and inhibin production (Liu *et al.*, 1998). In prepubertal mice, activin A stimulated these parameters in preantral follicles. Addition of FSH enhanced the effect of activin A, although treatment of FSH alone did not affect preantral follicle growth or steroidogenesis. In contrast, in adult mice FSH increased the diameter of preantral follicles, and also stimulated E_2 and inhibin secretion. Activin A did not stimulate growth of follicles from adult mice, but more interestingly, blocked the effect of FSH. These results indicate that activin A stimulates follicle development in the prepubertal mouse, but not in adults (Yokota *et al.*, 1997). Furthermore, in adult mice activin A might cause small preantral follicles to become dormant. Indeed, co-culture experiments of small and large preantral follicles revealed that the presence of the large preantral follicle inhibits the stimulation of growth of the small preantral follicle by FSH. Activin might be involved in this effect, since addition of follistatin to the culture medium blocked inhibitory effect of the large preantral follicle (Mizunuma *et al.*, 1999). The paradoxical action of activin A on folliculogenesis in immature and adult mice also holds for the action of TGFβ. This factor stimulates follicle growth of small preantral follicles derived from adult mice, while follicles derived from immature mice are not affected by TGFβ (Liu *et al.*, 1999).

In vitro studies have shown that communication between granulosa cells and the oocyte is essential for normal oocyte and follicle growth. Immature oocytes separated from their surrounding granulosa cells do not grow. Oocytes need to maintain gap junctions with granulosa cells to grow at a near normal rate (Tsafriri, 1997). It has been demonstrated that several specific connexins, proteins that form gap junctions, are involved. Follicles in mice lacking connexin 37 progress normally to the late secondary stage, but a limited number of small antral follicles are formed. The oocytes do not reach full size and are not competent to undergo meiosis (Simon *et al.*, 1997). In mice missing connexin 43, folliculogenesis can only proceed to the primary stage (Juneja *et al.*, 1999).

The role of androgens and oestrogens in preantral follicle development is still not completely clear. In cultured mouse preantral follicles, androgen treatment was shown to augment follicle growth, probably through conversion to oestrogens (Murray *et al.*, 1998).

However, in females lacking estrogen receptor α and β (αβERKO), preantral follicle development can occur, showing that oestrogens are not essential for preantral follicle growth (Couse *et al.*, 1999). Thus, oestrogens may be more important at later stages of development (see Paragraph 1.4.3).

IGF-I is expressed in granulosa cells from the primary follicle stage onward. The same expression pattern was found for IGF-I receptor (IGF-IR), except that IGF-IR was also expressed in granulosa cells of atretic follicles (Wandji *et al.*, 1998). Based on this expression pattern it was suggested that IGF-I might be important in follicle development. Indeed, preantral follicle development was impaired in IGF-I knockout females, since the formation of the thecal layer did not occur (Baker *et al.*, 1996). IGF-I is also important for later stages of follicular development as is described in the next paragraph.

1.4.3 Late follicular development

All large preantral and small antral follicles would undergo atresia, if not a proportion of these follicles is "rescued" by FSH signalling, a process referred to as cyclic recruitment. In mice and rats, the process of cyclic recruitment ensures that approximately 12 large preantral/small antral follicles are able to reach the preovulatory follicle stage. The selectivity of gonadotropin action probably requires local regulatory mechanisms that may serve to enhance or decrease FSH action in certain follicles. This aspect is discussed later in this paragraph.

After cyclic recruitment has occurred, the recruited follicles continue proliferation of the granulosa cells. This proliferation is accompanied by accumulation of fluid in spaces in between the granulosa cells, which will eventually lead to formation of one large fluid filled cavity, the antrum (Figure 1.3). From the moment that these fluid-filled spaces become apparent, the follicle is called an antral or tertiary follicle. As the follicle diameter enlarges by proliferation of granulosa cells and the accumulation of follicular fluid, the theca layer also undergoes morphological differentiation. Lying just outside the basement membrane, the theca interna acquires the morphological hallmarks of steroidogenesis, while the theca externa becomes richly vascularised. The final stage of follicle development, the preovulatory stage, is recognised by a meiotically competent oocyte (competent to complete the first meiotic division), surrounded by specialised granulosa cells, protruding from the follicle wall on a well-developed stalk into the large antral cavity. In response to the surge of LH, granulosa cells of preovulatory follicles cease dividing (Hirshfield, 1991) and initiate a program of terminal differentiation (Richards, 1994). Finally, ovulation occurs in response to the LH peak.

As mentioned above, FSH plays a major role during late follicular development. FSH is not only essential for antrum formation but also induces extensive proliferation of the granulosa cells, which can be shown by an increase of 5-bromodeoxyuridine (BrdU) labelling of these cells (Gaytan *et al.*, 1996). A balance of positive and negative regulators of the cell cycle controls cell cycle progression and proliferation. In granulosa cells, cyclin D2 acts as a positive regulator of cell cycle progression (Xiong *et al.*, 1992). In cyclin D2-deficient mice, granulosa cell proliferation is impaired and the follicles do not grow

beyond the preantral stage (Sicinski *et al.*, 1996), indicating a role of cyclin D2 in late follicle growth after cyclic recruitment. FSH not only stimulates granulosa cell proliferation, but also induces aromatase activity and LH receptor expression in granulosa cells (Richards *et al.*, 1976) and stimulates inhibin production (Lee *et al.*, 1982). The second gonadotropin, LH, stimulates androgen production by the theca cells, and testosterone is converted to oestrogens by the aromatase activity of the granulosa cells (Richards and Bogovich, 1982). Since oestrogens also influence follicle development, it can be stated that LH indirectly stimulates follicle growth via the action of oestrogens. LH, however, is also able to stimulate final follicle growth without the intermediate action of oestrogens. In GnRH-antagonist treated rats in which also oestradiol synthesis and action was inhibited, treatment with FSH and hCG resulted in an increase of the number of antral follicles in these animals compared to animals that only received FSH (Uilenbroek *et al.*, 1997). Furthermore, LH is essential for ovulation to occur, which will not be discussed in this chapter.

The importance of oestradiol for follicle development is shown in hypogonadotropic rats, in which it was shown that oestradiol was able to augment the effect of FSH treatment on antral follicle growth (Uilenbroek *et al.*, 1997). The mechanism by which oestradiol augments FSH action on follicular growth involves induction of gonadotropin receptors (Richards *et al.*, 1976; Hsueh *et al.*, 1984). In αβERKO females, follicles predominantly reach the small antral follicle stage, and only a few follicles containing a large antrum are detected. The same phenotype was found in females deficient in aromatase (ArKO), the enzyme that catalyses conversion of testosterone to oestrogens. These results indicate that oestrogens are essential for preovulatory follicle development (Fisher *et al.*, 1998; Couse *et al.*, 1999). This conclusion is supported by the induction of cyclin D2 and the concomitant reduction of $p27^{Kip1}$, a negative regulator of the cell cycle, by oestradiol in granulosa cells (Robker and Richards, 1998).

IGF-I is not only involved in early follicle development but also in later stages of follicle development. IGF-I-deficient female mice have an almost identical ovarian phenotype as the FSHβ knockout female, *i.e.* that folliculogenesis occurs until the small antral follicle stage. The only difference is that the theca layer appears to be absent in IGF-I-deficient females (Baker *et al.*, 1996). FSH and IGF-I maybe involved in an intrafollicular positive feedback loop, in which IGF-I enhances FSH action and FSH enhances IGF-I action by mutually complementary receptor up-regulation. This hypothesis is supported by the finding that in IGF-I-deficient females FSHR expression was reduced and could be augmented by treatment of these females with IGF-I (Zhou *et al.*, 1997). The induction of FSHR expression by IGF-I is subsequently positively reinforced by FSH-induced augmentation of IGF-I receptor expression (Adashi *et al.*, 1988; Zhou *et al.*, 1991). IGF-I is thus important in upregulating the sensitivity of ovarian follicles for FSH.

GDF9 not only stimulates preantral follicle growth and differentiation but also influences antral follicles. An *in vitro* study showed that GDF9 stimulated proliferation but suppressed FSH-induced differentiation of cultured granulosa cells from both small and large antral rat follicles (Vitt *et al.*, 2000). These results are in accordance with observations that, in large antral follicles, the granulosa cells closest to the basal lamina become more

differentiated in response to gonadotropins than cumulus cells that surround the oocyte. The relatively undifferentiated status of the cumulus cells is necessary for optimal oocyte development (Eppig *et al.*, 1998). Inhibition or prevention of differentiation of the cumulus cells could therefore be a major role of GDF9 secreted by the oocyte. Thus, GDF9 regulates several processes important for cumulus expansion and maintenance of an optimal oocyte microenvironment (Elvin *et al.*, 1999a). Furthermore, the effects of GDF9 could not be duplicated by BMP6 and BMP15, two other TGFβ family members which are also expressed by the oocyte (Elvin *et al.*, 1999a). However, BMP4 and BMP7, produced by theca cells, do have an effect on antral follicles. In rat granulosa cells, derived from antral follicles and grown in serum-free medium, FSH-induced E_2 production is increased by BMP4 and BMP7, while FSH-induced progesterone production was decreased (Shimasaki *et al.*, 1999). Sheep heterozygous for a naturally occurring null mutation in the *GDF9B* or *Bmp15* gene show an increased ovulation rate. A possible explanation is that 50% of the normal plasma level of GDF9B reduces effective follicle growth by which less oestrogens and inhibin are produced and by which suppressive effects on plasma FSH levels are delayed (Galloway *et al.*, 2000).

Inhibin and activins, other members of the TFβ family, are also important for antral follicle growth. Several knockout mouse models revealed the involvement of activins and inhibins in late follicle growth. Female mice lacking activin receptor type II (ActRII) are viable and display a block in folliculogenesis at a slightly later developmental stage compared to FSHβ-deficient females (Matzuk *et al.*, 1995). However, females deficient in activin B showed no impairment of follicle development, while folliculogenesis of females deficient in activin A and activin AB could not be studied since these animals die within 24 hours after birth (Matzuk *et al.*, 1995). Transgenic female mice overexpressing inhibin A, show a block in folliculogenesis at the early antral stage, a phenotype most likely caused by the suppressed serum FSH level (Pierson *et al.*, 2000). In transgenic mice overexpressing follistatin, an activin binding protein that inhibits activin action, the ovarian phenotype is depended on the level of overexpression. Female mice from two of the transgenic lines with the highest expression of the follistatin transgene are infertile or become infertile at 4 to 5 months of age. Most of these females showed a block in ovarian folliculogenesis at the large preantral stage, similar to the phenotype of FSHβ-deficient females (Guo *et al.*, 1998). The inhibitory effect of follistatin may be caused by suppression of the FSH level as a result of inhibition of activin bioactivity. However, possibly activin also has local, direct effects on ovarian follicles. Female α-inhibin-deficient mice primed with PMSG and hCG, have impaired folliculogenesis and the number of ovulated oocytes was greatly reduced compared to wild type females. After examination of the ovaries of the treated females, it was concluded that follicle development occurred until the antral stage, although some early preovulatory follicles were found. These mice develop also sex-cord stromal tumours from 6 weeks of age, and it is not clear whether these tumours have any effect on follicle development (Matzuk *et al.*, 1996). In addition, it remains to be elucidated whether the effects are caused by the absence of inhibin itself, or by the high levels of activins, which are found in the α-inhibin-deficient mice.

Figure 1.4A shows a schematic description of follicle development. Figure 1.4B shows

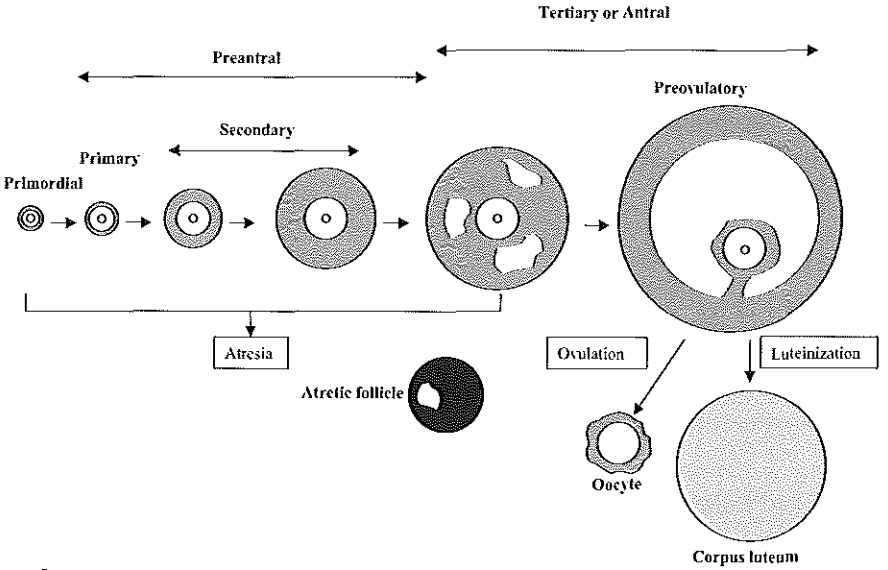

Figure 1.4A
Ovarian follicle development: Different stages and major events.
The different follicle stages are in **bold**. The major events are indicated by the text boxes.

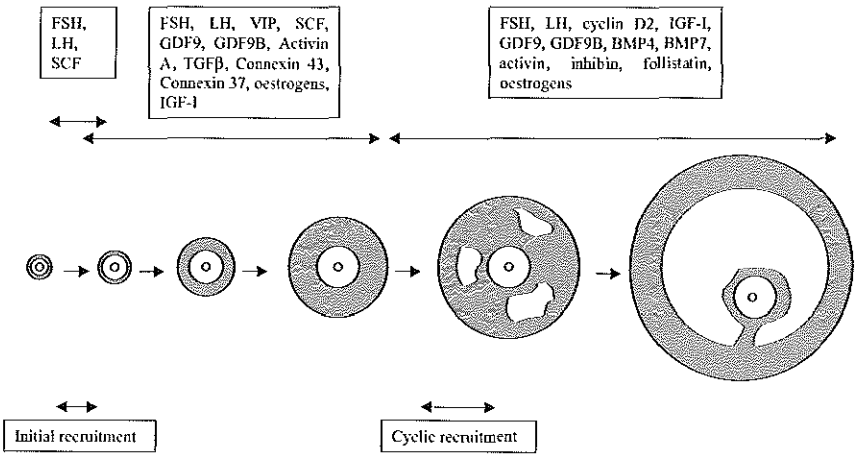

Figure 1.4B
Intra- and extraovarian factors influencing ovarian folliculogenesis at certain stages during follicle development.
Double arrows indicate the stages of follicular development which are affected by the factors mentioned in the boxes above the double arrows. The names of the different developmental stages of the ovarian follicle are shown in Figure 1.4A. For an exact description of the effects of the different factors see text of Paragraph 1.4.1-1.4.3. The boxes with initial and cyclic recruitment together with the double arrows indicate the moment that these processes occur during follicular development.

Table 1.2
classification of mouse follicles.

Follicle class	Diameter (µm)	Follicle type (Pedersen)	Other classifications	
1	<20	2	primordial	-
2	20-99	3a/3b	primary	preantral
3	100-169	4	secondary	preantral
4	170-219	5a	secondary	preantral
5	220-309	5b	secondary	preantral
6	310-369	6	tertiary	antral
7	>370	7-8	tertiary	antral

at what stages of follicular development initial and cyclic recruitment occur. Furthermore, Figure 1.4B shows when during follicular development factors mentioned in Paragraph 1.4.2 and 1.4.3 play a substantial role in follicular development.

For practical use a follicle classification is needed. In Chapters 2, 3, 4 and 5 of this thesis the ovarian follicles are classified according to their mean diameter. The follicles in mice are divided into 7 classes, which are shown in Table 1.2 and Figure 1.5A-F. In Chapter 5, however, another classification is used for rat follicles. Table 1.2 also shows how these classes can be compared to the frequently used follicle classification according to Pedersen (Pedersen and Peters, 1968).

1.5 Follicular atresia

More than 99% of the primordial follicles never reach the preovulatory stage, but undergo a degenerative process called atresia. The term "follicular atresia" refers to those processes during which the ovarian follicle loses its integrity and the oocyte degenerates (Ingram, 1962). Atresia occurs in ovarian follicles of all developmental stages, although the majority of atresia under normal physiological conditions occurs in large preantral and small antral follicles when continued growth is dependent upon gonadotropins (Figure 1.4A) (Kaipia and Hsueh, 1997).

Almost 20 years ago it was postulated that atresia involves apoptosis (programmed cell death) (Tsafriri and Braw, 1984), and by now a growing body of evidence has demonstrated that indeed apoptosis is the biochemical mechanism underlying follicular atresia (Hsueh *et al.*, 1994; Tilly, 1996). Apoptosis is an active cell "suicide" program found in all multi-cellular organisms, and takes place in tissues undergoing developmental changes or responding to alterations in physiological stimuli. Apoptosis affects single cells within a tissue, and is distinguished by characteristic morphological changes both in the nucleus and the cytoplasm. A typical nuclear change is chromatin condensation, resulting in the formation of dense zones of heterochromatin underlying the nuclear membrane (Wyllie,

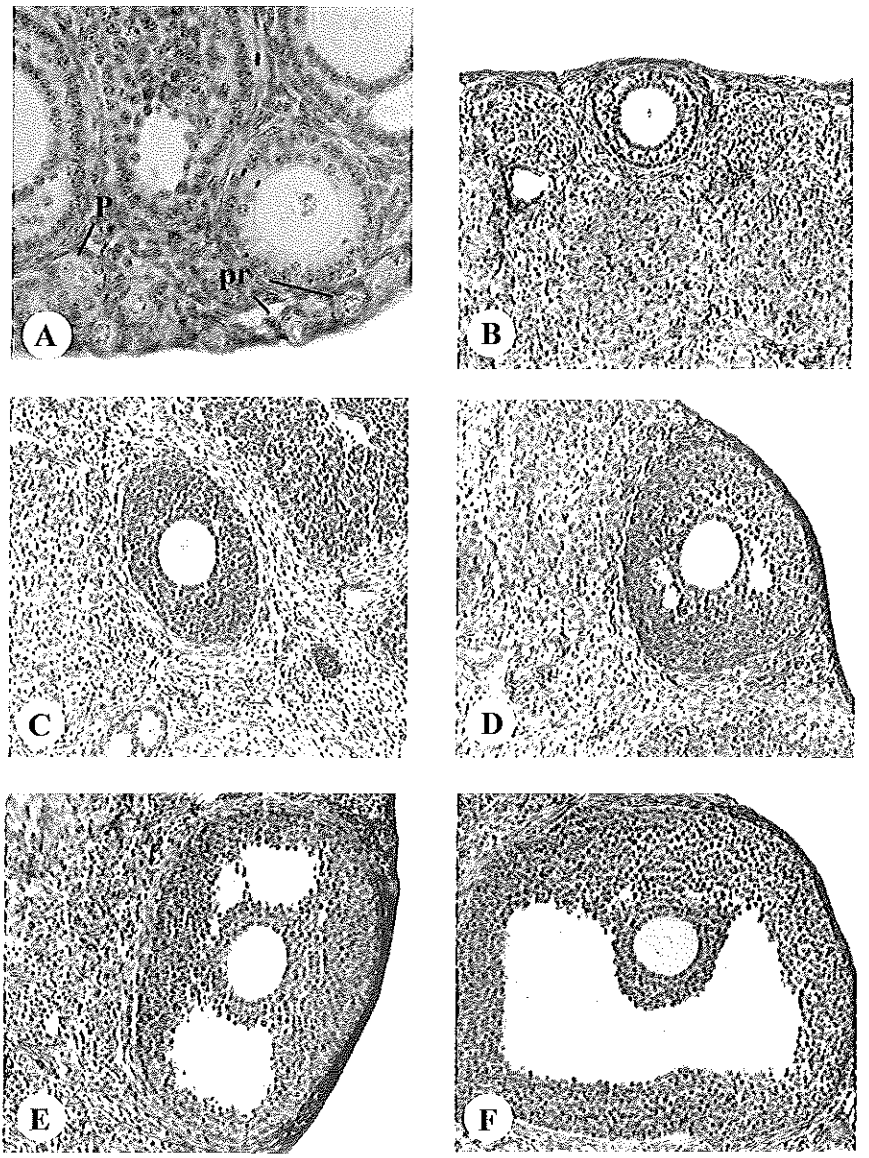

Figure 1.5
Examples of different classes of ovarian follicles. Classification was based on the mean diameter of the follicle.

A. Class 1 or Primordial follicle (pr) (diameter < 20 μm) and Class 2 or Primary follicle (P) (20 μm < diameter < 100 μm). Magnification x 400
B. Class 3 (100 μm < diameter < 170 μm)
C. Class 4 (170 μm < diameter < 220 μm)
D. Class 5 (220 μm < diameter < 310 μm)
E. Class 6 (310 μm < diameter < 370 μm)
F. Class 7 (diameter > 370 μm)
B-F, Magnification x 200.

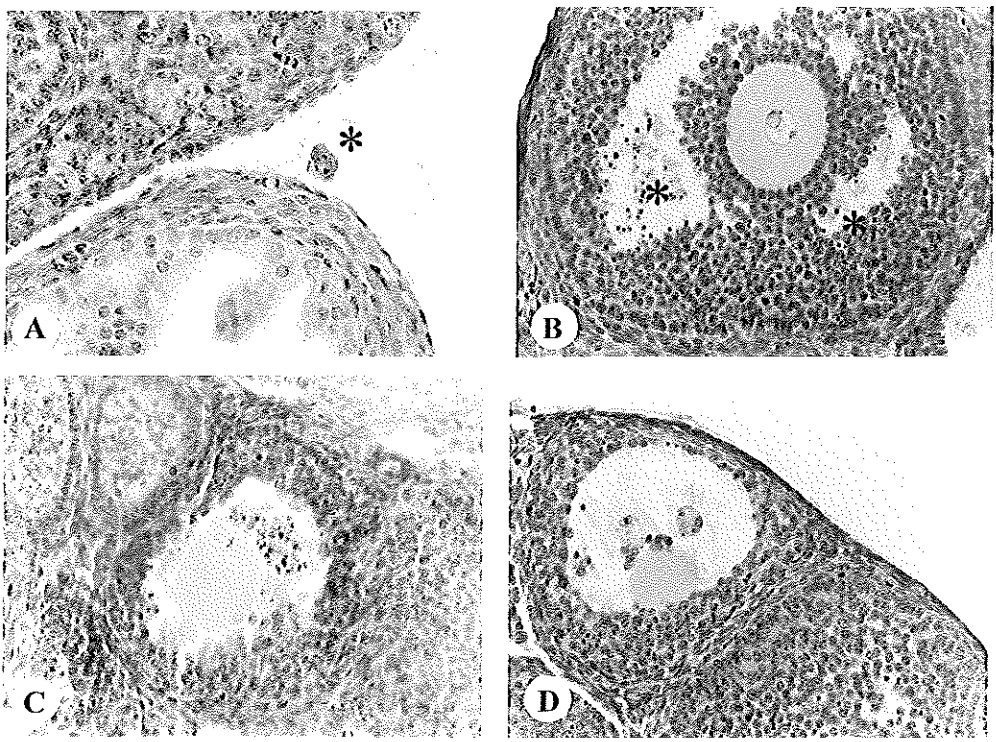

Figure 1.6
Ovarian follicle loss through extrusion of the primordial follicle or through atresia of preantral and antral follicles.

A. Extrusion of a primordial follicle from a prepubertal mouse ovary (*). Magnification x 400.

B. Small antral follicle in an early stage of atresia. Fragments of pyknotic granulosa cells are found in between the granulosa cells and in the antrum (*). Magnification x 400.

C. Small antral follicle in advanced stage of atresia. The oocyte has resumed maturation and a pseudo-maturation spindle has formed. Magnification x 400.

D. Small antral follicle in a late stage of atresia. Fragments of the oocyte are found in the antrum. Magnification x 400.

1987). Independent of chromatin condensation, calcium/magnesium-dependent endonuclease is activated, resulting in the cleavage of DNA between nucleosomal units (Wyllie *et al.*, 1980). The end result is the generation of DNA fragments in size multiples of 185-200 base pairs, which can be visualised as a distinct ladder of DNA bands after agarose gel electrophoresis and ethidium bromide staining (Wyllie, 1980; Arends and Wyllie, 1991). The DNA ladder pattern has been regarded as a hallmark of apoptosis, and provides a specific biochemical marker for apoptotic cell death. The cytoplasm of apoptotic cells is characterised by aggregation and disorientation of cytoplasmic organelles. Concomitantly, the plasma membrane shows shrinkage and blebbing, and the cell eventually breaks down into several membrane-bound apoptotic bodies (Kaipia and Hsueh, 1997).

Apoptotic bodies are phagocytised by neighbouring cells, and since phagocytosis usually takes place before the integrity of the plasma membrane is lost, there is no leakage of cytoplasmatic components and no inflammation reaction is induced (Hsueh *et al.*, 1994).

During atresia many morphological characteristics of apoptosis are found in the granulosa cells of the atretic follicle. The process of atresia usually differs in primordial, small preantral and large preantral/antral follicles and has been extensively described by Byskov (Byskov, 1978b). In primordial follicles, the oocyte usually shows signs of degeneration, while the pregranulosa cells do not seem to be affected. In the mouse, three different mechanisms exist by which primordial follicles are eliminated from the ovary. The oocyte may become necrotic/apoptotic, lytic, or leave the ovary through the surface epithelium (Figure 1.6A) (Byskov, 1978b). In mice, only oocyte lysis seems to occur predominantly during later stages in life, while necrosis and extrusion through the epithelium occur during the first days after birth. In atretic small preantral follicles, the granulosa cells become pyknotic and mitosis is rarely seen. The follicle and the oocyte become irregular and shrink, and the nucleus of the oocyte is often eccentrically placed and exhibits clumping of the chromatin. No maturation divisions occur in oocytes of atretic small preantral follicles. The fate of atretic small preantral follicles is unknown, but follicle cells as well as oocytes may be removed by phagocytosis, leaving no recognisable trace (Byskov, 1978b). In large preantral and antral follicles an identical pattern of atresia occurs. The appearance of pyknotic nuclei, in which chromatin condensation and shrinkage has taken place and which are recognisable as small dark dots, is observed for the granulosa cells that face the antral cavity and this is usually considered to be one of the first morphological signs of atresia. Fragments of the pyknotic nuclei are later extruded into the follicular fluid as dense particles (Figure 1.6B). As atresia progresses, the granulosa cell layer gradually becomes thinner. Dead granulosa cells are removed by invading leukocytes or macrophages. Accompanying the drastic changes in the granulosa cell layer, the follicle collapses and the oocyte often undergoes pseudomaturation, which becomes apparent with the formation of a pseudomaturation spindle (Figure 1.6C). The first stages of this process are comparable to the normal maturation process of oocytes. Finally, the oocyte often breaks up into fragments of uneven sizes (Figure 1.6D), and is removed by invading cells during the final stages of atresia (Byskov, 1978b).

Many of the features of apoptosis have been remarkably conserved through evolution, such that the existence of a universal pathway of execution of apoptosis has been proposed (Steller, 1995; Wyllie, 1995). Hormone signals may suppress or promote apoptosis in different cell types by activating similar intracellular pathways. Internucleosomal DNA fragmentation, one of the hallmarks of apoptosis, also occurs in granulosa cells of atretic follicles. Thus far, this DNA fragmentation is apparently confined to granulosa cells of follicles undergoing the transition to the antral stage of development, or to large antral follicles not selected for ovulation (Hughes and Gorospe, 1991; Billig *et al.*, 1994; Palumbo and Yeh, 1994; Tilly, 1996).

In *C. elegans*, three genes, *ced-9*, *ced-3*, and *ced-4*, are necessary for the execution of apoptosis. In vertebrate species, homologs of these genes have been identified and are believed to comprise a program of cell death analogous to that observed in *C. elegans*. Ver-

tebrate homologs of *ced-9* have been classified as *Bcl-2* family members, *ced-3* homologs as genes encoding caspases, while a putative *ced-4* homolog in vertebrates encodes apoptotic protease-activating factor-1 (Apaf-1). These gene families are found to be active during follicular atresia. Many members of the *Bcl-2* family are expressed in the ovary (reviewed in (Hsu and Hsueh, 2000)) and are involved in anti-apoptotic processes (*Bcl-2*, *Bcl-x_L*, *Bcl-w*, *Mcl-1*), or in the stimulation of apoptosis (*Bax*, *Bad*, *Bak*, *Bik*). A clear example of the involvement of the *Bcl-2* gene family in ovarian atresia is shown by a transgenic mice overexpressing Bcl-2 in the ovary (Hsu *et al.*, 1996), revealing suppression of follicular cell apoptosis and enhancement of folliculogenesis. Caspases, cysteine proteases that mainly promote apoptosis through proteolytic degradation of proteins essential for cellular survival, are also found in the ovary (Boone and Tsang, 1998). In caspase-2-deficient mice an excess number of oocytes was found, that were resistant to cell death following exposure to cytostatic drugs (Bergeron *et al.*, 1998). Expression of Apaf-1, the third factor important in the execution of the apoptotic process, is abundant in granulosa cells of early antral follicles, whereas *in vivo* gonadotropin priming completely suppresses Apaf-1 expression and granulosa cell apoptosis (Robles *et al.*, 1999). Based on information from studies on *C. elegans* homologs of Bcl-2, caspases and Apaf-1, it has become clear that these three factors involved in the execution of the apoptotic process interact with each other (Hsu and Hsueh, 2000). Presumably, Apaf-1 acts as a critical bridge protein to couple upstream regulatory events (mediated via *Bcl-2* family members) to downstream effector molecules (caspases) that carry out the cell death command (Song *et al.*, 1999; Zou *et al.*, 1999).

Follicular atresia and factors playing a role in this process have been extensively studied and many hormones and growth factors can effect follicular atresia. Several review articles about follicular atresia have appeared recently (Hsueh *et al.*, 1994; Tilly, 1996; Hsueh *et al.*, 1996; Kaipia and Hsueh, 1997; Hsu and Hsueh, 1997; Asselin *et al.*, 2000; McGee, 2000), and therefore only a few factors involved in follicular atresia will be discussed in this chapter. As discussed above, most follicles become atretic during the late preantral to the early antral stage when continued growth becomes dependent on gonadotropins, and follicles undergo cyclic recruitment. Thus, follicles are destined to become atretic unless they are selected.

Gonadotropins play a very important role in the recruitment of follicles and are effective inhibitors of apoptosis (Chun *et al.*, 1994; Tilly and Tilly, 1995). The anti-apoptotic actions of FSH and LH are probably mediated by IGF-I (Chun *et al.*, 1994), while the anti-apoptotic actions of the gonadotropins can be mimicked by treatment of follicles with neuropeptides, such as VIP, and protein kinase A activators (Flaws *et al.*, 1995). Oestrogens also seem to be anti-apoptotic (Billig *et al.*, 1993), while several other factors, such as GnRH-like peptides (Billig *et al.*, 1994), IL-6 (Gorospe and Spangelo, 1993), androgens (Billig *et al.*, 1993), TNF-α (Kaipia *et al.*, 1996), and Fas-ligand (Quirk *et al.*, 1995) can trigger apoptosis of ovarian cells through specific receptors in the ovarian follicle.

Since the progression of apoptosis in ovarian follicles is dependent on the co-operative regulation of different paracrine and autocrine factors, it is likely that none of these factors is singularly obligatory in the control of follicle growth or demise. Instead, a bal-

ance of these different survival and apoptotic factors may decide whether a follicle will continue development or undergo atresia (Hsu and Hsueh, 2000).

1.6 Ovarian cycle in the mouse

The ovarian cycle in rodents such as mice and rats is called the oestrous cycle. The name oestrous cycle is derived from the fact that at the moment that the preovulatory follicles are ready to ovulate, the female becomes receptive to the male, exhibiting pronounced behavioural changes that increase the probability of mating. This period is termed oestrus, and the female is said to be "in heat". The oestrous cycle in mice and rats is not bound to a certain season, as in other mammals, and therefore occurs throughout the year. Furthermore, ovulation is not dependent on overt nervous stimulation like in rabbits, and repeats itself every 4 to 5 days. On basis of the characteristics of the cell types in a vaginal smear, the oestrous cycle can be divided into several stages, pro-oestrus, oestrus, metoestrus, and dioestrus (Long and Evans, 1922). During the oestrous cycle, small antral follicles start to grow and either degenerate or reach the preovulatory stage and ovulate.

