ACTIVINS AND ACTIVIN RECEPTORS IN THE RAT TESTIS

ACTIVINES EN ACTIVINE RECEPTOREN IN DE TESTIS VAN DE RAT

PROEFSCHRIFT

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Aan mijn ouders
Voor Marjolein

CONTENTS

ABBREVIATIONS

AA	: amino acids
ACTH	: adrenocorticotropin
ActRI	: activin receptor type I
ActRII	: activin receptor type II
ActRIIB	: activin receptor type IIB
ALK	: activin receptor-like kinase
AMH	: anti-Müllerian hormone (Müllerian inhibiting substance)
AS	: anti-serum
ATP	: adenosine triphosphate
bFF	: bovine follicular fluid
BMP	: bone morphogenetic protein
bp	: base pair
BSA	: bovine serum albumin
cAMP	: adenosine cyclic-3':5'-monophosphate
cDNA	: complementary deoxyribonucleic acid
Ci	: curie
COS	: monkey kidney cell line
CRH	: corticotropin releasing hormone
DABA	: 3,5-diaminobenzoic acid dihydrochloride
DMEM	: Dulbecco's modified Eagle's medium
DNA	: deoxyribonucleic acid
DPP	: decapentaplegic gene product
EC	: embryonal carcinoma
EDF	: erythroid differentiation factor (activin-A)
EDTA	: ethylenedinitrilotetraacetate
EGF	: epidermal growth factor
ELISA	: enzyme linked immunosorbent assay
ES	: embryonic stem
FCS	: fetal calf serum
FGF	: fibroblast growth factor
FITC	: fluorescein isothiocyanate
FS	: follistatin
FSH	: follicle-stimulating hormone (follitropin)
g	: gravity
GH	: growth hormone
GnRH	: gonadotropin releasing hormone
h	: hour
hCG	: human chorionic gonadotropin
HPLC	: high pressure liquid chromatography
IFN-γ	: interferon-γ
IgE	: immunoglobulin E
IGF-I	: insulin-like growth factor I
IGF-II	: insulin-like growth factor II
IL-1	: interleukin-1
IRMA	: immuno radiometric assay
IU	: international unit

kb	: kilo base
Kd	: binding affinity
kDa	: kilo Dalton
LH	: luteinizing hormone (lutropin)
LHRH	: luteinizing hormone releasing hormone
LPS	: lipopolysaccharide
MEM	: Eagle's mimimal essential medium
mRNA	: messenger ribonucleic acid
Mv1Lu	: mink lung epithelial cell line
Mw	: molecular weight
NAM	: normal amphibian medium
NCAM	: neural cell adhesion molecule
oFF	: ovine follicular fluid
PAGE	: polyacrylamide gel electrophoresis
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction
PDGF	: platelet-derived growth factor
pFF	: porcine follicular fluid
pH	: acidity
PHA	: phytohaemagglutinin
PModS	: a peritubular myoid cell factor which modulates Sertoli cell function
R1881	: methyltrienolone (synthetic androgen)
Rb	: retinoblastoma gene product
RIA	: radioimmunoassay
RNA	: ribonucleic acid
SCCM	: Sertoli cell conditioned medium
SCF	: stem cell factor
SDS	: sodium dodecyl sulfate
SEM	: standard error of the mean
SSC	: sodium chloride/sodium citrate
TGF-α	: transforming growth factor α
TGF-ß	: transforming growth factor ß
TM3	: Leydig cell line
TM4	: Sertoli cell line
TNF-α	: tumor necrosis factor α
TRH	: thyroid-stimulating hormone releasing hormone
TSH	: thyroid-stimulating hormone
U	: unit
v/v	: volume/volume
w/v	: weight/volume
wt/vol	: weight/volume

CHAPTER 1

INTRODUCTION

1.1 Aim and scope of this thesis

Inhibin and activin are gonadal protein hormones, which were originally defined by their negative and positive feedback action on the release of follicle stimulating hormone (FSH) from the pituitary gland. However, recent studies revealed that inhibin and activin do not only control FSH release but can also affect the functions of a large number of other cell types, as will be discussed in **section 1.7**. This section is preceded by short descriptions of the testicular cell types (**section 1.2**), the structures of inhibins, activins and other members of the TGF--ß family of growth and differentiation factors (**sections 1.3** and **1.4**), and of receptors and non-receptor binding proteins for inhibin and activin (**sections 1.6** and **1.7**). Finally, events occurring after binding of activin to its receptor are discussed in **section 1.8**.

The aim of the experiments described in this thesis was the elucidation of intratesticular effects of activin. The expression of activin receptors, the secretion of activins and the effects of activins in the rat testis have been investigated.

In **Chapter 2**, the expression of activin receptor type II mRNA in different testicular cell types is described.

The secretion of inhibin and inhibin-like proteins by Leydig cells and Leydig cell tumors is described in **Chapter 3**.

Chapter 4 concentrates on the secretion of activin-B by Sertoli cells. This chapter also describes the effects of recombinant activin-A on Sertoli cell aromatase activity, FSH receptor mRNA expression and androgen receptor expression.

In **Chapter 5**, the secretion of activin-A by peritubular myoid cells and peritubular myoid cell lines is examined. Furthermore, the effects of recombinant activin-A on Sertoli cell inhibin and transferrin secretion is described. Finally, the effects of activin-A and another peritubular myoid cell secreted paracrine factor (PModS) will be compared in this chapter.

Chapter 6 forms the general discussion of the findings presented in the preceding chapters

1.2 The testis

The primary functions of the testis are the production of sperm and the synthesis of androgens. These processes take place in the seminiferous tubules and the interstitium, respectively. The seminiferous tubules are composed of developing germ cells enclosed by Sertoli cells, and are surrounded by peritubular myoid cells and a basal lamina. The interstitium contains Leydig cells, blood vessels, macrophages and lymph space (Fig.1). The normal functioning of the testis depends on interactions between the different testicular cell types (reviewed by Skinner, 1991), which are partially regulated by the pituitary hormones luteinizing hormone (LH) and FSH (Fig.2).

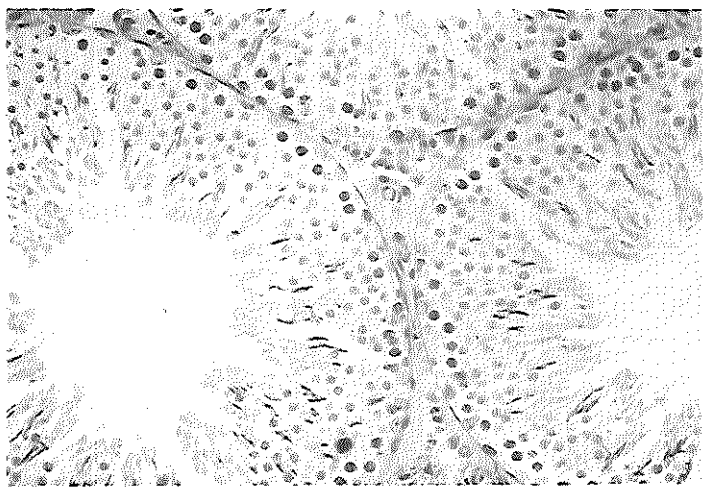

Fig.1. Cross-section of a testis from an adult rat. Three seminiferous tubules with Sertoli cells and developing germ cells can be seen. The tubules are surrounded by flat peritubular myoid cells and in the center of the tubules, a lumen is found, through which the mature spermatozoa leave the tubule. In between the tubules, the interstitium is located, in which Leydig cells are present.

1.2.1 Leydig cells

Interstitial Leydig cells differentiate from urogenital mesenchyme derived precursor cells and provide the male body with androgens by secretion of testosterone. Leydig cell function is regulated by LH, secreted by the pituitary gland. LH secretion is under positive control of luteinizing hormone releasing hormone (LHRH), also called gonadotropin releasing hormone (GnRH), from the hypothalamus. On the other hand testosterone inhibits LH secretion (Fig.2). Testosterone is an important regulator of Sertoli cell function, and therefore of spermatogenesis. Leydig cells also secrete renin, opioid dynorphin, oxytocin and pro-opiomelanocortin-derived peptides, which may regulate Sertoli cell function. On the other hand, Sertoli cells can influence Leydig cell function, as both stimulating and inhibitory effects have been described (for review see Skinner, 1991).

1.2.2 Peritubular myoid cells

Peritubular myoid cells are flat mesenchyme derived cells located around the seminiferous tubules. The origins of peritubular cells and Leydig cells are closely associated. Peritubular cells are present early in development and differentiate, partially in response to androgens, during the early stages of puberty. This differentiation is accompanied by an extreme flattening and cytoplasmic expansion. Together with the Sertoli cells peritubular cells produce an extracellular matrix, which supports the seminiferous tubule. Peritubular cells are also involved in the contraction of the seminiferous tubule, which may play a role in the release and transport of spermatozoa. Peritubular cells appear to be important for the regulation

of Sertoli cell function, since they secrete an androgen regulated factor (PModS), which influences many parameters of Sertoli cell function. It is hypothesized that PModS has an important role in the induction and maintenance of Sertoli cell differentiation (see chapter 5). Other paracrine factors secreted by peritubular cells are transforming growth factors (TGF) -α and -ß and insulin-like growth factor I (IGF-I), which can regulate both Sertoli cell and Leydig cell function (for review see Skinner, 1991).

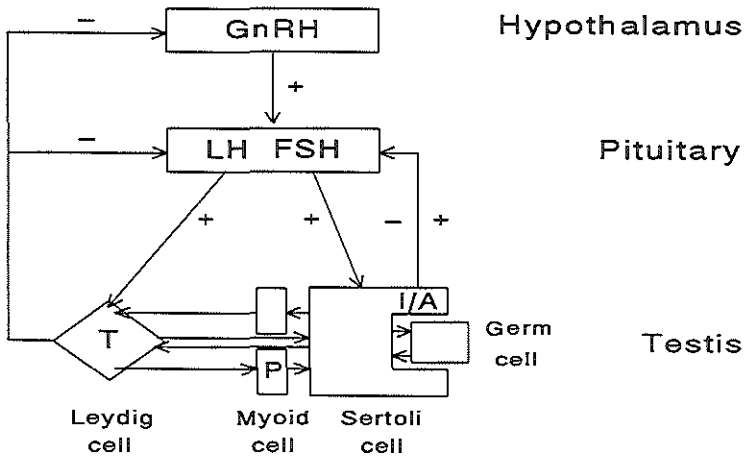

Fig.2. Schematic representation of the hypothalamus-pituitary-testis axis and the interactions between Leydig cells, peritubular myoid cells, Sertoli cells and germ cells (T=testosterone, P=PModS, I=inhibin, A=activin).

1.2.3 Sertoli cells

Sertoli cells are a population of epithelial cells derived from the mesonephric tubule. The cells proliferate rapidly during fetal and early postnatal life, but do no longer divide after approximately 16 days of age in rats. Sertoli cells then differentiate and become columnar cells extending from the basal lamina to the tubular lumen. Adjacent Sertoli cells become interconnected by tight junctional complexes, which form the blood-testis barrier. This barrier divides the tubules in a basal compartment, containing spermatogonia and early spermatocytes, and a luminal compartment, in which the more mature germ cells are located. The barrier prevents the passage of many substances and creates the unique microenvironment required for proper germ cell development. The impermeability of the barrier implies that the Sertoli cells provide components, necessary for germ cell survival, at the luminal side of the barrier. The Sertoli cells also provide physical support for the developing germ cells. Furthermore, regulatory interactions between Sertoli cells and germ cells have been suggested. The main regulators of spermatogenesis, FSH and testosterone, have their effects via the Sertoli cell that. Paracrine factors, which are possibly secreted by Sertoli cells, are IGF-I, IGF-II, interleukin-1 (IL-1), stem cell factor (SCF), TGF-α, TGF-ß, anti-Müllerian hormone (AMH), activin and inhibin (for review see Skinner, 1991). Both inhibin and activin play a role as gonadal feedback hormones of pituitary FSH release.

1.2.4 Spermatogenesis

Spermatogenesis is the process, by which immature germ cells develop to spermatozoa. This process takes place within the seminiferous tubules and is characterized by the sequential formation of different spermatogenic cell types (Fig.3). Early spermatogenic cells (gonocytes) are derived from primordial germ cells, which differentiate from cells of the primitive ectoderm. In the fetal rat, gonocytes proliferate actively until, at day 17 of gestation, they become quiescent in the G_o/G_1 phase of the cell cycle. Approximately one week after birth they differentiate into type A spermatogonia, which proceed through several mitotic divisions yielding intermediate and subsequently type B spermatogonia. After a last mitotic division type B spermatogonia become primary spermatocytes and enter meiosis. The first meiotic division yields secondary spermatocytes, which immediately enter the second meiotic division. This results in haploid spermatids, which elongate to form the mature spermatozoa. The latter process is called spermiogenesis.

The various germ cells are not randomly distributed in the seminiferous epithelium of the rat but are arranged in strict cellular associations. In a testicular cross-section, one or two generations of spermatogonia along the basement membrane, one or two generations of spermatocytes and one or two generations of spermatids bordering the lumen of the seminiferous tubule can be seen, depending on the tubular stage present in the section (Fig.3). In the rat spermatogenic cycle 14 stages have been defined, which cover approximately 13 days. A completely developed germ cell has passed through the different stages 4 times, which takes 52 days (for review see Parvinen, 1982).

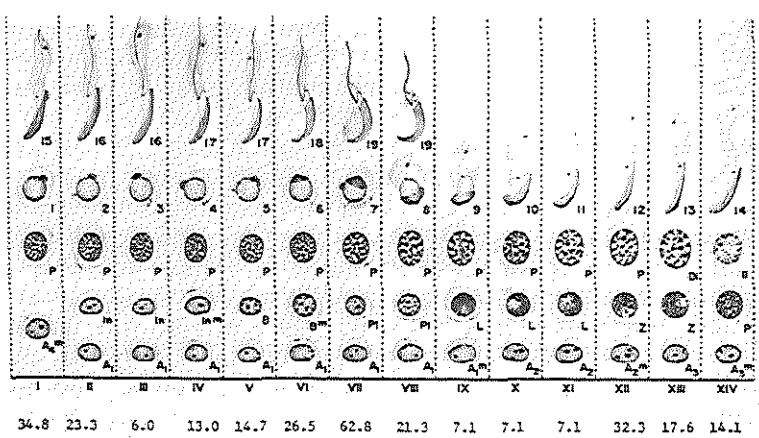

Fig.3. Diagram showing the arrangement of the germ cells in the 14 different stages (indicated by Roman numerals) of the seminiferous epithelial cycle in the rat (after Parvinen, 1982). The time covering each stage (hours) is indicated below the figure. Abbreviations are: A=type A spermatogonium; ^{m}=mitotic division; In=intermediate type spermatogonium; B=type B spermatogonium; Pl=preleptotene spermatocyte; L=leptotene spermatocyte; Z=zygotene spermatocyte; P=pachytene spermatocyte; Di=primary spermatocyte at diplotene stage; II=secondary spermatocyte; 1-19=spermatids at the successive stages of spermiogenesis (1-7=round spermatids; 8-19=elongating spermatids).

1.3 Inhibin and activin; from protein to gene

Since it became apparent that ovarian follicular fluid is a rich source of inhibin (de Jong and Sharpe, 1976), inhibins have been purified from follicular fluid of several species (reviewed by de Jong et al., 1990a). A schematic representation of the different inhibin-like proteins is shown in figure 4. Inhibin was first purified from bovine follicular fluid as a 58 kDa protein, based on its ability to suppress FSH release from cultured pituitary cells (Robertson et al., 1985). The purified protein was composed of 2 disulfide-linked polypeptide chains of 44 and 14 kDa, designated α- and ß-subunit, respectively. The 58 kDa inhibin protein can be transformed to a 32 kDa protein by acid precipitation (Robertson et al., 1985) or after incubation with peripheral plasma (McLachlan et al., 1986a). This drop in molecular weight results from a shortening of the 44 kDa α-subunit to 20 kDa, which represents the C-terminal part of the α-subunit precursor. Later on, 32 kDa inhibin has been purified from bovine (bFF), porcine (pFF) and ovine (oFF) follicular fluid (for review see de Jong et al., 1990a). After the purification of two forms of inhibin it appeared that 2 different inhibin ß-subunits exist ($ß_A$ and $ß_B$). Furthermore, a 65 kDa inhibin protein has been found in follicular fluid as well as larger molecular weight inhibin-like proteins (105 and 95 kDa)(Sugino et al., 1992). The latter proteins result from the combination of either a 50 kDa or a 40 kDa α-subunit with a 55 kDa $ß_A$-subunit. The processing of ovarian and testicular inhibins seem to be different, since in testicular homogenates only 32 kDa inhibin has been found, whereas in homogenates of the ovary several larger forms of inhibin have been detected (Grootenhuis et al., 1989).

Throughout the purification of inhibin from pFF two fractions, which stimulated the secretion of pituitary FSH release were observed. From one of these fractions a 24 kDa protein has been purified, which was found to be a heterodimer of the inhibin $ß_A$- and $ß_B$-subunit (Ling et al., 1986). At the same time a homodimer of two inhibin $ß_A$-subunits with FSH release stimulating activity was purified from pFF (Vale et al., 1986). The proteins were named activin-AB and activin-A, respectively. Recently, activin-B has also been purified from pFF (Nakamura et al., 1992a). Activin-A and the inhibin $ß_A$-subunit monomer were isolated from bFF (Robertson et al., 1992). In contrast, the inhibin α-subunit monomer has never been purified, although a 26 kDa α-subunit precursor protein, consisting of the 20 kDa C-terminal part of the α-subunit covalently linked to the pro-sequence of the N-terminal part of the same subunit, was isolated from bFF (Robertson et al., 1989; Sugino et al., 1989). This protein is formed after the N-terminal domain (αN) is cleaved from the entire α-subunit precursor, as has been shown by pulse-chase labelling experiments (Grootenhuis et al., 1990b). This αN protein was also purified from bFF (Robertson et al, 1989).

On the basis of the amino acid sequences of the inhibin α-, $ß_A$- and $ß_B$-subunit, oligonucleotide probes were synthesized to isolate cDNAs encoding these subunits from rat, porcine, bovine and human ovaries (reviewed by de Jong et al., 1990a). The protein structure obtained from these cDNAs confirmed that the subunits are separately synthesized as pre-pro-hormones. The precursors contain dibasic proteolytic cleavage sites, located next to the amino acids found in the N-terminal sequence of the purified proteins. From the nucleotide sequence of these cDNAs it is also clear that the inhibin subunits are very conserved between species;

the human, bovine, porcine and rat inhibin α-chains show a similarity of approximately 80%, the mature $ß_B$-chains differ only in 4 amino acids, whereas the mature $ß_A$-chains are completely identical (Esch et al., 1987a). The $ß_A$- and $ß_B$-subunit share about 70 % amino acid sequence identity. A significant similarity between the inhibin ß-subunits and the subunits of transforming growth factor-ß was also noticed, classifing inhibin and activin as members of the TGF-ß superfamily of growth and differentiation factors (see below).

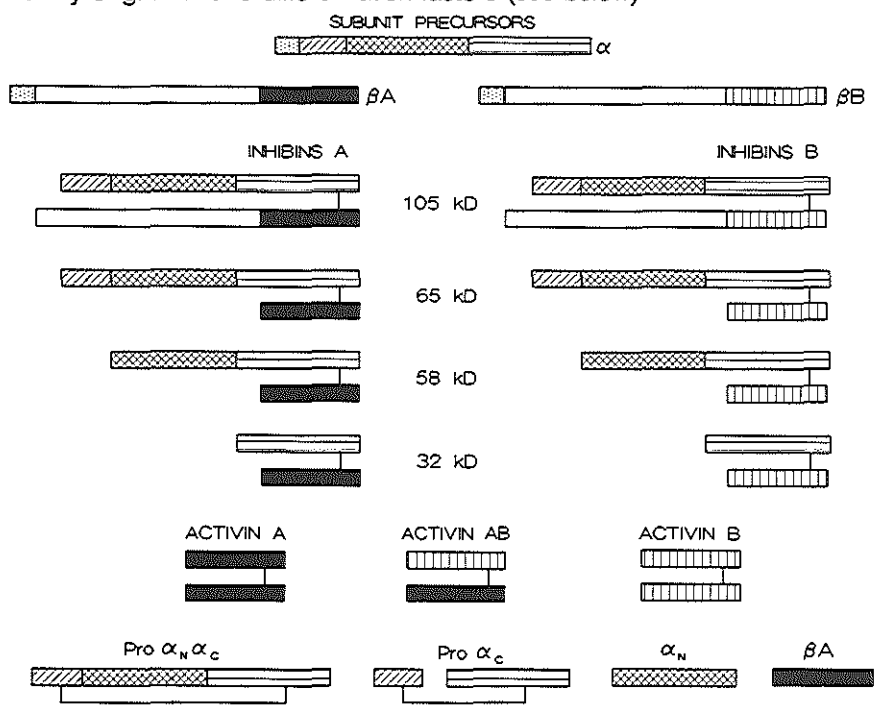

Fig.4. Schematic depiction of the inhibin-like proteins purified from follicular fluid. The inhibin α-subunit is synthesized as a large precursor, consisting of three parts, pro-α_N-α_C , separated by dibasic proteolytic cleavage sites and preceded by a signal sequence. In the α-subunit precursor possibly a single disulfide bridge between pro- and α_C-part exists. This bridge probably disappears when the α-subunit precursor combines with one of the ß-subunit precursors. Instead a single disulfide bridge between α_C and ß is formed (see section 1.4), resulting in a 105 kDa inhibin molecule. Proteolytic cleavage of the pro-part of the α- or ß-subunit results in 95 kDa (not shown) and 65 kDa inhibin, respectively. Further processing will give 58 kDa inhibin, which can be transformed to 32 kDa inhibin. When the α-subunit precursor does not combine with a ß-subunit, proα_C and α_N can arise from proteolytic cleavage of the precursor. The disulfide bridge between pro- and α_C-part probably prevents the formation of α_C mono- and dimers, which have never been detected. The $ß_A$- and $ß_B$-subunits can combine to form different activins. The pro-part of the ß-subunits contains 4 cysteine residues, which will possibly form disulfide bridges. This results in the absence of disulfide bridges between the pro-part and the mature part of the ß-subunit, which explains that ß-subunit monomers are found.

The inhibin subunit genes have also been isolated. The inhibin α-subunit gene is a single copy gene with an intron of approximately 1.5 kb as found in mouse (Su and Hsueh, 1992), rat (Feng et al., 1989b; Albiston et al., 1990) and

human (Stewart et al., 1986). The promoter region of this gene lacks TATA or CAAT boxes at the transcription start site but contains several potential cis-acting elements like cyclic AMP, glucocorticoid and estrogen response elements. The inhibin $ß_A$- and $ß_B$-subunit genes also contain one intron of 9.7 and 2.5 kb, respectively, in rat (Feng et al., 1989b) and human (Stewart et al., 1986; Mason et al., 1989). Again no TATA or CAAT boxes are present. The promoter region of the $ß_B$-subunit gene is extremely GC-rich, as is found primarily in housekeeping and growth related genes. The inhibin α- and $ß_B$-subunit genes have been assigned to human chromosome 2, regions q33→qter and cen→q13, respectively (Barton et al., 1989). Interestingly, the same chromosome contains the gene encoding activin receptor type II (Geurts van Kessel et al., non-published results). The inhibin $ß_A$-subunit gene has been mapped to human chromosome 7p15→p14.

1.4 The TGF-ß superfamily

Inhibins and activins are members of an extending family of structurally related growth and differentiation factors. This protein family includes the transforming growth factors-ß (TGF-ßs), with a variety of functions (reviewed by Massagué et al., 1992); anti-Müllerian hormone (AMH), which is required for the regression of the Müllerian duct in males (Cate et al., 1986); the bone morphogenetic proteins (BMPs), which can induce cartilage and bone formation (reviewed by Lyons et al., 1991); the Drosophila decapentaplegic gene product (DPP), which is required for dorsal-ventral axis formation, embryonic gut formation and morphogenesis of the imaginal discs (Padgett et al., 1987); and the Xenopus *Vg-1* gene product, which appears to be involved in the induction of endoderm on the vegetal pole of the oocyte (Weeks and Melton, 1987). Recently, new family members have been cloned: mouse growth and differentiation factor 1 (Lee, 1990), 3 and 9 (McPherron and Lee, 1993), mouse *Vgr-2* (Jones et al., 1992a) and Drosophila 60A (Wharton et al., 1991). These new members are most homologous to *Vg-1*. In 1993, *nodal*, which is expressed in mouse embryos at about the time of mesoderm formation (Zhou et al., 1993), *dorsalin-1*, that appears to regulate cell differentiation within the neural tube of chick embryos (Basler et al., 1993) and **GDNF** (glial cell line derived neurotrophic factor; Lin et al., 1993), were also added to the TGF-ß superfamily.

The TGF-ß superfamily is characterized by the conservated position of seven cysteine residues located near the C-terminal end of the molecule. *Vgr-2* and growth and differentiation factor 3 and 9 lack the 4th of these cysteine residues, which is probably necessary for dimer formation (see below). The TGF-ßs and the inhibin ß-subunits have two conserved cysteine residues extra. From the three-dimensional structure of TGF-ß2 and TGF-ß1, as determined by cristallography and nuclear magnetic resonance respectively, it has become clear that eight of these nine cysteine residues are important for the formation of intrachain disulfide bonds. The remaining cysteine residue (amino acid residue 77) is involved in the formation of dimers (Daopin et al., 1992; Schlunegger and Grütter, 1992; Archer et al., 1993). Mutation of this residue results in the secretion of biologically inactive TGF-ß monomers (Brunner et al., 1992). With this knowledge it can be speculated that cysteines 95 and 80 of the mature inhibin α and inhibin ß chain respectively are

important for dimerization. Substitution of cysteine residue 80 of activin-A by serine indeed results in the secretion of an activin-A monomer unable to stimulate FSH release by pituitary cells (Hüsken-Hindi et al., 1993).