The oestrous cycle is regulated by hormones secreted by the pituitary gland and ovarian follicles. At oestrus, an elevated level of FSH, due to the low levels of oestrogens and inhibin, "rescues" a cohort of small antral follicles from becoming atretic (cyclic recruitment) (Hirshfield and De Paolo, 1981). FSH-initiated cyclic recruitment is sometimes described interchangeably with the process of follicle selection (McGee and Hsueh, 2000). From the rescued cohort of small antral follicles approximately 12 follicles grow on to the preovulatory stage, while the remaining follicles undergo atresia. The exact mechanism of selection of the preovulatory follicles is not clear, but FSH-sensitivity of the follicles plays a role in this process, as is suggested by studies in the human (Fauser and Van Heusden, 1997). Increased FSH-sensitivity could be the result of enhanced FSH receptor expression or changes in local growth factors that augment or attenuate FSH responsiveness as suggested by bovine studies (Bao *et al.*, 1997; Evans and Fortune, 1997). During the metoestrus/dioestrus period the inhibin serum level gradually increases (Sander *et al.*, 1986), followed by an increase in oestradiol serum concentration at the end of dioestrus. The selected follicles grow until they reach the preovulatory stage at pro-oestrus. At the beginning of pro-oestrus oestradiol reaches peak levels, which triggers a LH and FSH surge on pro-oestrus. LH stimulates the oocyte of the preovulatory follicle to resume meiosis, while it also induces ovulation in the preovulatory follicles. The granulosa cells of the preovulatory follicles stop producing oestradiol and switch to the production of progesterone. The LH level rapidly declines at the end of pro-oestrus, while FSH concentration remains high until the next day. This extension of the FSH surge is often called the secondary FSH peak.

1.7 Anti-Müllerian hormone

In 1947 it was shown that during male foetal development a testicular factor distinct from testosterone caused regression of the Müllerian ducts, which form the anlagen of the uterus, the oviducts, and the upper part of the vagina (Jost, 1947; Munsterberg and Lovell-Badge, 1991). This factor was called anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), and was subsequently identified as a dimeric glycoprotein related to TGFβ (Cate *et al.*, 1986). Together with inhibins, activins, BMPs, and GDFs, AMH is a member of the TGFβ superfamily of peptide growth and differentiation factors (Massague, 1990). Members of this family are synthesised as precursor proteins that require proteolytic cleavage to generate a bioactive C-terminal domain. AMH is a 140 kDa dimeric glycoprotein, which is cleaved by endoproteases to generate a 110 kDa N-terminal and a 25 kDa C-terminal subunit (Pepinsky *et al.*, 1988). PC5 and furin, members of the proprotein convertase family, might be involved in proteolytic processing of AMH (Nachtigal and Ingraham, 1996). AMH differs from the other TGFβ family members in that the N-terminus can potentiate the activity of the C-terminal domain, and even appears to be required to obtain full bioactivity of AMH (Wilson *et al.*, 1993). Members of the TGFβ family signal via heterodimeric or heterotetrameric receptor complexes, containing type I and type II transmembrane serine/threonine kinase receptors (Cheifetz *et al.*, 1987; Massague, 1990). For AMH, until now only a type II receptor (AMHRII) has been identified (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b).

1.7.1 Expression of AMH and AMHRII

In male mice, AMH mRNA expression is first found on E12 in the foetal testis (Munsterberg and Lovell-Badge, 1991). At later developmental stages high expression of AMH mRNA is restricted to the testicular Sertoli cells, where it remains high during the entire foetal period (Munsterberg and Lovell-Badge, 1991; Taketo *et al.*, 1993). Studies in rats have shown that AMHRII mRNA expression is found in the mesenchymal cells surrounding the Müllerian ducts (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b), while also foetal Sertoli cells show AMHRII mRNA expression (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b). After birth AMH mRNA expression strongly decreases in the Sertoli cells from both mice and rats, until it becomes hardly detectable (Munsterberg and Lovell-Badge, 1991; Taketo *et al.*, 1993; Baarends *et al.*, 1995a). During the rat spermatogenic cycle a considerable level of AMH mRNA expression was also found in the Sertoli cells at stage VII, whereas it is much lower at other stages of the spermatogenic cycle (Baarends *et al.*, 1995a). In contrast to AMH, AMHRII mRNA expression remains elevated in the rat testis (Baarends *et al.*, 1994). In accordance with AMH mRNA expression, AMHRII shows a spermatogenic stage-specific expression: expression increases from Sertoli cells at stage XIII, becomes maximal during stages VI and VII, and declines to undetectable levels at stages XI-XII (Baarends *et al.*, 1995a).

In female foetuses no AMH expression is found (Munsterberg and Lovell-Badge, 1991; Hirobe *et al.*, 1992; Taketo *et al.*, 1993), and this lack of expression is essential for

normal differentiation of the female internal reproductive tract structures, since AMH would cause degeneration of the Müllerian ducts, the anlagen of the female genital tract. AMHRII mRNA expression, however, is found in the foetal rat ovary and the Müllerian duct cells (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b). Surprisingly, several days after birth, both AMH mRNA expression and AMH protein are detected in rat ovarian granulosa cells (Ueno *et al.*, 1989b; Hirobe *et al.*, 1992; Baarends *et al.*, 1995b). It was found that AMH expression is highest in granulosa cells from preantral and small antral follicles. Within these follicles AMH expression is not always evenly distributed, but sometimes highest in the granulosa cells immediately surrounding the antrum and around

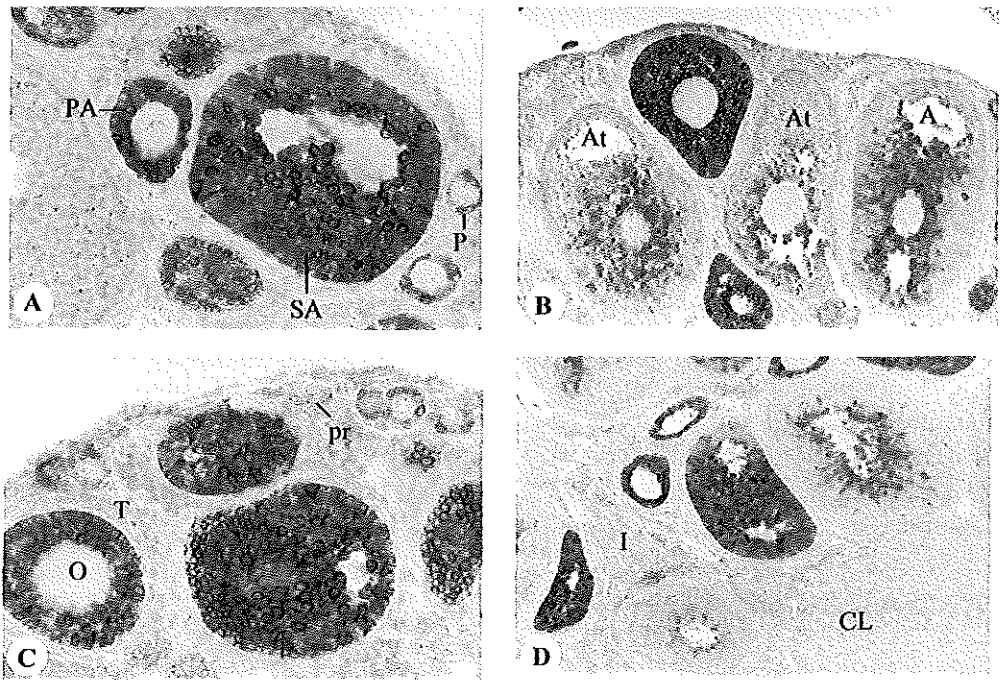

Figure 1.7
Immunohistochemical localisation of AMH in a 30-day-old and a 4 month-old C57Bl/6J mouse ovary.

A. Section of an ovary of a 30-day-old mouse. AMH is first found in granulosa cells from early primary follicles (P) and is highly expressed in granulosa cells of larger preantral (PA) and small antral (SA) follicles. Magnification x 400.

B. Section of an ovary of a 30-day-old mouse. AMH expression diminishes in antral (A) and atretic (At) follicles. The granulosa cells surrounding the antrum or the oocyte are the last cells to loose AMH expression. Magnification x 200.

C. Section of an ovary of a 30-day-old mouse. AMH expression is not present in primordial follicles (pr), oocytes (O) and theca cells (T). Magnification x 400.

D. Section of an ovary of a 4-month-old mouse. AMH expression is not found in the corpus luteum (CL) and the interstitium (I). Magnification x 200.

the oocyte (Baarends *et al.*, 1995b; Hirobe *et al.*, 1994; Hirobe *et al.*, 1992; Ueno *et al.*, 1989a). Some studies show that in rat, AMH expression can be found until the preovulatory stage (Ueno *et al.*, 1989a; Hirobe *et al.*, 1994), while other studies show that AMH disappears from the small antral follicle stage onward (Hirobe *et al.*, 1992; Baarends *et al.*, 1995b). Follicles showing signs of atresia also show decreased or no AMH expression, while AMH expression is never found in primordial follicles and corpora lutea (Ueno *et al.*, 1989a; Hirobe *et al.*, 1992; Hirobe *et al.*, 1994; Baarends *et al.*, 1995b). During the oestrous cycle no differences occurred in the expression pattern in follicles of the same class, although some heterogeneity has been observed in AMH mRNA expression in preantral and small antral follicles at oestrus and dioestrus (Baarends *et al.*, 1995b; Hirobe *et al.*, 1994).

Also in mice, AMH production starts a few days after birth. Immunohistochemistry studies showed that in C57BL/6J females AMH expression is first found in granulosa cells of primary follicles of 4-day-old females (Chapter 3 of this thesis). In Parkes and Albino Swiss female mice, AMH mRNA and AMH protein expression, respectively, were first found in granulosa cells of growing follicles at 6 days of age (Munsterberg and Lovell-Badge, 1991; Taketo *et al.*, 1993). In C57Bl/6J females, AMH protein expression is high in granulosa cells from preantral and small antral follicles (Figure 1.7A). AMH expression diminishes from the antral follicle stage onward, just as in atretic follicles. In both follicle types the granulosa cells surrounding the oocyte and the antrum are the last to loose AMH expression (Figure 1.7B) (Munsterberg and Lovell-Badge, 1991). No expression is found in primordial follicles, oocytes and theca cells (Figure 1.7C), corpora lutea and interstitium (Figure 1.7D).

In the ovary, AMHRII mRNA expression is always colocalized with AMH mRNA expression (results not shown), indicating that at least some of the effects of AMH are autocrine in nature.

1.7.2 Regulation of AMH expression

Several factors that are involved in sex determination and differentiation also appear to be important in regulation of AMH expression. Sry (sex-determining region of the Y), a transcription factor encoded by the *Sry* gene located on the Y chromosome and involved in testis determination, can cause an increase in *Amh* promoter activity. Moreover, a Sry binding region has been identified in the *Amh* promoter (Haqq *et al.*, 1994). However, since AMH and Sry mRNA expression only overlap a short time, it may not be very likely that Sry regulates AMH expression (Hacker *et al.*, 1995). Besides the Sry binding region, a second putative element is identified in the *Amh* promoter. This element (*AMH*-RE-1) contains a motif which resembles the half site for a number of nuclear hormone receptors (Shen *et al.*, 1994). The orphan nuclear receptor SF1 binds to this region and this SF1 binding site turned out to be essential for sex- and cell-specific *Amh* promoter activity in transgenic mice (Giuili *et al.*, 1997). Recently, a second SF1 binding site was found in the distal part of the human *Amh* promoter (Watanabe *et al.*, 2000). However, SF1 alone appears not be sufficient for *Amh* gene activation. The transcription factor WT1 was found

to associate with SF1, which resulted in a synergistic action between SF1 and WT1 on *Amh* activation. This synergism between WT1 and SF1 is antagonised by coexpression of Dax1 (DSS-AHC critical region on X) (Nachtigal *et al.*, 1998), as SF1, a member of the nuclear hormone receptor family and involved in the differentiation of the indifferent gonad in the female direction (Zanaria *et al.*, 1994). A third region in the *Amh* promoter encompasses a canonical SOX-binding (Sry-related HMG-box) site that can bind the transcription factor Sox9. Transfection studies revealed that Sox9 can cooperate with SF-1 in activating the *Amh* promoter (De Santa Barbara *et al.*, 1998). The *Amh* promoter also is a target for the zinc finger transcription factors GATA-4 and GATA-1 (Vigier *et al.*, 1998; Beau *et al.*, 2000). Multiple GATA binding sites were found in the *Amh* promoter and both GATA-1 and GATA-4 are able to bind these GATA binding sites (Beau *et al.*, 2000; Watanabe *et al.*, 2000). GATA-4 also indirectly enhanced *Amh* gene transcription through interaction with SF1 (Tremblay and Viger, 1999). Also steroid hormones may be involved in AMH regulation. A sequence nearly identical to the estrogen response element (ERE) has been identified in the *Amh* gene promoter, which can bind ERα (Guerrier *et al.*, 1990). Furthermore, 39 ERE half-sites were identified in the *Amh* gene promoter (Dresser *et al.*, 1995). However, the functionality of the ERE half-sites in the *Amh* gene has not been proven.

All factors (Dax1, Sox9, Sry, SF1, WT1, GATA-1, GATA-1 and oestrogens) that seem to be involved in *Amh* gene regulation are present at some point during the period of sex determination and differentiation in the male foetus (Armstrong *et al.*, 1993; Ikeda *et al.*, 1994; Hacker *et al.*, 1995; Swain *et al.*, 1996). This indicates that these factors are potentially involved in *Amh* gene regulation. Indications for the regulatory effect of oestrogens on prenatal AMH expression was given by a study in which male mice were exposed *in utero* to the synthetic estrogen diethylstilbestrol (DES). In these males, DES treatment increased AMH mRNA expression, probably indirectly through an increase in SF1 expression (Visser *et al.*, 1998). However, since αβERKO males develop a normal reproductive tract, oestrogens do not appear to be essential regulators of AMH expression (Couse *et al.*, 1999). Postnatally, SF1, Dax1, GATA-4, WT1 and oestrogens are found in testis and ovary. SF1 and Dax1 are expressed in both granulosa and theca cells of ovarian follicles (Ikeda *et al.*, 1996), while GATA-4 was only found in the granulosa cells of ovarian follicles (Heikinheimo *et al.*, 1997). Ovarian WT1 mRNA is expressed exclusively in the surface epithelium and granulosa cells of primordial, primary, and secondary follicles, and its level decreases during follicle growth (Hsu *et al.*, 1995). Oestrogens are produced by granulosa cells of follicles from the large preantral stage onward (Hillier, 1981). The presence of these factors at the moment that AMH expression is found in the ovary, could implicate that these factors are able to regulate AMH expression in the postnatal ovary. However, the precise regulation of AMH mRNA expression in the adult gonad has not been elucidated thus far. The sex-specific expression of AMH before birth suggests that a sex-specific factor needs to stimulate or inhibit the expression of AMH in the foetal testis or ovary or stimulate AMH expression in the postnatal ovary. A possible candidate might be FIGα, which is expressed predominantly by oocytes of primordial follicles, but also by oocytes of growing follicles. Low expression was also found in the postnatal testis (Liang

et al., 1997).

1.7.3 AMH signal transduction

AMH is a member of the TGFβ superfamily of growth and differentiation factors, which includes TGFβs, BMPs, inhibins, activins and GDFs (Massague, 1990). Members of this family signal through a family of transmembrane serine/threonine (S/T) kinase receptors (Cheifetz *et al.*, 1987; Massague, 1990) with a characteristic overall structure that includes a short, cysteine-rich extracellular domain, a single transmembrane domain, and an intracellular serine/threonine kinase domain (Miyazono *et al.*, 1994; Attisano and Wrana, 1996). These receptors can be divided into two classes, the type I and type II receptors (Cheifetz *et al.*, 1987; Massague, 1990). Type I receptors are distinguished by a highly conserved sequence known as the 'GS domain', which contains a repetitive glycine-serine motif between the transmembrane and kinase domains. The kinase domains of the type I receptors are relatively well conserved, while the type II receptors possess more distantly related kinase domains (Wrana, 1998). The mechanism of receptor activation by TGFβ has been very well characterised (Wrana *et al.*, 1994; Chen and Weinberg, 1995; Attisano and Wrana, 1996) and may serve as a model for other family members. Biochemical and genetic data have shown that signalling by TGFβ-like factors occurs through complexes of type I and type II S/T kinase receptors (Wrana *et al.*, 1992; Franzen *et al.*, 1993). In the absence of ligand, the TGFβ receptors appear to exist as independent monomers at the cell surface (ten Dijke *et al.*, 1996). TGFβ binding to the type receptor II leads to recruitment of the type I receptor into presumably a heterodimeric complex, although there are some indications that the type I and type II receptors may in fact form a heterotetrameric complex (Chen and Derynck, 1994; Weis-Garcia and Massague, 1996). Within this complex, the type II receptor, which is a constitutively active kinase, phosphorylates the type I receptor on serine and threonine residues within the GS domain (Wrana *et al.*, 1994; Wieser *et al.*, 1995). Once phosphorylated, the type I receptor is activated to signal to downstream targets (Figure 1.8).

Recent studies have identified downstream signalling targets of type I S/T kinase receptors, which belong to a family of signal transduction molecules related to proteins encoded by the *Drosophila* gene *Mothers against dpp (Mad)* and the *C. elegans sma* genes. Until now, 9 vertebrate members of this family have been identified, which are called Smads. Sequence analysis of members of this family indicated the presence of three domains: a conserved amino-terminal MH1 (MAD homology 1) domain, a highly conserved carboxy-terminal MH2 domain, and a central, divergent, non-conserved region. The MH domains appear to be critical to the function and regulation of the protein (Wrana, 1998). The identified Smads can be divided into three subclasses: pathway-specific, common-mediator, and inhibitory Smads (Attisano and Wrana, 1998). The pathway-specific Smads (Smad1-3, Smad5 and Smad8-9) become activated upon phosphorylation by the type I receptor (Hoodless *et al.*, 1996; Kretzschmar *et al.*, 1997). Upon phosphorylation, the pathway-specific Smad is released from the type I receptor, upon which this Smad protein forms a hetero-oligomer with the common-mediator Smad4 (Hoodless

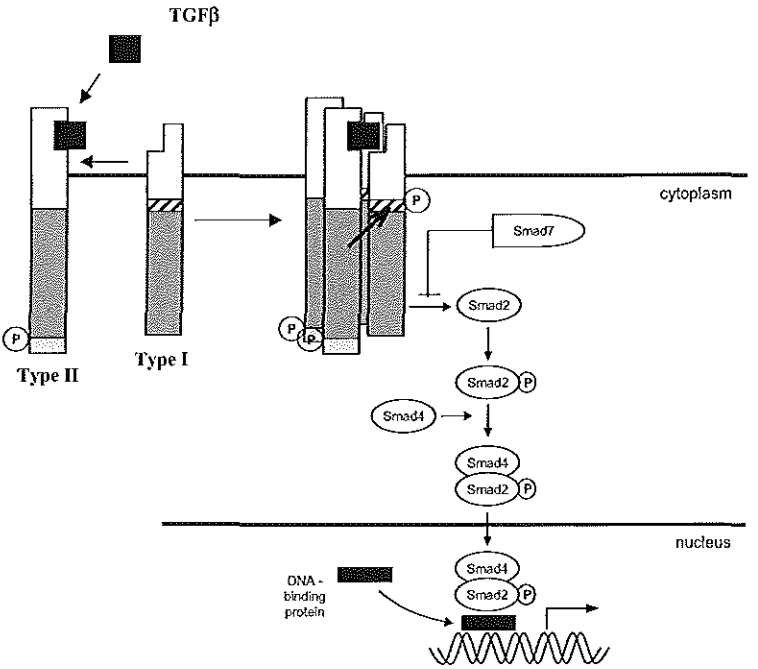

Figure 1.8
TGFβ signalling model.

After binding of TGFβ to its type II receptor, the type I receptor is recruited and a heterodimeric or heterotetrameric receptor complex is formed. The type I receptor is phosphorylated by the type II receptor, which activates the type I receptor to phosphorylate pathway-specific Smad2. This phosphorylation leads to heterodimerisation of Smad2 with the common-mediator Smad4. The hetero-oligomeric complex translocates to the nucleus where it binds to DNA. The inhibitory Smad7 can also bind to the TGFβ type I receptor and thereby prevents phosphorylation of the pathway-specific Smad2 (modified from Visser, 1998).

et al., 1996; Wu *et al.*, 1997), the only Smad protein identified in different species as a common-mediator. The hetero-oligomeric complex then translocates to the nucleus where it regulates gene transcription, either directly after DNA binding or as a cofactor in association with DNA-binding proteins (Massague, 1996; Heldin *et al.*, 1997). Smad6 and Smad7 belong to the inhibitory Smads. These Smads also interact with the type I receptor but their association of the inhibitory Smads to the type I receptor is more stable than observed for the pathway-specific Smads. By binding to the type I receptor, the inhibitory Smads interfere with the phosphorylation of the pathway-specific Smads, and thereby prevent the formation of active heterodimeric Smad complexes (Hayashi *et al.*, 1997; Imamura *et al.*, 1997) (Figure 1.8).

In contrast to the wealth of information that is available about the signalling mechanism of TGFβ, not much is known about AMH signalling. Thus far only the AMH type II receptor was cloned and identified (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b; Grootegoed *et al.*, 1994). This type II receptor is very specific for AMH, since AMHRII-deficient

and AMH/AMHRII-deficient animals have the exact phenotype as the AMH-deficient animals. Humans with a mutation in either AMH or AMHRII present with the persistent Müllerian duct syndrome (PMDS) (Behringer *et al.*, 1994; Mishina *et al.*, 1996). Furthermore, female AMHRII-deficient mice overexpressing hAMH have normal reproductive tracts and ovaries (Mishina *et al.*, 1999). Similar to TGFβ receptor, the AMH receptor complex probably also contains a type I receptor, although numerous attempts to identify this type I receptor seem to have failed. Possible candidates for the AMH type I receptor are activin receptor-like kinase 2 (ALK2) or ALK6 (Visser, 1998), two of the type I receptors that already have been cloned (He *et al.*, 1993). A recent study showed that by co-immunoprecipitation of known type I receptors with AMHRII ALK6 was the only type I receptor interacting in a ligand-dependent manner with AMHRII (Gouedard *et al.*, 2000). Smads through which AMH could signal to the nucleus remain to be identified, although the previous study also showed that anti-Müllerian hormone activates the Smad1 pathway (Gouedard *et al.*, 2000).

1.7.4 Functions of AMH

Function in Müllerian duct regression

During mammalian embryogenesis, two pairs of genital systems are developed. The Müllerian ducts have the potential to develop into oviducts, uterus, and the upper part of the vagina, while the Wolffian ducts can develop into the vasa deferentia, epididymides, and the seminal vesicles. Several experiments performed by Jost (Jost, 1947; Jost, 1953) showed that the foetal testes produce a hormone that inhibits the development of the Müllerian ducts. Many years later AMH was purified to homogeneity (Picard and Josso, 1984; Budzik *et al.*, 1985) based upon its ability to cause the regression of foetal female rat urogenital tracts in organ culture (Picon, 1969). The phenotype of male AMH-deficient and/or AMHRII-deficient mice further demonstrated the function of AMH in mammalian sexual development. Regression of the Müllerian ducts does not occur in these males and therefore they develop an uterus, oviducts and upper part of a vagina, in addition to the male reproductive system (Behringer *et al.*, 1994; Mishina *et al.*, 1996). Furthermore, female transgenic mice ectopically overexpressing human AMH (hAMH) show partial or complete regression of the Müllerian ducts. The stage of regression was positively correlated with the level of serum hAMH (Lyet *et al.*, 1995).

Function in gonadal development and function

In addition to its clear function during foetal sex differentiation, it has been suggested that AMH might also play a role in gonadal development and function. The study on freemartins gave a first indication for a possible role of AMH in gonadal development. Freemartins are female calves, which were *in utero* exposed to hormones of the male twin through fusion of the foetal blood vessels. These females not only show regression of the Müllerian ducts but also development of testicular cord-like structures in the ovaries (Jost *et al.*, 1972). Exposure of rat ovaries to AMH also resulted in the development of such cord-like structures (Vigier *et al.*, 1987). In hAMH overexpressing transgenic female mice,

however, not even 1% of the animals developed gonadal tubule formation (Lyet *et al.*, 1995), while only in adult but not in prepubertal αβERKO females cord-like structures are formed concomitant with an increased AMH level (Couse *et al.*, 1999). It is not clear whether AMH acts directly or indirectly upon the ovary to promote tubule formation. In αβERKO mice Sox9 expression is elevated, and this may have caused the formation of testis cord-like structures (Couse *et al.*, 1999).

For male mice, it was suggested that AMH has an effect on Leydig cells. Male mice that overexpress hAMH have impaired Leydig cell function, and AMH- and AMHRII-deficient males incidentally show Leydig cell hyperplasia (Behringer *et al.*, 1994; Lyet *et al.*, 1995; Mishina *et al.*, 1996), indicating that AMH might play a role in the control of Leydig cell differentiation and function by Sertoli cells. Several recent studies confirm the possibility that AMH might directly affect Leydig cell function (Racine *et al.*, 1998; Rouiller-Fabre *et al.*, 1998; Teixeira *et al.*, 1999).

The function of AMH in the postnatal ovary has been an enigma for many years. From the expression pattern of AMH and AMHRII in granulosa cells of preantral and antral follicles, it has been suggested that AMH might play a role in follicle development and/or selection (Ueno *et al.*, 1989a; Hirobe *et al.*, 1994; Baarends *et al.*, 1995b). Several *in vitro* studies showed that AMH affects FSH-stimulated events in the ovarian follicle. AMH decreases the aromatase activity and LH receptor number of cultured granulosa cells from rats and pigs (di Clemente *et al.*, 1994a), while it is also able to inhibit progesterone synthesis by cultured human granulosa/luteal cells (Kim *et al.*, 1992). Another indication for a role of AMH in follicle development/function came after examining AMH and AMHRII mRNA expression during the oestrous cycle in the rat. It was found that at oestrus, when FSH levels are high and cyclic recruitment of follicles takes place of which eventually some go on to become preovulatory, both AMH and AMHRII mRNAs are downregulated in some but not all large preantral and small antral follicles (Baarends *et al.*, 1995b). This heterogeneous expression pattern might indicate that AMH has a function in follicle recruitment (Baarends *et al.*, 1995b). From the ovarian phenotype of AMH-deficient mice it was concluded that AMH is not essential for ovarian function, since these females are fertile and have macroscopically normal ovaries (Behringer *et al.*, 1994). In this thesis, however, several new concepts on the function of AMH in the postnatal ovary are presented.

1.8 Aim and scope of this thesis

In mice, primordial follicles are formed during the first few days after birth. In some of these follicles, growth is immediately initiated (initial recruitment), while other primordial follicles remain dormant for many months. After initial recruitment the follicle grows to larger developmental stages, but is eventually destined to become atretic, unless it is selected to grow on to the preovulatory stage (cyclic recruitment). Since AMH is produced by follicular granulosa cells almost as soon as follicle growth is initiated, and since heterogeneous AMH expression is found in follicles undergoing cyclic recruitment, we

postulated that AMH might play a role in both initial and cyclic recruitment. Therefore, this thesis focuses on the role of AMH in these two important steps of follicular development.

In Chapter 2, a detailed study of the ovarian follicle dynamics in AMH-deficient female mice is described. The results indicate that AMH indeed has modulating effects at two important regulatory steps during follicle growth and development: initial and cyclic recruitment.

The role of AMH in initial recruitment was investigated using an ovary organ culture, and is described in Chapter 3. Results show that *in vitro* in the presence of exogenous AMH less primordial follicles start to grow than in the absence of added AMH.

It is postulated that FSH is essential in the process of cyclic recruitment, and that cyclic recruitment may depend on the FSH sensitivity of the follicles. Chapter 4 elaborates on the relation between AMH and FSH and their action on follicle growth. Furthermore, *in vivo* and *in vitro* studies are described that indicate that AMH partially determines the FSH sensitivity of recruitable follicles in the mouse.

Atresia is characterised by certain morphological features of the follicle, which become apparent when the atretic process is well on its way. Chapter 5 describes a method of detecting markers of future atresia that become visible before morphological markers of the atretic process are detectable. The possible role of AMH during cyclic recruitment was investigated by determining the relation between BrdU, a marker of future atresia, and AMH expression at the moment that cyclic recruitment occurs in the rat.

In the General Discussion (Chapter 6) the two known functional aspects of AMH in the ovary are discussed. Furthermore, this discussion elaborates on a possible role AMH could play in causing female infertility in humans.

Chapter 2

CONTROL OF PRIMORDIAL FOLLICLE RECRUITMENT BY ANTI-MÜLLERIAN HORMONE IN THE MOUSE OVARY

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Abstract

The dimeric glycoprotein anti-Müllerian hormone (AMH) is a member of the transforming growth factor β (TGFβ) superfamily of growth and differentiation factors. During male fetal sex differentiation, AMH is produced by Sertoli cells and induces degeneration of the Müllerian ducts, which form the anlagen of part of the internal female genital system. In females, AMH is produced by the ovary, but only postnatally. The function of AMH in the ovary is, however, still unknown. Female AMH null mice were reported to be fertile, with normal litter size, but this does not exclude a more subtle function for ovarian AMH.

To investigate the function of AMH in the ovary, the complete follicle population was determined in AMH null mice, in mice heterozygous for the AMH null mutation and in wild type mice of different ages: 25 days, 4 months and 13 months. In the present study we found that ovaries of 25-day- and 4-month-old AMH null females, compared to wild type females, contain more preantral and small antral follicles. In addition, in 4- and 13-month-old AMH null females smaller numbers of primordial follicles were found. Actually, in 13-month-old AMH null females almost no primordial follicles could be detected, coinciding with a reduced number of preantral and small antral follicles in these females. In almost all females heterozygous for the AMH null mutation the number of follicles fell in between the numbers found in wild type and AMH null females. In 4-month-old AMH null females, serum inhibin levels were found to be higher while FSH levels were lower, compared to wild type females. In contrast, inhibin levels were lower in 13-month-old AMH null females and FSH levels unchanged compared to wild type females. Furthermore, the weight of the ovaries was twice as high in the 4-month-old AMH null females compared to ovaries of age-matched wild type females.

We conclude that AMH plays an important role in primordial follicle recruitment, such that more primordial follicles are recruited in AMH null mice than in wild type mice; the mice heterozygous for the AMH null mutation take an in between position. Consequently, the ovaries of AMH null females, and also of females heterozygous for the AMH null mutation, will show a relatively early depletion of their stock of primordial follicles. The female AMH null mouse may thus provide a useful model to study regulation of primordial follicle recruitment and the relation between follicular dynamics and ovarian ageing.

INTRODUCTION

Anti-Müllerian hormone (AMH), also called Müllerian inhibiting substance (MIS), is a member of the transforming growth factor β (TGFβ) superfamily of peptide growth and differentiation factors (Cate *et al.*, 1986). During male fetal sex differentiation, AMH is synthesised by the testicular Sertoli cells and induces degeneration of the Müllerian ducts, which form the anlagen of the uterus, the oviducts and the upper part of the vagina (Munsterberg and Lovell-Badge, 1991). During female fetal development, no ovarian AMH production occurs (Hirobe *et al.*, 1992). However, AMH mRNA expression is detected in ovarian granulosa cells from postnatal day 3 onwards (Hirobe *et al.*, 1992). Immunohistochemical (Ueno *et al.*, 1989a; Ueno *et al.*, 1989b) and mRNA *in situ* hybridisation (Hirobe *et al.*, 1992; Baarends *et al.*, 1995b) studies in rats revealed specific expression of AMH and its type II receptor (AMHRII) in granulosa cells of mainly non-atretic preantral and small antral follicles, while the signal is lost in non-atretic large antral follicles and atretic follicles of all size classes (Baarends *et al.*, 1995b). During the oestrous cycle, no marked changes are detected in the patterns of AMH and AMHRII mRNA expression, except at oestrus, when a heterogeneous decrease in expression of both mRNAs is found in non-atretic large preantral and small antral follicles compared to the more homogeneous expression pattern on other days of the cycle (Baarends *et al.*, 1995b). In cultured rat granulosa cells, exogenous AMH inhibits biosynthesis of aromatase and decreases LH receptor number (di Clemente *et al.*, 1994a). Furthermore, AMH opposes proliferation of cultured granulosa-luteal cells (Kim *et al.*, 1992; Seifer *et al.*, 1993).

The marked changes in AMH and AMHRII mRNA expression and the reported effects of AMH in *in vitro* culture systems indicate that AMH may play a role during follicle development in the postnatal ovary. However, little is known about the exact function of AMH in the ovary. In the present study, the function of AMH in the postnatal ovary has been studied with the help of an AMH null mouse model generated by Behringer et al. (Behringer *et al.*, 1994). Since in female animals, AMH and its sole receptor (AMHRII) (Mishina *et al.*, 1996; Mishina *et al.*, 1999) are predominantly found in the postnatal ovary, non-ovarian effects of the deletion of the *amh* gene are unlikely to occur, and therefore the AMH null mouse provides an excellent model for examining the function of AMH in the mouse ovary. AMH null males develop Müllerian duct derivatives, including oviducts, a uterus, and a vagina, in addition to a complete male reproductive system. AMH null females have macroscopically normal uteri, oviducts and ovaries. Furthermore, these females are fertile and have litters of normal size (Behringer *et al.*, 1994). Yet, we hypothesised that AMH might be involved in subtle and long-term aspects of control of follicle development and/or follicle selection. To investigate whether AMH exerts effects on the composition of the follicle population, we determined the entire follicle population in wild type mice [AMH (+/+)], mice heterozygous for the AMH null mutation [AMH (+/-)] and AMH null mice [AMH (-/-)] at 25 days, 4 months and 13 months of age. Furthermore, other parameters of reproductive function, such as serum FSH and inhibin levels and ovarian and uterine weights, were also determined.

We found that AMH is an important regulator of primordial follicle recruitment and

that ovaries of AMH null mice show depletion of primordial follicles at an earlier age.

Materials and Methods

Animals

A single male and a single female AMH (+/-) mouse were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). This pair was used to generate F1 mice. Next, several F1 AMH (-/-) males and females were crossbred with female and male C57BL/6J mice to obtain AMH (+/-) mice on a C57BL/6J genetic background. Subsequently, these mice were used to make AMH (+/+), AMH (+/-) and AMH (-/-) mice, which were used for follicle population analysis. The mice were kept under standard animal housing conditions in accordance with the NIH Guidelines for the Care and Use of Experimental Animals. Lights were on from 7.30 am to 8.30 pm. AMH (+/+) mice, AMH (+/-) mice and AMH (-/-) mice of 25 days (n = 4), 4 months (n = 4) and 13 months of age (n = 5) were used for this study. The 4- and 13-month-old animals were killed on the day of oestrus, which was determined by placing the females individually with an AMH (+/+) male of proven fertility in the afternoon, followed by a check for a copulatory plug the next morning. Females with a copulatory plug were killed on the same day at 4 pm by decapitation, after which blood was collected immediately. Females of 25 days old, which are still prepubertal, were also killed at 4 pm. After bleeding, the ovaries were removed, weighed and fixed overnight in Bouin's fluid for histological examination of the follicle population. The uterus was also removed and weighed.

The fixed ovaries were embedded in paraffin after routine histological procedures and 8 µm sections were mounted on slides and stained with hematoxylin and eosin. Blood samples were stored overnight at 4°C and centrifuged the following day at 3000 rpm for 15 min at 4°C. Serum samples were stored at -20°C until assayed for FSH and inhibin.

Determination of mouse AMH genotype

To analyse the genotype of the mice, genomic DNA was isolated by incubating tail tissue overnight at 55°C in 500 ml 50 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 900 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) and 0.5% (w/v) SDS. The next day, 500 µl phenol (Merck, Darmstadt, Germany) dissolved in Tris-HCl (pH 8.0) was added, and the mixture was shaken vigorously for 15 sec. Subsequently, the solution was centrifuged at 13000 rpm for 10 min. To the supernatant 200 µl phenol and 200 µl chloroform-isoamylalcohol (24:1) were added. After shaking vigorously the solution was centrifuged for 10 min at 13000 rpm. To the supernatant 2 vol. 100% ethanol were added, and the precipitated DNA was washed with 70% ethanol. After the DNA was air-dried for a few minutes, it was dissolved in 100 µl TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA, pH 8.0). Approximately 1 µg DNA was used in the PCR reactions. Primers KO568 (5'-GGAACACAAGCAGAGCTTCC-3') and KO810 (5'GAGACAGAGTCCATCACG-ACC-3')

were used to determine the presence of the wild type allele and were suggested by the Jackson Laboratory (http://lena.jax.org/resources/documents/imr/ protocols/Amh_KO.html). Primer KO568 anneals to nucleotide sequence 568-587 located in exon 1 of the *amh* gene (numbering according to Genbank sequence: accession number X63240), while primer KO810 anneals to antisense sequence 810-789 located in intron 1, resulting in a PCR product of 243 basepairs. In animals containing the AMH null allele, part of exon 1, intron 1 and exon 2 are replaced by the pCM1 neo-cassette. Therefore, the PCR product is only produced in DNA of AMH (+/+) and AMH (+/-) animals, which contain the complete wild type allele. Primers p126 (5'-CTCGTCAAGAAGGCGATA-3') and p127 (5'-GGGATCG-GCAT-TGAACA-3') were used to determine the presence or absence of the AMH null allele, *i.e.* the pCM1 neo-cassette (Thomas and Capecchi, 1987). Primer p126 anneals to the antisense sequence 1057-1041, while primer p127 anneals to nucleotide sequence 265-281 in the pCM1 neo-cassette, resulting in a PCR product of 793 basepairs. Only AMH (+/-) and AMH (-/-) animals, which contain the pCM1 neo-cassette, will produce this PCR product. For the PCR reaction, 50 ng of primers KO568 and KO810, or 100 ng of primers p126 and p127 were used in a PCR buffer containing 1.2 mM dithiothreitol, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM deoxy-NTPs (Pharmacia, Uppsala, Sweden), 0.5 mM spermidine (Sigma, St. Louis, MO, USA) and 0.2 U Supertaq (Sphearo Q, Leiden, The Netherlands) in a total volume of 25 µl.

The PCR reaction mixtures were preheated at 94°C for 5 min followed by 30 PCR cycles (1 min denaturation at 94°C, 1 min annealing at 58°C for the wild type allele and 1 min annealing at 62°C for the null allele, 1 min extension at 72°C) and a final extension step of 10 min at 72°C. The DNA control PCR was carried out as previously described by Slegtenhorst et al. (Slegtenhorst-Eegdeman *et al.*, 1998). The products were electrophoresed on a 1.5% agarose gel.

Oestrous cycle length

Daily vaginal smears were taken of female mice belonging to the three different genotypes for a continuous period of 40 days, beginning at three to four months of age (n = 10 per group), to assess cycle length and the regularity of the cycle. Dried smears were examined microscopically and the stage of the oestrous cycle was determined according to the criteria of Allen (Allen, 1922). The females were housed individually in cages placed next to a cage containing an adult AMH (+/+) male.

Ovarian histology and follicle counting

Serial 8 µm sections of both ovaries were used for follicle counting. All primordial follicles were counted in every second section. Based on the mean diameter of the follicle, which was determined by measuring two perpendicular diameters in the section in which the nucleolus of the oocyte was present, the growing follicles were divided into two classes, *i.e.* small and large follicles. The small follicle class contains preantral 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 number of large follicles was very small in females of all three ages. This was expected, since in immature 25-day-old animals growing follicles become atretic before they reach this stage of follicular development (Sander *et al.*, 1986) and the cycling animals of 4 and 13 months of age were killed on the day of oestrus, a time point during the oestrous cycle when no large follicles have developed yet (Welschen and Rutte, 1971; Osman, 1985).

Non-atretic and atretic follicles were counted separately. The criteria for atresia were the presence of pyknotic nuclei in the granulosa cells and/or degeneration of the nucleus of the oocyte (Byskov, 1974a; Osman, 1985). For all three genotypes we found degenerating, *i.e.* fragmented oocytes, which are the remnants of atretic follicles (Byskov, 1974a). No diameter could be determined of these follicle remnants because the layer of granulosa cells could not very well be distinguished from the surrounding interstitial tissue. To prevent double counting of these follicle remnants, which we called atretic oocytes, these oocytes were counted in every tenth section. The atretic oocytes were classified as a separate group.

In addition, the total number of fresh corpora lutea was counted in the 4- and 13-month-old animals. Newly formed corpora lutea could be distinguished from the older ones by their smaller size of luteal cells.

Measurements of serum FSH and inhibin

Serum FSH was determined by radioimmunoassay using rat FSH as a ligand and antibodies against ovine FSH (Dullaart *et al.*, 1975). All results are expressed in terms of NIADDK-rat FSH-RP-2. The intra-assay variation was 8.8%, and all samples were measured in one assay.

Serum inhibin-like immunoreactivity was estimated using the method described by Robertson et al. (Robertson *et al.*, 1988), with a bovine follicular fluid preparation with arbitrary potency of 1 U/μg protein as standard (Grootenhuis *et al.*, 1989). The antibody was raised against purified 32 kDa bovine follicular fluid inhibin and cross-reacts with free α subunits. All samples were run in the same assay and the intra-assay coefficient of variation was 8.2%.

Statistical analysis

Results are presented as the mean ± SEM. The data were evaluated for statistical differences by 1-way analysis of variance (ANOVA), followed by a Duncan's new multiple range test using SPSS 7.5 (SPSS Inc., Chicago, IL, USA) computer software. Differences were considered significant at $P \leq 0.05$.

Results

Oestrous cycle length

Vaginal smears were taken daily for a period of 40 days to determine the length and regularity of the oestrous cycle in AMH (+/+), AMH (+/-) and AMH (-/-) female mice. No differences were found between these three groups of mice, in either the length or the regularity of the oestrous cycle. In all three groups some animals showed at least one prolonged oestrous cycle (varying from 8 to 22 days) in which several metoestrus or dioestrus smears were obtained. However, no consistent differences between the three genotypes were observed. In all groups regular oestrous cycles with a length of 4 to 6 days were found (results not shown).

Weights of ovaries and uterus

Uterine and ovarian weights were determined to obtain an indication whether AMH had some effect on development of the uterus and the ovary. Furthermore, the uterine weight is an estrogen-sensitive parameter and thus may also give an indication for ovarian function.

At all ages no statistical difference was found in uterine weight between the three genotypes (Table 2.1). No difference in the weight of the ovaries between the three groups was found at 25 days and 13 months of age. At the age of 4 months, however, the weight of the two ovaries was about 1.8-fold higher in the AMH (-/-) females than in AMH (+/+) mice. It is of interest to note that the ovarian weight of AMH (+/-) females fell in between the weights of the ovaries of AMH (+/+) and AMH (-/-) mice (Figure 2.1).

Table 2.1
Uterine weight in AMH (+/+), AMH (+/-) and AMH (-/-) female mice of 25 days, 4 months and 13 months of age.

Age	Genotype	Uterus weight (mg)
25 days (n = 4)	AMH (+/+)	16.0 ± 3.0
	AMH (+/-)	17.0 ± 3.0
	AMH (-/-)	11.0 ± 1.0
4 months (n = 4)	AMH (+/+)	130.0 ± 8.0
	AMH (+/-)	114.9 ± 9.2
	AMH (-/-)	88.9 ± 7.0
13 months (n = 5)	AMH (+/+)	210.5 ± 32.2
	AMH (+/-)	213.8 ± 18.7
	AMH (-/-)	165.6 ± 24.5

Values represent the mean ± SEM.

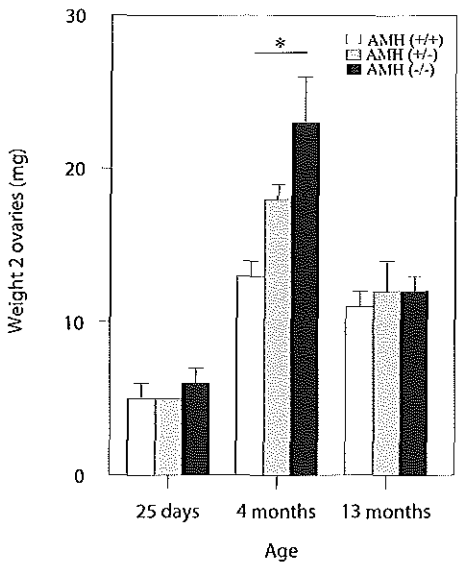

Figure 2.1
Ovarian weight in 25-day-, 4-month- and 13-month-old AMH (-/-), AMH (+/-) and AMH (+/+) female mice.

The combined weight of both ovaries is given. At 4 months of age, ovarian weight was significantly higher in AMH (-/-) females than in AMH (+/+) females.
Data represent the mean ± SEM (n = 4-5). *Asterisk* indicates a statistically significant difference ($P \leq 0.05$).

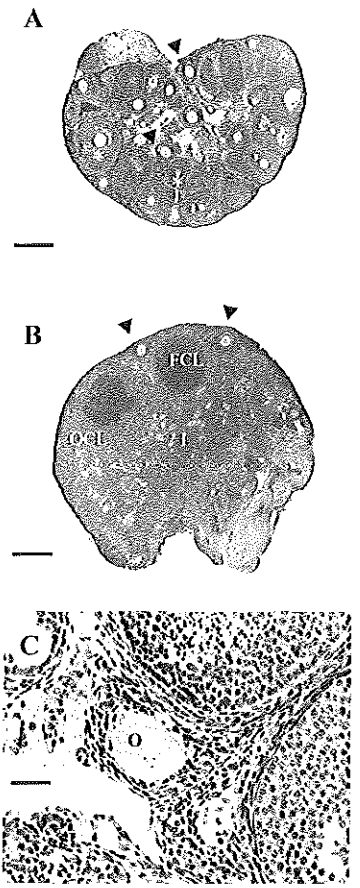

Figure 2.2
Photomicrographs of ovarian histology of AMH (-/-) female mice.

A. Ovary of a 25-day-old AMH (-/-) female mouse. Many non-atretic (arrowhead) and atretic (*asterisk*) small follicles are present, while no corpora lutea can be detected. Scale bar 250 µm.

B. Ovary of a 13-month-old AMH (-/-) female mouse. The ovary mainly consists of interstitium (I), old (OCL) and fresh corpora lutea (FCL). Only a few small follicles are present (arrowhead). Scale bar 250 µmm.

C. Atretic oocyte from a 25-day-old AMH (-/-) mouse ovary. The oocyte (AO) has resumed its meiotic division and has lost its round shape. Around the oocyte no clear granulosa and theca cell layers can be distinguished. Both the granulosa cell layer (GC) and theca cell layer (TC) are present in the follicle adjacent to the atretic oocyte. Scale bar 50 µm.

Ovarian morphology

The ovaries of all genotypes contained primordial, preantral and small antral follicles, both non-atretic and atretic. In 4- and 13-month-old animals both fresh and old corpora lutea were present, indicating an active oestrous cycle. At all ages atretic oocytes were found in the interstitium (Figure 2.2C). Figures 2.2A and 2.2B illustrate that, in a 25-day-old AMH (-/-) mouse ovary many small follicles are found, whereas a 13-month-old AMH (-/-) mouse ovary contains very few small follicles. At this latter age, the main part of the ovary consists of interstitial tissue and corpora lutea. For ovaries of age-matched AMH (+/+) and AMH (+/-) females similar histology was observed, as reported previously by Behringer et al. (Behringer *et al.*, 1994).

Follicle counts

To test the hypothesis that AMH might play a role in follicle growth and development or selection the entire follicle population in 25-day-, 4- and 13-month-old AMH (+/+), AMH (+/-) and AMH (-/-) female mice was determined (Figures 2.3A-C). Note the differences in scale of the y-axis between the different graphs in Figures 2.3A-C.

In 25-day-old females no significant difference in the number of primordial follicles between AMH (+/+), AMH (+/-) and AMH (-/-) females was detected (Figure 2.3A). However, compared to the ovaries of AMH (+/+) females of 4 months and 13 months old, the ovaries of age-matched AMH (-/-) females contained a significantly smaller number of primordial follicles (Figures 2.3B and 2.3C). In fact, at 13 months of age the number of primordial follicles was reduced in AMH (-/-) females to 38 ± 15, while in the AMH (+/+) females the average number of primordial follicles was 225 ± 52 (Figure 2.3C).

For the category of non-atretic small follicles the AMH (-/-) females at the age of 25 days showed a marked increase of approximately 1.5-fold compared to the AMH (+/+) mice (Figure 2.3A). In 4-month-old AMH (-/-) females an approximately 3-fold increase in the number of non-atretic small follicles was found compared to the AMH (+/+) females (Figure 2.3B). This 3-fold increase in follicle numbers was also found for the atretic small follicles. In contrast, significantly less non-atretic and atretic small follicles were counted in the 13-month-old AMH (-/-) females (Figure 2.3C). The number of atretic oocytes in 4-month-old AMH (-/-) females was about 4-fold larger than in AMH (+/+) females (Figure 2.3B). In the three groups of mice at the three different ages an average number of 0.9 ± 0.2 non-atretic large follicles and 0.8 ± 0.2 atretic large follicle were found (results not shown).

In almost all instances, the number of follicles present in AMH (+/-) females fell in between the numbers found in the AMH (+/+) and AMH (-/-) females (Figures 2.3A-C).

Number of fresh corpora lutea

To determine whether there are any differences between the three genotypes in the number of ovulations during the last oestrous cycle, the number of fresh corpora lutea

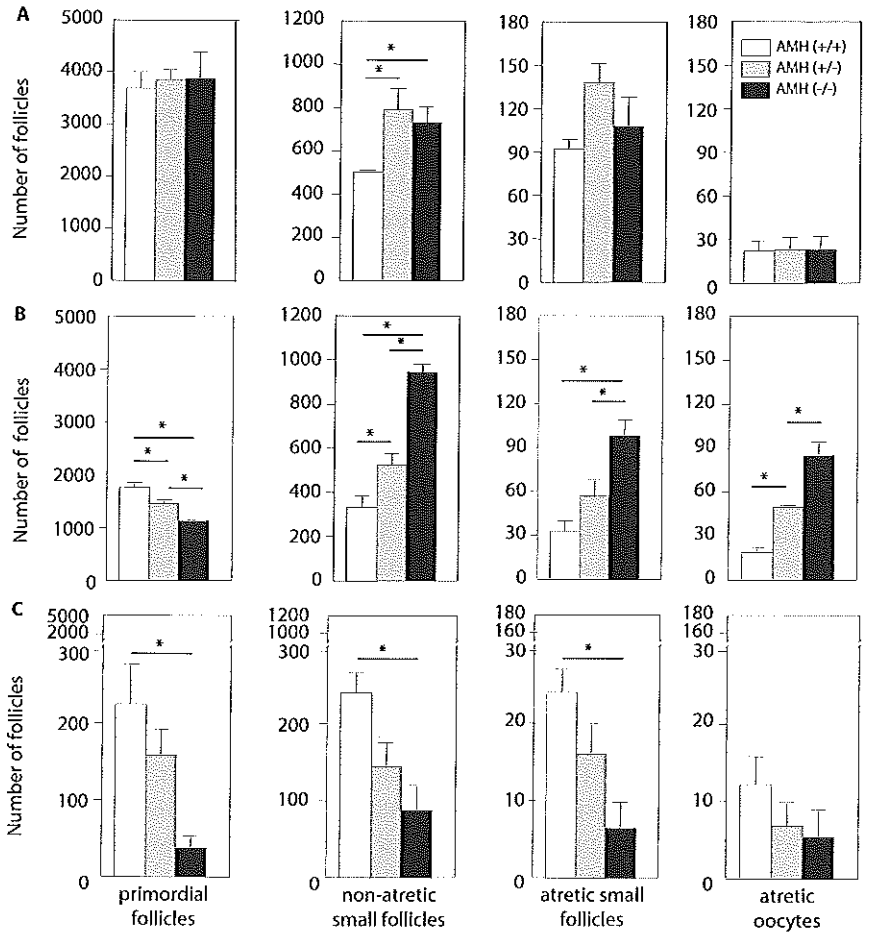

Figure 2.3
Follicle population in 25-day-, 4-month- and 13-month-old AMH (-/-), AMH (+/-) and AMH (+/+) female mice.

A. Follicle population in 25-day old AMH (-/-), AMH (+/-) and AMH (+/+) female mice. Significant more non-atretic small follicles were detected in AMH (+/-) and AMH (-/-) females than in AMH (+/+) females. No significant difference in the number of primordial follicles, atretic small follicles and atretic oocytes was observed between the three groups of mice.

B. Follicle population in 4-month-old AMH (-/-), AMH (+/-) and AMH (+/+) female mice. Significant less primordial follicles and significantly more non-atretic small follicles were found in AMH (-/-) and AMH (+/-) females than in AMH (+/-) females. Significantly more atretic small follicles were found in AMH (-/-) females than in the two other groups of females, while significantly more atretic oocytes were found in AMH (-/-) and AMH (+/-) females than in AMH (+/+) females.

C. Follicle population in 13-month-old AMH (-/-), AMH (+/-) and AMH (+/+) female mice. Significant less primordial and non-atretic and atretic small follicles were found in AMH (-/-) females than in AMH (+/+) females.

Data represent the mean ± SEM (n = 4-5). *Asterisks* indicate a statistically significant difference ($P \leq 0.05$).

Table 2.2
Serum inhibin and FSH levels in AMH (+/+), AMH (+/-) and AMH (-/-) female mice of 25 days, 4 months and 13 months old.

Age	Genotype	Inhibin α-subunit (U/ml)	FSH (ng/ml)
25 days (n = 4)	AMH (+/+)	33.9 ± 5.8	24.1 ± 5.3
	AMH (+/-)	28.9 ± 4.8	17.7 ± 1.5
	AMH (-/-)	25.0 ± 2.2	15.0 ± 1.8
4 months (n = 4)	AMH (+/+)	20.6 ± 1.6	39.7 ± 2.6
	AMH (+/-)	35.2 ± 1.5 [a]	31.5 ± 2.8
	AMH (-/-)	43.5 ± 4.0 [a]	27.9 ± 2.9 [a]
13 months (n = 5)	AMH (+/+)	21.5 ± 1.6	28.8 ± 2.6
	AMH (+/-)	12.6 ± 3.8 [a]	31.3 ± 2.9
	AMH (-/-)	11.5 ± 0.9 [a]	37.9 ± 3.8

Values represent the mean ± SEM.
[a] Indicates a significant difference from AMH (+/+) females in the same age group ($P < 0.05$), evaluated by Duncan's new multiple range test.

were counted in the 4- and 13-month-old females. At both 4 and 13 months of age no significant differences between the three groups of mice were found and an average number of 10 fresh corpora lutea was present (results not shown).

Measurement of serum FSH and inhibin levels

In 4-month-old AMH (-/-) females the serum inhibin level was 2-fold higher than in AMH (+/+) females of the same age, while in 13-month-old AMH (-/-) females the inhibin serum level was 2-fold lower (Table 2.2). No statistically significant difference was found in inhibin values in 25-day-old females of the three genotypes. For the serum FSH level a statistically significant difference was found only for AMH (-/-) females of 4 months old, which showed a decreased level as compared to AMH (+/+) animals (Table 2.2).

Discussion

Although AMH has a clear function during male fetal development (Jost, 1953), its action in the female is still being unravelled. While in the female AMHRII mRNA expression is already present in the ovary and the mesenchymal cells surrounding the Müllerian ducts before birth (Baarends *et al.*, 1994; di Clemente *et al.*, 1994b), AMH mRNA expression is only found after birth in the ovary. Postnatally, both mRNA species are highly expressed in granulosa cells of mainly non-atretic preantral and small antral follicles (Hirobe *et al.*, 1992; Baarends *et al.*, 1995b). This expression pattern does not change during the oestrous cycle, except at oestrus when a more heterogeneous pattern becomes apparent

and the expression of AMH mRNA is lower in some non-atretic large preantral and small antral follicles. This expression pattern may indicate a role for AMH in the regulation of follicle selection or maturation (Baarends *et al.*, 1995b). Such a role is supported by the findings that AMH has inhibitory effects on granulosa cell proliferation (Kim *et al.*, 1992; Seifer *et al.*, 1993), aromatase activity and LH receptor expression (di Clemente *et al.*, 1994a).

In the present study, a possible role of AMH in the ovary was examined by comparing the entire ovarian follicle population of AMH (+/+) mice with the follicle population of AMH (-/-) and AMH (+/-) mice. Ovaries of 25-day-, 4- and 13-month-old females were examined. These ages were chosen to represent three different stages in the reproductive life of the female mouse. At 25 days of age female mice are prepubertal and do not have oestrous cycles, at 4 months of age the females are at the height of their reproductive life and at 13 months of age the females have almost reached reproductive cessation (Gosden *et al.*, 1983).

Upon characterisation of the complete follicle population in AMH (+/+), AMH (+/-) and AMH (-/-) animals, the results indicate that the number of primordial follicles decreases with age in the three groups of mice. However, in AMH (-/-) females the pool of primordial follicles decreases faster than in AMH (+/+) mice. While in 25-day-old females no significant differences were found in the number of primordial follicles between AMH (+/+) and AMH (-/-) females, at 4 and 13 months of age the number of primordial follicles is significantly smaller in AMH (-/-) females. The absence of a difference in the primordial follicle number in prepubertal mice indicates that AMH may not have a role in the pronounced wave of attrition of follicles that occurs before regular oestrous cycles start (Pedersen, 1970). One way by which the smaller number of primordial follicles in AMH (-/-) females of 4 and 13 months old may be explained, is a loss of primordial follicles from the stock through degeneration by attrition. The extent of this phenomenon, however, is difficult to measure since adequate morphological markers of degeneration in primordial follicles are lacking (Edwards *et al.*, 1977). Furthermore, degenerated primordial follicles may be eliminated very rapidly from the ovary, preventing an accurate determination of their number (Hirshfield, 1994). An alternative explanation for the smaller number of primordial follicles found in 4- and 13-month-old AMH (-/-) females compared to AMH (+/+) females, could be an increased number of oestrous cycles within a certain time period in AMH (-/-) females. However, no difference in the length or the regularity of the oestrous cycle between the three genotypes at 3-4 months of age was detected. Yet another explanation would be that in AMH (-/-) animals more primordial follicles are recruited to enter the pool of growing follicles. This explanation is favoured on the basis of the present observations of increased numbers of non-atretic and atretic small follicles in AMH (-/-) females of 25 days and 4 months old compared to the age-matched AMH (+/+) control mice.

The larger number of small follicles in 4-month-old AMH (-/-) females might lead to a larger number of ovulations per cycle and subsequently to larger litters. However, we have not found any difference in size of the litters derived from AMH (-/-), AMH (+/-) or AMH (+/+) females (results not shown), which is consistent with the previously pub-

lished report (Behringer *et al.*, 1994). However, to exclude the possibility of increased embryonic death *in utero* in 4-month-old AMH (-/-) females, we determined the number of ovulations by counting the number of fresh corpora lutea, which emerge from the preovulatory follicles after recent ovulation. Again no difference was detected in the number of fresh corpora lutea between the ovaries of 4-month-old AMH (-/-) females and the age-matched females from the two other groups, which excludes a higher ovulation rate in the absence of AMH. These results suggest that in the absence of AMH action increased atresia of small follicles may result in loss of an increased percentage of growing follicles, so that finally the number of these follicles which reach the preovulatory stage is approximately equal in AMH (-/-) and AMH (+/+) mice. Indeed, in 4-month-old AMH (-/-) ovaries we found significantly more atretic small follicles than in AMH (+/+) ovaries. However, the number of non-atretic small follicles showed a similar increase. Thus, in the AMH (-/-) females more atretic small follicles are found probably because in these animals more non-atretic small follicles are being produced. In addition, the number of atretic oocytes, which are thought to arise from atretic follicles, is larger in AMH (-/-) females of 4 months of age, indicating that in AMH (-/-) females the larger number of small non-atretic follicles is compensated by more atretic follicles. Thus, increased atresia results in similar numbers of preovulatory follicles and ovulations in AMH (-/-) and AMH (+/+) female mice. Higher rates of atresia may also exist at other days of the cycle, which we have not studied, and therefore further studies are necessary to elucidate this point.

From 4 to 13 months of age the number of non-atretic small follicles in AMH (-/-) females shows a marked decline. This is in accordance with the very small number of primordial follicles that are still present in the ovaries of 13-month-old AMH (-/-) females. This smaller number of non-atretic small follicles is not associated with a smaller number of fresh corpora lutea still being formed in these females. This may be explained by more efficient growth of follicles to the preovulatory stage as indicated by the significantly lower percentage of atretic small follicles in the 13-month-old AMH (-/-) females.

It is of interest to note that, in almost all animals the number of follicles per class in the AMH (+/-) females fell in between the numbers found for AMH (+/+) and AMH (-/-) females. For other gene knockout animal models, such as activin null mice or FSH null mice (Kumar *et al.*, 1997; Matzuk *et al.*, 1995), such a gene dosage effect has not been reported. Moreover, Müllerian duct regression in male AMH (+/-) mice occurs to the same extent as in AMH (+/+) males (Behringer *et al.*, 1994). The AMH gene dose dependency observed in the present study may indicate that ovarian AMH production or secretion is not under stringent feedback control, but rather depends on the intrinsic activity of the gene itself.

The results presented herein indicate that AMH exerts an inhibitory effect on recruitment of primordial follicles into the pool of growing follicles. A direct effect of AMH on the primordial follicles themselves cannot be excluded, although no information is available which clearly demonstrates AMH type II receptor expression in these follicles. Alternatively, the primary action of AMH may be autocrine in nature, since granulosa cells of small follicles express both AMH and its type II receptor. The small follicles may be stimulated by AMH to produce (a) factor(s) which act(s) on primordial follicles.

Little is known about the factors that control the release of primordial follicles from their quiescent state. Primordial follicles can be considered as the ovarian follicle stock and this reserve of dormant follicles is not renewable (Gosden *et al.*, 1983). Throughout neonatal life and continuing through the reproductive life span, follicles leave the stock of primordial follicles in a continuous stream and begin to grow, thus depleting the original pool of primordial follicles with age (Gosden *et al.*, 1983; Hirshfield, 1994). There are indications that the recruitment of primordial follicles into the growing pool is influenced by the total size of the non-growing pool of primordial follicles itself (Cran and Moor, 1980). Thus, a decrease in the number of remaining primordial follicles is accompanied by an increase in their depletion rate (Hirshfield, 1994). Despite the fact that the gonadotropins FSH and LH predominantly act on recruited, antral follicles (Hirshfield, 1991), several studies show that gonadotropins influence the size of the primordial follicle pool. Hypophysectomy, for example, slows the loss of primordial follicles implicating a role of pituitary factors, such as the gonadotropins, in the control of growth initiation of primordial follicles (Meredith *et al.*, 1986). FSH may accelerate the initiation of the growth of primordial follicles. Unilateral ovariectomy in aged rats causes increased FSH serum levels and concomitant increased loss of primordial follicles (Meredith *et al.*, 1992), while in ageing rats, where primordial follicles are lost at an accelerated rate (Richardson *et al.*, 1987), both LH and FSH levels are increased compared to the levels in young females (Marcus *et al.*, 1993). In transgenic mice that maintain a chronically elevated serum LH level, the primordial follicle pool is depleted faster (Flaws *et al.*, 1997). A direct effect of FSH and LH on the primordial follicles themselves, however, appears to be unlikely, since several studies were unsuccessful in detecting full-length LH and FSH receptor mRNA expression in primordial follicles (Teerds and Dorrington, 1995; O'Shaughnessy *et al.*, 1996; Oktay *et al.*, 1997; O'Shaughnessy *et al.*, 1997). In contrast, gonadotropins are not essential for the initiation of primordial follicle growth. In FSH null females primordial follicles are recruited and grow into preantral follicles (Kumar *et al.*, 1997), and primordial follicle recruitment also occurs in cultured new-born mouse ovaries without the addition of gonadotropins to the culture medium (Eppig and O'Brien, 1996).

Despite their advanced age and the small number of primordial follicles present in their ovaries, we found that the 13-month-old females of the three different genotypes had not yet reached the period of acyclicity. Although the oestrous cycle in these animals was not checked with the help of vaginal smears, we conclude this from the fact that in the ovaries of 13-month-old females an average of 10 fresh corpora lutea was found, which is the same average number as found in 4-month-old females.

The ovaries from 4-month-old AMH (-/-) mice were heavier than the ovaries from control animals. Although most often the total mass of the corpora lutea largely determines the weight of an ovary, the number of corpora lutea was similar in the animals of the three genotypes, suggesting that the increase in ovarian weight in the AMH (-/-) females is caused by the 3-fold larger number of small follicles. In 25-day- and 13-month-old animals no significant differences were observed in ovarian weight between the three genotypes, which is in line with the much smaller differences in the number of small follicles

found in these animals. No difference was found between the three groups of mice for the uterine weight, which indicates that the estrogen levels in the AMH (+/+), AMH (+/-) and AMH (-/-) are similar.