Another striking feature, which the family members have in common, is that the homologous C-terminal part of the molecule, which forms homo- or hetero-dimers to obtain a mature growth factor, is preceded by a monobasic or dibasic cleavage site, a large N-terminal domain and a signal peptide. With regard to the N-terminal domain it is clear that it is involved in the regulation of biological activity and proper folding of the C-terminal domain. The N-terminal domain of TGF-ß (also called latency associated peptide) keeps TGF-ß in a latent, inactive form if it remains associated with the C-terminal domain after the proteolytic cleavage (Gentry and Nash, 1990). In the latent TGF-ß complex a 135-180 kDa unrelated protein, known as latent TGF-ß binding protein, is disulfide linked to the N-terminal domain (Wakefield et al., 1989; Kanzaki et al., 1990). Activation of latent TGF-ß can occur by the cooperation between two cell types and appears to involve plasmin, since plasmin inhibitors prevent TGF-ß activation (Sato et al., 1990). This activation seems self-regulating; the generation of TGF-ß stimulates plasminogen activator inhibitor I, which decreases plasmin activity. Retinoids enhance the production of plasminogen activator and therefore activate latent TGF-ß in endothelial cells (Kojima and Rifkin, 1993). In osteoblast-like cells, dexamethasone can activate latent TGF-ß by inducing the secretion of lysosomal proteases (Oursler et al., 1993). *In vitro*, activation of latent TGF-ß is accomplished by acid, alkali or heat treatment (Lawrence et al., 1985; Pircher et al., 1986; Brown et al., 1990). Only when mature TGF-ß is released from the latent complex it can interact with the TGF-ß receptor(s) (Wakefield et al., 1988). In contrast to the situation with TGF-ß, the N-terminal domain of AMH enhances the bioactivity of the C-terminal domain (Wilson et al., 1993). It has been suggested that association of the N-terminal and C-terminal domains of AMH is necessary for complete biological activity. Other TGF-ß family members, like activin (Huylebroeck et al., 1990) and bone morphogenetic protein (Wang et al., 1990), do not remain associated with their N-terminal domain after cleavage and require no additional activation step.

The N-terminal domain is also required for proper folding and secretion of the C-terminal domain. Deletion of the entire N-terminal region of activin-A and TGF-ß1 prevents the dimerization and secretion of biologically active homodimers (Gray and Mason, 1990). The N-terminal domain of both TGF-ß1 and TGF-ß2 contains three potential sites of N-linked glycosylation. Deletion of 162 amino acid residues of the TGF-ß1 precursor, which include these glycosylation sites (Sha et al., 1991), or treatment of Chinese hamster ovary cells, that produce recombinant TGF-ß1, with the glycosylation inhibitor tunicamycin (Sha et al., 1989) abolishes TGF-ß secretion. This suggests that glycosylation of the N-terminal domain of TGF-ß is involved in secretion. The mutation of mannose-6-phosphate (M-6-P) containing glycosylation sites in the TGF-ß precursor (predominantly the first two of the three N-linked glycosylation sites in the TGF-ß1 precursor and the third in the TGF-ß2 precursor) drastically reduces the secretion of TGF-ß (Brunner et al., 1992). The binding to M-6-P receptors is thought to be important for intracellular transport. The N-terminal domains of the inhibin α-, $ß_A$- and $ß_B$-subunit contain one potential N-linked glycosylation site (Esch et al., 1987a), but its relevance for processing is still unknown.

1.5 Receptors for members of the TGF-ß superfamily

Because of the structural conservation of the members of the TGF-ß superfamily, one can suggest that there is also homology between the receptors for the members of this protein family. Receptors for several members of the TGF-ß superfamily have been characterized and show indeed homology.

1.5.1 Activin receptors

Chemical cross-linking studies with radioiodinated activin-A showed the presence of multiple binding sites for activin-A on several cell types as summarized in table I. The cross-linked complexes resemble the TGF-ß type I, II and III receptors (see section 1.5.2).

Table I. Activin binding sites detected with chemical cross-linking

Cell type[reference]	Mw cross-linked complex	Affinity constant
COS + ActRII cDNA[1]	84 kDa	1.8×10^{-10}M
AtT20 corticotroph[1]	84, 78, 65 kDa	?
Friend erythroleukemia[2]	140, 76, 67 kDa	3.1×10^{-10}M
K562/erythr. progenitor[3]	80, 73 kDa	?
Fetal osteoblasts[4] (TGF-ß binding sites)	>200, 73 kDa (>200, 85, 65 kDa)	4×10^{-9}M, 4×10^{-10}M (?)
Aortic endothelial cells[5]	160, 80, 65 kDa	?
P19 embryocarcinoma[6]	200, 80, 65, 41 kDa	1×10^{-9}M, 5×10^{-11}M

*The references for these experiments are: **1.** Mathews and Vale, 1991; **2.** Hino et al., 1989; **3.** Shao et al., 1992a; **4.** Centrella et al., 1991; **5.** McCarthy and Bicknell, 1993; **6.** Mathews et al., 1991.*

Though activin-binding sites (Kd 1-4 x 10^{-10}M) have been identified on several other activin responsive cells (Campen and Vale, 1988a; Sugino et al., 1988a; Kondo et al., 1989; Lapolt et al., 1989), a real breakthrough was the expression cloning of activin receptor type II from AtT20 mouse corticotropic cells (Mathews and Vale, 1991). The cloned cDNA encodes a protein of 513 amino acid (AA) residues comprising a signal peptide (19 AA), an extracellular ligand-binding domain with a high cysteine content (116 AA), a transmembrane domain (26 AA) and an intracellular domain (352 AA), which has a potential kinase domain of 300 amino acids in common with other protein kinases. The intracellular domain is characterized by 12 subdomains with a number of highly conserved amino acids. The sequences in two of these subdomains (VIB and VIII) predict that the activin receptor is a serine/threonine kinase, although additional tyrosine specificity is not excluded. The activin receptor has no tyrosine residue in the autophosphorylation region between subdomains VII and VIII, as is found in tyrosine kinases. Potential autophosphorylation sites are predicted at Ser^{352} and Thr^{356} in the autophosphorylation region, and at several Ser and Thr residues in the C-terminal tail of the receptor. Compared with all known protein kinase sequences the activin receptor

has the highest homology with an orphan nematode membrane receptor, Daf-1 (Georgi et al., 1990), and other receptors for the TGF-ß superfamily (see Fig.5). All those receptors have short, conserved inserts in the kinase domain sequence between subdomains VI-A and VI-B and between subdomains X and XI that are not present in other protein kinases. This suggest that they belong to a new, unique subfamily of protein kinases.

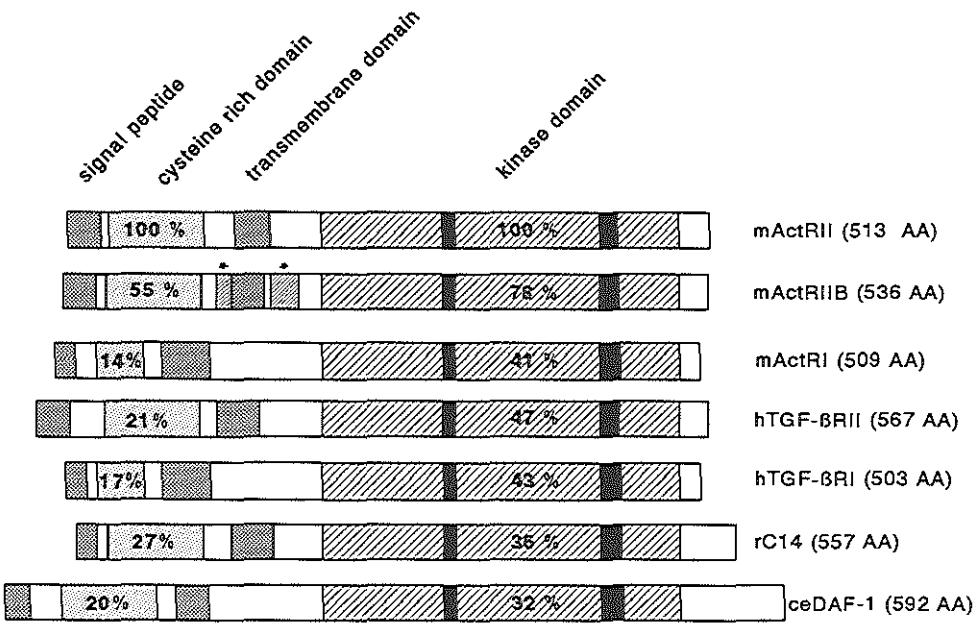

Fig.5. Receptors for the TGF-ß superfamily. Murine activin receptor type II (mActRII; Mathews and Vale, 1991), type IIB (mActRIIB; Attisano et al., 1992) and type I (mActRI; Ebner et al., 1993a), human TGF-ß receptor type II (hTGF-ßRII; Lin et al., 1992) and type I (hTGF-ßRI; Franzén et al., 1993), the putative rat anti-Müllerian hormone receptor (rC14; Baarends et al., 1994) and Caenorhabditis elegans Daf-1 gene product (ceDAF-1; Georgi et al., 1990) have been alined. Important domains in the receptors are indicated at the top of the figure. The conserved inserts in the kinase domain sequence are indicated with black boxes. The similarity of the extracellular domain and kinase domain of the receptors with that of mActRII is shown. Asterisks indicate the sites of alternative splicing in mActRIIB, which result in 4 receptor isoforms; ActRIIB1 containing both indicated regions, ActRIIB2, which only contains the extracellular region, ActRIIB3, which only contains the intracellular region and ActRIIB4, in which both regions are absent.

Cross-linking of ^{125}I-activin to COS cells transfected with the cloned activin receptor cDNA results in an 84 kDa complex. The dissociation constant of this receptor amounts to 1.8 x 10^{-10}M for activin-A binding. Binding of iodinated activin-A can be competed by activin-B with slightly reduced potency compared with activin-A. However, the activin-B preparation used had lost part of its potency in several bioassays. Inhibin-A can compete for activin-A binding with approximately 10-fold lower potency, whereas TGF-ß1 has no effect on activin binding to this receptor (Mathews and Vale, 1991).

Type II activin receptor cDNA has also been cloned from rat (Shinozaki et al., 1992; Feng et al., 1993), human (Donaldson et al., 1992; Matzuk and Bradley, 1992b), chicken (Ohuchi et al., 1992), and Xenopus (Kondo et al., 1991; Nishimatsu et al., 1992a). It turned out that this receptor is highly conserved between species. Furthermore, the gene encoding mouse activin receptor type II has been cloned (Matzuk and Bradley, 1992a). It comprises 11 exons and spans more than 66 kilobases, due to two large introns (intron 1 (over 40 kb)) and intron 4 (12.9 kb)). The 5' region of the gene lacks TATA or CCAAT boxes, often found at the transcription start site, but several transcription factor binding sites are present.

Polymerase chain reaction with degenerated oligonucleotides constructed according to sequences in homologous regions in kinase domains II and VIB of activin receptor type II (ActRII) and Daf-1, yielded a second activin receptor (ActRIIB). This receptor was cloned from mouse testes, mouse erythroleukemia cells and mouse 3T3 fibroblasts (Attisano et al., 1992). Four isoforms of this receptor, generated by alternative splicing, have been cloned (Fig.5). The first difference between these isoforms concerns nucleotides 414 to 437, introducing a deletion of 8 amino acids in the extracellular proline-rich region immediately before the transmembrane region. A second difference is found in the region of nucleotides 566 and 638 and includes 24 amino acids immediately preceding a proline-rich sequence between the transmembrane region and the kinase domain. These differences are due to alternative splice events at intron/exon boundaries, which are located at identical positions in the ActRII gene (introns 3 and 4). This suggests that several alternatively spliced forms of ActRII may also exist (Matzuk and Bradley, 1992a). The cloned ActRII (Mathews and Vale, 1991) contains a sequence corresponding to the extracellular alternatively spliced segment but lacks a sequence corresponding to the intracellular alternatively spliced segment, and is therefore most homologous to ActRIIB2. Two of the alternatively spliced isoforms of ActRIIB (IIB3 and IIB4), both lacking the 8 extracellular amino acids, have a dissociation constant for activin-A binding similar to ActRII (3-4 x 10^{-10}M), whereas the other two isoforms (IIB1 and IIB2) have a higher affinity for activin-A (Kd 1 x 10^{-10}M). ^{125}I-activin binding to all activin receptor isoforms is competed by inhibin-A at a relatively high (8 nM or higher) concentration, suggesting low affinity of inhibin for these receptors. No competition is found with BMP-4 or TGF-ß1.