The large number of small follicles in the 4-month-old AMH (-/-) mice may also explain the changes in serum levels of FSH (decrease) and inhibin α-subunit (increase) in these animals. In mouse preantral follicles, both inhibin A and inhibin B are produced (O'Shaughnessy and Gray, 1995; Smitz and Cortvrindt, 1998), while small antral follicles mainly produce inhibin B and large antral follicles inhibin A (Merchenthaler *et al.*, 1987; Meunier *et al.*, 1988). Despite the fact that only the inhibin α-subunit level was measured in the serum samples, we suggest that the increased number of small follicles in the AMH (-/-) females is also responsible for an increase in bio-active dimeric inhibin A or B, which in turn suppresses both FSH synthesis and FSH release from the pituitary gland (De Jong, 1988). The reduced FSH level in AMH (-/-) females of 4 months of age might prevent the larger number of small antral follicles in these animals to develop to the preovulatory follicle stage. Indeed, if the FSH level was not lower in AMH (-/-) females, an increase in litter size in these mice might have been expected. The larger number of growing follicles accompanied by a decrease in FSH is an intriguing observation, since FSH stimulates preantral follicle development (Arendsen de Wolff-Exalto, 1982; Van Cappellen *et al.*, 1989; McGee *et al.*, 1997), although the vast majority of the effects of FSH are on the recruited, antral pool of follicles (Hirshfield, 1991). A possible explanation might be that small follicles might be more sensitive to FSH in the absence of AMH. Alternatively, AMH may be instrumental in inhibition of the production and/or activity of (a) factor(s) important for preantral follicle development. Candidates for such factors are GDF9 (growth and differentiation factor 9) and BMP15 (bone morphogenetic protein 15), which both are members of the TGFβ superfamily. GDF9 is an oocyte-specific growth factor and in mice lacking GDF9 there is an early block in folliculogenesis at the one-layer primary follicle stage (Dong *et al.*, 1996). Recently it has been shown that recombinant GDF9 is able to enhance growth and differentiation of cultured small preantral follicles (Hayashi *et al.*, 1999). BMP15 is most closely related to and shares a coincident expression pattern with GDF9, which could imply that also BMP15 is necessary for preantral follicle development (Dube *et al.*, 1998). Thus, AMH might have an effect on expression of GDF9 and/or BMP15, thereby influencing preantral follicle development.

In conclusion, the study of ovaries of AMH null mice indicates that ovarian AMH directly or indirectly prevents or inhibits primordial follicles to enter the pool of growing follicles. As impaired recruitment of primordial follicles may be the underlying cause of human diseases such as premature ovarian failure (Christin-Maitre *et al.*, 1998), it will be of great interest to study the role of AMH in the physiology of the human ovary.

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Chapter 3

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

Abstract

Recruitment of primordial follicles is essential for female fertility, but the exact mechanisms of regulation are largely unknown. From earlier studies in anti-Müllerian hormone-deficient mice, it was concluded that anti-Müllerian hormone (AMH) might be involved in the regulation of primordial follicle recruitment. In the present study, we tested this hypothesis by culturing ovaries from 2-day-old C57Bl/6J mice for 2 or 4 days in the presence or absence of AMH. Ovaries of 2-day-old mice contain many primordial follicles and some naked oocytes, but no follicles at later stages of development. Immunohistochemistry showed that both AMH and inhibin α-subunit protein are first found in granulosa cells of primary follicles of 4-day-old ovaries, which is almost immediately after follicle growth is initiated. Using histology and immunohistochemistry, it was revealed that follicular development occurred to the same extent in cultured ovaries, as in in vivo *ovaries of comparable age. Furthermore, AMH type II receptor (AMHRII) mRNA expression was found in both cultured ovaries and freshly isolated ovaries of 2-, 4- and 6-day-old mice. After 2 days of culture, the number of growing follicles found in ovaries cultured in the presence of AMH was reduced to approximately 59 % of the number in ovaries cultured in the absence of AMH, and this reduction reached 66 % after 4 days of culture. This observation was supported by a lower inhibin α-subunit mRNA expression level in AMH-treated ovaries, compared to ovaries cultured without AMH. AMHRII mRNA expression level was not influenced by AMH.*

In conclusion, in cultured neonatal mouse ovaries, AMH is able to inhibit initiation of primordial follicle growth. This is the first ovarian growth factor for which such an inhibiting effect has been demonstrated.

INTRODUCTION

In mammals, the pool of primordial follicles is established during foetal development (human) (Peters, 1969a) or just after birth (mouse, rat) (Peters, 1978; Ueno *et al.*, 1989a), and this pool of primordial follicles constitutes the complete supply of oocytes available to a female during the remainder of her reproductive life. Factors that directly control the initiation of primordial follicle growth, a process also known as initial recruitment, remain largely unknown, although it was recently shown that stem cell factor (SCF, or Kit-ligand) can induce primordial follicle growth in the rat ovary (Parrott and Skinner, 1999).

Study of anti-Müllerian hormone-deficient ovaries revealed that also anti-Müllerian hormone (AMH) is involved in regulation of primordial follicle recruitment (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 foetal development (Jost, 1947). In the female, mRNAs encoding AMH and its type II receptor (AMHRII) are expressed postnatally by granulosa cells of mainly non-atretic preantral and small antral follicles (Hirobe *et al.*, 1992; Baarends *et al.*, 1995b). In addition, AMHRII mRNA expression is also found in the prenatal ovary (Baarends *et al.*, 1994). In cultured rat granulosa cells exogenous AMH inhibits biosynthesis of aromatase and decreases LH receptor number (di Clemente *et al.*, 1994a). Furthermore, AMH opposes the proliferation of cultured granulosa-luteal cells (Kim *et al.*, 1992; Seifer *et al.*, 1993). More insight in the ovarian function of AMH was gained after examination of the follicle population of AMH-deficient (AMHKO) female mice. The ovaries of these female mice contain less primordial follicles and more growing follicles than the ovaries of their wild type littermates. Therefore, it was concluded that AMH might inhibit primordial follicle recruitment (Durlinger *et al.*, 1999).

The present study examined the ability of AMH to inhibit primordial follicle recruitment in cultured neonatal mouse ovaries. Cultured ovaries were compared to *in vivo* ovaries of corresponding ages. To evaluate the quality of the cultured ovaries, AMH and inhibin α-subunit protein expression were compared between freshly isolated and cultured ovaries. Primordial follicle recruitment was characterised by determining the number of primordial and growing follicles in the neonatal ovaries after 2 and 4 days of culture in the presence or absence of added AMH. In addition, induction of primordial follicle growth was investigated by determining AMHRII and inhibin α-subunit mRNA expression in both cultured and freshly isolated ovaries, while also the expression of mRNAs encoding oocyte-specific markers growth and differentiation factor 9 (GDF9) and zona pellucida protein 3 (ZP3) were investigated in these ovaries.

The present study revealed that AMH is an important inhibitor of primordial follicle recruitment.

MATERIALS AND METHODS

AMH production

In the ovarian culture experiments recombinant rat AMH was used. Human embryonic kidney 293 (HEK293) cells were stably transfected with a cDNA encoding His-tagged rat AMH inserted in the pRrCMV expression vector, The AMH cDNA contained an optimised cleavage site that yields maximal amounts of cleaved mature recombinant 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% (v/v) fetal calf serum (FCS), penicillin (400 IU/ml), streptomycin (0.4 mg/ml) and neomycine 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 concentrated approximately 35-fold using a Centriprep10 filter system (Millipore Corp., Bedford, MA, USA). Proteins with a molecular mass above 10 kDa were concentrated.

The amount of AMH was measured by an ELISA, using the Biorad TMB Peroxidase EIA substrate kit (Biorad, Life Science Groups, Hercules, CA, USA). The primary antibody, pentaHis monoclonal antibody (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, USA), was used in a 1:1000 dilution. The amount of AMH was calibrated in every ELISA using a constant, standard preparation of His-tagged AMH. The amount of AMH used for calibration was estimated using SDS PAGE (4-15% Tris-HCl precast gel) (Biorad) and the 6x His-protein ladder (Qiagen), followed by western blotting and visualisation of the His-tag using the pentaHis monoclonal antibody. The concentradted supernatant of wild type HEK293 cells was used as the 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 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 1:2000 dilution and a secondary peroxidase-conjugated mouse-anti-goat/sheep antibody (Sigma) in a dilution of 1:10000.

The AMH preparations were shown to be fully bioactive using a Müllerian duct regression assay (Nachtigal and Ingraham, 1996) (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 (Figure 3.1). 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 fluid for morpho-

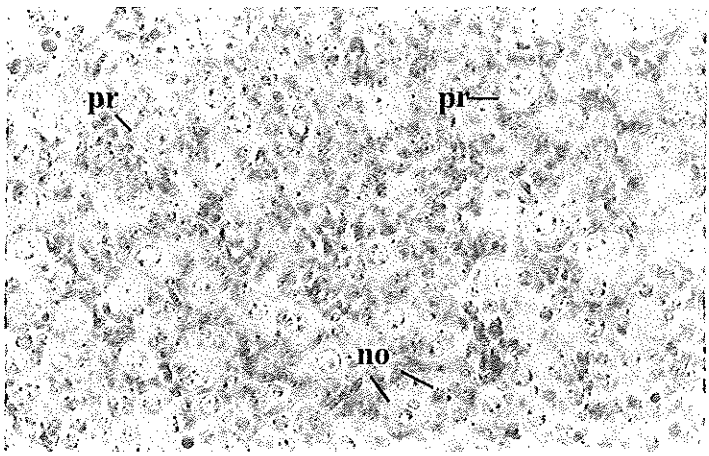

Figure 3.1
Photomicrograph of a 2-day-old mouse ovary.

Many primordial follicles (pr) and some "naked" oocytes (no) are present. A "naked" oocyte is a small oocyte, which is not surrounded by pregranulosa cells, while a primordial follicle consists of a small oocyte that is surrounded by one layer of pregranulosa cells. Magnification x 400.

logical 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, USA). Each well contained 0.5 ml DMEM/F-12 medium with GLUTAMAX-I (Gibco) supplemented with 1% (v/v) charcoal-stripped fetal calf serum (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, USA) 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 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 37°C 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

ng/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 fluid or snap frozen in liquid nitrogen and stored at -80 C. Ovaries of 2-, 4- and 6-day-old C57Bl/6J mice were used for comparison of the development of follicles in the culture experiments.

Ovarian histology and follicle counting

To investigate the development of ovarian follicles in the cultured ovaries, the ovaries were fixed for 2 h in Bouin's fluid, embedded in paraffin after routine histological procedures, and 8 mm sections were mounted on slides and stained with hematoxylin and eosin.

Primordial follicles are in rest 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 ≤ 20 µm were classified as primordial follicles, while follicles with a mean diameter > 20 µm were classified as growing follicles (primary and secondary follicles). The mean diameter of the follicles was determined by measuring two perpendicular diameters (Van Cappellen *et al.*, 1989). All follicles were counted in every second section. We did not attempt to distinguish between non-atretic and atretic follicles, since at this stage of follicle development, atresia is not immediately 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 postnatal 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 in the absence or presence of AMH. 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 mm sections were made. Sections were mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma). After deparaffinisation, sections were quenched for 20 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 37°C. For AMH immunohistochemistry the preincubation step was followed by

incubation at 4°C overnight with primary polyclonal antibody MIS (C-20; Santa Cruz), diluted 1:1000 in 5% BSA in PBS. The antibody is raised against a peptide mapping at the carboxy terminus of human AMH, which differs from corresponding mouse sequence, by a single amino acid. For inhibin α-subunit immunohistochemistry the preincubation step was followed by incubation at 37°C 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. Hematoxylin 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

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 -80°C. For each condition total RNA was isolated from 20 ovaries 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 -80°C.

RNase protection assay

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'-GACTTCCACGGTTGCCTTG-3', annealing to nucleotide sequence 735-753 of the *Zp3* gene (numbering according to GenBank sequence), and 5'-GCAGTCCAG-CCTTCC-ACAG-3', annealing to antisense sequence 1174-1156, a 440bp fragment was generated. For inhibin α-subunit PCR reaction primers 5'-GCCATCCCAACA-CATACG-3', annealing to sense sequence 374-391, and 5'-GAAACTGGGAGGGTGTACG-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 [^{32}P]-UTP-labelled 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'-CTGCCATGGAACACTTGCTC-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-labelled anti-sense RNA probe. Mouse AMHRII anti-sense probe was generated as described before (Visser *et al.*, 1998). The control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA probe was synthesised using a construct containing 163 bp AccI-Sau3AI fragment of the rat GAPDH cDNA.

RNase protection assays of total RNA, extracted from fresh and cultured mouse ovaries, were performed as described by Baarends (Baarends *et al.*, 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 arbitrary units are expressed as ratios after division by the corresponding GAPDH values.

In the assay, 5 µl of the RNA samples was hybridised 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. Two separate RNase protection assays were performed.

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, USA) 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 3.2 shows the result of this analysis. No AMH is found in lane 1, containing concentrated control medium, whereas two bands representing AMH protein is found 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

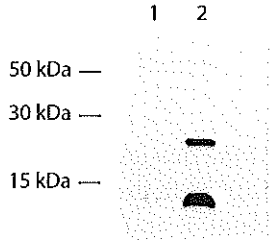

Figure 3.2
Western blot analysis of concentrated wild type HEK293 and rat AMH-producing HEK293 cells.

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).

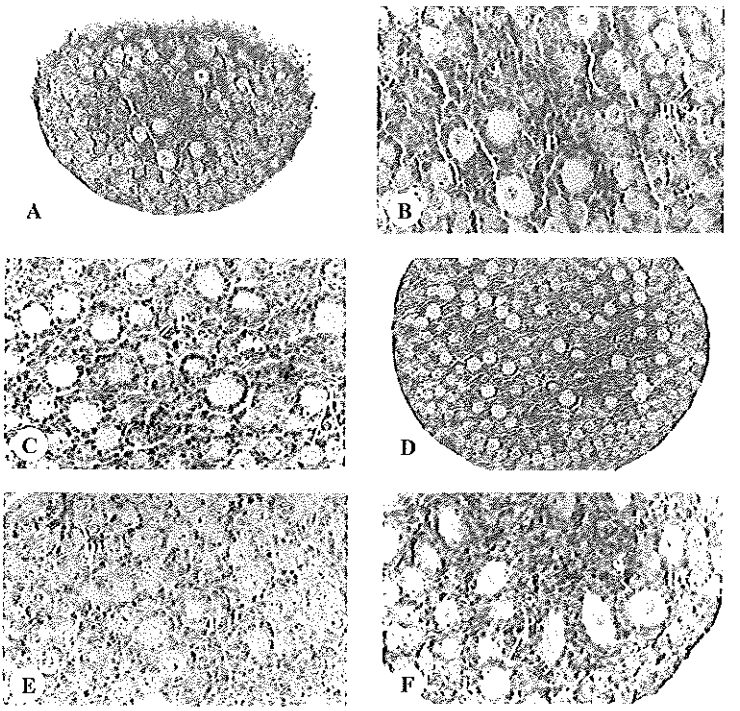

Figure 3.3
Photomicrographs of cultured and non-cultured neonatal mouse ovaries.

A-B. Ovary of a 2-day-old mouse cultured for 2 days in the presence of exogenous AMH (A). The present culture conditions had no detrimental effect on tissue quality, since almost no necrotic and apoptotic cells were found. Magnification x 200. After 2 days of culture the ovaries contained many primordial follicles (pr) and primary follicles (P) of different sizes (B). Magnification x 400.

C-D. Ovary of a 2-day-old mouse cultured for 4 days in the presence of exogenous AMH. In addition to primordial (pr) and primary (P) follicles, also some early secondary follicles are found (S) (C). Magnification x 400. 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 (D). Magnification x 200.

E. Ovary of a 4-day-old mouse. Many primordial (pr) and some primary (P) follicles are found. Magnification x 400.

F. Ovary of a 6-day-old mouse. Besides primordial (pr) and primary (P) follicles, also some early secondary (S) follicles are present. Magnification x 400.

kDa). The absence of larger bands indicates complete cleavage of the proform of AMH.

Ovary organ culture and ovarian morphology

The culture system was evaluated by comparing the morphology of the ovaries that were cultured for either 2 or 4 days in the presence or absence of added AMH with the morphology of *in vivo* ovaries of corresponding age, which were isolated from 4- and 6-day-old mice, respectively.

Histological examination of the ovaries after 2 or 4 days of culture indicated that the

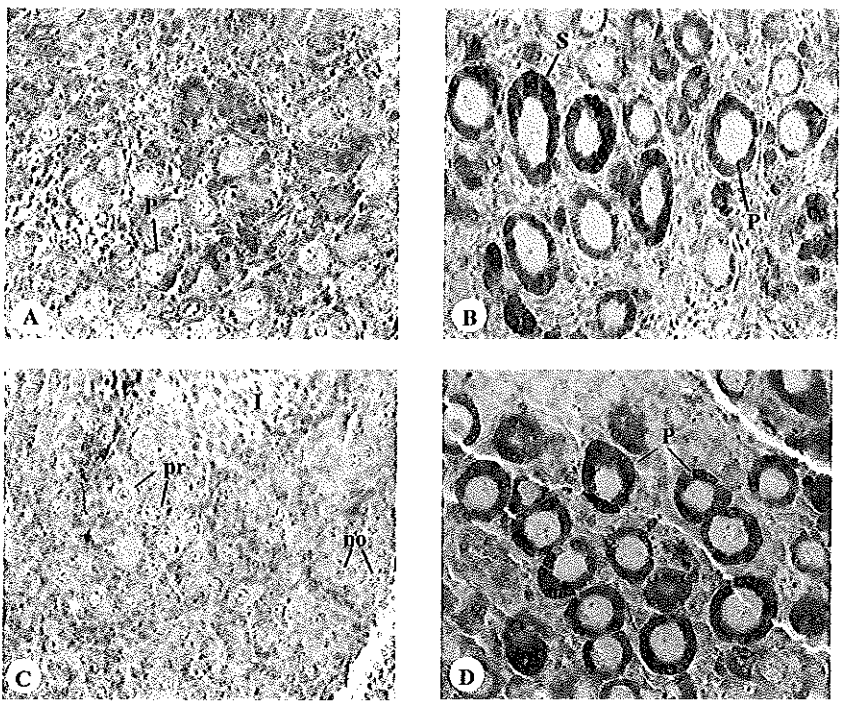

Figure 3.4
Immunohistochemical localisation of AMH in freshly isolated and cultured neonatal C57Bl/6J mouse ovaries.

A. Section of a 4-day-old mouse ovary. AMH protein expression is found in several granulosa cells of primary follicles (P). Some background staining is present in oocytes and interstitial cells. Magnification x 400.

B. Section of a 6-day-old mouse ovary. Abundant AMH protein expression is found in granulosa cells of primary follicles (P) and early secondary follicles (S). Magnification x 400.

C. Section of a 2-day-old mouse ovary. No positive staining is found in naked oocytes (no), primordial follicles (pr) and interstitial tissue (I). Magnification x 400.

D. Section of a 2-day-old mouse ovary cultured for 2 days in the presence of added AMH. High AMH protein expression is found in granulosa cells of primary follicles (P). Magnification x 400.

culture conditions had no pronounced detrimental effect on tissue quality. Almost no necrosis or apoptotic cells were found (Figure 3.3A). After 2 days of culture the ovaries contained many primordial follicles but also some primary follicles of different sizes (Figure 3.3B), while after 4 days of culture, the ovaries contained in addition to primordial and many primary follicles also some early secondary follicles (Figure 3.3C). Most primordial follicles were located in the cortical part of the ovary, while the primary and secondary follicles (taken together referred to as growing follicles) were mostly found in the central part of the ovary (Figure 3.3D). Primary follicles in ovaries cultured for 2 days reached a larger diameter than primary follicles in 4-day-old ovaries (compare Figure 3.3B

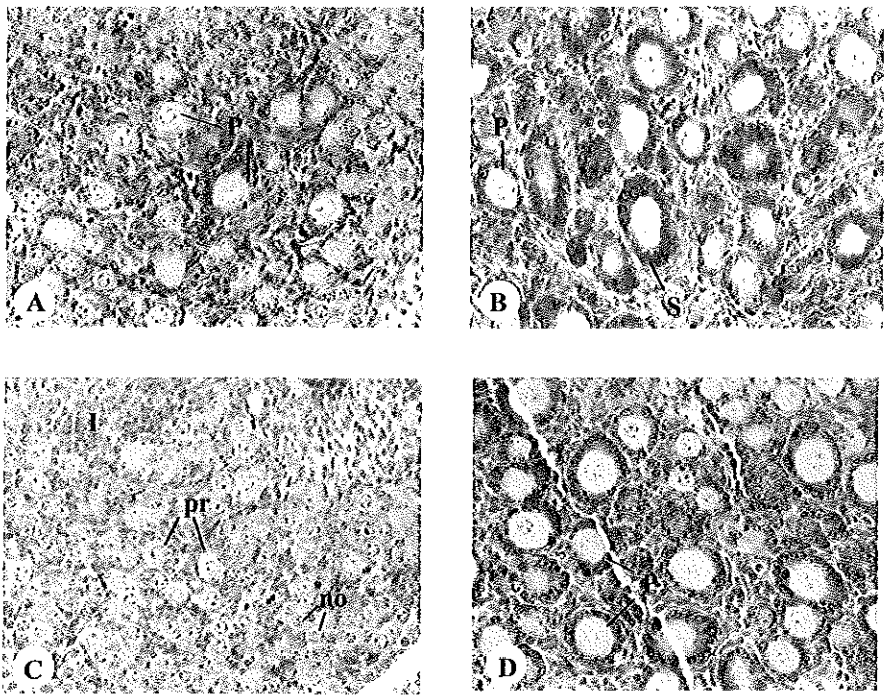

Figure 3.5
Immunohistochemical localisation of inhibin α-subunit in freshly isolated and cultured neonatal C57B/6J mouse ovaries.

A. Section of an ovary of a 4-day-old mouse. Low inhibin α-subunit protein expression is found in granulosa cells of primary follicles (P). Some background staining is found in the oocytes. Magnification x 400.

B. Section of an ovary of a 6-day-old mouse. High inhibin α-subunit protein expression is found in the granulosa cells of primary follicles (P) and early secondary follicles (S). Magnification x 400.

C. Section of an ovary of a 2-day-old mouse. No inhibin α-subunit protein expression is found in naked oocytes (no), primordial follicles (pr) and interstitial tissue (I). Magnification x 400.

D. Section of an ovary of a 2-day-old mouse cultured for 2 days in the presence of added AMH. High inhibin α-subunit protein expression is found in the granulosa cells of primary follicles (P). Magnification x 400

with Figure 3.3E), while follicle development in ovaries cultured for 4 days was not different from ovaries from 6-day-old mice (compare Figure 3.3C with Figure 3.3F).

AMH and inhibin α-subunit immunohistochemistry

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

AMH protein expression was first found in a few granulosa cells of primary follicles, from 4-day-old mice (Figure 3.4A). In larger primary follicles all granulosa cells show AMH protein expression. High AMH protein expression is found in the granulosa cells of primary and early secondary follicles from 6-day-old mice (Figure 3.4B). No positive immunostaining is found in naked oocytes, primordial follicles or interstitial tissue (Figure 3.4C). The AMH expression pattern was identical in the cultured ovaries as in the freshly isolated ovaries, *i.e.* expression was found in granulosa cells of primary and secondary follicles. However, since primary follicles reached a larger diameter in 2-day-old ovaries cultured for 2 days the expression level of AMH was higher than that in fresh ovaries of 4-day-old animals (compare Figures 3.4A and D). 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). Some background staining was found in the oocytes and in the interstitium (Figure 3.4A).

The inhibin α-subunit protein expression pattern was identical to the AMH protein expression (Figure 3.5A-D).

No difference was found in expression level or expression pattern of both AMH and inhibin α-subunit between ovaries cultured in the presence or absence of added AMH (results not shown).

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 determined in ovaries that were cultured for 2 or 4 days in the absence or presence of added AMH.

No difference was found in the total number of follicles per ovary, when the ovaries were derived from the same female (results not shown). However, some variation did occur between ovaries isolated from different animals. This inter-animal variation was mainly due to a difference in the number of primordial follicles. Furthermore, we found that removal of the peri-ovarian sac resulted in loss of primordial follicles, since approximately 3000 primordial follicles were found in 2-day-old ovaries with the peri-ovarian sac in place, and about 1400 after removal of the peri-ovarian sac (N = 2). 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

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.

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

The numbers of follicles found in two independent experiments are pooled and shown as mean ± SEM ($P<0.05$).

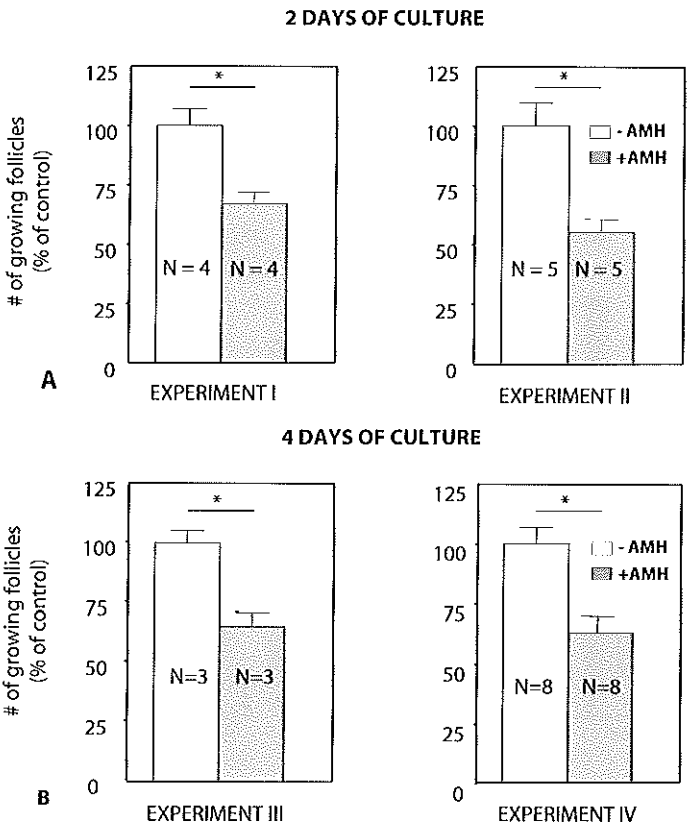

Figure 3.6
Number of growing follicles in 2-day-old mouse ovaries cultured for 2 days (A) or 4 days (B) in the absence or presence of AMH.

Data of four independent experiments are shown (I-IV). The number of growing follicles found in ovaries cultured in the presence of AMH are 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).

of culture (1401 ± 79).

Table 3.1 shows the number of primordial and growing follicles after 2 and 4 days of culture in the presence or absence of added AMH. After 2 and 4 days of culture, significantly less growing follicles were found in the AMH-treated ovaries compared to the control ovaries. After normalisation, we found that after 2 days of culture the number of

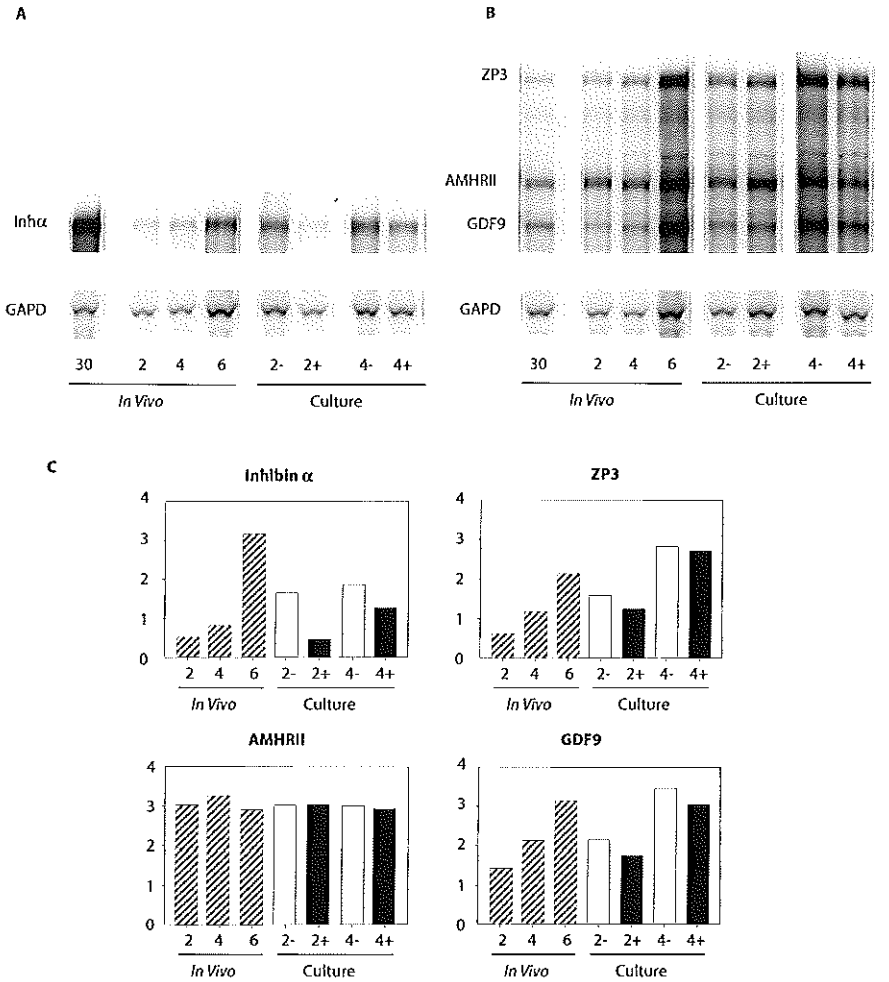

Figure 3.7
Effect of AMH on expression of GDF9, ZP3, AMHRII and inhibin α-subunit mRNAs.

A-B. Phosphorlmage 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 mice, and in 2-day-old ovaries cultured for 2 or 4 days in the presence or absence of added 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 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 normalised to that of GAPDH mRNA.

growing follicles cultured in the presence of added MH was approximately 59 ± 4 % of the control (Figure 3.6A), while after 4 days of culture this was approximately 66 ± 6 % (Figure 3.6B).

After 2 days of culture, for both treatment groups, most growing follicles reached a diameter of 30 mm 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.

RNase protection assay

The mRNA expression level of the granulosa cell markers, inhibin α-subunit and AMHRII, and of the oocyte-specific markers, GDF-9 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.

PhosphorImage analysis of the RNase protection assay is shown in Figures 3.7A and 3.7B. 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 3.7C). 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 73% was found, compared to the ovaries cultured in the absence of AMH. After 4 days of culture this decrease was 32% (Figure 3.7C).

Both oocyte-specific markers (GDF9 and ZP) showed an increased expression in the freshly isolated ovaries from day 2 to day 6, while no clear difference was found in mRNA expression level between ovaries cultured in the presence or absence of added AMH (Figure 3.7C).

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 and even for years in the human. 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 stem cell factor (SCF) can stimulate the recruitment of primordial follicles (Parrott and Skinner, 1999). This is the first study in which it was shown that an ovarian growth factor plays an important role in initiation of primordial follicle growth. In our study of ovaries from AMH-deficient females it was suggested that AMH also 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 an *in vitro* 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), certain primordial follicles located in the central part of the ovary are the first to initiate growth.

After 2 and 4 days of culture, less growing follicles were found in ovaries cultured in the presence of added 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 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.

AMH indeed inhibits the recruitment of primordial follicles, rather than causing deceleration of early primary follicle growth, since no difference in follicle diameter was observed after culture in the absence or presence of added AMH was found. 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 showed that AMHRII mRNA expression, essential for an effect of AMH, is present in ovaries of 2-day-old mice. In the rat , ovarian AMHRII mRNA expression is even found before birth (Baarends *et al.*, 1995b). In addition, *in situ* hybridisation studies showed ovary-specific AMHRII expression in foetal ovaries, although exact localisation of the expression to the pregranulosa cells was not possible (Baarends *et al.*, 1995b) (Durlinger *et al.*, unpublished observations). At later stages of ovarian development, AMHRII expression is limited to the granulosa cells of mainly non-atretic preantral and small antral follicles (Baarends *et al.*, 1995b) (Durlinger *et al.*, unpublished observations).

Not only AMH but also inhibin α-subunit protein is produced, almost as soon as primordial follicle growth is initiated. RNase protection assay showed that both after 2 and 4 days of culture inhibin α-subunit expression level is decreased in ovaries cultured in the presence of added AMH, supporting the hypothesis that AMH inhibits initiation of primordial follicle growth.

A functional role for the early production of inhibin α-subunit is not clear. More insight in the role of inhibin in ovarian follicle development could come from detailed analysis of the follicle population of inhibin-deficient females, before they develop ovarian tumours (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, independent of the size of the pool of primordial follicles. This observation is in accordance with the hypothesis that a reduced

number of primordial follicles leads to an increased rate of loss of the remaining primordial follicles, or in other words, that the number of growing follicles is inversely correlated with the number of primordial follicles present in the ovary (Krarup *et al.*, 1969). Several studies support this hypothesis. In an *in vitro* study in which prenatal and neonatal mouse ovaries were placed in culture it was also shown that the number of primordial follicles that start to grow is unrelated to the number of primordial follicles present in the ovary (Byskov *et al.*, 1997). Also destruction of primordial germ cells by busulphan, thereby reducing the number of primordial follicles, results in an earlier exhaustion of the primordial follicle pool (Hirshfield, 1994), again indicating that the number of primordial follicles that initiate growth is independent of the size of the primordial follicle pool.

Although the amount of AMH (800 ng/ml) used in the culture experiments causes complete Müllerian duct regression *in vitro* (900 ng/ml) (Nachtigal and Ingraham, 1996) (Durlinger *et al.*, unpublished observations), the outgrowth of primordial follicles was not completely blocked. Furthermore, in AMH-deficient females not all primordial follicles initiated growth. These observations indicate that AMH is not the only regulator of initial recruitment. Indeed, the stimulatory effect of SCF on the initiation of primordial follicle growth (Parrott and Skinner, 1999) shows that primordial follicle growth is under the influence of both stimulatory and inhibitory regulation. It would be interesting to culture neonatal ovaries in the presence of both SCF and AMH to see whether the positive effect of SCF on initial recruitment is abolished by AMH. Besides AMH and SCF, probably many other, as yet unidentified, factors are involved, whose role in initial recruitment is still not established. A possible candidate might be GDF9, another member of the TGFβ family of growth and differentiation factors. GDF9 is found in oocytes of early ovarian follicles from the primary stage onward (Hayashi *et al.*, 1999). GDF9 is essential for follicle development beyond the primary stage, since GDF9-deficient females show a block in folliculogenesis at this stage (Dong *et al.*, 1996). Whether GDF9 also has an effect on the pool of primordial follicles is still not clear, since in GDF9-deficient animals no estimation of the size of the primordial follicle pool was made. An *in vitro* study in which neonatal rat ovaries were cultured in the presence or absence of GDF9, however, showed that GDF9 increased inhibin α-subunit expression (Hayashi *et al.*, 1999). Since inhibin α-subunit is a marker for early follicle growth, this might indicate that GDF9 stimulates initial recruitment. However, this result needs to be supported by actual follicle counts. A search for receptors of hormones and growth factors expressed by either the oocyte or the pregranulosa cells of the primordial follicles might help to get more insight in the process of initial recruitment.

In conclusion, using an ovarian organ culture it has been shown that AMH inhibits the initiation of primordial follicle growth

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Chapter 4

Effects of anti-Müllerian hormone and follicle-stimulating hormone on follicle development in the mouse ovary

Abstract

Ovarian follicle growth is under the influence of many hormones and growth factors. Follicle-stimulating hormone (FSH) is an important regulator of ovarian follicle growth. This gonadotropic hormone is essential for follicle development from the small antral follicle stage onward, while preantral follicles are not dependent on, but sensitive to FSH. Therefore, factors affecting the sensitivity of ovarian follicles to FSH are important for both preantral and antral follicle growth. Studies on ovaries from anti-Müllerian hormone-deficient females suggested that anti-Müllerian hormone (AMH) might attenuate the sensitivity of ovarian follicles to FSH. This suggestion is supported by the observation that exogenous AMH inhibits FSH-stimulated processes in cultured granulosa cells.

The effects on ovarian follicle development of AMH and FSH, and the combined action of these hormones were studied in three different experiments using gene knockout mice, deficient for AMH and FSHβ. Experiment 1 showed that in vitro *FSH-stimulated preantral follicle growth is inhibited in the presence of AMH. Experiment 2 showed that in immature AMH-deficient females more follicles start to grow under the influence of exogenous FSH than in their wild type littermates. In Experiment 3, the effect of the absence of FSH on AMH regulation of ovarian follicle growth was determined by crossbreeding FSHβ-deficient mice with AMH-deficient females. Comparison of the follicle population of 4-month-old FSHβ-/AMH-deficient females with the follicle population of 4-month-old wild type, FSHβ-deficient females, and AMH-deficient females, revealed that the effect of AMH-deficiency on the recruitment of primordial follicles in the mouse ovary is increased in the absence of FSH.*

In conclusion, these studies show that AMH reduces FSH-sensitivity of preantral and small antral follicles, and that AMH is a dominant regulator of early follicle growth.

INTRODUCTION

The gonadotropin follicle-stimulating hormone (FSH) is required for follicular growth from the large preantral to the preovulatory stage. This is most clearly shown by the phenotype of FSHβ-deficient mice, which are infertile due to a block in folliculogenesis at the large preantral stage (Kumar *et al.*, 1997). During the oestrous cycle, large preantral and small antral follicles undergo cyclic recruitment (McGee and Hsueh, 2000), a process by which several follicles are recruited and of which some can grow to the preovulatory follicle stage, while the non-selected follicles will undergo atresia (Osman, 1985). The level of serum FSH is very important in this process. The elevated level of FSH at oestrus prevents some, but not all follicles to become atretic, so that they are able to continue growth (Hirshfield and De Paolo, 1981). Thus, during cyclic recruitment the sensitivity of the follicles to FSH appears to be of central importance. Studies in the bovine show that increased FSH-sensitivity could be a result of enhanced FSH receptor expression (Bao *et al.*, 1997; Evans and Fortune, 1997). Although preantral follicle development can occur in the absence of FSH (Kumar *et al.*, 1997), there is growing evidence that also preantral follicles are sensitive to FSH. In prepubertal rats, for example, treatment with GnRH-antagonist for several days decreases the number of preantral follicles (Van Cappellen *et al.*, 1989), while in women with gonadotropin resistant ovary syndrome, follicle development is limited to primordial and a few primary follicles (Talbert *et al.*, 1984).

The development of ovarian follicles, however, is not only regulated by FSH but also intra-ovarian factors, such as oestrogens and growth factors, also are involved. Activins, inhibins, and growth and differentiation factor 9 (GDF9), all members of the transforming growth factor-β (TGFβ) family of growth and differentiation factors, can influence follicular development (Findlay, 1993; Dong *et al.*, 1996; Matzuk *et al.*, 1996). We showed recently that anti-Müllerian hormone (AMH), another member of the TGFβ family, inhibits the initiation of primordial follicle growth (Durlinger *et al.*, 1999). From the same study it was also suggested that AMH is able to inhibit FSH-stimulated follicle growth. This idea is supported by inhibitory effects of AMH on FSH-stimulated actions on cultured granulosa cells (Kim *et al.*, 1992; Seifer *et al.*, 1993; di Clemente *et al.*, 1994a).

In the present study, three different experimental approaches were used to examine the effects of AMH and FSH alone, and their combined effect, on ovarian follicle growth in the mouse. In an *in vitro* study FSH-stimulated growth of preantral follicles was studied in the presence or absence of AMH. In an *in vivo* experiment, immature AMH-deficient (AMHKO) females and their wild type littermates were treated with GnRH-antagonist and FSH after which follicle growth was examined. Finally, follicle growth was studied in 4-month-old AMH-/FSHβ-deficient (FAKO) females, by comparing the follicle population of the FAKO females with the follicle population in AMHKO, FSHβ-deficient (FSHβKO), and wild type females of the same age.

From these studies it can be concluded that AMH inhibits FSH-stimulated follicle growth in the mouse, suggesting that AMH at least partially determines the sensitivity of ovarian follicles for FSH. Therefore, AMH may play an important role during cyclic recruitment. Furthermore, the increased primordial follicle recruitment and subsequent preant-

ral follicle growth in AMHKO females is augmented in the absence of FSH, as is shown by the FAKO ovarian phenotype.

Materials and methods

Animals

Different strains of mice were used in the three different experiments described in this paper. In Experiment 1, F1 female offspring from C57Bl/6J females and CBA/J males from Harlan Winkelmann GmbH (Bohren, Germany) were used to collect preantral follicles for follicle culture. For Experiment 2, wild type and AMH-deficient female mice on a C57Bl/6J background were generated as described previously (Durlinger *et al.*, 1999). In Experiment 3, female and male mice heterozygous for both FSHβ and AMH null allele were used to obtain AMH-/ FSHβ-deficient (FAKO), FSHβ-deficient (FSHβKO), AMH-deficient (AMHKO), and wild type female mice. Male FSHβKO mice which were used in the breedings were kindly donated by M. Matzuk and R. Kumar. All strains were kept on a C57Bl/6J background. Animals were kept under standard animal housing conditions in accordance with the NIH Guidelines for the Care and Use of Experimental Animals.

In Experiment 1, the effect of AMH on FSH-stimulated preantral follicle growth was tested in an *in vitro* experiment. Therefore, ovaries were collected from 21- to 23-day-old B6CBA female mice. The mice were anesthetized with ether, and blood was collected by puncture of the ophthalmic venous plexus. After clotting, blood was centrifuged for 15 min at 4000x g, and serum was collected and stored at -20 C until it was used in the follicle culture medium. The ovaries were used to collect preantral follicles (Rose *et al.*, 1999).

In Experiment 2, the growth of ovarian follicles under the influence of exogenous FSH was examined. Therefore, females of 25 days old were divided into four groups, each group containing 6 wild type and 6 AMHKO animals. Group A was treated from day 25 to day 28 with saline. Group B received during the same period 200 µg GnRH-antagonist per injection (Org 30276; NV Organon, Oss, The Netherlands). Two groups of mice received GnRH-antagonist from day 25 to day 31. In addition, one of these two groups received saline (group C), while the other group 2.5 IU FSH (Puregon, NV Organon) per injection (group D) from day 29 to day 31. All injections were given twice a day at 09.00 h and at 21.00 h and the animals were sacrificed by decapitation 12 h after receiving the last injection. The different treatment schedules are shown in Figure 4.1.

Experiment 3 was performed to determine the combined effect of FSH and AMH on ovarian follicle development. Therefore, FAKO females of 4 months of age and their wild type, FSHβKO and AMHKO were killed by decapitation at 16.00 h. Wild type and AMHKO females were killed on the day of oestrus.

In Experiments 2 and 3 after decapitation, ovaries were removed, weighed, and fixed overnight in Bouin's fluid for histological examination of the follicle population. The uteri were also weighed. The fixed ovaries were embedded in paraffin after routine histo-

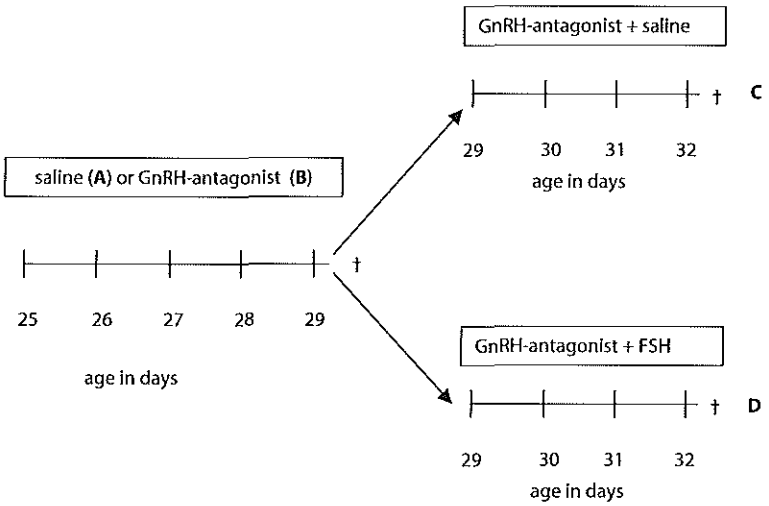

Figure 4.1
Treatment of immature wild type or AMHKO females with saline, GnRH-antagonist, or GnRH-antagonist in combination with saline or FSH.

Group A and group B were treated twice a day from day 25 to day 28, with saline or GnRH-antagonist, respectively. The animals were killed on day 29.

Group C and D were treated twice a day from day 25 to day 29 with GnRH-antagonist, and from day 29 to day 31 with GnRH-antagonist in combination with either saline or FSH, respectively. The animals were killed on day 32.

logical procedures, 8 µm sections were mounted on slides and stained with haematoxylin and eosin. In Experiment 2, blood samples were collected from all animals. The samples were kept overnight at 4°C and centrifuged the following day at 3000 rpm for 15 min at 4°C. Serum samples were stored at -20°C until assayed for FSH, inhibin A or inhibin B.

Determination of mouse AMH and FSH genotype

To determine the genotype of the mice used in Experiments 2 and 3, several PCR reactions had to be carried out. The AMH genotyping was described previously (Durlinger *et al.*, 1999). The only difference is that the genomic DNA was isolated using a different method described earlier (Slegtenhorst-Eegdeman *et al.*, 1998).

Primers FSHβ-FOR (5'-TTCAGCTTTCCCCAGAAGAG-3') and FSHβ-REV (5'-CTGCT-GACAAAGAGTCTATG-3') were used to determine the presence of the wild type *FSHβ* allele. Primer FSHβ-FOR anneals to nucleotide sequence 32-51 located in exon 1 of the *FSHβ* gene (numbering according to GenBank sequence, accession number U12932), whereas primer FSHβ-REV anneals to antisense sequence 278-259 located in intron 1 of the *FSHβ* gene, resulting in a PCR product of 247 bp. In animals carrying the *FSHβ* null allele, exons 1and 2, and most of exon 3 are replaced by the PGK-Hprt expression cassette. Primer HPRT-FOR (5'-CCTGCTGGATTACAT-TAAAGCACT-3') and HPRT-

REV (5'-GTCAAGGCA-TATCCAACAACAAA-3') were used to determine the presence of the *FSHβ* null allele, *i.e.* the PGK-hprt expression cassette (Kumar *et al.*, 1997). Primer HPRT-FOR anneals to the nucleotide sequence 318-341, whereas primer HPRT-REV anneals to the antisense sequence 669-646 in the PGK-hprt expression cassette, resulting in a PCR product of 352 bp.

For the PCR reactions 25 pmol of all primers was used, while the PCR reactions were executed as described previously (Slegtenhorst-Eegdeman *et al.*, 1998). An annealing temperature of 45°C for the *wild type* allele and of 55°C for the *FSHβ* null allele were used. The PCR products were electrophoresed on a 1.5 % agarose gel.

Ovarian histology and follicle counting

Serial 8 µm sections of the ovaries were used for follicle counting. Follicle counting was performed as described previously (Durlinger *et al.*, 1999). In Experiment 2, both ovaries were used for determining the follicle population. With the exception of primordial follicles (diameter ≤ 20 µm), all follicles were included into the study. The follicles were divided in four groups on basis of their mean diameter, which was determined by measuring two perpendicular diameters in the section in which the nucleolus of the oocyte was present. The four groups of follicles were: small preantral follicles (100-170 µm), large preantral follicles (171-220 µm), small antral follicles (221-310 µm) and large antral follicles (311-370 µm).

In Experiment 3 only the right ovary of each animal was used for follicle counting, since we did not observe a difference in the composition of the follicle populations of the right and left ovaries of the same female (unpublished observations). In this study, all follicles, both non-atretic and atretic, were counted, including the primordial follicles. The follicles were divided into three groups on basis of their mean diameter (µm); primordial follicles (diameter ≤ 20 µm), small follicles (21-310 µm), and large follicles (diameter > 310 µm). The group of small follicles contains all preantral and small antral follicles, while in the large follicle group all large antral follicles are found. Also the number of atretic oocytes (Durlinger *et al.*, 1999) was determined.

AMH production

In the follicle culture experiment (Experiment 1) recombinant rat AMH was used. Human embryonic kidney 293 (HEK293) cells were stably transfected with a cDNA encoding His-tagged rat AMH inserted in the pRrCMV expression vector. The AMH cDNA contained an optimised cleavage site that yields maximal amounts of cleaved recombinant 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 neomycine 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, USA).

The amount of AMH was measured by ELISA, using the Biorad TMB Peroxidase EIA substrate kit (Biorad, Life Science Groups, Hercules, CA, USA). The primary antibody, pentaHis monoclonal antibody (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, USA), was used in a 1:1000 dilution. The amount of AMH was calibrated in every ELISA using a constant, standard preparation of His-tagged AMH. The amount of AMH used for calibration was estimated using SDS PAGE (4-15% Tris-HCl precast gel) (Biorad) and the 6x His-protein ladder (Qiagen), followed by western blotting and visualisation of the His-tag using the pentaHis monoclonal antibody. 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 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 1:2000 dilution and a secondary peroxidase-conjugated mouse-anti-goat/sheep antibody (Sigma) in a dilution of 1:10000 (data not shown).

The AMH preparations were shown to be fully bioactive using a Müllerian duct regression assay (Nachtigal and Ingraham, 1996) (data not shown).

Follicle collection and follicle culture

For each culture, the ovaries of at least 4 animals were collected aseptically and transferred into L-15 Leibovitz medium (Gibco) supplemented with 0.3% BSA (Sigma), 5 µg/ml insulin (Sigma), 2 mM glutamine (Gibco), 10 µg/ml transferrin (Sigma), 50 µg/ml L-ascorbic acid (Sigma) and 2 ng/ml selenium (Sigma) at 37°C. Preantral follicles with a diameter of 135-210 µm were isolated by needle dissection (29-gauge 1/2-in) and collected in αMEM medium (Gibco) supplemented with 0.3% BSA, 5 µg/ml insulin, 2 mM glutamine, 10 µg/ml transferrin, 50 µg/ml L-ascorbic acid and 2 ng/ml selenium. Follicles from different ovaries were pooled in the collection medium and incubated in a humidified incubator, gassed with 5% CO_2 in air at 37°C, until enough follicles were isolated for the culture.

Follicles with a normal morphological appearance, *i.e.* a central spherical oocyte, high density of granulosa cells, and a theca cell layer enclosing the entire follicle, were divided into two groups with a diameter of 135-165 µm or 165-210 µm, and were subsequently individually cultured in Millicell-CM culture plate inserts (Millipore) with 250 µl αMEM culture medium supplemented with 5% immature mouse serum. Half of the number of follicles was cultured in the presence of rat AMH, produced by HEK293 cells. To induce follicle growth, 100 mIU/ml (10 ng/ml) recombinant human FSH (Puregon; NV Organon) was included into the culture medium after the first 24 h of culture. Culture medium was exchanged on culture days 1 and 4. Follicles were cultured up to 5 days in a humidified incubator gassed with 5% CO_2 in air at 37°C. The diameter of the follicles was measured on day 0, 1, 4 and 5 using x100 magnification and a calibrated micrometer. In addition, on the same days the survival rate of the follicles was checked by evaluation of degeneration (blackening of the follicle) and bursting (loss of oocyte).

Hormone analyses

In Experiment 2 serum FSH was determined by RIA using rat FSH as ligand and antibodies against ovine FSH (Dullaart *et al.*, 1975). All results are expressed in terms of NIDDK rat FSH RP-2. The intra-assay variation was 7.2%, and all samples were measured in one assay.

Inhibin A and inhibin B were measured using kits purchased from Serotec Limited, Oxford, UK. Suitability of the Serotec assays for measuring mouse inhibin A and B dimers was investigated previously (Smitz and Cortvrindt, 1998). This investigation was needed, since the standard material used in these kits was extracted from human follicular fluid and calibrated against recombinant human 32 kDa inhibin A and B forms. The antibodies used in the Serotec kits were able to detect bovine, ovine, porcine, mouse and rat inhibin A in serum (McConnell *et al.*, 1996). The intra-assay of the inhibin A assay was 9% and of inhibin B assay 15%. All samples were measured in one assay.

Statistical analysis

Results are presented as the mean ± SEM. The data were evaluated for statistical differences either by one-way ANOVA, followed by Duncan's new multiple range test or by Independent-Samples T Test using SPSS9 (SPSS, Inc., Chicago, IL, USA) computer software. Differences were considered significant at $P \leq 0.05$.

Results

Experiment 1:
Effect of AMH on FSH-stimulated preantral follicle growth *in vitro*

To test the effect of AMH on FSH-stimulated preantral follicle growth *in vitro*, two groups of preantral follicles with a different mean diameter were cultured in the presence or absence of added AMH.

In both cultures, addition of exogenous AMH caused inhibition of FSH-stimulated preantral follicle growth in a time-dependent manner, indicated by the significant smaller diameter of follicles cultured in the presence of added AMH at days 4 and 5 of culture (Figure 4.2). During the follicle culture, the increase in diameter was mainly the result of an increase in the number of granulosa cells (data not shown). The follicles with the larger size at the start of the culture maintain a larger diameter during the culture than the follicles with a smaller starting size.

No difference was found in the survival rate of the follicles, when cultured in the presence or absence of added AMH (results not shown).

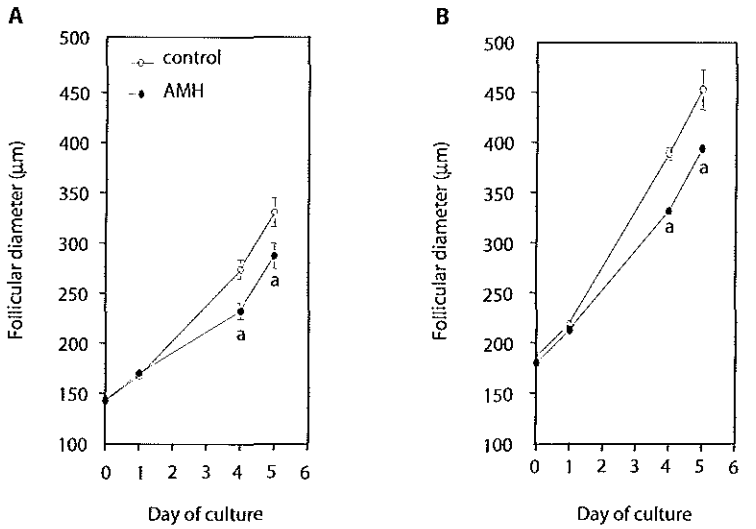

Figure 4.2
Effect of AMH on FSH-stimulated preantral follicle growth in vitro.

A. Culture of preantral follicles with a diameter between 135 and 165 mm at day 0 of culture

B. Culture of preantral follicles with a diameter between 165 and 210 mm at day 0 of culture.
After 4 and 5 days of culture, the diameter of follicles cultured in the presence of AMH was significantly reduced compared to that in the absence of AMH.

Data represent the mean ± SEM (N ranging between 15 and 45). A significant difference from follicles cultured in the absence of AMH is indicated by the letter a ($P \leq 0.05$).

Experiment 2:
FSH-stimulated follicle growth in AMHKO females *versus* wild type females

To determine whether the presence or absence of AMH affects the number of follicles that grow under the influence of FSH, immature wild type and AMHKO female mice were treated with either GnRH-antagonist, or GnRH-antagonist in combination with FSH. The different treatment schedules are shown in Figure 4.1.

Serum FSH level

To determine the effect of GnRH-antagonist treatment on the endogenous serum FSH level, and to compare this level between wild type and AMHKO females, serum FSH was measured in groups A, B, and C. The serum FSH level of group D could not be determined, since the antibody used in the RIA partly cross-reacts with the exogenous FSH.

Comparing the serum FSH level of group B with that of group A, shows that GnRH-antagonist treatment resulted in a significantly reduced serum FSH level in both genotypes (Table 4.1). Treatment with GnRH-antagonist for 7 days (group C) instead of 4 days (group B) did not significantly lower the serum FSH level further. In all three treatment groups no significant difference in serum FSH level between wild type and AMHKO

Table 4.1
Serum FSH level in immature wild type and AMHKO females of different treatment groups.

Treatment group	FSH (ng/ml)	
	wild type	AMHKO
A (saline, d 25-28)	14.8 ± 1.4	14.5 ± 1.3
B (GnRH-ant, d 25-28)	5.3 ± 0.4 [a]	5.4 ± 1.1 [a]
C (GnRH-ant, d 25-31)	4.9 ± 0.4	4.7 ± 0.3

Values represent the mean ± SEM (n =6).
[a] indicates a significant difference from group A. Values were evaluated by Independent-Samples T Test (P≤ 0.05).
Animals were killed at day 29 (groups A and B) or at day 32 (group C).

Table 4.2
Uterine and ovarian weights in immature wild type and AMHKO female mice of different treatment groups.

Treatment group	Uterine weight (mg)		Weight 2 ovaries (mg)	
	wild type	AMHKO	wild type	AMHKO
A (saline, d 25-28)	13.2 ± 0.4	14.4 ± 2.1	4.5 ± 0.2	4.5 ± 0.4
B (GnRH-ant, d 25-28)	11.9 ± 0.3[a]	10.4 ± 1.1	3.2 ± 0.3[a]	4.0 ± 0.2[*]
C (GnRH-ant, d 25-31 + saline d 29-31)	7.3 ± 1.1[a]	7.7 ± 0.4[a]	2.3 ± 0.2	3.6 ± 0.2[*]
D (GnRH-ant d 25-31 + FSH, d 29-31)	8.2 ± 0.7	7.6 ± 0.7	4.5 ± 0.3[c]	6.3 ± 0.5[c]

Values represent the mean ± SEM (n = 6).
[a,c] indicate a significant difference from groups A and C, respectively. * indicates a significant difference from wild type females within the same treatment group. Values were evaluated by Independent-Samples T Test (P ≤ 0.05).
Animals were killed at day 29 (groups A and B) or at day 32 (groups C and D).

females within the same treatment group was found.

Ovarian weight

GnRH-antagonist treatment decreased ovarian weight in wild type females but not in AMHKO females (group B *versus* group A), while FSH treatment increased the ovarian weight in both genotypes (group D *versus* group C) (Table 4.2). Within the treatment groups, a significant difference in ovarian weight between the two genotypes was only found when a low serum level of FSH was present (groups B and C). In both groups the ovarian weight was significantly higher in AMHKO females, compared to the ovarian weight of their wild type littermates.

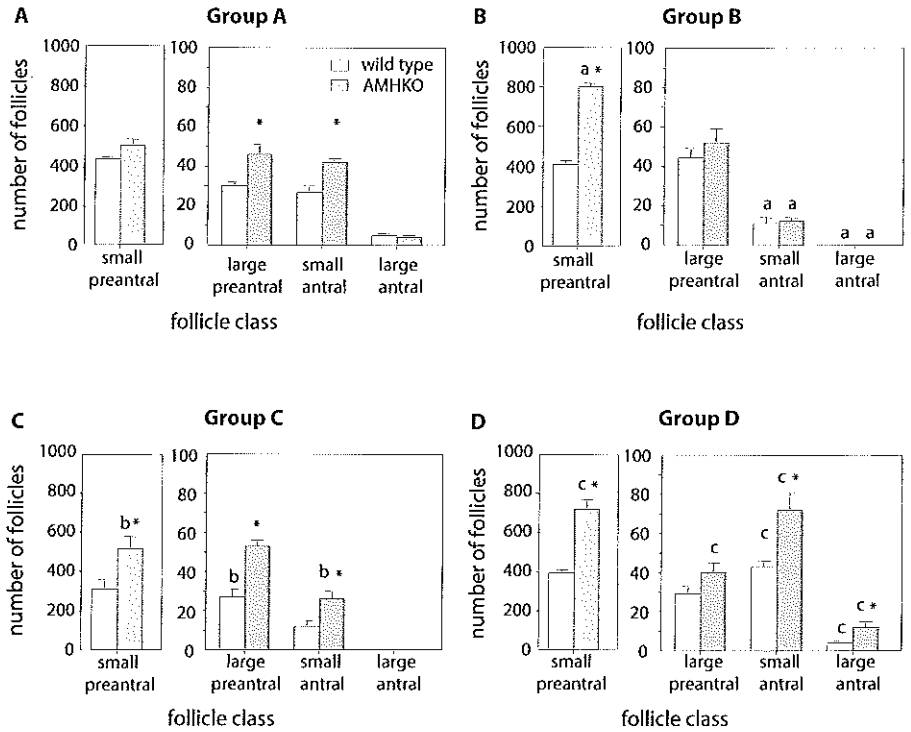

Figure 4.3
Number of small preantral, large preantral, small antral and large antral follicles in wild type and AMHKO female mice after treatment with either saline, GnRH-antagonist + saline, or GnRH-antagonist + FSH.

Wild type and AMHKO females were treated with saline (A) or GnRH-antagonist (B) from day 25 to day 28, or animals were treated with GnRH-antagonist from day 25 to day 28 and from day 29 to day 31 with saline (C) or FSH (D).

In all groups, except group A, a significant difference was found in the number of small preantral follicles between wild type and AMHKO females. In both group A and C significantly more large preantral and small antral follicles were found in AMHKO females compared to wild type females. In group D, significantly more small antral and large antral follicles was found in AMHKO females compared to wild type females.

Data represent the mean ± SEM (n = 6). a, b, c indicates a significant difference from group A, group B, and group C, respectively. * indicates a significant difference from wild type females within the same treatment group ($P \leq 0.05$).

Follicle counts

The total number of small preantral, large preantral, small antral, and large antral follicles in both wild type and AMHKO females of the four different treatment groups are shown in Figure 4.3A-D.

In mice treated with saline from day 25 to day 28 (group A), the follicles reached the large antral stage in both genotypes, and no significant difference was found in the number of large antral follicles between wild type and AMHKO females (Figure 4.3A).

The number of both large preantral and small antral follicles was significantly higher in AMHKO females than in wild type females, while no significant difference was found between the two genotypes for the number of small preantral follicles (Figure 4.3A). The decrease of serum FSH level in group B resulted in the expected decrease in the number of antral follicles in both genotypes, but in the AMHKO females also in a large increase in the number of small preantral follicles (Figure 4.3B). The longer exposure to a low serum FSH level in treatment group C had a stronger inhibitory effect on the number of follicles of all classes in wild type females (Figure 4.3C). However, notwithstanding the low serum FSH level, the number of small antral follicles was increased in AMHKO ovaries of treatment group C. Administration of FSH resulted in the presence of large antral follicles and in an increase in the number of small antral follicles in both genotypes (Figure 4.3D). In the AMHKO mice, however, stimulation of follicle growth was much stronger, both in the number of follicles and in the extent of their development as indicated by their size.

Serum inhibin A and inhibin B levels

Preantral and antral follicles produce both inhibin A and inhibin B. Serum levels of both factors were determined in all treatment groups, to see whether the different treatments had an effect on the serum inhibin A and inhibin B levels.

In general, the serum inhibin B level was higher than the serum inhibin A level (Table 4.3). Treatment of the animals with GnRH-antagonist resulted for both genotypes in a decrease of serum inhibin A and inhibin B levels, while treatment with FSH resulted for both genotypes in a strong increase in both inhibin A and B.

Within all treatment groups, no significant difference in the concentrations of both inhibins was found between wild type and AMHKO females, with the exception of the serum inhibin B level in group B, which was slightly but significantly higher in AMHKO

Table 4.3
Serum inhibin A and inhibin B levels in immature wild type and AMHKO females of different treatment groups.

Treatment group	Inhibin A (ng/l)		Inhibin B (ng/l)	
	wild type	AMHKO	wild type	AMHKO
A (saline, d 25-28)	24 ± 3	16 ± 2	72 ± 9	52 ± 5
B (GnRH-ant, d 25-28)	< 5[a]	< 5[a]	13 ± 3[a]	30 ± 6[a*]
C (GnRH-ant, d 25-31 + saline, d 29-31)	< 5	< 5	10 ± 2	14 ± 2
D (GnRH-ant, d 25-31 + FSH, d 29-31)	59 ± 19[c]	90 ± 17[c]	266 ± 85[c]	317 ± 60[c]

Values represent the mean ± SEM (n =6).
[a,c] indicate a significant difference from group A and C, respectively. * indicates a significant difference from wild type females within the same treatment group. Values were evaluated by Independent-Samples T Test ($P \leq 0.05$).
Animals were killed at day 29 (groups A and B) or at day 32 (groups C and D).

females than in wild type females.