Activin receptor type IIB protein has been purified from the mouse embryonal carcinoma cell line P19 by affinity chromatography (Nakamura et al., 1992b). This 70 kDa protein binds recombinant activin-A with high affinity (Kd 3.5 x 10^{-10}M); this binding can be competed by native porcine activins A, AB and B with similar potency. Bovine inhibin-A is 10-fold less potent and TGF-ß does not compete for activin-A binding to this receptor. The receptor can phosphorylate itself and its ligand on serine, threonine and tyrosine (ratio 85:5:15). Activation of the receptor by the addition of activin does not change the phosphorylation pattern. Since only the first 18 N-terminal amino acids have been sequenced it is not possible to specify which isoform of ActRIIB was purified. The same authors claim that they have also purified an ActRII protein, with specificity for phosphorylation of serine, threonine and tyrosine, from COS cells transfected with ActRII cDNA, but results were not shown. Others demonstrated that ActRII, transfected in COS cells, can be phosphorylated on serine residues. This phosphorylation is slightly increased after addition of activin-A (Ichijo et al., 1993), suggesting that ActRII

undergoes ligand depending autophosphorylation on serine residues. Nevertheless, these authors do not completely eliminate the possibility of threonine/tyrosine kinase activity, since these phosphoamino acids are susceptible to dephosphorylation. The absence of autophosphorylation on tyrosine residues in both ActRII and ActRIIB has also been found by Mathews and Vale (1993) using normal and mutant receptors, which lack a lysine in kinase domain II that is required for kinase activity. Activin-A treatment did not change the phosphorylation state of either type II receptor. Furthermore, immunoprecipitation of activin type II receptor coprecipitated a type I receptor from AtT20 cells, P19 embryonal carcinoma cells and Chinese hamster ovary cells, suggesting the formation of a heterodimeric complex of activin type I and type II receptors like was found for the TGF-ß receptors (see section 1.5.2).

Rat (Legerski et al., 1992; Feng et al., 1993) and Xenopus (Hemmati-Brivanlou et al., 1992; Mathews et al., 1992; Nishimatsu et al., 1992a) ActRIIB have also been cloned. Again a strong conservation between species was found. Finally, a *Drosophila* activin receptor has been cloned (Childs et al., 1993). The kinase domain of this receptor is 61% and 60% identical to mouse activin receptor II and IIB2, respectively. Outside of the kinase domain the similarity among these receptors is very limited, although the spacing of 10 cysteine residues in the extracellular domains of the receptors is well conserved.

1.5.2 TGF-ß receptors

Chemical cross-linking studies with radioiodinated TGF-ß1 showed that several membrane associated TGF-ß binding proteins exist (reviewed by Massagué, 1992). The most important ones, with apparent molecular weights of 55, 80 and 280 kDa, have been termed type I, II and III receptors, respectively. The type I and type II receptors are glycoproteins with N-linked carbohydrate groups, which are not required for cell surface expression or TGF-ß binding. Both receptors discriminate between various isoforms of TGF-ß in receptor competition assays, with relatively high affinities for TGF-ß1 and TGF-ß3 and much lower affinity for TGF-ß2. The type III receptor, also called betaglycan, is a membrane protein which contains a large number of glycosaminoglycan groups. This receptor has similar affinities for all TGF-ß isoforms.

The TGF-ß type II (Lin et al., 1992) and III (López-Casillas et al., 1991; Wang et al., 1991) receptor have been cloned by expression cloning using labelled TGF-ß1. The type III receptor cDNA encodes an 853 amino acid protein with a large extracellular domain, containing at least one site for glycosaminoglycan addition, a transmembrane domain and an intracellular domain of 41 amino acid residues with no known signalling motive. Expression of this receptor in L6 myoblasts which lack the type III receptor leads to an increased ligand binding to the type II receptor. This suggests that the type III receptor is not a signalling molecule but presents the ligand to its signal transducing receptor(s). This model is confirmed by López-Casillas et al. (1993), who showed that type II and type III receptors can be immunoprecipitated as a complex, independent of the presence of a type I receptor. This complex is formed at the cell surface only in the presence of TGF-ß. The type III receptor allows high affinity binding of TGF-ß1 to a large population of otherwise relatively inert type II receptors, with limited effects on binding to the type

I receptor. The binding of TGF-ß2 to the type I and type II receptor, which is almost absent without the type III receptor, is similar to that of TGF-ß1 in the presence of the type III receptor. This is accompanied by an increase in biological activity, rendering TGF-ß2 nearly as potent as TGF-ß1. Therefore, it is concluded that the type III receptor increases TGF-ß binding and eliminates biological differences between TGF-ß isoforms.

In contrast to the type III receptor, the type II receptor is involved in signal transduction as became clear after the cloning of the human type II receptor (Lin et al., 1992). It is a 567 amino acid protein with a signal sequence (23 AA), a cysteine-rich extracellular domain (136 AA), a single transmembrane domain (30 AA) and a cytoplasmic domain with predicted serine/threonine kinase activity (378 AA). A fusion protein of the cytoplasmic domain of this receptor (Asn-192 to Lys 567) and glutathion S-transferase can phosphorylate itself on serine and threonine residues. At the protein level mink (Wrana et al., 1992) and rat (Tsuchida et al., 1993) type II receptors are very homologous to the human type II receptor showing 94.2 % and 91.5 % similarity, respectively. The affinity of the type II receptor for TGF-ß1 and TGF-ß3 is higher than for TGF-ß2 as demonstrated by cross-linking studies (Lin et al., 1992; Wrana et al., 1992). The involvement of the type II receptor in TGF-ß signalling appears from studies with mink lung epithelial cell (Mv1Lu) mutants (Wrana et al., 1992) and human hepatoma cell mutants (Inagaki et al., 1993) missing this receptor. Transfection of the TGF-ß type II receptor restores growth inhibition by TGF-ß1 in these cells. The involvement of kinase activity in TGF-ß signalling has been demonstrated by Ohtsuki and Massagué (1992) using serine/ threonine protein kinase inhibitors.

Studies with Mv1Lu mutants reveal that the TGF-ß type I receptor plays an important role in signal transduction: TGF-ß binding to the type II receptor alone is insufficient to signal growth inhibition and early gene responses in Mv1Lu cells. The formation of a complex between the type I receptor and a type II receptor with an intact kinase domain is necessary for signal transduction (Wrana et al., 1992). Additionally, the type I receptor requires the type II receptor to bind TGF-ß, but an intact type II receptor kinase domain is not essential for binding (Wrana et al., 1992). The tight interaction between both receptors is evident from the co-immunoprecipition of the type I and type II receptor from human hepatoma cells when TGF-ß1 is added to the cells (Inagaki et al., 1993). In the same way, type I and type III receptor have been co-immunoprecipitated with the type II receptor from rat-1 cells (Moustakas et al., 1993). Inactiviation of the type II receptor in Mv1Lu cell lines by overexpression of a dominant negative truncated type II receptor, abolishes the growth inhibitory effect of TGF-ß1 and TGF-ß2. Nevertheless, this has no effect on induction of fibronectin, plasminogen activator inhibitor I and Jun B and on the cell surface binding of TGF-ß1 to the type I receptor (Chen et al., 1993). This suggests that the type I receptor is involved in signal transduction with a signalling pathway distinct from the type II receptor. Since Wieser et al. (1993) found a diminished TGF-ß signalling with a truncated type II receptor in Mv1Lu cells, this conclusion can be questioned and ascribed to an incomplete block of the type II receptor signalling, due to the addition of an insuficient amount of truncated receptor. Moreover, transfection of a truncated type II receptor in Mv1Lu mutants restores TGF-ß1 binding to the type I receptor but fails to make the cells responsive to TGF-ß (Wieser et al., 1993). Finally, a truncated

type II receptor inhibits the TGF-ß stimulated transcription of several TGF-ß responsive genes in neonatal cardiac myocytes (Brand et al., 1993).

A potential type I receptor (**TSK 7L**) has been cloned from a murine cell line, which contains large quantities of type I receptor protein (Ebner et al., 1993a). Low stringency hybridization of a cDNA library with a probe corresponding to the kinase domain of ActRII yielded a cDNA encoding a 509 amino acids polypeptide with a signal peptide (17 AA), a cysteine rich extracellular domain (123 AA), a transmembrane domain (27 AA) and a cytoplasmic domain with possible serine/threonine kinase activity (363 AA). The cytoplasmic domain shares 32.7 and 33.1% amino acid sequence identity with the TGF-ß and activin type II receptors, respectively. This receptor does not bind TGF-ß1, TGF-ß2, activin or inhibin, when expressed in quail QT-6 cells, but decreases the binding of TGF-ß1 to the type II receptor present on these cells. Cotransfection of this receptor with the human type II receptor in human 293 cells also decreases the binding of TGF-ß1 to the type II receptor, whereas some binding to the type I receptor is present. Cotransfection of this murine receptor with the murine type II receptor results in a decrease of TGF-ß1 binding to the type II receptor and a strong increase of binding to the type I receptor. This suggests that there is a species dependent interplay between the TGF-ß receptors type I and II. Independent of this study, the human homologue of this receptor has been cloned from a hepatoma cell line (**SKR1**; Matsuzaki et al., 1993) and from placenta (**ALK-2**; ten Dijke et al., 1993). Although not classified as a potential TGF-ß type I receptor because of lack of TGF-ß binding, it has a similarity of 98.2 % with the mouse type I receptor. In search for the AMH receptor the rat homologue (**R1**) of this potential TGF-ß type I receptor has been cloned, showing a similarity of 97.4 % with that of mouse (He et al., 1993). The same receptor has also been cloned from adult rat brain (**ActX1R**) and is highly expressed in adult rat brain, pituitary gland and lung but its expression is low in the adult rat testis (Tsuchida et al., 1993b). In a later study it has been shown that, in the presence of an activin type II (or IIB) receptor, this potential TGF-ß type I receptor binds activin-A and inhibits activin binding to the type II receptor (Ebner et al., 1993b). The affinity of this receptor for activin-A is much higher than for TGF-ß1. Interaction of this receptor with ActRII or ActRIIB generates an active signalling complex, which stimulates the expression of a luciferase reporter gene construct (Attisano et al., 1993). Therefore, this receptor is classified as an activin type I receptor rather than a TGF-ß type I receptor.

Recently, a functional 503 amino acids TGF-ß type I receptor (**ALK-5**), with an overall structure similar to the TGF-ß type II receptor, has been cloned from human erythroleukemia cells by polymerase chain reaction with degenerated primers based on conserved regions in the serine/threonine kinase receptors (Franzén et al. 1993). This receptor binds TGF-ß1 and restores the TGF-ß1 response in Mv1Lu cells lacking a type I receptor. In contrast to the activin type I receptor (see above), transfection of the TGF-ß type I receptor increases TGF-ß1 binding to the type II receptor. The interaction between type I and type II receptor also appears from their co-immunoprecipitation using antibodies against type I or type II receptor, which indicates that they form a heteromeric complex. Another argument for this receptor being the TGF-ß type I receptor is its loss of TGF-ß binding after dithiothreitol treatment, which has been described as a property of the type I receptor. The rat homologue of this receptor (**R4**) shows 96.2 % similarity

(He et al., 1993). This receptor restores TGF-ß gene response in Mv1Lu mutants lacking an endogenous type I receptor and has an intrinsic serine/threonine kinase activity, which is essential for signal transduction (Bassing et al., 1994). In contrast to the type II receptor, which phosphorylates mainly on serine, this type I receptor phosphorylates mainly on threonine. A model for the interaction between TGF-ß receptors type I, II and III is depicted in figure 6.

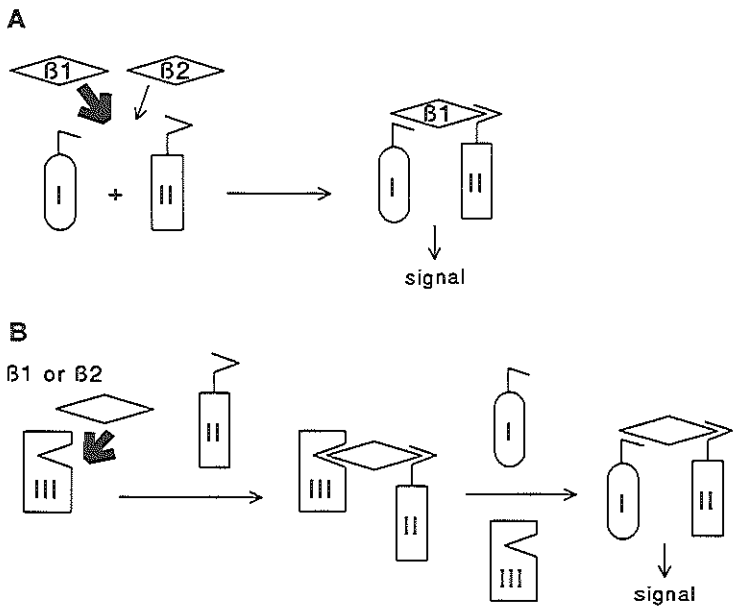

Fig.6. Model for the formation of TGF-ß receptor complexes (after López-Casillas et al., 1993). A. TGF-ß1 interacts with the type II receptor. Possibly a conformational change results in binding of the type I receptor. A high affinity receptor signalling complex is formed, in which the kinase domains of both the type I and type II receptor is essential. In this model TGF-ß2 is a weak agonist. It remains to be determined whether the type I receptor signal activates the type II receptor. It is also not clear if just two receptors are part of the complex. Furthermore, it has to be elucidated whether type I and type II receptors form homodimers or heterodimers with other receptors (e.g. activin receptors). B. The type III receptor binds TGF-ß1 or TGF-ß2 and presents it to the type II receptor. This complex is obligatory for high affinity binding of TGF-ß2. The type I receptor displaces the type III receptor and the high affinity receptor complex shown in A is formed.