Uterine weight

Uterine weight was determined as an oestrogen-sensitive parameter, and thus gives an indication of ovarian function.

GnRH-antagonist treatment for 4 days caused a slight, but significant, decrease in the weight of the uterus in wild type females, while no difference was found in AMHKO females (Table 4.2). Longer administration of GnRH-antagonist did cause a significant decrease of uterine weight in both genotypes. Treatment with FSH did not change the uterine weight in both genotypes. No significant difference was found in the weight of the uterus between wild type and AMHKO females within the four different treatment groups.

Experiment 3: Comparison of follicle growth in 4-month-old wild type, AMHKO and FSHβKO, and FAKO females

To examine the effect of the absence of FSH on AMH regulated follicle growth, the follicle population of 4-month-old FSHβ-/AMH-deficient (FAKO) female mice was compared with the follicle population in wild type, FSHβKO, and AMHKO female littermates.

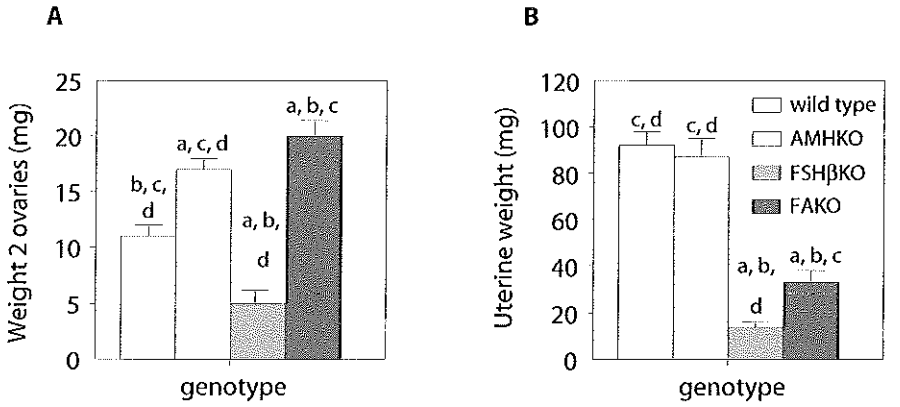

Figure 4.4
Uterine and ovarian weight in 4-month-old wild type, AMHKO, FSHβKO, and FAKO female mice.

A. The combined weight of both ovaries is given. Ovarian weight was significantly different between all phenotypes.

B. No difference in uterine weight was found between wild type and AMHKO females. Uterine weight of both FSHβKO and FAKO females was dramatically reduced. The uterine weight of FSHβKO females was even significantly lower than in FAKO females.

Data represent the mean ± SEM (n = 4). a, b, c, d indicate a significant difference from wild type, AMHKO, FSHβKO, and FAKO females, respectively ($P \leq 0.05$).

Uterine and ovarian weight

In FSHβKO mice, ovarian weight was strongly decreased, which is in line with previous studies (Kumar *et al.*, 1997), while ovarian weight was increased in AMHKO mice, confirming our previous results (Durlinger *et al.*, 1999) (Figure 4.4A). In FAKO females, the effect of the absence of AMH on ovarian weight was further increased, resulting in a significantly larger ovarian weight than in AMHKO females.

Absence of FSH caused a large decrease in uterine weight in FSHβKO and FAKO females, in agreement with a role for FSH in stimulation of oestrogen production by the ovary. This decrease, however, was less pronounced in FAKO females (Figure 4.4B). The absence of only AMH did not affect uterine weight.

Ovarian morphology and follicle counts

To determine the relation between the effect of AMH and FSH on ovarian activity *in vivo*, ovarian morphology and the entire follicle population in 4-month-old FAKO female mice were examined and compared with that in wild type, AMHKO, and FSHβKO female mice of the same age. Wild type and AMHKO females were killed on the day of oestrus.

The ovaries of all genotypes contained primordial, preantral, and small antral follicles, both non-atretic and atretic (Figure 4.5). Atretic oocytes (Durlinger *et al.*, 1999) were also found in all genotypes. No large antral follicles were found in FSHβKO and FAKO females, due to the lack of FSH in these animals, while in wild type and AMHKO females large follicles were absent, since at oestrus these follicles have not developed yet in mice with a normal oestrous cycle (Osman, 1985; Welschen and Rutte, 1971). In wild type and AMHKO females numerous fresh and old corpora lutea were present (Figure 4.5A). The ovaries of FSHβKO females were small due to the lack of corpora lutea, but contained follicles until the small antral stage (Figure 4.5C). In contrast, despite the lack of corpora lutea, ovaries of FAKO females were large and contained many small preantral follicles, but also small antral follicles (Figure 4.5D-E). Furthermore, in these ovaries many remnants of atretic follicles were found, as hollow spaces in the interstitium (Figure 4.5F)

As can be seen in Figure 4.6A-D absence of AMH alone or in combination with the absence of FSH has strong effects on the number of the different follicle classes. More non-atretic small follicles are found in the absence of AMH, and this effect is strongly increased in the additional absence of FSH (Figure 4.6C). The number of small follicles is much lower in the presence of AMH, as is shown by the follicle numbers found in wild type and FSHβKO mice. The effect of AMH and FSH on atretic small follicles and atretic oocytes was not different from the effect of AMH and FSH on non-atretic small follicles. (Figures 4.6B and 4.6D). The increase in the number of small follicles in AMHKO and FAKO females is reflected by a concomitant decrease in the number of primordial follicles (Figure 4.6A).

Discussion

Quantitative analysis of the follicle population in AMHKO females revealed that AMH

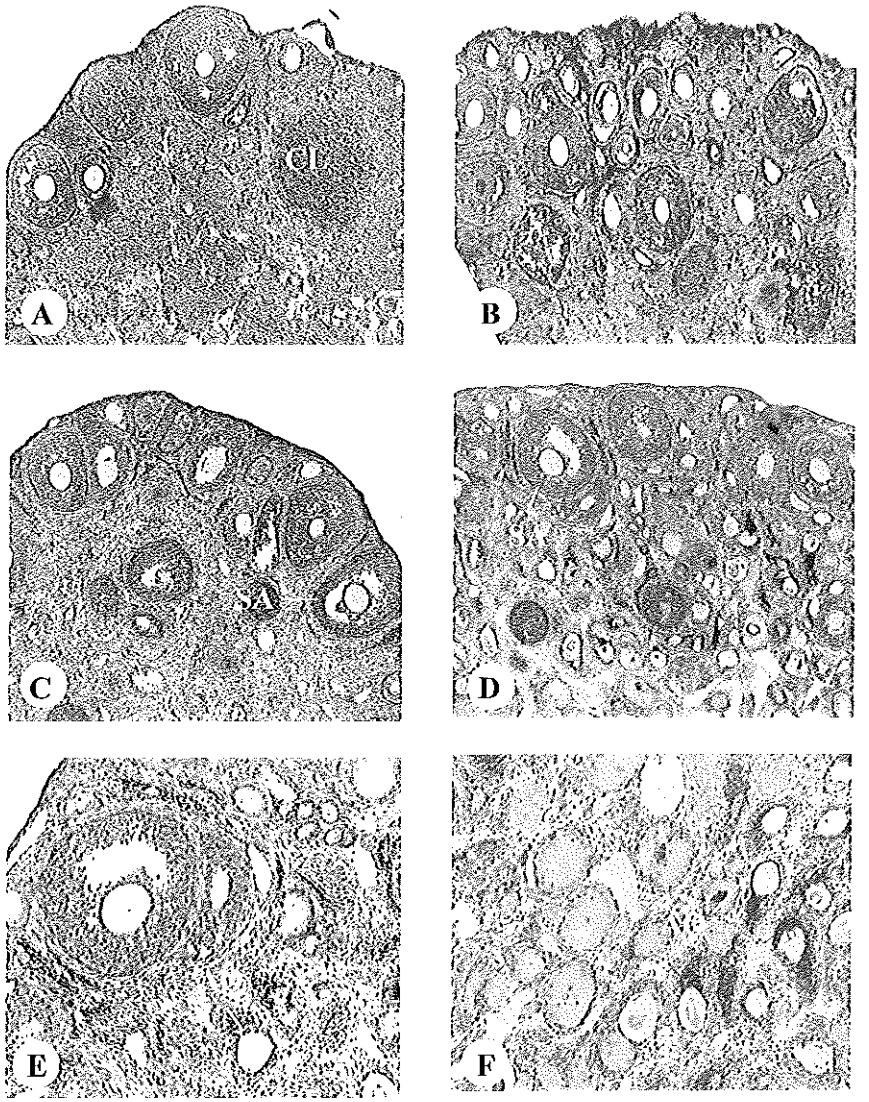

Figure 4.5
Photomicrographs of ovarian sections of 4-month-old wild type, AMHKO, FSHβKO, and FAKO female mice.

A-B. Section of an ovary of (A) a wild type mouse and of (B) an AMHKO mouse Follicles of different developmental stages are found in both genotypes. In the wild type ovary a fresh corpus luteum (CL) is shown. Magnification x 100.

C-D. Section of an ovary of (C) a FSHβKO mouse and of (D) a FAKO mouse In both FSHβKO and FAKO females follicle development occurs to the small antral stage (SA). Magnification x 100.

E-F. Section of an ovary of a FAKO mouse. Besides small antral follicles, many remnants of atretic oocytes are found in the interstitium as hollow spaces (AO). In some the degenerating oocyte is still present (*). Magnification x 200.

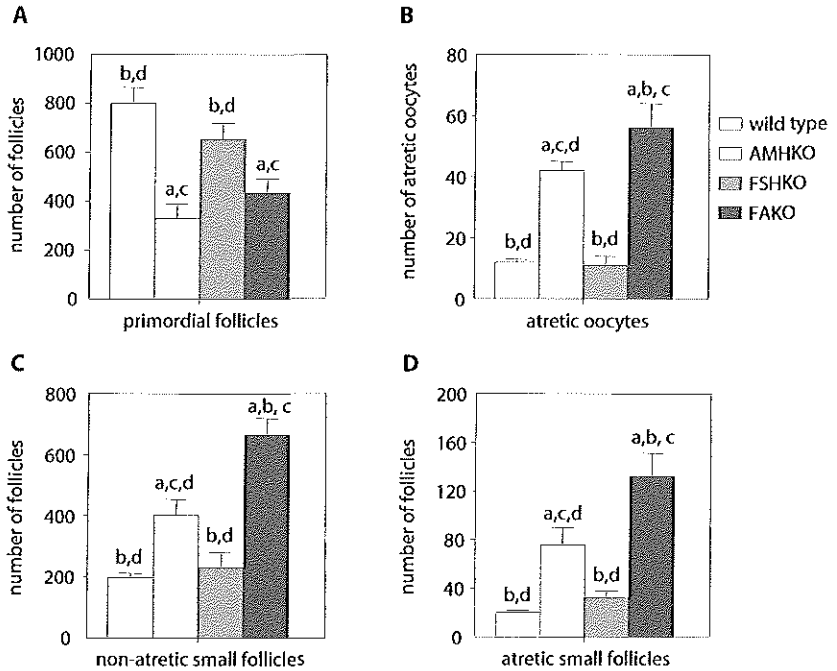

Figure 4.6
Follicle population in 4-month-old wild type, AMHKO, FSHβKO, and FAKO female mice.

A. Number of primordial follicles. No significant difference was found in the number of primordial follicles between wild type and FSHβKO females, and between AMHKO and FAKO females. Significantly less primordial follicles were found in AMHKO and FAKO females compared to wild type and FSHβKO females.

B-D. Number of atretic oocytes (B). Number of non-atretic small follicles (C). Number of atretic small follicles (D). No significant differences in atretic oocytes and non-atretic and atretic small follicles were determined between wild type and FSHβKO females. However, these numbers were significantly higher in both AMHKO and FAKO females than in wild type and FSHβKO females, with a higher number in FAKO females compared to AMHKO females.

Data represent the mean ± SEM (n = 4). a, b, c, d indicate a significant difference from wild type, AMHKO, FSHβKO, and FAKO females, respectively ($P \leq 0.05$).
Note the difference in scale of the Y-axis among the different graphs.

inhibited the recruitment of primordial follicles (Durlinger *et al.*, 1999). The same study also gave indications for an effect of AMH on the sensitivity of growing follicles to FSH, since more preantral and small antral follicles were found in 4-month-old AMHKO females, in spite of a relatively low serum FSH level, than in wild type females. Although growth of preantral and small antral follicles is not dependent on FSH (Kumar *et al.*, 1997), it has been shown that these follicles are sensitive to FSH (McGee *et al.*, 1997; Van Cappellen *et al.*, 1989).

In the present study, Experiment 1 clearly shows that addition of AMH to the culture medium inhibits FSH-stimulated follicle growth of preantral follicles *in vitro*, for follicles with a diameter of 135-165 μm and 165-210 μm at the start of the culture. AMH is not the

only member of the TGFβ family of growth and differentiation factors that can affect preantral follicle growth *in vitro*. Oocyte-derived GDF9 augments FSH-stimulated preantral follicle growth from 6-day-old rats, as was shown by an increase in follicle diameter and an increase in protein content of follicles cultured in the presence of both FSH and GDF9 (Hayashi *et al.*, 1999). For mouse preantral follicles, this stimulatory effect of GDF9 was only found for follicles with a diameter between 165-210 µm and not for follicles with a diameter between 135-165 µm (Dr. Uschi Rose, personal communication). In preantral follicles from immature mice a significant increase in diameter was observed when the follicles were cultured with activin A or both activin A and FSH (Yokota *et al.*, 1997). The stimulatory effect of activin A could be suppressed by follistatin in a dose-dependent manner (Liu *et al.*, 1998) and was not found in preantral follicles from adult mice (Yokota *et al.*, 1997). These studies all suggest that, at least in immature mice, several hormones and growth factors regulate preantral follicle growth.

The results of the *in vivo* experiment, in which the serum FSH level of immature AMHKO and wild type females was lowered by GnRH-antagonist or increased by treatment with FSH (Experiment 2), also demonstrate that AMH inhibits FSH-stimulated follicle growth. Comparing follicle numbers between GnRH-antagonist treated animals (group B) and control animals (group A) reveals that in both genotypes mainly small and large antral follicles are affected by the decrease in serum FSH level. This again indicates that antral follicle growth is dependent on FSH while preantral follicle growth is not, as was already shown by studies on hypophysectomised mice (Wang and Greenwald, 1993) or by FSHβKO mice (Kumar *et al.*, 1997). The rise in the number of small preantral follicles in AMHKO females in group B compared to group A can be explained by loss of inhibitory action of AMH on the recruitment of primordial follicles in these animals, and by an accumulation of these follicles, since further development is inhibited by the low serum FSH level in group B. In AMHKO females, growth of large preantral and small antral follicles is less affected by longtime exposure to low serum FSH level than in wild type females (group C *versus* group B), as indicated by the maintenance of the number of large preantral follicles and even a small increase in the number of small antral follicles, which is not found in the wild type animals. Treatment of animals with large amounts of exogenous FSH on top of GnRH-antagonist treatment (group D) stimulated small and large antral follicle growth in both wild type and AMHKO females. This stimulatory effect, however, was more pronounced in AMHKO females than in wild type females both in numbers and in developmental stage. Collectively, these follicle data show that in the absence of AMH, follicle growth is more sensitive to stimulation by FSH.

In adult mice the ovarian weight is mainly determined by the mass of corpora lutea present in the ovary. Thus, the anovulatory ovaries of FSHβKO females that do not contain corpora lutea show a five-fold decrease in weight compared to the wild type ovaries (Kumar *et al.*, 1997) (and present results). In a previous study, however, we showed that also the number of follicles could have a major impact on ovarian weight. Notwithstanding similar numbers of corpora lutea, the ovaries of 4-month-old AMHKO females contain about three times more preantral and small antral follicles than ovaries from wild type females, resulting in an almost two-fold increase in ovarian weight (Durlinger *et*

al., 1999) (present results). This observation is supported by the ovarian weight of FAKO females, which is even further augmented by the very high number of small follicles. The differences in follicle population of the immature animals in Experiment 2 are not always reflected in the ovarian weight. Probably the differences in follicle number were too small to cause significant differences in ovarian weight.

In Experiment 2, serum inhibin levels were determined to see whether these levels are good parameters for the composition of the ovarian follicle population. In a follicle culture experiment using immature mouse follicles it was shown that inhibin A is predominantly produced by large antral follicles and inhibin B by preantral and small antral follicles (Smitz and Cortvrindt, 1998). The differences in the amount of serum inhibin A and inhibin B found between different treatment groups in Experiment 2 partly support this observation. In both groups A and D, where the ovaries contain some large antral follicles, but mostly preantral and small antral follicles, a higher serum inhibin B than inhibin A level is found. Furthermore, reducing the number of large antral follicles to zero by GnRH-antagonist treatment (groups B and C) resulted in a non-detectable level of serum inhibin A. These two observations again show that predominantly large antral follicles produce inhibin A. It is likely that inhibin B is mainly produced by small antral follicles, since the serum inhibin B level was mainly affected by a change in the number of small antral follicles (group A *versus* group D). The large difference in both serum inhibin A and inhibin B levels between groups A and D is caused by a super-stimulation of inhibin production by high amounts of exogenous FSH, since inhibin production is directly stimulated by FSH.

The uterine weight can be used as an indication of oestrogen production by the ovaries, since the uterine epithelium is strongly stimulated by oestradiol (Galand *et al.*, 1971). Oestrogens are mainly produced by large antral and preovulatory follicles, although small antral and even preantral follicles are able to produce small amounts of oestrogens. The changes in uterine weight clearly underline the histological observations in the ovaries. Thus, the low FSH level in GnRH-antagonist-treated immature mice is accompanied by a decrease in uterine weight. The continued GnRH-antagonist treatment in the FSH treated group (group D) ensures that the LH level remained low, resulting in low thecal androgen production which is needed for ovarian oestrogen output. Notwithstanding the increased number of large follicles in the FSH-treated animals, oestrogens and therefore uterine weight remained low.

In Experiment 3, large differences existed in the uterine weights between wild type and AMHKO females on the one hand, and FSHβKO and FAKO females on the other hand. Uterine weights in FSHβKO and FAKO females are low, reflecting the absence of oestrogen-producing large antral follicles. However, the slight increase in uterine weight in FAKO females as compared to FSHβKO females may reflect increased oestrogen production by the much higher (four-fold) number of small follicles.

Going back to the respective roles and interaction of AMH and FSH in the ovary, the crossbreeding Experiment 3, FSHβKO and AMHKO females clearly indicated that AMH is a dominant regulator of early follicle growth. FSH does not appear to be involved in primordial follicle growth, since in both AMHKO and FAKO females less primordial and

more growing follicles were found. Indeed, even more growing follicles are found in ovaries of FAKO females, probably as a result of accumulation of these follicles, since the absence of FSH causes a block of follicular growth at the large preantral/small antral follicle stage. It was already known that FSH is not necessary for preantral follicle growth, but since preantral follicles are sensitive to FSH one could expect that a total loss of FSH production in mice would lead to some minor effects on preantral follicle growth. Comparison of the follicle population between wild type and FSHβKO females revealed, however, that preantral follicle growth occurs to the same extent in both genotypes. Also no effect was seen on the primordial follicle pool. This is in contrast to several studies in which the pool of primordial and growing follicles was influenced by increasing or decreasing gonadotropin levels (Meredith *et al.*, 1992; Wang and Greenwald, 1993). The only difference between these experimental animal models and the model used in this study, the FSHβKO mouse model, is that in the present model only FSH production is absent, while in the other models both FSH and LH and/or others factors produced by the pituitary are affected. For LH, it has been shown that this hormone can stimulate primordial follicle recruitment, since in mice overexpressing LH the outgrowth of primordial follicles is increased (Flaws *et al.*, 1997). Until now, no data are available about other factors from the pituitary gland which might exert an effect on recruitment of primordial follicles.

The question remains, by what mechanisms AMH inhibits the stimulatory effect of FSH on follicle growth. The inhibitory mechanism could involve an effect of AMH on FSH receptor expression. A change in the expression of the FSH receptor may change the sensitivity of a follicle to FSH, as was demonstrated by studies in the bovine (Bao *et al.*, 1997; Evans and Fortune, 1997). However, several studies have shown that AMH can inhibit FSH-stimulated and also cAMP-stimulated aromatase activity (di Clemente *et al.*, 1994a; Rouiller-Fabre *et al.*, 1998; Teixeira *et al.*, 1999), indicating that the molecular site of AMH action lays downstream of the FSH receptor.

Regulation of the sensitivity of follicles to FSH by AMH could be important during cyclic recruitment, since FSH is a crucial regulator of this process. A possible role of AMH in cyclic recruitment is indicated by the presence at oestrus of a group of non-atretic large preantral/small antral follicles with high AMH mRNA expression and, a group with much lower AMH mRNA expression, whereas these follicles were otherwise indistinguishable (Baarends *et al.*, 1995b). The increased sensitivity to FSH of the follicles with low AMH mRNA expression may allow these follicles to be selected for continued growth and ovulation in the next oestrous cycle. This phenomenon will be discussed in the next chapter of this thesis.

From the present study it can be concluded that AMH is important for ovarian follicle growth. Besides inhibiting the outgrowth of primordial follicles, AMH is also able to inhibit FSH-stimulated follicle growth. Ovarian follicle growth is under the influence of many growth regulatory factors, and it appears that AMH is one of these regulatory factors.

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Chapter 5

A FUNCTIONAL ROLE FOR ANTI-MÜLLERIAN HORMONE IN CYCLIC RECRUITMENT OF FOLLICLES DURING THE RAT OESTROUS CYCLE*

* Parts of this study have already been published (Uilenbroek *et al.*, 1998) or are in press (Durlinger *et al.*, 2001)

INTRODUCTION

Anti-Müllerian hormone (AMH) is produced by the postnatal ovary, where it is expressed by granulosa cells of mainly preantral and small antral follicles as shown by *in situ* hybridisation (Baarends *et al.*, 1995b). AMH is absent in primordial follicles, and following transient expression in preantral and small antral follicles, AMH disappears when a small antral follicle continues to grow or becomes atretic (Baarends *et al.*, 1995b). In the postnatal ovary, AMH inhibits the initiation of primordial follicle growth (Durlinger *et al.*, 1999) (Chapter 3 of this thesis), a process also known as initial recruitment (McGee and Hsueh, 2000). Furthermore, AMH has been shown to inhibit FSH-stimulated follicle growth (Chapter 4 of this thesis). Studies on cultured ovarian tissue and cells showed that AMH inhibits FSH-stimulated biosynthesis of aromatase, decreases FSH-stimulated LH receptor number (di Clemente *et al.*, 1994a), and attenuates the proliferation of cultured granulosa-luteal cells (Kim *et al.*, 1992; Seifer *et al.*, 1993).

During each oestrous cycle of the rat, a large group of follicles (about 60 per ovary) are recruited from the pool of large preantral and small antral follicles. A small number of these follicles (12-14) will continue to grow to the preovulatory stage, while the other follicles become atretic (Osman, 1985). During the rat oestrous cycle, this process of follicle recruitment, also called cyclic recruitment, takes place at oestrus, and is dependent on the secondary FSH surge present on the early morning of oestrus. Inhibition of this surge by administration of inhibin results in a decreased number of antral follicles the next day (Hirshfield and De Paolo, 1981), while administration of FSH on oestrus morning results in superovulation (van Cappellen *et al.*, 1997). Although FSH plays an important role in this process, a number of growth factors might also be involved in modulating the effects of FSH on large preantral and small antral follicles for FSH. One of these growth factors might be AMH, which has been shown to inhibit general FSH-dependent processes in granulosa cells. We have observed previously that, at oestrus, but not other stages of the oestrous cycle, two groups of non-atretic large preantral and small antral follicles were present: follicles with a high expression of AMH and follicles with a low expression of AMH (Baarends *et al.*, 1995b). High expression of AMH may cause a lower sensitivity to FSH and subsequent atresia, while follicles expressing a low amount of AMH may be more sensitive to the growth stimulatory effect of FSH.

It was the aim of the present study to investigate whether AMH is involved in cyclic recruitment. In addition, we planned to reinvestigate the hypothesis that non-atretic large preantral and small antral follicles with a high expression of AMH at oestrus become atretic, while follicles of the same size, but with a low expression of AMH, become preovulatory. To distinguish between follicles that are on their way to become atretic and those that are able to continue growth, a histochemical marker was needed that indicates forthcoming atresia. Therefore, in the first part of this study we searched for a marker of future atresia, which shows clear expression before any morphological sign of atresia is detectable. To find such a marker, an *in vivo* animal model was used, in which atresia of preovulatory follicles could be studied in a predictive and chronological order. It was already known that blockage of the preovulatory surge of LH with pentobarbital prevents

ovulation, causing the 10-12 preovulatory follicles to become gradually atretic (Braw and Tsafriri, 1980; Uilenbroek *et al.*, 1980). In the present study, a GnRH-antagonist was used to block the preovulatory LH surge and subsequent ovulation. In the first experiment, this model was evaluated, and it was concluded that the model is suitable for the study of atresia in preovulatory follicles in a predictable and chronological order. In the second experiment, we found that a decreased proliferative activity of granulosa cells, detected by using 5-bromodeoxyuridine (BrdU) labelling, is a useful marker of future atresia. In the third experiment, the AMH mRNA expression, measured by *in situ* hybridisation, and the BrdU labelling index were determined in all non-atretic follicles present at oestrus morning and metoestrus morning. The correlation between AMH mRNA expression and BrdU labelling index of the granulosa cells was determined in those follicles in which selection takes place (diameter 276-400 μm). It appeared that, at oestrus morning, the AMH mRNA expression in these follicles is negatively correlated with the BrdU labelling index, indicating that follicles with a high AMH mRNA expression become atretic, while those with a low AMH mRNA expression become preovulatory.

In conclusion, using an *in vivo* model in the rat, it was found that a low BrdU labelling index of granulosa cells can be used as a marker for future atresia. Furthermore, at oestrus there is a negative correlation between AMH mRNA expression and BrdU labelling index in those follicles that undergo cyclic recruitment, suggesting that AMH is indeed involved in the process of cyclic recruitment.

Materials and methods

Animals

The experiments were performed using 3-4-month-old Wistar rats (RP-1 strain). The animals were locally bred and housed under standard conditions of light (lights on from 05.00 h to 19.00 h) and temperature (20-23°C). Vaginal smears were taken daily. Only animals showing at least two successive 5-day oestrous cycles were used. The University Animal Ethics Committee approved the protocol for the handling of animals in this study.

Treatment and tissue preparation

In the first experiment, animals were given a subcutaneous injection with 20 μg GnRH-antagonist (Org 30276, Organon NV, Oss, The Netherlands) at 10.00 h on the day of pro-oestrus (P). This dose lowered the preovulatory LH surge and blocked ovulation (Meijs-Roelofs *et al.*, 1990). To prevent ovulation the next day, a second injection was given at 10.00 h the following morning (P+24). Following this treatment, vaginal smears revealed the presence of nucleated cells at P+96 and cornified cells and the presence of ova in the oviduct at P+120. To study whether the rate of atresia could be delayed, pro-oestrus animals were given 0.5 IU hCG 2 h after administration of 20 μg GnRH-antago-

nist at P and P+24. To study whether the rate of atresia could be accelerated, animals were given a larger amount of GnRH-antagonist (100 µg) at P and P+24. Animals were killed at P, P+24, P+48, P+72, or P+96 hours. Per time point, three animals were investigated. At autopsy, the ovaries were dissected out and fixed overnight in Bouin's fluid. After fixation, the ovaries were embedded in paraffin and serial sections (10 µm) were stained with haematoxylin and eosin. These sections were subsequently used for histological examination of the preovulatory follicles.

In the second experiment, GnRH-antagonist treated animals were killed at P, P+7, P+14, P+24, P+27, P+31, or P+48 (three animals per time point). In addition to these three animals, two extra animals per time point received next to the GnRH-antagonist injections at P and P+24, a single intraperitonial injection with 5-bromodeoxyuridine (BrdU; Boehringer Mannheim, Germany) dissolved in saline (100 mg/kg body weight) two hours before the animals were killed. The ovaries were fixed in buffered 4% formaldehyde (pH 6.8, 16-24 hours at room temperature) for histological evaluation and immunohistochemistry. After fixation, the ovaries were embedded in paraffin and serial sections (10 µm) were stained with haematoxylin and eosin. Sections used for immunohistochemistry were mounted on slides coated with 3-aminopropyltriethoxysilane (APES; Sigma, St Louis, USA).

In the third experiment, one animal was killed at oestrus at 10.00 h and one at metoestrus at 10.00 h. Two hours before the animals were killed a single intraperitoneal injection with 5-bromodeoxyuridine was given. The ovaries were dissected out and fixed overnight in 4% formaldehyde at 4°C. Serial sections of 10 µm thickness were made. Every fifth section of both ovaries was stained with haematoxylin and eosin. Both adjacent sections were mounted on microscopical slides coated with APES: one for *in situ* hybridisation of AMH mRNA, the other for BrdU labelling. From the sections stained with haematoxylin and eosin, it appeared that by counting the follicles with a visible oocyte at every fifth section, all non-atretic follicles would be incorporated into the study.

Histology

In Experiments 1 and 2, non-atretic and atretic preovulatory follicles were counted and classified. Atresia of antral follicles was classified as described by Osman (Osman, 1985). Atretic follicles were designated as early atretic (Stage I) and late atretic (Stage II). In Stage I degenerative changes are only present in the granulosa layer, while in Stage II, changes are also present in the oocyte, which shows signs of resumption of meiosis e.g. formation of a pseudo-maturation spindle. Follicles in Stage I can be further subdivided into Stage Ia and Stage Ib. At Stage Ia an overall shrinkage of the granulosa wall is apparent at a low magnification, and detailed degenerative changes, such as pyknosis of granulosa cell nuclei, are seen at a higher magnification in scattered small areas (Figure 5.1 C-D). At Stage Ib many pyknotic cells are found throughout the entire granulosa layer. Many nuclear fragments (apoptotic bodies) are seen at the periphery of the antrum (Figure 5.1E-F). Stage II can also be subdivided in Stage IIa, in which the degenerating oocyte is still surrounded by an envelope of degenerating cumulus cells (Figure 5.1 G-H), and

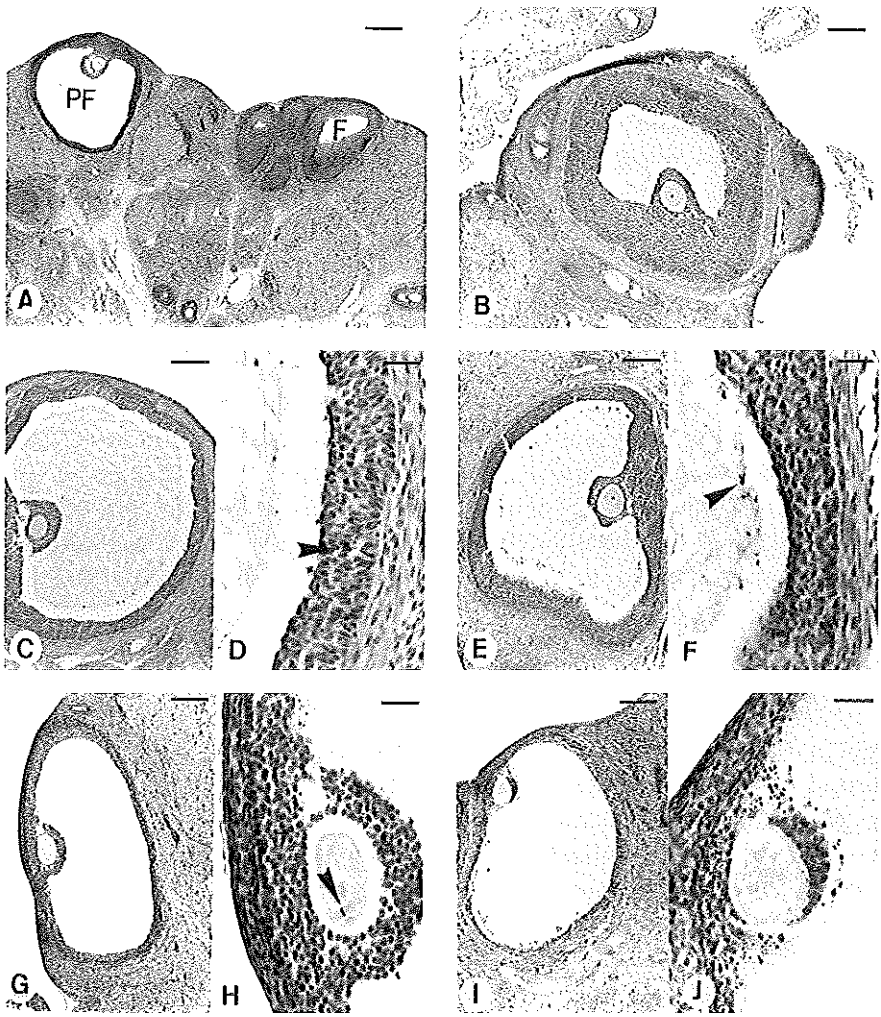

Figure 5.1
Histology of preovulatory follicles at different stages of atresia.