Besides the type I, II and III receptors the existence of several other membrane associated TGF-ß binding proteins has been demonstrated (reviewed by Massagué, 1992). Vascular endothelial cells express a disulfide-linked dimer of 95 kDa subunits (endoglin), that binds TGF-ß1 and TGF-ß3 but not TGF-ß2 (Cheifetz et al., 1992). Interestingly, the transmembrane domain and the relative short cytoplasmic domain of endoglin and the TGF-ß type III receptor are remarkably similar (71% amino acid similarity). Like the type III receptor, endoglin seems to present TGF-ß to signalling receptors, by the formation of a heteromeric complex with the type II and type I receptor (Yamashita et al., 1994). Three proteins attached to the membrane by phospholipid anchors also bind either TGF-ß1 or TGF-ß2. Furthermore, a TGF-ß binding protein of approximately 400 kDa has been

purified from bovine liver. This receptor, also refered to as type V receptor, autophosphorylates on serine. The amino acid sequence of one of the peptides generated by cyanogen bromide cleavage of the purified type V receptor revealed homology with the putative ATP binding site of the type II receptors for activin and TGF-ß and the Daf-1 gene product (O'Grady et al., 1992). Finally, a 64 kDa protein, that binds TGF-ß, activin and inhibin (type IV receptor), is found on pituitary tumor cells (Cheifetz et al., 1988).

1.5.3 Other related receptors

The putative anti-Müllerian hormone (AMH) type II receptor has been cloned from a 21-day-old rat Sertoli cell cDNA library (Baarends et al., 1994). The overall structure of this receptor, including a cysteine rich extracellular domain and a kinase domain with inserts between subdomains VI-A and VI-B and between subdomains X and XI, reveals homology with ActRII, ActRIIB, TGF-ßRII and Daf-1. Expression of this receptor is detected in the mesenchyme adjacent to the Müllerian ducts and in the female gonads at days 15 and 16 of rat embryonic development. At day 19, when in male embryos the Müllerian duct is almost completely degenerated, the expression in the female urogenital ridge is high, whereas it is absent in the male urogenital ridge. Postnatally, the receptor is specifically expressed in testis and ovary. In an effort to clone the AMH type II receptor from fetal rat urogenital ridge cDNA, two unidentified members of the TGF-ß receptor family (**R2** and **R3**) have been cloned (He et al., 1993). With respect to their size (505 and 503 amino acid residues), the presence of a short series of tandem Ser/Gly residues and other conserved amino acids immediately preceding the kinase domain (type I box) and the similarity of their kinase domains with that of the activin type I receptor (64% and 79%) these receptors are candidate type I receptors. Interestingly, in situ hybridization showed that receptor **R2** is expressed in the seminiferous tubule. The human homologues of these receptors (**ALK-4** and **ALK-1**) have also been cloned as well as another unknown potential serine/threonine kinase receptor (**ALK-3**) of 532 amino acid residues (ten Dijke et al., 1993). Recently, it has been shown that **ALK-1** (**R3**, **TSR-1**) binds both activin-A and TGF-ß1, if cotransfected with activin or TGF-ß type II receptors, respectively (Attisano et al., 1993). These complexes do not activate the reporter construct activated by the activin type I/II receptor complex. It remains to be elucidated whether these complexes are active in other systems.

Paralkar et al. (1991) demonstrated several binding sites for bone morphogenetic protein 2B (BMP-2B) on MC3T3 E1 osteoblasts (apparent Mw 200 kDa, 70 kDa and 60 kDa) and 3T3 fibroblasts (200 kDa, 90 kDa and 60 kDa), using chemical cross-linking of radiolabelled BMP-2B. Although these binding sites again resemble the binding sites reported for TGF-ß, indicating that BMP receptors may belong to the same receptor family, mammalian BMP receptors have not been cloned yet. However, recently Estevez et al. (1993) cloned a TGF-ß receptor family member (*daf-4*) from the nematode *Caenorhabditis elegans*, which binds human BMP-2 and BMP-4. This 744 amino acids receptor molecule inhibits dauer larva formation and promotes nematode growth. It has been speculated that this receptor forms a heterodimeric complex with the *daf-1* receptor.

1.6 Non-receptor binding proteins for inhibin and activin

1.6.1 Follistatin

Parallel to the purification of inhibin from porcine and bovine follicular fluid, follistatin has been purified, based on its ability to suppress FSH secretion from pituitary cells (Robertson et al., 1987; Ying et al., 1987b). The potency of follistatin to suppress FSH release *in vivo* (DePaolo et al., 1991a) and *in vitro* (Robertson et al., 1987 and 1990; Ying et al., 1987b; Wang et al., 1990) is 10-30% of that of inhibin. In bovine follicular fluid three bioactive follistatin proteins, with molecular weights of 31, 35 and 39 kDa, have been detected (Robertson et al., 1987). In porcine follicular fluid 32 and 35 kDa follistatins have been found (Ying et al., 1987b). Amino acid sequences from purified tryptic fragments of porcine follistatin were used to construct oligonucleotide probes for screening of a porcine ovarian cDNA library (Esch et al., 1987b). The cloning of cDNAs encoding porcine, rat, sheep and human follistatin revealed a similarity of more than 97% at the protein level (Shimasaki et al., 1989; Tisdall et al., 1992). The *Xenopus* homologue of human follistatin has a similarity of 84%, indicating strong conservation between species (Tashiro et al., 1991).

Cloning of the human and porcine follistatin genes (Shimasaki et al., 1988a and 1988b) revealed that the follistatin gene is a single copy gene of approximately 6 kb, consisting of six exons. The first one encodes the 29 amino acid residues of the signal peptide. Two different follistatin mRNAs can be generated by alternative splicing in the intron between exon 5 and 6. This results in 2 follistatin proteins of 315 amino acid residues (FS-315) and 288 amino acid residues (FS-288). The latter protein is missing the last 27 C-terminal amino acid residues encoded by exon 6 and mainly exists as a 31 kDa core protein and a 35 kDa glycosylated form. Recombinant human FS-315 is expressed as a 35 kDa core protein and a glycosylated form of 38 kDa (Inouye et al., 1991a). Western blotting of purified porcine follistatin with antibodies against different C-terminal peptides has demonstrated that FS-315 is processed to FS-300 resulting in a 32 kDa core protein (Inouye et al., 1991a). *In vivo*, there is a preference (probably more than 95%) for splicing of the mRNA encoding FS-315 in several organs, which explains the low abundance of FS-288 in follicular fluid (Michel et al., 1990). Nevertheless, in an *in vitro* pituitary bioassay recombinant human FS-288 is equipotent with inhibin and much more potent than recombinant human FS-315 or purified porcine follistatin.

The interest in follistatin grew when this entity turned out to be a binding protein for activin-A (Nakamura et al., 1990), which neutralizes the action of activin (Asashima et al., 1991a; Kogawa et al., 1991a; Xiao et al., 1992a). The Kd for the activin-follistatin complex is estimated between 1.3×10^{-10}M and 9.1×10^{-10} M (Nakamura et al., 1990; Kogawa et al., 1991a; Xiao et al., 1992a). A two-fold molar excess of follistatin is necessary for maximum inhibition of activin-A action, suggesting that both ß-subunits of activin have to bind follistatin for neutralization (Carroll et al., 1989; Asashima et al., 1991a; Shimonaka et al., 1991; Xiao et al., 1992a). In dispersed pituitary cell monolayer cultures follistatin suppresses activin-A stimulated FSH secretion and synthesis (Caroll et al., 1989; Wang et al., 1990; Kogawa et al., 1991a) as well as the increased FSH-ß mRNA levels (Carroll et al., 1989). Therefore, Kogawa et al. (1991a) suggested that the inhibition of pituitary

FSH release by follistatin is accomplished by the neutralization of locally produced activin (see also section 1.7.1). As *Xenopus* follistatin inhibits the mesoderm inducing activity of *Xenopus* activin-A, -AB and -B, follistatin is a binding protein for all the known activin forms (Fukui et al., 1993).

Site directed mutagenesis showed that the loss of Asn-linked carbohydrate side chains of follistatin has no effect on activin-A binding or the FSH suppressing activity of follistatin (Inouye et al., 1991b). However, the introduction of two amino acids between the second and third amino acid of follistatin or replacement of the third amino acid (cysteine) by serine results in a complete loss of both activin-A binding and FSH suppression. The affinities of glycosylated and non-glycosylated FS-315, FS-300 and FS-288 for activin-A are essentially similar ($5.4\text{-}6.8 \times 10^{-10}$M). Nevertheless, FS-288 has a high affinity for heparan sulfate side chains of membrane proteoglycans, whereas FS-315 has no affinity for the cell surface (Sugino et al., 1993). This can explain the higher potency of FS-288 in inhibiting FSH release, since cell matrix bound follistatin is more effective in neutralizing endogenous secreted activin.

Ligand blotting revealed that follistatin binds both activin-A and inhibin-A (Shimonaka et al., 1991), probably via the common ß-subunit. Recently, binding of ^{125}I labelled activin-A to a protein of approximately 37 kDa in human serum and follicular fluid has been demonstrated (Schneyer et al., 1992). The radiolabelled activin could be displaced by both activin-A and inhibin-A, suggesting that this binding protein is follistatin. This has been confirmed by Krummen et al. (1993) who found a relatively small amount of a 120 kDa complex when radiolabelled activin-A, but not inhibin-A was incubated with human serum. This complex eluted with a retention time similar to that of activin-A bound to purified follistatin. A relatively large amount of a similar complex was found after incubation of human follicular fluid with radiolabelled activin-A or inhibin-A. Preabsorption of the follicular fluid with antiserum to porcine follistatin prevented the formation of these complexes. Based on these results follistatin is characterized as a minor activin/inhibin binding protein in human serum and a major binding protein in follicular fluid. Binding of activin-A and inhibin-A to follistatin in immature female rat serum has also been found (Woodruff et al., 1993). Follistatin binding inhibits not only activin-A stimulated FSH release by pituitary cells but also activin-A stimulated hemoglobin production by K562 cells as well as activin-A immunodetection by ELISA. Follistatin does not interfere with immunodetection of inhibin-A by RIA (Krummen et al., 1993).

Follistatin mRNA has been found in a large number of tissues (reviewed by Michel et al., 1993) and is often colocalized with the inhibin ß-subunits. This strengthens the hypothesis that these proteins interact in the paracrine or autocrine regulation of many tissues. One of the tissues in which follistatin and inhibin ß-subunit mRNAs are colocalized is the testis. Follistatin mRNA is detected in rat testes (Michel et al., 1990) and human follistatin cDNA has been cloned from a human testis cDNA library (Shimasaki et al., 1988a). Follistatin protein is localized in nuclei of spermatocytes and spermatids around meiosis (Kogawa et al., 1991b), where also the inhibin $ß_A$-subunit is found (Shaha et al., 1989). Leydig cells are also positive for follistatin, whereas in Sertoli cells no follistatin protein has been detected (Kogawa et al., 1991b). In contrast, in situ hybridization demonstrated high expression of follistatin mRNA in Sertoli cells during stages IX-XI of the seminiferous epithelial cycle (Kaipia et al., 1992).

1.6.2 α_2-Macroglobulin

Another binding protein for activin and inhibin is α_2-macroglobulin. With a serum concentration between 2000 and 4000 mg/l in healthy humans, depending on age and sex, α_2-macroglobulin is one of the major proteins in serum. The protein is highly conserved as it is found in mammals, reptiles, birds, amphibians and fish. The molecule has a molecular weight of 725 kDa and, in its native state, consists of four 185 kDa polypeptide subunits held together by interchain disulfide and non-covalent bonds. Interaction of α_2-macroglobulin with several cytokines, including TGF-ß has been reported. The binding of TGF-ß to α_2-macroglobulin has no effect on TGF-ß bioactivity and is suggested to regulate the clearance of TGF-ß (reviewed by James, 1990).