A. Ovarian histology 3 days after blocking ovulation with GnRH-antagonist (P+72). The ovaries show preovulatory follicles in advanced stages of atresia (PF). Another set of antral follicles (F) is present, which will ovulate between P+96 and P+120.

B. Healthy preovulatory follicle at pro-oestrus.

C-D. Stage Ia of atresia. Note the presence of pyknotic granulosa cells in scattered small areas of the granulosa layer (➤).

E-F. Stage Ib of atresia. The complete granulosa layer is affected by degeneration. Note apoptotic bodies at the periphery of the antrum (➤).

G-H. Stage IIa of atresia. The granulosa layer is thin, and in the oocyte, which is still surrounded by cumulus cells, a pseudo-maturation spindle is visible (➤).

I-J. Stage IIb of atresia. The oocyte lays almost solitary in the antrum, while many macrophages are present in the antrum.

Stage IIb, in which the oocyte lies almost solitary in the antrum without any surrounding cumulus cells. No or only a few nuclear particles are seen in the antrum, but macrophages are usually present (Figure 5.1 I-J).

In Experiment 3, all non-atretic follicles of both ovaries of the two rats were counted. At both oestrus and metoestrus morning one animal was examined. Six follicle groups were distinguished based on their diameter as described previously (Osman, 1985): Class 0 follicles from 100 to 275 µm (preantral follicles with at least three layers of granulosa cells), Class 1 (276-350 µm), Class 2 (351-400 µm), class 3 (401-450 µm), Class 4 (451-575 µm) and Class 5 (>575 µm). Classes 1 and 2 are small antral follicles, and Classes 4 and 5 are large antral follicles capable to ovulate. The follicular size was calculated from two perpendicular diameters of the follicle in which the nucleolus of the oocyte was visible.

Immunohistochemistry of BrdU

For BrdU immunostaining, sections were quenched in 3% (v/v) H2O2 for 15 min, washed with PBS and incubated with 0.1% (w/v) pronase (Boehringer Mannheim) in distilled water at 37°C for 10 min. After rinsing in PBS at 4°C, the sections were treated with 2N HCl for 30 min at 37°C, and neutralised with 0.1 M borate buffer (1 part 0.1 M Borax and 5 parts 0.1 M boric acid, pH 8.5). After rinsing in PBS, sections were subsequently incubated with anti-BrdU (1:25; Sigma) in PBS containing 4% (v/v) normal goat serum (Sigma) for 30 min at 37°C. Immunoreactive sites were visualised with DAB. Sections were counter-stained with haematoxylin.

BrdU labelling index of the granulosa cells in Experiment 2 was determined for an area opposite the oocyte and for an area adjacent to the oocyte (Figure 5.2A). The labelling

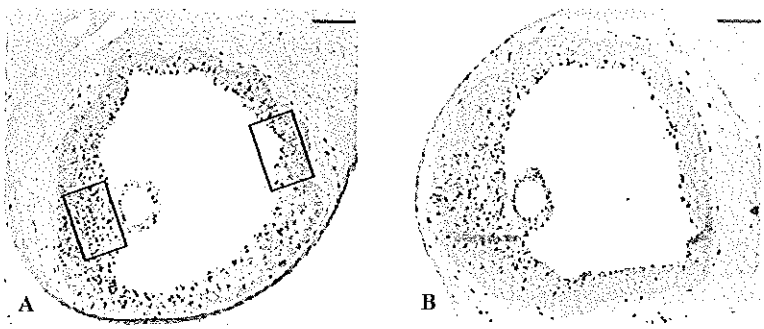

Figure5.2
Immunohistochemical localisation of BrdU in preovulatory follicles after GnRH-antagonist treatment.

A. Preovulatory follicle at pro-oestrus (P). The BrdU labelling index was determined in the designated areas with the help of an eyepiece with a grid.

B. Preovulatory follicle at P+14.

Scale bar = 100 µm.

index of a follicle is represented by the number of labelled granulosa cells expressed as a percentage of all granulosa cells counted in the designated areas. Cells were counted with the aid of an ocular with a grid.

In situ hybridisation of AMH mRNA

In situ hybridisation of AMH mRNA was performed as described earlier (Baarends *et al.*, 1995b). Briefly, after deparaffination and digestion with proteinase K (Boehringer Mannheim), sections were postfixed with paraformaldehyde. After blocking non-specific binding with triethanolamine, sections were incubated with ^{35}S-labeled antisense RNA probes specific for AMH. Hybridisation was carried out overnight at 55°C. Slides were washed with high stringency. After dehydration, slides were dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, USA) and dried, followed by exposure at 4°C for 1 week. After developing, the sections were counter-stained with haematoxylin and mounted. To minimise variation, all sections were used in a single *in situ* hybridisation. The amount of hybridisation was expressed in arbitrary units. Based on the amount of silver grains, five categories were distinguished: 0 = no silvergrains above background, 0.5 = some expression, 1 = clear expression, 2 = high expression, and 3 = very high expression. Within the follicle, the expression of AMH can be variable. Granulosa cells surrounding the oocyte often showed a higher expression than the granulosa cells around the antrum (periluminal granulosa cells). Therefore, AMH mRNA expression was presented as the mean of the expression found in cumulus cells and that in periluminal cells.

Statistical analysis

Results are expressed as means ± SEM. The data were evaluated for statistically significant differences by one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test using SPSS 7.5 computer software (SPSS inc., Chicago, USA). A difference was considered to be significant if $P < 0.05$.

Results

Experiment 1: Atresia of preovulatory follicles

To evaluate the *in vivo* model of induction of atresia of preovulatory follicles, numbers of non-atretic and atretic preovulatory follicles at P, P+24, P+48, P+72 and P+96 were counted in animals in which ovulation was blocked by injecting 20 µg GnRH-antagonist (Table 5.1). At P and P+24 all preovulatory follicles had a non-atretic appearance. At P+48 and P+72 the preovulatory follicles were in an early stage of atresia (Stage Ia and Ib), while at P+96 two types of large follicles were present: non-atretic and atretic follicles. The non-atretic follicles were recently formed and would ovulate the following day, while

Table 5.1
Number of non-atretic and atretic preovulatory follicles after blockage of ovulation with GnRH-antagonist. GnRH-antagonist (20 µg) was given at 10.00 h at pro-oestrus (P) and the next day (P+24).

Day of autopsy	Non-atretic	Early atretic		Late atretic	
		Stage Ia	*Stage Ib*	*Stage IIa*	*Stage IIb*
P	11.7 ± 0.3	0	0	0	0
P+24	11.7 ± 0.9	0	0	0	0
P+48	0	4.7 ± 1.8	7.3 ± 1.5	0	0
P+72	0	3.7 ± 2.7	7.0 ± 2.5	0.7 ± 0.3	0
P+96	14.7 ± 0.6	1.0 ± 1.0	0	7.0 ± 1.0	3.3 ± 0.7
P+48 *	7.0 ± 1.5	1.3 ± 0.3	3.7 ± 0.7	0	0
P+48 #	0	0	0	7.7 ± 0.9	5.0 ± 1.0

Values represent mean ± SEM (n = 3).
*0.5 IU hCG and #100 µg GnRH-antagonist were given at P and P+24

the atretic follicles contained oocytes showing resumption of meiosis, indicative of late atresia (Stage IIa and IIb).

Administration of a subovulatory dose of hCG (0.5 IU) at P and P+24 to rats treated with 20 µg GnRH-antagonist at P and P+24 partially prevented the occurrence of atresia at P+48, compared to rats treated with only 20 µg GnRH-antagonist. Exposure to a larger dose of GnRH-antagonist (100 µg) at P and P+24, resulted in a lower serum concentration of LH at P+24 (0.20 ± 0.01 ng/ml versus 0.72 ± 0.09 ng/ml P<0.001), accompanied by more rapid atresia as indicated by the late stage of atresia found in the preovulatory follicles present at P+48 (Table 5.1) (Uilenbroek *et al.*, 1998).

From this experiment we concluded that blockage of ovulation at pro-oestrus by administration of a GnRH-antagonist provides a suitable model that allows the study of atresia of preovulatory follicles in a predictable chronological order. The rate by which the follicles become atretic appears to depend on the level of circulating LH after GnRH-antagonist administration.

Experiment 2:
BrdU immunostaining and labelling index

In the second experiment a more detailed analysis was made of the period between P and 48 hours after the first injection with GnRH-antagonist (P+48), to investigate whether in this period markers of future atresia could be found. Similarly to Experiment 1, the preovulatory follicles at P+24 were non-atretic, while at P+48 all preovulatory follicles were in an early stage of atresia (Stage Ia and Ib) (Table 5.2). With the exception of a single preovulatory follicle at P+14, the first pyknotic granulosa cells were found 27 hours after

Table 5.2
Number of non-atretic and atretic preovulatory follicles after blockage of ovulation with GnRH-antagonist. GnRH-antagonist (20 µg) was given at 10.00 h at pro-oestrus (P) and the next day (P+24). Values are means ± S.E.M. for three rats.

Day of autopsy	Non-atretic	Early atretic	
		Stage Ia	Stage Ib
P	11.3 ± 1.3	0	0
P+7	12.6 ± 0.3	0	0
P+14	12.7 ± 0.9	0.3 ± 0.3	0
P+24	12.7 ± 0.3	0	0
P+27	10.7 ± 0.3	1.3 ± 0.3	0.3 ± 0.3
P+31	6.7 ± 0.9	3.3 ± 0.7	2.0 ± 1.0
P+48	0	3.7 ± 1.2	6.7 ± 1.3

Values represent mean ± SEM (n = 3).

the first injection with GnRH-antagonist (P+27).

Since atresia of follicles is accompanied by a decrease in granulosa cell proliferation (Gaytan *et al.*, 1996), BrdU labelling index of granulosa cells was studied as a possible marker for future atresia. BrdU is a thymidine analogue that is incorporated into DNA during the S phase of the cell cycle (Gratzner, 1982). Nuclear BrdU was found in granulosa and theca cells of all developmental follicle classes, including the preovulatory follicles (Figure 5.2A). Primordial and primary follicles did not show any BrdU labelling. Some cells of corpora lutea also showed immunostaining, as did macrophages in the interstitium and in the antrum of follicles at more advanced stages of atresia. Background staining was very low and restricted to the corpora lutea and erythrocytes, probably caused by endogenous peroxidase activity. In the oocytes no staining was present. The cumulus cells were the last granulosa cells to lose their BrdU immunoreactivity during atresia, and BrdU incorporation into the nuclei of granulosa cells was absent during advanced stages of atresia (results not shown). At P+14 a clear reduction in the number of BrdU labelled granulosa cells was found (Figure 5.2B).

Figure 5.3 shows the BrdU labelling index of granulosa cells in the vicinity of the oocyte and granulosa cells more distant from the oocyte, at the different time points after the first GnRH-antagonist injection, as a percentage of the BrdU labelling index of time point 0 hours (control). At almost all time points, the labelling index of the granulosa cells in the vicinity of the oocyte was higher than that of the other granulosa cells. The BrdU labelling index of the granulosa cells both in the vicinity of the oocyte and more distant from the oocyte was significantly decreased at P+14, when no morphological signs of atresia were present.

The results of this experiment show that the earliest morphological sign of atresia was present at P+27, while at P+14 the BrdU labelling index was already significantly reduced

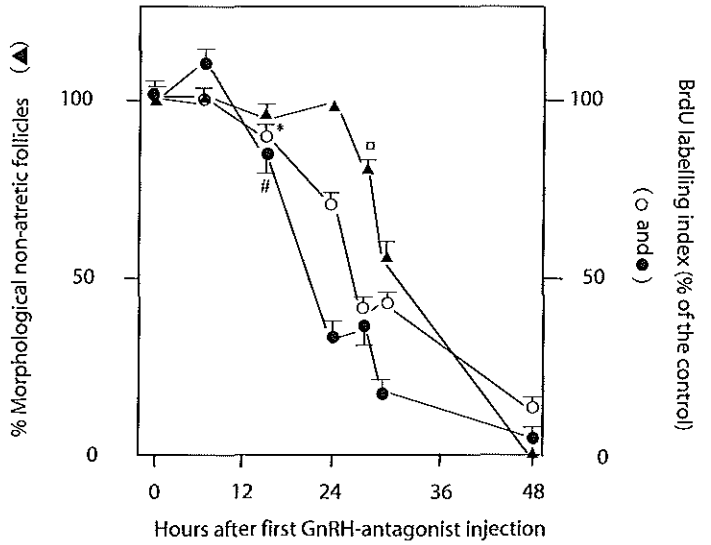

Figure 5.3
Morphology versus BrdU labelling index of granulosa cells of preovulatory follicles at several time points after GnRH-antagonist treatment.

Percentage of morphologically non-atretic preovulatory follicles (- ▲ -) and BrdU labelling index of granulosa cells from preovulatory follicles at different time points after GnRH-antagonist treatment as a percentage of the BrdU labelling index at 0 hours (control).
Animals were injected with BrdU two hours before sacrifice and the ovaries were processed for detection of BrdU incorporation. The BrdU labelling index decreases during the process of atresia. This decrease is somewhat delayed in granulosa cells in the vicinity of the oocyte (- ○ -) compared to the BrdU labelling index of other granulosa cells (- ● -).
Values are given as mean percentages ± SEM. (n=8). *, #, ¤: value of indicated time point and time points following this time point differ significantly ($p < 0.05$) from the value of time point 0.

in granulosa cells from follicles destined to become atretic. Thus, the BrdU labelling index can be regarded as a suitable marker for future atresia.

Experiment 3:
Relation between AMH mRNA expression and BrdU labelling index in small antral follicles

The quantification of the mRNA expression of AMH in granulosa cells of non-atretic follicles of the various size classes present at 10.00 h at oestrus and metoestrus is depicted in Figure 5.4A-B. On oestrus morning most follicles are preantral (<275 μm) or small antral (276-400 μm) and the expression of AMH of the small antral follicles varies between 1.0 and 1.5 units. At metoestrus morning, more medium-sized antral follicles (401-450 μm) are present than at oestrus, and the expression of AMH mRNA decreased from a relatively high expression in preantral follicles (2.5 units) to a lower expression in large antral follicle (0.5 units).

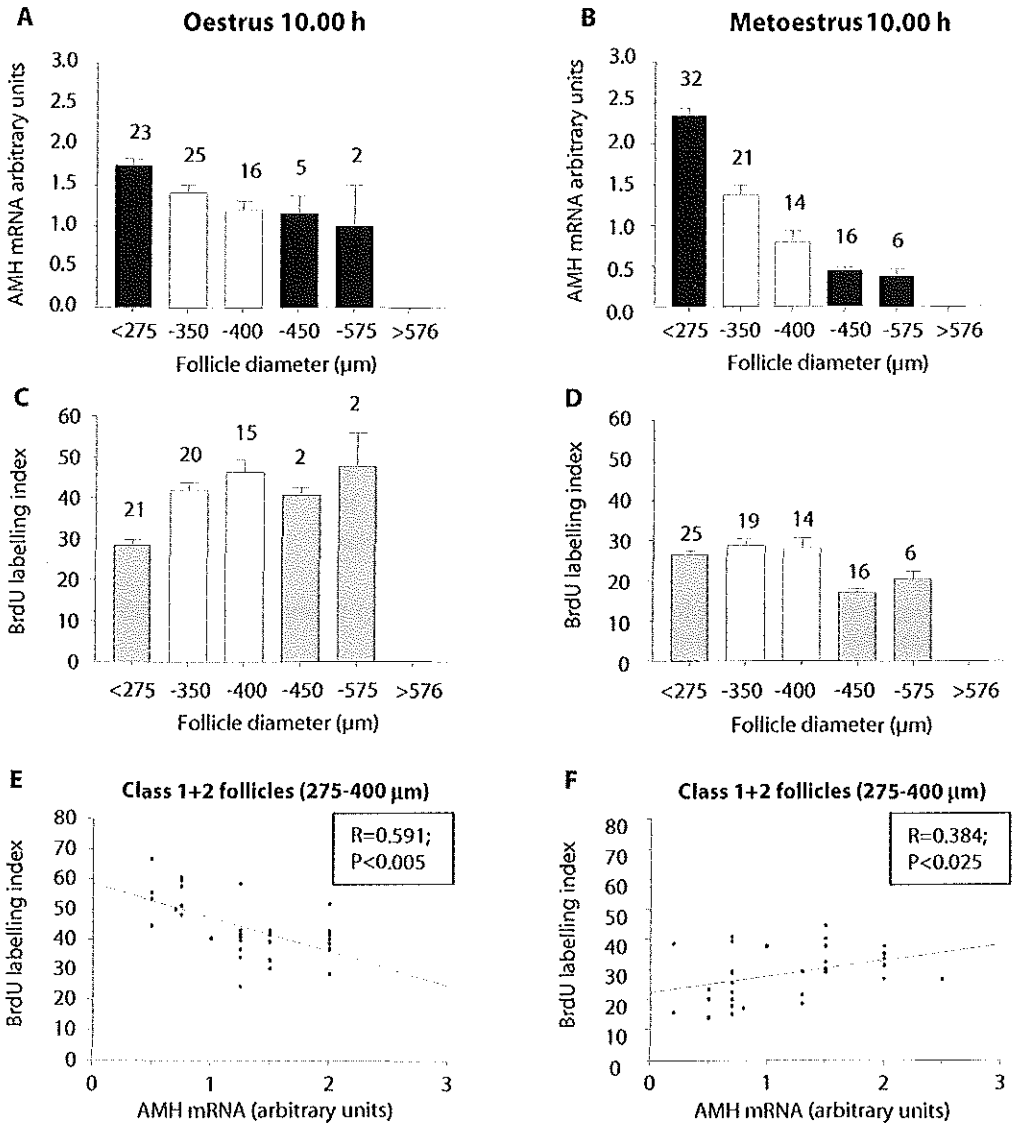

Figure 5.4
The relation between AMH mRNA expression and BrdU labelling in different follicle classes at the morning of oestrus and metoestrus.

A-D. AMH mRNA expression (A-B) and BrdU labelling index of granulosa cells (C-D) in non-atretic follicles of various sizes at oestrus (10.00 h) and metoestrus (10.00 h). AMH mRNA is expressed in arbitrary units ranging from 0.0 to 3.0. BrdU labelling index is expressed as the percentage positive cells per total number of granulosa cells. Numbers above the columns are the number of follicles counted.

E-F. The correlation between AMH mRNA expression and BrdU labelling index for Class 1 and 2 follicles (275-400 µm) at oestrus (10.00 h) and metoestrus (10.00 h).

The BrdU labelling index in the preantral and antral follicles at oestrus and metoestrus is shown in Figure 5.4C-D. Except for preantral follicles, the BrdU labelling index in all follicles at oestrus is higher than that at metoestrus.

The relation between AMH mRNA expression and BrdU labelling index in the granulosa cells in small antral follicles (275-400 µm) was investigated (Figure 5.4E-F). Follicles of that size were chosen, since from this group of follicles certain follicles are selected to grow on to the preovulatory stage, while the other follicles become atretic (van Cappellen *et al.*, 1993). At oestrus 10.00 h, a negative correlation between AMH mRNA expression and BrdU labelling index was found ($R=0.591$, $P<0.005$) (Figure 5.4E). This negative correlation indicates that at oestrus morning, follicles are present with both a high AMH mRNA expression level and a low BrdU labelling index, or with a low AMH mRNA expression level and a high BrdU labelling index. At metoestrus 10.00 h, however, the correlation between AMH mRNA expression and BrdU labelling index had changed and was significantly positive ($R=0.384$, $P< 0.025$) (Figure 5.4F).

Figure 5.5 gives an example of two non-atretic small antral follicles of the same size, on which *in situ* hybridisation for AMH mRNA and BrdU immunohistochemistry was performed. In these follicles, a clear difference in AMH mRNA expression level is found; relatively high in one of these follicles and lower in the other. In the follicle with low expression, the granulosa cells surrounding the oocyte are the last to lose their AMH

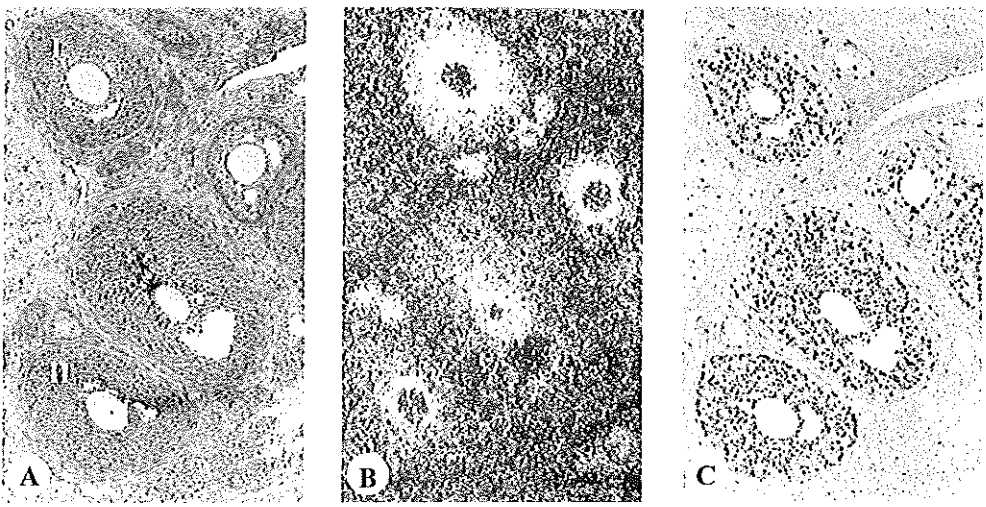

Figure 5.5
Localisation of AMH mRNA expression and BrdU labelling in an ovarian section at oestrus 10.00 h.

A. Ovarian section stained with haematoxylin and eosin. Magnification x 100.

B. Darkfield view of AMH mRNA expression. Magnification x 100.

C. Immunohistochemical localisation of BrdU. Magnification x 100.

Note the presence of two small antral follicles: follicle I is a Class 1 follicle with a high AMH mRNA expression and a BrdU labelling index of 40 %, while follicle II is a Class 1 follicle with a low AMH mRNA expression and a BrdU labelling index of 58 %.

mRNA expression (Figure 5.5B) The BrdU labelling of these follicles is shown in Figure 5.5C. A clear staining is found in the granulosa cells of all growing follicles. The BrdU labelling index of these follicles were 40 % and 58 %.

These results indicate that at oestrus morning, small antral follicles with a high AMH mRNA expression level are on their way of becoming atretic, indicating that AMH might be involved in the process of cyclic recruitment.

Discussion

The normal fate of an ovarian follicle is death by atresia. During the oestrous cycle, however, a limited number of follicles is recruited to continue growth and will reach the preovulatory follicle stage. This process of cyclic recruitment (McGee and Hsueh, 2000) is mainly regulated by FSH. In rats it has been shown that FSH is the main survival factor for small antral follicles (Chun *et al.*, 1996). Blocking the secondary FSH peak, normally occurring on oestrus around 06.00 am, resulted in absent recruitment of small antral follicles (Hirshfield and De Paolo, 1981), while administration of FSH on oestrus resulted in superovulation (van Cappellen *et al.*, 1993). In monkeys, immunoneutralisation of the actions of circulating oestrogen during the midfollicular phase leads to sustained elevation of circulating FSH, allowing the development of multiple preovulatory follicles (Zeleznik *et al.*, 1987). The mechanism by which certain follicles are selected to grow, and others are not, is still not exactly known. It seems, however, that sensitivity of the follicle to FSH is important. Bovine studies suggest that enhanced FSH receptor (FSHR) expression could be important in augmenting the sensitivity of a follicle for FSH (Evans and Fortune, 1997). Certain ovarian growth factors are able to augment FSH responsiveness of a follicle by stimulating FSHR expression. In insulin-like growth factor I (IGF-I)-deficient female mice, FSHR expression is significantly reduced, suggesting that IGF-I is able to increase FSHR expression (Zhou *et al.*, 1997).

In this chapter, it is suggested that also AMH might play a role in determining the sensitivity of ovarian follicles to FSH. This hypothesis was based on the finding that, at oestrus in the rat, when a high serum FSH level is present, two populations of AMH mRNA expressing follicles exist (Baarends *et al.*, 1995b). One population of non-atretic large preantral and small antral follicles showed high AMH mRNA expression, while another population of the same-sized follicles showed low AMH mRNA expression. We postulate that the level of AMH expression influences the sensitivity of the follicle for FSH, causing follicles with a high amount of AMH protein to become atretic, and the follicles with a low amount of AMH to escape atresia and become preovulatory. Part of the results found in Chapter 4 of this thesis support the hypothesis that AMH lowers the sensitivity of follicles for FSH. When treating prepubertal wild type and AMHKO female mice with both GnRH-antagonist and FSH more follicles start to grow in the AMHKO females than in wild type females. Furthermore, it has been shown that AMH inhibits FSH-stimulated follicle growth *in vitro*.

The aim of this study was to test the hypothesis that high AMH mRNA expression

at oestrus morning results in atresia of these follicles. Since a low BrdU labelling index appeared to be a marker of future atresia, we correlated the AMH mRNA expression with the BrdU labelling index in the granulosa cells of those follicles from which selection takes place (276-400 µm) (van Cappellen *et al.*, 1993). Follicles smaller than 275 µm have a high AMH expression and form the stock of follicles that are invariable present during every day of the oestrous cycle. Follicles larger than 400 µm present at oestrus have failed to ovulate and are destined to become atretic, while those present at metoestrus have been selected during the previous oestrous cycle and will soon become preovulatory.

The present results demonstrate that at oestrus indeed a negative correlation exists between AMH mRNA expression and BrdU labelling index, although no clear distinction between two populations of follicles can be made. A negative correlation between AMH and BrdU labelling index at oestrus is in agreement with the hypothesis that follicles with a high AMH expression become atretic, while follicles with a low AMH expression become preovulatory. Such a negative correlation was not present at metoestrus, consistent with the notion that at that stage cyclic recruitment of follicles already has taken place.

The conclusion that AMH plays a role in the cyclic recruitment of follicles at oestrus should be taken with some caution. For future studies, that should include more animals, it may be desirable to use immunohistochemistry employing an AMH antibody to detect AMH protein expression. AMH immunohistochemistry displays less interexperimental variability. However, also quantification of immunohistochemical signals is a complex issue. It may be worthwhile to investigate whether the pattern of AMH protein expression in follicles that are destined to become atretic is different from the expression in future preovulatory follicles.

In conclusion, the results presented in this chapter suggest that AMH is an important candidate determinant of FSH sensitivity of recruitable follicles, and thereby could play a role in control of cyclic recruitment.

Chapter 6

GENERAL DISCUSSION

6.1 Introduction

Female reproductive success in mammalian species depends on correct prenatal development of the ovaries, which harbour the female gametes, from primordial germ cells to mature oocytes. In contrast to the continuous proliferation of spermatogonia during male reproductive life, proliferation of female germ cells only occurs within a certain fetal period, leading to the formation of a pool of female germ cells of limited size. Correct maturation of the oocytes, and proliferation and differentiation of the somatic cells surrounding the oocyte (folliculogenesis), are also important for female fertility. Thus, a better understanding of factors involved in fetal ovarian development and in folliculogenesis during reproductive life, may improve clinical diagnosis and treatment of female infertility owing to ovarian failure.

The major objective of this thesis was to get more insight in the role of anti-Müllerian hormone (AMH), an ovarian growth factor, in the postnatal ovary. In this chapter, current knowledge on the action of AMH in the postnatal mouse ovary will be briefly summarised. Furthermore, we speculate about possible roles of AMH in the postnatal ovary, with respect to human female fertility and the onset of menopause.

6.2 AMH action in the postnatal mouse ovary

In the female mouse ovary, AMH is first produced a few days after birth by granulosa cells of early primary follicles (Chapter 3 of this thesis). Subsequently, high AMH protein expression is found in granulosa cells of preantral and small antral follicles. Since the discovery of also AMH type II receptor (AMHRII) expression in the ovary (Baarends *et al.*, 1994), questions were raised about a possible function of AMH in the postnatal ovary. Recent studies, described in this thesis, give some insight on the function of AMH in the ovary.

Transgenic mice in which AMH expression has been altered provide good tools to study the effect of AMH on the functioning of the postnatal ovary. The first transgenic mice that were generated in which AMH expression was altered, were mice chronically overexpressing hAMH (Behringer *et al.*, 1990). Female mice overexpressing AMH are infertile. Most of these mice have a blind ending vagina, and lack uterus and oviducts. Although the ovaries are present at birth, they contain less germ cells, and in most of the animals the ovaries are devoid of germ cells and develop cord-like structures within two weeks after birth. In adult females the ovaries are degenerated. Only in those mice with the lowest level of serum AMH, ovarian development is not affected. Thus, in mice, the presence of AMH during the fetal period when ovarian and female germ cell development occurs, clearly has an impact on this development. However, much information cannot be drawn from this mouse model, when investigating the possible role of AMH during postnatal follicle development and functioning.

A few years ago, AMH-deficient (AMHKO) mice were generated (Behringer *et al.*, 1994). Male AMHKO mice show a clear phenotype, since they retain Müllerian duct deriv-

atives, while in some male mice also Leydig cell hyperplasia is observed. These males are fertile but their fertility is impaired. In female AMHKO mice, the lack of AMH production does not affect fertility, and no clear ovarian phenotype was reported. A study of the entire follicle population in AMHKO females, however, revealed that loss of AMH production affects the primordial follicle pool, since in the ovaries of 4- and 13-month-old AMHKO females less primordial follicles were found than in the ovaries of their wild type littermates (Durlinger *et al.*, 1999). This increased recruitment of primordial follicles in AMHKO females also leads to an increased number of growing follicles in AMHKO mice of 25 days and 4 months of age. An *in vitro* study subsequently showed that AMH is able to inhibit the initiation of primordial follicle growth in cultured neonatal mouse ovaries (Chapter 3 of this thesis). AMH is therefore important for the initiation of primordial follicle growth, a process also known as initial recruitment (McGee and Hsueh, 2000). Besides AMH, a second factor, stem cell factor (SCF), seems to have a direct effect on primordial follicle recruitment (Parrott and Skinner, 1999).

The regulation of initial recruitment is still unclear. The hypothesis that it is a pre-programmed feature of the ovary (Edwards *et al.*, 1977) does not seem to hold, since it is clearly affected by SCF and AMH. AMH has an inhibitory effect on initiation of primordial follicle growth, while SCF stimulates this process. Thus, it seems likely that initial recruitment is under influence of inhibitory and stimulatory factors, including SCF and AMH coming from growing follicles. Probably more factors are involved, since SCF and AMH *in vitro* do not, respectively, completely stimulate or block the recruitment of the entire pool of primordial follicles.

This inhibitory effect of AMH on primordial follicle recruitment might implicate that absence of, or reduced, AMH production in the female could result in an earlier depletion of the stock of primordial follicles. Therefore, it is expected that the generation of preovulatory follicles ceases at a younger age in AMHKO females than wild type females, since the size of the pool of primordial follicles is inversely correlated with the end of the reproductive life of the female. This is clearly shown in *e.g.* Bax-deficient females. Bax is involved in the execution of apoptosis, or programmed cell death, in a multitude of cell types. In Bax-deficient females, the stock of primordial follicles shrinks much slower than in wild type females, resulting in sustained ovarian function in aged mice (20-22 months) (Perez *et al.*, 1999).

A possible early cessation of the generation of preovulatory follicles in AMHKO females, through an increased loss of primordial follicles, was investigated by the determination of the number of corpora lutea in ovaries in aged (16-17 months old) AMHKO females and their wild type and heterozygous littermates (Table 6.1). The presence of corpora lutea indicates that ovulations have occurred within two weeks prior to sacrifice. In 56 % of the aged AMHKO females no corpora lutea were found, compared to absence of corpora lutea in 29 % of the heterozygous females and 18 % of the wild type females. These results provide an indication that indeed AMHKO females reach the age of ovulatory failure earlier than wild type females. Most, but not all, ovaries lacking corpora lutea were devoid of growing follicles, indicating that not only an exhaustion of the follicular reserve causes the failure of generating corpora lutea, but also other factors are involved in

Table 6.1
Number of wild type female mice, female mice heterozygous for AMH mutation and AMHKO female mice of 16-17 months of age with corpora lutea or no corpora lutea present in their ovaries.