Binding of activin and inhibin to a high molecular weight protein in human serum has been reported (Krummen et al., 1993). This protein has been identified as α_2-macroglobulin based on several criteria: radiolabelled activin and inhibin bind to purified α_2-macroglobulin; the complexes formed elute with similar retention times on HPLC as complexes formed after incubation of radiolabelled activin and inhibin with human serum and preadsorption of human serum with α_2-macroglobulin antiserum inhibits the formation of this complex. A relatively large amount of ^{125}I-activin-follistatin complex has been found in human serum, indicating that α_2-macroglobulin is a major activin/inhibin binding protein in human serum. In contrast, α_2-macroglobulin is a minor binding protein in human follicular fluid. In serum of immature female rats α_2-macroglobulin also binds activin and inhibin (Woodruff et al., 1993). Binding of α_2-macroglobulin to activin or inhibin has no effect on their ability to alter pituitary FSH release or hemoglobin production by K562 cells *in vitro*. Also no effect on immunodetection of activin by ELISA or immunodetection of inhibin by RIA has been found (Krummen et al., 1993).

Binding of inhibin to α_2-macroglobulin or follistatin might be the reason for the fact that Vaughan and Vale (1993) could not obtain an inhibin signal in human and porcine follicular fluid, using a highly sensitive and specific two-site IRMA for inhibin-A, in spite of the presence of inhibin bioactivity and α-subunit immunoreactivity as measured by RIA. In the IRMA antibodies against synthetic peptides corresponding to amino acid residues 1 to 27 of the mature inhibin α-subunit and amino acid residues 81 to 113 of the mature inhibin $ß_A$-subunit were used. RIA and IRMA used the same antibody against the inhibin α-subunit, suggesting that inhibin binding proteins interfered with the binding of inhibin to the antibody against the inhibin $ß_A$-subunit used in the IRMA. Using other two-site immunoassays with antibodies against synthetic peptides, the inability to measure inhibin in biological fluids due to the presence of binding proteins has also been reported by others (Betteridge and Craven, 1991; Groome, 1991). However, Baly et al. (1993) developed a two-site ELISA, based upon antibodies against recombinant inhibin-A and activin-A, in which α_2-macroglobulin and follistatin do not interfere.

In the testis the main α_2-macroglobulin secreting cell is the Sertoli cell; α_2-macroglobulin has been found in culture medium of Sertoli cells (Cheng et al., 1990) and not in culture medium of peritubular myoid cells (Zwain et al., 1993) or germ cells (Grima et al., 1992). Coculture of Sertoli cells and peritubular myoid cells (Zwain et al., 1993) or addition of germ cell-conditioned medium to Sertoli cell cultures (Grima et al., 1992) stimulates α_2-macroglobulin secretion by Sertoli cells.

In contrast, FSH and testosterone have no effect on α_2-macroglobulin secretion (Grima et al., 1992; Zwain et al., 1993). However, cultured rat seminiferous tubule segments at stages XIII-I secrete increased amounts of α_2-macroglobulin in response to a high dose (50 ng/ml) of FSH (Kangasniemi et al., 1992). Furthermore, basal secretion of α_2-macroglobulin by Sertoli cells varies throughout the seminiferous cycle. The physiological relevance of the interaction between α_2-macroglobulin and activin or inhibin is not clear but could concern the clearance of both hormones.

1.7 Expression and effects of inhibin, activin and related proteins

Expression of inhibin subunits, follistatin and activin receptors has been detected in several organs. To elucidate the possible roles of these proteins within these organs a large number of experiments have been performed. Both expression of inhibin and activin related proteins and their effects in different systems will be discussed in this section.

1.7.1 Pituitary gland and hypothalamus

The effects of inhibin and activin on pituitary and hypothalamic function are summarized in table II, and are discussed in more detail in the following subsections.

Table II. Effects of inhibin and activin on pituitary and hypothalamic function

	Inhibin	Activin
Gonadotrophs		
FSH release (basal and GnRH-stimulated)	↓	↑
Basal LH release	↓ =	=
GnRH-stimulated LH release	↓rat ↑sheep	=rat ↓sheep
GnRH receptors	↓rat ↑sheep	↑rat ↓sheep
Number of FSH cells	?	↑
Follistatin mRNA	=	↑
Corticotrophs		
ACTH release	?	↓
Proopiomelanocortin mRNA	=	↓
Somatotrophs		
GH release + biosynthesis	=	↓
Growth	?	↓
Lactotrophs		
Prolactin release	?	↓
Posterior lobe		
Oxytocin release	=	↑
Hypothalamus		
CRF release	=	↑

The following symbols are used: ↑ stimulating effect; ↓ inhibiting effect; = ineffective; ? when effect is not examined

1.7.1.1 Inhibin and gonadotropin release

As discussed in section 1.3, inhibin has been purified on basis of its ability to inhibit FSH release from dispersed pituitary cells. Inhibin affects not only FSH secretion but also FSH synthesis (for review see de Jong, 1988) by decreasing FSH ß-subunit mRNA expression in rat (Attardi et al., 1989; Carroll et al., 1989) and sheep (Mercer et al., 1987) gonadotropic cells. This decrease can be partially ascribed to a decrement in transcription rate of the FSH ß-subunit gene (Clarke et al., 1993). It has also been found that inhibin decreases the half-life of FSH ß-subunit mRNA in the absence of transcriptional inhibitors (Attardi and Winters, 1993). Therefore, it is postulated that inhibin induces (a) labile protein(s) that accelerates the degradation of FSH ß-subunit mRNA.

In vivo studies using intact and castrated male or female rats (Ying et al., 1987a; Carroll et al., 1991b; DePaolo et al., 1991a; Rivier and Vale 1991a; Rivier et al., 1991a,b; Robertson et al., 1991), hypothalamic-pituitary-disconected ewes (Mercer et al., 1987) and ovariectomized ewes (Findlay et al., 1987) show that administration of inhibin decreases FSH secretion within 4-6 h, through a mechanism partially independent of GnRH. Suppression of serum FSH levels in male rats appears to be less effective than in female rats (Robertson et al., 1991).

Another way to demonstrate the physiological role of inhibin is immunoneutralization of endogenous inhibin. These studies reveal that, in the male rat, inhibin regulates serum FSH levels during early postnatal development until the time of puberty, but not in the adult animal (Culler and Negro-Vilar, 1988; Rivier et al., 1988). Inhibin immunoneutralization in adult male rats only results in increased FSH secretion after the selective destruction of Leydig cells with ethane dimethyl sulfonate (EDS). This suggests that in the adult male rat a Leydig cell factor, probably testosterone, is the main factor regulating serum FSH levels (Culler and Negro-Vilar, 1990). In the adult male rhesus monkey and the prepubertal bull inhibin seems to play a role in regulation of serum FSH levels (Medhamurthy et al., 1991; Kaneko et al., 1993).

In contrast to the situation in male rats, immunoneutralization of endogenous inhibin in female rats shows that inhibin regulates serum FSH levels from day 20 onwards (Rivier et al., 1986; Culler and Negro-Vilar, 1988). In the adult cyclic female rat inhibin immunoneutralization results in an increased ovulation rate (Rivier and Vale, 1989; Sander et al., 1991) as it does in ewes (Forage et al., 1987; Findlay et al., 1989a; Mizumachi et al., 1990; Wrathall et al., 1990), gilts (Brown et al., 1990) and heifers (Morris et al., 1993), probably because of increased serum FSH levels. In heifers inhibin immunoneutralization results in an increased twin-calving rate (Morris et al., 1993). Although in the female mink inhibin immunoneutralization also increases serum FSH levels, litter number and weight decreases (Ireland et al., 1992). This can be explained by altered uterine function, uterine "crowding" or direct effects of inhibin antibodies on embryonic development. Surprisingly, immunization of ewes against the inhibin α_N-subunit impairs fertility in ewes without affecting serum FSH levels (Findlay et al., 1989b).

Inhibin suppresses FSH release *in vitro* and *in vivo* but its effect on LH release is controversial. Some studies report slightly diminished basal LH release *in vitro* (Farnworth et al., 1988a; Attardi et al., 1989; Gregg et al., 1991), others document no effect on basal but only on GnRH-stimulated LH release *in vitro* (Fukuda et al., 1987; Campen and Vale, 1988b; Farnworth et al., 1988b; Kotsuji et

al., 1988; Jakubowiac et al., 1990; Muttukrishna and Knight, 1990). Finally, no effects of inhibin on LH secretion *in vivo* were found (DePaolo et al., 1991a; Rivier et al., 1991a,b; Robertson et al., 1991). Inhibin does not alter LH ß-subunit mRNA expression *in vivo* and *in vitro* (Mercer et al., 1987; Attardi et al., 1989; Carroll et al., 1989 and 1991b), suggesting that its primary action is on FSH biosynthesis and secretion.

The effect of inhibin on GnRH stimulated LH release is, in contrast to the effect on FSH release, species dependent. In rat, pig and sheep GnRH-stimulated FSH release is inhibited by inhibin administration, whereas LH release is inhibited in rat (Fukuda et al., 1987; Campen and Vale 1988b; Farnworth et al., 1988b; Kotsuji et al., 1988) and pig (Jakubowiac et al., 1990), but stimulated in sheep (Muttukrishna and Knight, 1990; Gregg et al., 1991). This difference is probably due to different effects of inhibin on GnRH receptor numbers; inhibin reduces the number of GnRH receptors (Wang et al., 1988) and the GnRH-induced up-regulation of GnRH receptors (Wang et al., 1989) in rat pituitary cells, but increases the amount of GnRH binding sites (Laws et al., 1990; Gregg et al., 1991) and GnRH receptor mRNA (Sealfon et al., 1990) in ovine pituitary cells. The nature of these species differences remains unclear. Complementary data from Braden et al. (1990) show that, in pituitary cells from rats, inhibin does not suppress basal levels of GnRH receptors, whereas GnRH-induced up-regulation of GnRH receptors is blocked by an inhibition of receptor synthesis. Inhibin immunoneutralization in female rats enhances GnRH-induced gonadotropin release by pituitary cells isolated from these rats (Culler, 1992). This indicates that *in vivo* inhibin can partially block GnRH action. Nevertheless, inhibin administration *in vivo* does not change LH levels (see above), suggesting that the inhibin effects on GnRH action *in vivo* are not very pronounced. Finally, inhibin decreases FSH serum levels further in rats treated with gonadotropin antagonists (Rivier and Vale, 1991a), indicating that inhibin can have its effect on FSH secretion independent of the GnRH drive.

1.7.1.2 Activin and gonadotropin release

Activin has been discovered during the purification of inhibin due to its stimulating effect on pituitary FSH release (see section 1.3). Recombinant activin-A stimulates FSH secretion from rat (Kitaoka et al., 1988; Schwall et al., 1988a; Huylebroeck et al., 1990) and ovine (Gregg et al., 1991; Muttukrishna and Knight, 1991) pituitary cells, without effect on LH release. Furthermore, activin-A stimulates FSH ß-subunit mRNA expression (Carroll et al., 1989; Attardi and Miklos, 1990), possibly by a stabilization of this mRNA (Carroll et al., 1991a). Yet, Attardi and Winters (1993), were not able to confirm an effect on FSHß mRNA stability. Interestingly, addition of activin-A to perifused rat pituitary cells elicites a much larger increase of FSH ß-subunit mRNA expression than administration to pituitary cells in culture dishes (Weiss et al., 1992), probably because the latter cells are already stimulated by auto- or paracrine factors like activin-B (see below). In agreement with this observation, stimulation of FSH secretion by rat pituitary cells is higher at a low than at a high cell density (Kitaoka et al., 1989; Katayama et al., 1990). Activin-A stimulated FSH secretion is enhanced by somatostatin, which is most effective at high cell density (Kitaoka et al., 1989). This suggests that somatostatin can inhibit the secretion of a factor that suppresses activin action, e.g. follistatin which is present in the pituitary (see below). Furthermore, activin-A

stimulated FSH release is enhanced by testosterone and progesterone, but not by estradiol (Miyake et al., 1993). Finally, activin-A seems to act as a growth factor within the pituitary, for it increases the number of FSH immunoreactive cells (Katayama et al., 1990). This effect is restricted to small, undifferentiated FSH-containing cells, whereas in the more differentiated larger FSH-containing cells activin stimulates FSH release (Katayama et al., 1991). The growth factor-like action of activin-A is blocked by follistatin secreted from folliculo-stellate cells (Katayama et al., 1992).

In vivo, activin-A specifically stimulates FSH synthesis and secretion in immature (Schwall et al., 1989; Carroll et al., 1991b) and adult (Carroll et al., 1991b; Rivier and Vale, 1991b) female rats, but it has little or no effect in immature and adult male rats (Carroll et al., 1991b). However, in the adult male macaque activin-A significantly stimulates basal FSH release (McLachlan et al., 1989). The *in vivo* effects of activin appear to be partially independent of GnRH, since in ovariectomized adult female rats treated with a GnRH antagonist, activin-A still elevates serum FSH levels (Rivier and Vale, 1991b).

Like described for inhibin, the effect of activin-A on GnRH-stimulated FSH release is consistingly stimulating, whereas its effect on GnRH-stimulated LH release seems species dependent. In rat pituitary cells, activin-A has no effect on GnRH-stimulated LH release (Kitaoka et al., 1988; Schwall et al., 1988b), although a stimulation of GnRH receptor number has been reported (Braden and Conn, 1992). In ovine pituitary cells, activin-A suppresses the number of GnRH receptors (Gregg et al., 1991) and GnRH-stimulated LH release (Muttukrishna and Knight, 1991). In the adult male macaque GnRH-stimulated FSH and LH release are both increased by activin-A treatment (McLachlan et al., 1989). Interestingly, it has been demonstrated that activin-A can stimulate GnRH release from a murine GnRH-secreting neuronal cell line, which is antagonized by inhibin (González-Manchón et al., 1991). Whether activin can stimulate GnRH release *in vivo* remains to be clarified.

The effects of activin on pituitary function can be explained by the presence of ActRII (chapter 2) and ActRI (Tsuchida et al., 1993) in the pituitary. Inhibin α- and β_B-subunit mRNAs are also present within the rat anterior pituitary (Meunier et al., 1988a) and activin-B protein has been found in cultured rat anterior pituitary cells (Bilezikjian et al., 1993a). Expression of both inhibin α- and β_B-subunit has been localized in gonadotrophs and is stimulated by ovariectomy. This increase of inhibin subunit mRNA expression is prevented by estrogen treatment (Roberts et al., 1989a). In contrast, castration of adult male monkeys decreases inhibin β_B-subunit mRNA expression in the pituitary (Attardi et al., 1992). In rat pituitaries inhibin subunits are costored with FSH and LH in secretory granules (Roberts et al., 1992). The presence of the inhibin β_B-subunit in the pituitary, suggests an autocrine role for activin-B in regulating FSH secretion. Indeed, basal and activin-B stimulated FSH secretion from dispersed pituitary cell cultures is inhibited by a monoclonal antibody to activin-B, without effect on LH secretion (Corrigan et al., 1991). Activin-A stimulated FSH release is not blocked by this antibody. Concordantly, expression of FSH ß-subunit, but not LH ß-subunit or α-subunit decreases during incubation with this antibody. Furthermore, the increase in FSH secretion found after ovariectomy of hypophysectomized rats bearing a pituitary transplant under their kidney capsule, is partially prevented by immunoneutralization with a monoclonal antibody

to activin-B (DePaolo et al., 1992). The same antibody also partially decreases the secondary FSH surge in normal cyclic rats. Nevertheless, activin-B is found to be less potent in stimulating pituitary FSH release than activin-A or activin-AB (Nakamura et al., 1992a).

Follistatin mRNA has also been detected in rat pituitary (Michel et al., 1990) and has been localized in gonadotrophs and folliculo-stellate cells (Kaiser et al., 1992). The protein has been purified from bovine pituitary homogenates (Kogawa et al., 1991a) and culture medium of pituitary folliculo-stellate cells (Gospodarowicz and Lau, 1989). In culture medium from anterior pituitary cells follistatin has also been detected (Bilezikjian et al., 1993b). In dispersed pituitary cell monolayer cultures activin-A stimulates follistatin mRNA expression, whereas inhibin-A has no effect (DePaolo et al., 1993). Concordantly, activin-A stimulates the secretion of immunoreactive follistatin from anterior pituitary cultures, which is inhibited by inhibin-A (Bilezikjian et al., 1993b). Since FSH secretion is stimulated by activin-A treatment, follistatin seems part of an intrinsic pituitary ultrashort feedback system preventing overexposure to activin-A rather than totally inhibiting its activity. Serum Ovariectomy increases follistatin expression in normal pituitaries and pituitaries placed under the kidney capsule to remove hypothalamic input, which could be the result of increased pituitary $ß_B$-subunit expression (DePaolo et al., 1993). The increase in pituitary follistatin mRNA expression after gonadectomy of 21 day old rats is stronger in males than in females and parallels or precedes the increase of FSH biosynthesis and secretion. Estradiol replacement in ovariectomized female rats does not reverse this increase, but stimulates a further increase in pituitary follistatin mRNA levels. In contrast, testosterone replacement in castrated male rats decreases pituitary follistatin mRNA expression, towards but not completely back to baseline levels found in intact males (Kaiser and Chin, 1993). These results indicate that the gonads may have an effect on the paracrine interaction between activin and follistatin in the pituitary.

1.7.1.3 Other effects of activin and inhibin in the pituitary and hypothalamus

Gonadotrophs are not the only activin responsive pituitary cell type. Suppression of adrenocorticotropin (ACTH) secretion by primairy anterior pituitary cell cultures has been described (Vale et al., 1986), suggesting an effect on corticotrophs. A similar effect, accompanied by a decrease in proopiomelanocortin mRNA expression, has been found in AtT20 mouse corticotropic cells (Bilezikjian et al., 1991), from which also ActRII was cloned (Mathews and Vale, 1991). In this system inhibin has no effect on basal or activin-A induced ACTH secretion. In contrast to these studies, Kitaoka et al. (1988) reported that activin-A has no effect on basal or corticotropin releasing hormone (CRH) stimulated ACTH release. *In vivo*, intraventricular administration of activin-A in male rats stimulates circulating ACTH levels, due to a stimulation of hypothalamic CRH release, whereas inhibin administration has no effect (Plotsky et al., 1991). Inhibin $ß_A$-subunit immunostaining has been located in nerve terminals in the CRH-rich region of the paraventricular nucleus (PVN), indicating the possible physiological relevance of these findings. Immunostaining for the inhibin $ß_A$-subunit has also been found in nerve terminals connected to oxytocin containing neurons, indicating a role for activin in the control of oxytocin secretion from the posterior lobe of the pituitary. Infusion of activin-A, but not inhibin, into the PVN of male rats can indeed cause

oxytocin secretion. Concordantly, antiserum against the inhibin $ß_A$-subunit can attenuate suckling-induced oxytocin secretion in female rats (Sawchenko et al., 1988). Binding of ^{125}I-labelled activin has been demonstrated in the PVN of female rats as well as in several other brain regions (Jakeman et al., 1992). Together with the presence of ActRII (Mathews and Vale, 1991), ActRI (Attisano et al., 1993) and inhibin α-, $ß_A$- and $ß_B$-subunit mRNAs (Meunier et al., 1988a), these obsevations predict several other functions for activin in the brain. Additionally, follistatin has been found in the cerebral cortex (Michel et al., 1990).

Effects of activin on somatotrophs have also been described. In primary cultures of rat anterior pituitary cells, activin-A inhibits both basal and growth hormone releasing hormone stimulated growth hormone (GH) secretion (Vale et al., 1986; Kitaoka et al., 1988; Bilezikjian et al., 1990), somatotroph growth and GH biosynthesis (Billestrup et al.,1990). Inhibin is not able to reverse the effect of activin-A on GH biosynthesis. In response to activin-A treatment, somatotroph cytosolic free calcium concentration increases (Tasaka et al., 1992).

Basal prolactin secretion from primary rat pituitary cells is not affected by activin-A, whereas TRH-stimulated prolactin secretion is inhibited (Kitaoka et al., 1988). Furthermore, the prolactin secretion by the rat pituitary cell line GH4 and its proliferation are inhibited by activin-A but much stronger by TGF-ß (Ramsdell et al., 1991). Effects on TSH release from thyrotrophs have not been found (Kitaoka et al., 1988).

These results indicate that activin, and to a lesser extend inhibin, can play an important role in the regulation of pituitary cell function. As paracrine factors in the pituitary and the brain they can affect the secretion of several pituitary hormones.

1.7.2 Testis

1.7.2.1 Expression of inhibin subunits, activin receptors and follistatin in the testis

In the adult rat testis expression of inhibin α- and $ß_B$-subunit mRNA has been amply demonstrated (Esch et al., 1987a; Meunier et al., 1988a; Feng et al., 1989a; Krummen et al., 1989), whereas conflicting results on the presence of $ß_A$-subunit mRNA were reported. The expression of testicular inhibin α-subunit mRNA is high until day 20-25 postnatally and declines gradually thereafter (Meunier et al., 1988a; Keinan et al., 1989; Klaij et al., 1992). This correlates with a decrease of inhibin α-subunit immunostaining in Sertoli cells and decreasing inhibin plasma levels between days 15 and 90 of postnatal life (Rivier et al., 1988). The expression of total testicular inhibin $ß_B$-subunit mRNA decreases slightly after day 14 (Feng et al., 1989a; Klaij et al., 1992). The ratio between the 4.2 and 3.5 kb inhibin $ß_B$-subunit transcripts found in the testis, changes during the first 3 weeks of life, resulting in expression of mainly the 3.5 kb mRNA at day 21 (Klaij et al., 1992). These messengers are transcribed from different transcription initiation sites (Feng and Chen, 1993), and are both actively translated (Klaij et al., 1992). It is still obscure whether the protein products derived from these mRNAs differ in biological function. Testicular inhibin α-subunit mRNA expression is decreased 7 days after hypophysectomy of both immature and mature rats, and is restored after FSH replacement. Supplementation of testosterone has no effect on inhibin α-subunit expression (Keinan et al., 1989; Krummen et al., 1990). These results demonstrate

that the inhibin α-subunit gene is regulated by FSH. These treatments have no effect on inhibin β_B-subunit mRNA expression (Krummen et al., 1990). In contrast, Feng et al. (1989a) reported an increase of inhibin β_B-subunit mRNA expression 10 days after hypophysectomy of immature and mature rats. However, neither FSH nor testosterone treatment has an effect on the expression of this mRNA, indicating that these hormones are not important for the regulation of the inhibin β_B-subunit.

Inhibin subunit mRNAs are expressed in a stage-specific manner as found by studying dissected seminiferous tubules (Bhasin et al., 1989; Kaipia et al., 1991), stage-synchronized testes (Klaij et al., 1994) and in situ hybridization (Kaipia et al., 1992). As shown in figure 7, inhibin α-subunit mRNA expression is high in stages XIV-IV and low in stages VI-VIII. A similar tendency has been demonstrated for the secretion of immunoreactive inhibin from dissected seminiferous tubules (Gonzalez et al., 1989a). Since FSH receptor mRNA levels showes the same cyclic expression pattern (Heckert and Griswold, 1991; Kliesch et al., 1992), the stage specific variation in inhibin α-subunit expression could be secondary to changes in response to FSH. In contrast to the results of Kaipia et al. (1991), Klaij et al. (1994) found two peaks in the expression of the inhibin α-subunit; at stages V and XIII. The reason for the difference between these results is not clear, but could result from increased α-subunit expression in Leydig cells (see below), which are absent in the study of Kaipia et al. (1991). Expression of inhibin β_B-subunit mRNA is maximal during stages XIII-III and minimal at stages VI-VII. The expression of the inhibin β_A-subunit is restricted to stages VIII-XI and its distribution over the stages is identical to that of follistatin expression (Kaipia et al., 1992). These stages represent approximately 15% of the spermatogenic cycle, explaining the difficulty in the detection of the inhibin β_A-subunit mRNA in whole testis homogenates.

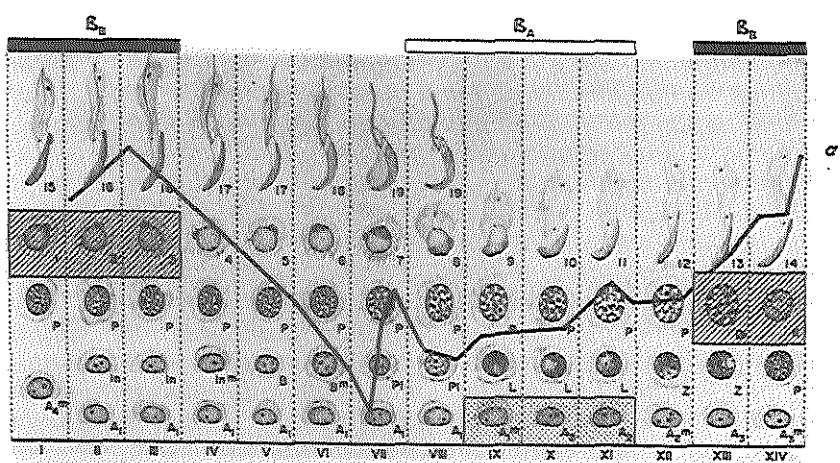

Fig.7. Expression of the inhibin subunits and activin receptors throughout the cycle of the seminiferous epithelium. Expression of the inhibin α-subunit (line), β_A-subunit (open bar) and β_B-subunit (closed bar) in Sertoli cells has been indicated. Only the stages in which the inhibin β_B-subunit is highly expressed have been marked. The expression of ActRII (stripped bars) and ActRIIB (dotted bar) in germ cells has also been indicated. The figure is composited from data of chapter 2; and from Kaipia et al., 1991, 1992 and 1993.