Genotype	corpora lutea	no corpora lutea
Wild type (n = 17)	15	2
Heterozygote (n = 14)	10	4
AMHKO (n = 16)	7[a]	9[a]

[a] indicates a significant difference from wild type females ($P < 0.05$), evaluated with χ^2 test.

this process. Changes in the neuroendocrine regulation, through the hypothalamus and the pituitary gland, of preovulatory follicle development and ovulation are probably also of influence (Nelson and Felicio, 1990).

The study of the AMHKO females also gave indications for a role of AMH in determining the sensitivity of ovarian follicles to FSH (Durlinger *et al.*, 1999). In AMHKO females at an age of 4 months more growing follicles were present than in wild type females, despite a lower serum FSH level (Durlinger *et al.*, 1999). An inhibitory effect of AMH on FSH-induced processes was already shown by several *in vitro* studies. In cultured granulosa cells, exogenous AMH inhibits biosynthesis of aromatase and decreases LH receptor number (di Clemente *et al.*, 1994a), while AMH also opposes proliferation of cultured granulosa-luteal cells (Kim *et al.*, 1992; Seifer *et al.*, 1993). The idea that AMH inhibits FSH action was supported by an *in vitro* study in which it was shown that AMH inhibits FSH-stimulated growth of mouse preantral follicles (Chapter 4 of this thesis). Furthermore, an *in vivo* study in which follicle growth was stimulated by FSH in AMHKO females and their wild type littermates revealed that under the influence of FSH more follicles start to grow in AMHKO females than in wild type females (Chapter 4 of this thesis). The study of the ovarian phenotype of AMH-/FSHβ-deficient (FAKO) females also revealed that AMH is a much more dominant regulator of early follicle growth than FSH, since in FAKO females the ovarian phenotype caused by AMH-deficiency is even more pronounced (Chapter 4 of this thesis).

The inhibitory effect of AMH on FSH-stimulated follicle growth might be very important during cyclic recruitment. In mice and rats, a group of follicles is recruited from the pool of large preantral and small antral follicles, to continue growth to the preovulatory stage. This process of cyclic recruitment takes place at oestrus, when FSH reaches a peak level. This FSH peak has been shown to be important for the number of follicles that are recruited (Hirshfield and De Paolo, 1981; van Cappellen *et al.*, 1993). It is thought that, depending on the stage of development, each follicle requires a certain concentration of FSH to continue growth, or in other words, each follicle has its own FSH threshold level and this threshold level should be surpassed to ensure growth to the preovulatory stage. The concept of the FSH threshold is developed after studies in the human (Brown, 1978).

Therefore, it is thought that in rodents at oestrus a high sensitivity of large preantral and small antral follicles to FSH is important for the recruitment of these follicles. Although all follicles have an equal potential to reach the preovulatory stage, it is thought that only those follicles that are sufficiently sensitive to FSH can respond accurately to the hormone. Thus, factors influencing sensitivity to FSH are important during cyclic recruitment. Since it has been shown that AMH is able to inhibit FSH-stimulated follicle growth, AMH might be one of the factors involved in decreasing FSH-sensitivity. The existence at oestrus in rats of two populations of small antral follicles, one with a high AMH mRNA expression level and one with a low AMH mRNA expression level, supports the idea of a role of AMH in cyclic recruitment (Baarends *et al.*, 1995b). More indications for a role of AMH in cyclic recruitment come from a preliminary study in the cycling rat (Chapter 5 of this thesis). This study shows that at oestrus the small antral follicles with a low AMH mRNA expression level are likely to be rescued from atresia and are selected for continuing growth and development toward the preovulatory stage, while the ones with a high AMH mRNA expression level will become atretic.

In conclusion, several studies on the function of AMH in the postnatal ovary, indicate that AMH has at least two functions in the postnatal mouse ovary. First, AMH plays a role during initial recruitment, when resting primordial follicles are initiated to grow, and second, it may modify preantral follicle growth. This latter effect could be important during cyclic recruitment, when certain large preantral and small antral follicles are recruited to grow on to the preovulatory follicle stage. Figure 6.1 gives a schematic representation of AMH action during folliculogenesis in the postnatal ovary.

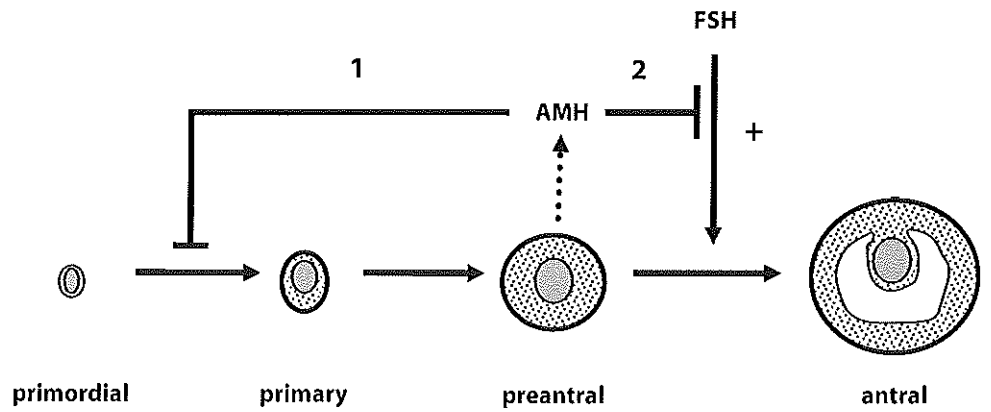

Figure 6.1
Effects of AMH in the postnatal ovary.
AMH produced by preantral and small antral follicles in the postnatal ovary has two sites of action in the postnatal ovary. It inhibits initial recruitment (1), while it also inhibits the stimulatory effect of FSH on the growth of preantral and small antral follicles (2).

6.3 AMH AND FERTILITY IN WOMEN

In a recent review by Elvin and Matzuk (1998) and in Chapter 1 of this thesis, a description of several mouse models is given, with spontaneous or induced mutations in their genome, causing ovarian failure resulting in female infertility. These mouse models are tools to study specific mechanisms of infertility, and this knowledge can be used to understand causes of human female infertility. In many of these models an impaired development of the ovary results in infertility, and also the formation of the germ cells is often disturbed. Furthermore, improper development of ovarian follicles is a major cause of female infertility in several mouse models.

In most women, a clear temporal order in events related to their fertility is found. At a mean age of 30 years, when the remaining follicle reserve has become a fraction of its original number, the first signs of decreasing fertility are detected. Thereafter, a further decrease in both oocyte quantity and quality dictates the subsequent events leading to the end of fertility, including decrease of fertility, increased abortion rate, the beginning of cycle irregularity, and, when almost no follicles are left in the ovaries, the occurrence of menopause (te Velde *et al.*, 1998).

In this thesis, the AMHKO mouse model and several other experimental approaches have been used to show that AMH plays an important role in the functioning of the postnatal ovary, and therefore, one may consider the impact on human female fertility of changed AMH expression in the postnatal ovary.

In contrast to mice and rats, in the human female AMH protein expression is first detected at the end of fetal life in granulosa cells of preantral follicles (Rajpert-De Meyts *et al.*, 1999). In ovaries subsequent to this stage of development, strong AMH expression is found in granulosa cells of preantral and small antral follicles, while in large antral follicles the expression was variable. Primordial, primary, very large antral follicles and atretic follicles were always negative (Rajpert-De Meyts *et al.*, 1999) (our own unpublished observations). Overall, serum AMH concentration in women is low. Peak AMH serum level is found during puberty, the level drops in adult females and is undetectable after menopause (Lee *et al.*, 1996; Cook *et al.*, 2000). The low serum concentration might be a technical problem (detection limit) in using the AMH serum level in studying the relation between serum AMH level and female infertility. However recently, a new highly sensitive enzyme-linked immunosorbent assay for AMH has been developed, by which the detection of a very low concentration of AMH in the serum is possible (Long *et al.*, 2000).

A possible equivalent of the AMH overexpression mouse model in the human are women suffering from Müllerian aplasia, also referred to as the Mayer-Rotinansky-Küner-Hauser (MRKH) syndrome (Shokeir, 1978; Golan *et al.*, 1989). Most of these women have a blind ending vagina and are diagnosed with primary amenorrhea (Shokeir, 1978; Lindenman *et al.*, 1997). Closer examination of these women reveals abnormal Müllerian duct differentiation, ranging from complete lack of Müllerian duct derivatives to the formation of a bicornuate uterus and septate vagina (Golan *et al.*, 1989; Visser, 1998). In contrast to female mice with a chronic high level of AMH, these women have functioning ovaries, since at puberty they develop normal secondary sex characteristics. The ovar-

ian phenotype of these women remains to be studied and provides a possible source of information about the role of AMH in the postnatal ovary.

However, identification of the direct cause of Müllerian aplasia has appeared to be complicated, because of the variability of the phenotype. Inspection of patients with complete Müllerian aplasia and their pedigrees provides evidence for autosomal inheritance of this defect (Shokeir, 1978). Müllerian aplasia might be caused by aberrant expression of the *AMH* gene in the female before birth, resulting from mutations in the *AMH* gene promoter or mutation of factors regulating *AMH* gene expression. Until now, however, no reports have been published of gene mutations related to AMH gene expression causing Müllerian aplasia. Furthermore, it is quite likely that aberrant expression of AMH is not the most frequent cause of Müllerian aplasia, but that dysfunction of genes involved in the formation of the Müllerian ducts, or genes involved in the patterning of the Müllerian duct (*Hoxa9-Hoxa13*) can be a major cause of Müllerian aplasia (Taylor *et al.*, 1997; Block *et al.*, 2000).

The inhibiting effect of AMH on initial recruitment, as has been shown in the mouse by both *in vivo* and *in vitro* studies, might also play a role in the human female. The occurrence of menopause, the definite cessation of menses, in women coincides with an almost complete loss of the last follicles from their ovaries, while the period prior to menopause (perimenopause) is also characterised by a low number of follicles present in the ovaries (Richardson *et al.*, 1987). Thus, the number of follicles in the ovary of a woman reaching the end of her reproductive period is the major determinant of the timing of both the perimenopause and the menopause. Post-menopausal females are infertile due to the exhaustion of the follicular reserve, while females in perimenopause, which still contain follicles in their ovaries, have a reduced fertility of which the cause is not completely known (Richardson and Nelson, 1990). Probable causes could be an age-related reduction in oocyte quality (Gaulden, 1992; Eichenlaub-Ritter, 1998) and/or changes in production pattern of follicle growth affecting hormones produced by the hypothalamus and pituitary gland (Wise *et al.*, 1997; Wise *et al.*, 1999).

Through its inhibitory effect on primordial follicle recruitment, AMH may regulate the efficiency of the use of the primordial follicle pool and therefore may be involved in the determination of the age of menopause in women. This could have implications for the start of menopause in women with mutations in the genes encoding AMH or AMHRII. Thus, the determination of the age at (peri)menopause and AMH serum concentration in female relatives of boys or males presenting with Persistent Müllerian Duct Syndrome (PMDS), might provide information on the relevance of AMH action in influencing ovarian function.

Complete loss of ovarian follicles and subsequent infertility is also found in women suffering from premature ovarian failure (POF). POF causes amenorrhea, infertility, sex steroid deficiency and elevated gonadotropins in women younger than 40 years (Nelson *et al.*, 1996). Known causes of POF are, *e.g.*, radiation exposure and chemotherapy (Conway, 1997), autoimmunity (Addison's disease; myasthenia gravis) (Hoek *et al.*, 1997) or chromosomal abnormalities (Powell *et al.*, 1994). In most cases, however, no etiology of POF can be identified. Aberrant expression of follicle growth regulating factors, such as

AMH, also could result in early loss of the ovarian follicle stock. Furthermore, idiopathic POF consists of afollicular and follicular forms. Examination of ovarian biopsies from patients with idiopathic POF revealed that in about 40% of these patients their ovaries still contain follicles (Van Kasteren, 2000). Folliculogenesis is obviously disturbed in these females, and the cause of this disturbance could, again, be abnormal functioning of follicle growth regulating factors.

As suggested before in Chapters 4 and 5 of this thesis, AMH might play a role during cyclic recruitment, by lowering the FSH-sensitivity of follicles undergoing cyclic recruitment in rodents. During the human menstrual cycle, a cohort of antral follicles also escapes atresia, due to the positive action of FSH on survival of the growing follicle. Among this group about 10 antral follicles, one grows faster than the rest of the cohort and emerges as the dominant follicle, which will eventually ovulate. Although the exact mechanism causing this one follicle to emerge as the dominant one is unknown, this follicle is likely to be more sensitive to FSH (Fauser and Van Heusden, 1997). FSH-sensitivity might be augmented by enhanced FSH and/or LH receptor expression, or an increase in local growth factors that increased FSH responsiveness, as suggested by studies in the bovine (Xu *et al.*, 1995; Bao *et al.*, 1997; Evans and Fortune, 1997). It is suggested that a decrease in local growth factors that diminish FSH responsiveness, such as AMH, also could be important in determining FSH sensitivity of follicles undergoing cyclic recruitment.

Altered FSH-sensitivity might be one of the causes of 'gonadal failure' in terms of an inadequate number of recruited follicles, in woman undergoing controlled ovarian hyperstimulation with gonadotropins. Most of the females showing such a gonadal failure are older than 38 years, although much younger women have the same problem. Many of these women show a reduction in the number of follicles present in their ovaries. This decline in follicular reserve leads to fewer follicles being available for maturation by hyperstimulation with gonadotropins (Ferraretti *et al.*, 2000), and therefore can be seen as the major cause of the poor response. However, mechanisms other than depletion in ovarian follicle reserve can be involved in a poor response, mainly in the group of young women that still have a considerable number of follicles in their ovaries. Aberrant expression of intra-ovarian factors influencing the sensitivity of follicles to FSH could be involved in the poor response of these females to ovarian hyperstimulation with gonadotropins. If, for example, AMH expression would be very high in these females, than the sensitivity of follicles to FSH could be low. Determination of the serum AMH concentration in these women, and correlating this concentration to FSH-sensitivity of the remaining follicle pool, might provide some information on the role of AMH in determining FSH-sensitivity of follicles.

In conclusion, in the studies of the mouse, indications were found that AMH is involved in two steps during follicular growth and development: the initial recruitment of primordial follicles into the pool of growing follicles, and the subsequent preantral follicle growth, possibly affecting the cyclic recruitment of large preantral or small antral follicles to grow on to the preovulatory stage. AMH may have this role in the human ovary as

well. It is therefore of great importance to enhance our knowledge of the role of AMH in the ovary, which may lead to valuable information concerning the role of dysregulation of AMH function as a cause of infertility in women, and the possible treatment of this infertility.

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SUMMARY

Anti-Müllerian hormone (AMH) is a dimeric glycoprotein, and belongs to the transforming growth factor β (TGFβ) family of growth and differentiation factors. This family also includes the inhibins and activins, growth and differentiation factor 9 (GDF9), and many other factors. During male fetal development AMH is produced by the testicular Sertoli cells, and is essential for regression of the Müllerian ducts. Following the period in which the Müllerian ducts are sensitive to AMH, the human ovaries also start to produce AMH. In the human female, AMH is first found in the ovary during the last weeks of fetal life, while in rodents like mice and rats AMH production starts a few days after birth. In the ovary the granulosa cells of mainly non-atretic preantral and small antral follicles express AMH. Follicular development in the female ovary is under the influence of many hormones and growth factors, of which several are produced by the ovary itself. Because of the clear and specific expression pattern of AMH in the postnatal ovary, it was suggested that AMH might be one of the ovarian-derived growth factors involved in the regulation of follicle growth and development.

This thesis focuses on the role of AMH in the control of ovarian follicle growth and development in the postnatal mouse ovary.

In the General introduction (Chapter 1), gonad and germ cell formation in the mammalian female are described, on basis of observations in female mice, with the focus on the most important factors involved in these processes. Furthermore, ovarian follicle growth and development and the most important of the many hormones and growth factors that tightly control ovarian follicle development are described. Finally, in Chapter 1 emphasis is on AMH, one of the ovarian growth factors.

In the experiments described in Chapter 2, the role of AMH in the postnatal ovary was investigated with the help of AMH-deficient (AMHKO) female mice. A close examination of the ovarian follicle population of AMHKO females revealed that less primordial follicles are present in ovaries of 4- and 13-month-old AMHKO females than in wild type females. These results present proof that AMH exerts an inhibitory effect on the recruitment of primordial follicles. Furthermore, indications were found for a possible inhibitory effect of AMH on the sensitivity of follicles to follicle-stimulating hormone (FSH), since in AMHKO females more growing follicles were found than in wild type females, despite a lower serum FSH level in the AMHKO animals.

The inhibitory effect of AMH on the initiation of primordial follicle growth was investigated also in an *in vitro* study in which neonatal wild type mouse ovaries (with endogenous AMH expression) were cultured in the presence of added (exogenous) AMH (Chapter 3). Ovaries of 2-day-old mice predominantly contain primordial follicles, some 'naked' oocytes and no growing follicles, and therefore provide a good model to investigate the effect of AMH on primordial follicle recruitment. Immunohistochemical examination of AMH and inhibin α-subunit protein expression in freshly isolated neonatal ovaries revealed that both proteins are first found in granulosa cells of early primary follicles of 4-day-old mice, indicating that both AMH and inhibin α-subunit are markers of

early follicle growth. Culture of 2-day-old mouse ovaries up to 4 days had no detrimental effect on the overall ovarian morphology and follicle development. In the cultured ovaries, AMH and inhibin α-subunit protein expression were also found in granulosa cells from the early primary stage onward. After 2 and 4 days of culture in the presence of added AMH, less growing follicles were found than in ovaries cultured in the absence of exogenous AMH. These findings were supported by a significant lower level of inhibin α-subunit mRNA expression after 2 and 4 days of culture in ovaries cultured in the presence of exogenous AMH. No effect was found on AMH type II receptor (AMHRII) mRNA expression and on the expression of selected oocyte markers: zona pellucida protein 3 (ZP3) and growth and differentiation factor 9 (GDF9).

The separate and combined effects of AMH and FSH on ovarian follicle development were studied as described in Chapter 4. Three different experimental approaches were used. An *in vitro* study, in which isolated preantral mouse follicles were cultured in the presence of added AMH, showed that AMH is able to inhibit FSH-stimulated preantral follicle growth. Indications for this inhibitory effect of AMH on FSH-stimulated follicle growth were also found in an *in vivo* study using immature wild type and AMHKO female mice. This study showed that in AMHKO females that were treated with FSH more follicles are growing than in wild type females. Finally, a comparative study of ovaries from 4-month-old wild type, AMHKO, FSHβ-deficient (FSHβKO), and AMH-/FSHβ-double-deficient (FAKO) females revealed that in FAKO females, like in AMHKO females, less primordial follicles and more growing follicles are found than in wild type females. Therefore, it can be concluded that AMH is a more dominant regulator of early follicle growth than FSH. Furthermore, this study revealed that the absence of FSH does not influence the recruitment of primordial follicles or their subsequent growth.

In Chapter 5, experiments on the possible role of AMH during cyclic recruitment are described. During cyclic recruitment, a process that occurs at oestrus in rodents, several large preantral and small antral follicles are 'rescued' from atresia and thus are able to continue growth. Indications for such a role of AMH first came from a study on cycling rats, which showed that at oestrus two populations of large preantral and small antral follicles exist, one with a high AMH mRNA expression level and the other with a relatively low AMH mRNA expression level. It was suggested that follicles with a low mRNA expression level are able to continue growth, while follicles with a high AMH mRNA expression level would become atretic. Using a model of GnRH-antagonist-treated cycling rats, it was found that the BrdU labelling index (based on incorporation of 5-bromodeoxyuridine, as a measure of DNA replication and cell proliferation) of granulosa cells of follicles that are destined to become atretic decreases before atresia becomes morphologically apparent, and therefore this index can be used as a marker for future atresia. Finally, using the decrease of the BrdU labelling index as a marker of future atresia, it was shown that on oestrus morning a negative correlation exists between the AMH mRNA expression level and the BrdU labelling index of the granulosa cells of follicles undergoing cyclic recruitment. These results indicate that, on the day of oestrus in the rat, follicles with a high AMH expression level are likely to become atretic, while follicles with a low AMH mRNA expression level are likely to grow on, suggesting that AMH is involved in cyclic recruit-

ment.

The General discussion (Chapter 6) summarises our current knowledge about the roles of AMH in ovarian follicle growth and development, mainly based on experiments using mice and rats. It concludes that AMH can affect ovarian follicle growth and development in two ways: it can inhibit recruitment of primordial follicles and subsequent preantral follicle growth. This effect on preantral follicle growth might be important during the process of cyclic recruitment.

In addition, this chapter elaborates on the clinical significance of the present work, in the context of improvement of diagnosis and therapy of human subfertility, infertility, and premature ovarian ageing.

SAMENVATTING

Het anti-Müllerse gang hormoon (AMH) is een lid van de TGFβ familie van groei- en differentiatiefactoren. Tot deze familie behoren o.a. ook de inhibines en activines, en groei- en differentiatiefactor 9 (GDF9). Tijdens de ontwikkeling van de mannelijke foetus in zoogdieren wordt AMH geproduceerd door de Sertoli cellen in de zaadballen (testikels of testes). Via locale diffusie werkt deze factor in de omgeving van de testes als een hormoon, en veroorzaakt het verdwijnen van de gangen van Müller, waaruit in de vrouwelijke foetus de eileiders, baarmoeder en het bovenste deel van de vagina gevormd worden. In de vrouwelijke foetus van de mens wordt AMH ook in de eierstokken (ovaria) geproduceerd, maar dit begint pas tijdens de laatste weken van de foetale ontwikkeling, nadat de periode waarin de Müllerse gangen gevoelig zijn voor de werking van AMH is verstreken. In ovaria van muizen en ratten komt de AMH productie niet eerder dan een aantal dagen na de geboorte op gang. Deze productie vindt plaats in de granulosa cellen van voornamelijk niet-atretische preantrale en klein-antrale follikels. Follikelgroei en -ontwikkeling in de ovaria staan onder invloed van verschillende hormonen en groeifactoren, waarvan een aantal door de ovaria zelf gemaakt worden. Het duidelijke en specifieke expressiepatroon van AMH in postnatale ovaria suggereerde dat AMH één van de ovariële groeifactoren zou kunnen zijn, betrokken bij de regulatie van follikelgroei en -ontwikkeling.

In de experimenten beschreven in dit proefschrift werd de mogelijke rol van AMH in de regulatie van follikelgroei en -ontwikkeling in de ovaria van de muis onderzocht, en de resultaten worden besproken.

In de Algemene Inleiding (Hoofdstuk 1) wordt de ontwikkeling van de vrouwelijke geslachtsorganen en de vorming van de kiemcellen in de muis beschreven. De nadruk wordt hierbij gelegd op de belangrijkste factoren die betrokken zijn bij deze processen. Verder wordt de groei en ontwikkeling van ovariële follikels, en de werking van de hormonen en groeifactoren die het meest bij deze processen betrokken zijn, beschreven. In het laatste deel van de Algemene Inleiding wordt uitvoerig ingegaan op aspecten van AMH, een van de ovariële groeifactoren en tevens het onderwerp van dit proefschrift.

In de experimenten beschreven in Hoofdstuk 2 werd de mogelijke rol van AMH in postnatale ovaria onderzocht, met behulp van vrouwelijke AMH-deficiënte (AMHKO) muizen. Na uitvoerige bestudering van de totale follikelpopulatie in AMHKO muizen werd gevonden dat de ovaria van 4 en 13 maanden oude AMHKO vrouwtjes minder primordiale follikels bevatten dan hun wild type nestgenoten. Deze resultaten laten zien dat AMH een remmend effect heeft op de rekrutering (initiatie van groei en ontwikkeling) van primordiale follikels. Verder werden er tijdens deze studie aanwijzingen gevonden voor een remmend effect van AMH op de gevoeligheid van follikels voor het follikel-stimulerend hormoon (FSH). Deze conclusie is gebaseerd op het feit dat in 4 maanden oude AMHKO vrouwtjes meer groeiende follikels werden gevonden dan in wild type vrouwtjes van dezelfde leeftijd, ondanks een lagere serum FSH concentratie in de AMHKO vrouwtjes.

Het remmend effect van AMH op de initiatie van de groei van primordiale follikels

werd bestudeerd in een *in vitro* studie, waarin ovaria van pasgeboren wild type vrouwtjes werden gekweekt in de aan- of afwezigheid van toegevoegd AMH. Deze experimenten zijn beschreven in Hoofdstuk 3. Ovaria van 2 dagen oude muizen bevatten voornamelijk primordiale follikels en 'naakte' oocyten, maar geen groeiende follikels. Vanwege deze samenstelling van de follikelpopulatie zijn deze ovaria uiterst geschikt om het effect van AMH op de rekrutering van primordiale follikels te testen. De eiwitten AMH en inhibine α-subunit werden met behulp van immunohistochemie aangetoond in granulosa cellen van vroeg primaire follikels, in ovaria van 4 dagen oude muizen. Dit laat zien dat zowel AMH als inhibine α-subunit markers zijn voor vroege follikelgroei. Het kweken van 2 dagen oude ovaria gedurende 4 dagen had geen schadelijk effect op de algehele morfologie van het ovarium en op de follikelontwikkeling. In de gekweekte ovaria werd AMH en inhibine α-subunit eiwit ook gevonden in de granulosa cellen vanaf het vroeg primaire follikel stadium. In de ovaria gekweekt in aanwezigheid van toegevoegd AMH werden zowel na 2 als 4 dagen kweek minder groeiende follikels aangetroffen dan in ovaria gekweekt zonder toegevoegd AMH. Deze resultaten worden ondersteund door een significant lager niveau van inhibine α-subunit mRNA expressie in ovaria die 2 of 4 dagen gekweekt waren in de aanwezigheid van toegevoegd AMH. Geen effect van AMH werd gevonden op het AMH receptor type II (AMHRII) mRNA expressie niveau, en op het expressie niveau van twee onderzochte oocyte markers: zona pellucida 3 (ZP3) mRNA en groei- en differentiatiefactor 9 (GDF9) mRNA.

De onafhankelijke en gecombineerde effecten van AMH en FSH op ovariële follikelontwikkeling staan beschreven in Hoofdstuk 4. Drie verschillende experimentele benaderingen werden uitgevoerd. Een *in vitro* studie waarin geïsoleerde preantrale follikels werden gekweekt in de aan- of afwezigheid van toegevoegd AMH heeft aangetoond dat AMH in staat is om FSH-gestimuleerde follikelgroei te remmen. Aanwijzingen voor dit remmend effect van AMH werden ook gevonden in een *in vivo* studie, waarin prepuberale wild type en AMHKO muizen werden behandeld met FSH. In deze studie werden meer groeiende follikels gevonden in AMHKO vrouwtjes dan in wild type vrouwtjes. In een tweede *in vivo* studie werd de follikelpopulatie in ovaria van 4 maanden oude AMH-/FSHβ-dubbel-deficiënte (FAKO) vrouwtjes vergeleken met de follikelpopulatie in AMHKO, FSHβ-deficiënte (FSHβKO) en wild type vrouwtjes. Deze studie laat zien dat in ovaria van FAKO vrouwtjes nog minder primordiale follikels en nog meer groeiende follikels gevonden worden dan in ovaria van AMHKO vrouwtjes. Hieruit kan worden geconcludeerd dat, vergeleken met FSH, AMH een meer dominante regulator is van vroege follikelgroei. Verder kan de conclusie getrokken worden dat de afwezigheid van FSH geen invloed heeft op rekrutering van primordiale follikels en de daaropvolgende follikelgroei.

In Hoofdstuk 5 wordt een experiment beschreven dat de mogelijke rol van AMH in de rekrutering van grote preantrale en kleine antrale follikels beschrijft. Deze rekrutering vindt in knaagdieren plaats op de dag van oestrus, en voorkomt dat een aantal grotere preantrale en klein-antrale follikels atretisch worden. Aanwijzingen voor een dergelijke rol van AMH zijn gebaseerd op de waarneming dat twee populaties van de grotere preantrale en klein antrale follikels in de rat op oestrus voorkomen: één met een hoog AMH

mRNA expressie niveau en de ander met een laag AMH mRNA niveau. Follikels met een laag AMH mRNA expressie niveau zouden door kunnen groeien, terwijl follikels met een hoog AMH mRNA expressie niveau atretisch zouden kunnen worden. Met behulp van een experimenteel model waarin ratten met een normale ovariële cyclus met GnRH-antagonist werden behandeld, werd gevonden dat de *BrdU labelling index* (een experimentele methode om celdelingsaktiviteit te meten) van granulosa cellen van follikels die op weg zijn atretisch te worden een duidelijke daling laat zien voordat morfologische kenmerken van atresie in deze follikels zichtbaar worden. De *BrdU labelling index* van de granulosa cellen kan dus worden gebruikt als marker voor toekomstige atresie. Vervolgens werd aangetoond dat op de ochtend van oestrus een negatieve correlatie bestaat tussen de *BrdU labelling index* en het expressie niveau van AMH mRNA van granulosa cellen van follikels die rekrutering ondergaan. Dit resultaat is wederom een aanwijzing voor het feit dat, in de rat op oestrus, follikels met een hoog AMH mRNA expressie niveau waarschijnlijk atretisch worden, en follikels met een laag AMH mRNA expressie niveau kunnen doorgroeien. Deze resultaten suggereren opnieuw dat AMH betrokken is bij de rekrutering van de grotere preantrale en klein-antrale follikels tijdens de ovariële cyclus.

In de Algemene Discussie wordt de huidige kennis over de mogelijke rol van AMH bij de regulatie van de groei en ontwikkeling van ovariële follikels, die op basis van gegevens uit onderzoek aan muizen en ratten is verzameld, samengevat. De gegevens laten zien dat AMH de groei en ontwikkeling van ovariële follikels op twee manieren kan beïnvloeden. AMH remt allereerst de rekrutering van primordiale follikels, en vervolgens ook nog de groei en ontwikkeling van preantrale follikels. Dit remmend effect op de groei en ontwikkeling van preantrale follikels zou belangrijk kunnen zijn tijdens de rekrutering van deze follikels tijdens de ovariële cyclus.

Het laatste deel van de Algemene Discussie gaat in op de klinische relevantie van het werk gepresenteerd in dit proefschrift, met betrekking tot het verbeteren van het stellen van een diagnose en het vaststellen van een toe te passen therapie, in geval van subfertiliteit of infertiliteit, maar mogelijk ook om meer inzicht te krijgen in oorzaken van vroegtijdige veroudering van de ovaria.

List of publications

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(toch nog een lang dankwoord geworden)

Curriculum vitae

Alexandra Lilian Louise Durlinger werd geboren op 31 oktober 1969 te Maastricht. In 1988 behaalde zij aan het Trichtercollege in haar geboorteplaats het VWO diploma. In datzelfde jaar begon zij met de studie Biologie aan de Universiteit Utrecht. Tijdens deze studie werd bij de Projectgroep Vergelijkende Endocrinologie van de Universiteit Utrecht onderzoek verricht aan de expressie van het gonadotroop hormoon tijdens de larvale ontwikkeling van de Afrikaanse meerval (Dr. J. Peute en Prof.dr. H.J.Th. Goos). Bij de Projectgroep Experimentele Embryologie van de Universiteit Utrecht werd een morfologische studie verricht aan diverse aspecten van morfogenetische plasma's in drie mollusken: *Lymnae stagnalis, Physa fontinalis* en *Bithynia tentaculata* (Dr. R. Dohmen en Prof.dr. J.A. van den Biggelaar). In augustus 1994 werd het doctoraaldiploma Biologie behaald. Van oktober 1994 tot januari 1995 werkte zij als voorlichtingsmedewerker bij het 'Phuket Gibbon Rehabilitation Project' in Phuket, Thailand.

Van oktober 1995 tot januari 2000 was zij werkzaam bij de Afdeling Endocrinologie & Voortplanting van de Erasmus Universiteit Rotterdam, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd onder supervisie van Dr.ir. A.P.N. Themmen, Dr. J.Th.J. Uilenbroek en Prof.dr. J.A. Grootegoed.