Cyclic variations in seminiferous tubule mRNA expression are probably based on cell-cell interaction within the tubule. It has been demonstrated that depletion of late spermatids *in vivo* decreases serum inhibin α-subunit immunoreactivity and increases serum FSH levels (Allenby et al., 1991), suggesting that late spermatids stimulate the secretion of biologically active inhibin. In this study depletion of early spermatids has no effect on serum inhibin levels, which contrasts with the observation that addition of early spermatids or early spermatid conditioned medium to Sertoli cells from 20-day-old rats *in vitro* increases inhibin secretion (Pineau et al., 1990). However, it should be questioned if this latter experiment provides a good model, because *in vivo* Sertoli cells from 20-day-old rats are never exposed to early spermatids. Spermatocytes may inhibit inhibin subunit expression, since a decrease in numbers of pachytene spermatocytes is followed by increased inhibin α-subunit mRNA expression (Kaipia et al., 1991) and the appearance of pachytene spermatocytes suppresses inhibin α- and β_B-subunit mRNA expression (Klaij et al., 1994). The appearence of B spermatogonia or preleptotene spermatocytes seems to stimulate inhibin α- and β_B-subunit mRNA expression (Klaij et al., 1994).

Experiments using in situ hybridization (Roberts et al., 1989b) and immunocytochemistry (Merchenthaler et al., 1987; Rivier et al., 1988; Roberts et al., 1989b; Saito et al., 1989; Shaha et al., 1989) demonstrated that the testicular expression of inhibin subunits is mainly localized in Sertoli cells. However, expression has also been found in other testicular cell types as indicated in table III. Also *in vitro*, Sertoli cells express inhibin subunits and secrete immunoreactive and bioactive inhibin, as was demonstrated by the purification of 32 kDa inhibin-B from Sertoli cell culture medium (Grootenhuis et al., 1990a). FSH, but not testosterone stimulates inhibin α-subunit mRNA expression in Sertoli cell cultures from immature rats, without effect on inhibin β_B-subunit mRNA (Toebosch et al., 1988; Keinan et al., 1989; Klaij et al., 1990). This effect can be mimicked by dibutyryl cAMP, indicating that the FSH effect is mediated by cAMP (Klaij et al., 1990). In conformity, the promoter region of the inhibin α-subunit gene, but not of the inhibin β_B-subunit gene, contains a cAMP-responsive element (Feng et al., 1989b). As a result, FSH also stimulates the secretion of immunoreactive inhibin from Sertoli cell cultures (Bicsak et al., 1987; Morris et al., 1988; Toebosch et al., 1988; Risbridger et al., 1989a) and cultured segments of seminiferous tubules (Gonzalez et al., 1988). The effect of FSH on the secretion of inhibin bioactivity is controversial. Several authors reported an increase in the secretion of biologically active inhibin (Le Gac and de Kretser, 1982; Ultee-van Gessel et al., 1986; Toebosch et al., 1988; Risbridger et al., 1989a). However, Grootenhuis et al. (1990b) demonstrated that FSH does not stimulate the secretion of immunoreactive or bioactive 32 kDa inhibin. Instead, the secretion of a highly immunoreactive but biologically inactive 29 kDa inhibin α-subunit precursor protein (proα_c) is stimulated. The discrepancy between these studies is probably due to differences in culture temperature, cell purity and bioassay conditions. Moreover, differences in bioactivity could result from changes in follistatin secretion. The FSH-stimulated secretion of immunoreactive inhibin by cultured immature Sertoli cells is attenuated by adenosine, which probably suppresses cAMP accumulation (Conti et al., 1988). Besides FSH, epidermal growth factor (EGF) stimulates the secretion of immunoreactive inhibin from Sertoli cell cultures (Morris et al., 1988) and cultured segments of seminiferous tubules

(Gonzalez et al., 1989b). In contrast, insulin inhibits the secretion of immunoreactive inhibin from cultured tubules (Gonzalez et al., 1989c), whereas IGF-1 has no effect on inhibin secretion by cultured Sertoli cells (Toebosch et al., 1988). In cultures of Sertoli cells from 14-day-old rats inhibin $ß_B$-subunit mRNA expression is suppressed decreased by addition of phorbol ester or calcium ionophore. A parallel decrease of bioactive inhibin secretion and an increase of proα_c secretion have been observed. This indicates that the level of $ß_B$-subunit mRNA expression is rate limiting for the production of bioactive inhibin and can be regulated by protein kinase C or intracellular calcium (Klaij et al., 1992).

Table III. Localization of inhibin subunits, activin receptors and follistatin in the rat testis

	α	$ß_A$	$ß_B$	ActRII	ActRIIB	Fol
Leydig cells						
Embryo	++	+++	-	?	?	?
Day 12 pn	+	++	+++	?	?	?
Day 21 pn	+	-	-	+	?	?
Adult	+	-	-	+	?	+
Peritubular myoid cells	++	+++	+	++	-	?
Sertoli cells	+++	+	++	++	+	+
Germ cells						
A_1 and A_2 spermatogonia	?	?	?	-	++	-
Late spermatocytes	+	+	?	+++	-	+
Early spermatids	?	?	?	+++	-	+

The following symbols are used: + present (the relative amount is indicated by the number of symbols); - absent; ? not determined

Since Sertoli cells express inhibin ß-subunit mRNAs, these cells might be capable of secreting activin. Indeed, high levels of immunoreactive activin have been demonstrated in testis homogenates (Shintani et al., 1991). Furthermore, a 25 kDa fraction of Sertoli cell conditioned medium can stimulate the release of FSH from pituitary cells in culture (Grootenhuis et al., 1989). Finally, the presence of activin-B in Sertoli cell conditioned medium has been confirmed by the "animal cap bioassay", immunoprecipitation and Western blotting (chapter 4). These observations indicate that, in the immature rat, Sertoli cells can secrete activin. The ratio of the expression of inhibin α and $ß_B$ subunit mRNAs in total testes of rats of various ages is constant from day 21 onwards (Klaij et al., 1992). This suggests that activin is also produced by Sertoli cells in the adult testis, provided that translation efficiencies of these mRNAs do not change.

Initially, Leydig cells have not been considered as a source of inhibin or activin. However, in the embryonic rat, at 20 days of gestation, a strong signal for $ß_A$-subunit mRNA can be seen over the interstitial tissue (Roberts et al., 1991). In testis from 12-day-old rats immunostaining and mRNA for the inhibin α-, $ß_A$- and $ß_B$-subunit have been detected in Leydig cells (Roberts et al., 1989b). In mature rats only the inhibin α-subunit has been observed. Moreover, inhibin α-subunit immunostaining has been found in human (Bergh and Cajander, 1990; Vannelli et al., 1992) ovine (Veeramachaneni et al., 1989) and rat (Teerds et al., 1991) Leydig

cells. In the Leydig cell line TM3 inhibin $ß_A$- and, to a lesser extent, $ß_B$-subunit mRNA has been detected, in the absence of inhibin α-subunit mRNA (Lee et al., 1989). Conditioned medium from these cells stimulates FSH release from pituitary cells, indicating activin bioactivity. A similar effect has been found with conditioned medium from Leydig cells of 17-day-old rats and 2 to 3 weeks old pigs. From these results it is concluded that Leydig cells secrete activin. A later study (Mather et al., 1992) confirmed the secretion of bioactive and immunoreactive activin from immature rat Leydig cells. However, between 19 and 21 days of age activin production by Leydig cells becomes undetectable (Mather et al., 1992). In Leydig cells from 21-day-old rats and mature rats only inhibin α-subunit mRNA is expressed and in the culture medium from these cells only inhibin α-subunit protein has been found (chapter 3). Nevertheless, others report the secretion of biologically active inhibin by Leydig cells from adult rats (Risbridger et al., 1989b).

LH stimulates the secretion of immunoreactive inhibin by Leydig cells from immature (chapter 3) and mature (chapter 3; Risbridger et al., 1989b) rats, probably by a calcium-mediated mechanism (Simpson et al., 1991). This can partially explain the increase in serum immunoreactive inhibin levels found after *in vivo* administration of LH or hCG in rats (Sharpe et al., 1988; Drummond et al., 1989) and men (McLachlan et al., 1988; Burger et al., 1990). LH replacement to adult hypophysectomized rats results in an increase of testicular inhibin α-subunit mRNA expression, whereas in immature rats no effect has been found (Krummen et al., 1990). However, from a study with mature rats, in which Leydig cells were destructed, it is concluded that normally Leydig cells contribute little to the intratesticular and blood levels of inhibin in the adult rat (Maddocks and Sharpe, 1989).

In homogenates of human Leydig cell tumors inhibin immunoreactivity has also been detected (de Jong et al., 1990b). In contrast to normal Leydig cells, tumor Leydig cells can secrete biologically active inhibin, although in total RNA from these cells expression of inhibin ß-subunits was not detected (chapter 3). Nevertheless, in a recent study, expression of a low amount of inhibin $ß_A$- and $ß_B$-subunit mRNA has been found in poly(A^+) RNA from several tumor Leydig cells. In these tumor cells ActRII mRNA is also expressed (Chen et al., 1993). Addition of cAMP derivatives strongly suppresses the expression of the inhibin $ß_A$-subunit mRNA. Finally, peritubular myoid cells have been indicated as an activin-A producing cell in the testis (chapter 5). Also in this study $ß_A$-subunit mRNA expression is inhibited by a cAMP analogue.

1.7.2.2 Effects of inhibin and activin in the testis

In the testis, several effects of inhibin and activin have been described, as summarized in table IV. Activin and inhibin have been postulated as regulators of Leydig cell steroidogenesis. In primary cultures of testicular cells from 7-day-old rats, activin-A inhibits LH-stimulated testosterone production, although it is ineffective when added alone. In the same cultures and in cultures of testis cells from adult hypophysectomized rats, inhibin enhances LH-stimulated testosterone secretion, without effect on basal testosterone secretion (Hsueh et al., 1987). Activin-A also inhibits human chorionic gonadotropin (hCG) stimulated testosterone and cAMP formation in cultures of Leydig cell from normal adult rats, without effect on hCG binding. Furthermore, it inhibits forskolin- and 8-bromo-cAMP-induced

testosterone formation and the conversions of pregnenolone and progesterone to testosterone (Lin et al., 1989). This suggests that activin acts on multiple steps in the steroidogenic pathway, including coupling between LH receptor and G-protein or G-protein and adenylate cyclase and 17-hydroxylase activity. In this culture system, inhibin has no effect on basal or hCG-stimulated testosterone production but reverses the inhibitory action of activin. In immature porcine Leydig cells activin-A inhibits cholesterol side chain cleavage cytochrome P450 ($P450_{scc}$) activity and stimulates 3ß-hydroxysteroid dehydrogenase/isomerase (3ßHSD) activity (Mauduit et al., 1991). No effect has been found on hCG binding or low density lipoprotein metabolism. The net result of these effects is a decrease in hCG-or 8-bromo-cAMP-stimulated dehydroepiandrosterone accumulation and a slight increase in testosterone secretion. The discrepancies between the studies described are probably due to species differences and/or different culture conditions.

Table IV. Effects of inhibin and activin on testicular function

	Inhibin	Activin
Leydig cell		
LH-stimulated testosterone neonatal rat	↑	↓
LH-stimulated testosterone adult rat	↑	↓
$P450_{scc}$ activity pig	?	↓
3ßHSD activity pig	?	↑
Germ cells		
Number In and B spermatogonia *in vivo*	↓	?
Spermatogonial proliferation *in vitro*	=	↑
Sertoli cells		
Reaggregation	=	↑
FSH-stimulated aromatase activity	?	↓
Androgen receptor expression	?	↓
Transferrin secretion	?	↑
Inhibin secretion	?	↑

Inhibin and activin may also have a regulatory role in the spermatogenic process. In adult mice and hamsters it has been demonstrated that intratesticular inhibin administration reduces the number of differentiating intermediate and B-type spermatogonia (van Dissel-Emiliani et al., 1989). In accordance with these results, DNA synthesis in intermediate and B-type spermatogonia is decreased after treatment of immature rats with a partially purified inhibin preparation (Franchimont et al., 1981). The inhibin $ß_B$-/α-subunit mRNA ratio is low at stages I-VI (Bhasin et al., 1989; Klaij et al., 1994) and correlates with relatively high amounts of bioactive inhibin in stage I (Klaij et al., 1994). An increase in inhibin secretion during these stages may be a regulatory mechanism to prevent excessive germ cell numbers. Furthermore, binding of fluorescein isothiocyanate (FITC)-labelled inhibin has been found on isolated spermatogonia, spermatocytes and spermatids, indicating a possible role for inhibin in the control of spermatogenesis (Woodruff et al., 1992). Recently, inhibin α-subunit immunostaining has been found in coated pits and vesicles of human spermatocytes, suggesting receptor mediated endocytosis of the inhibin α-subunit (Vannelli et al., 1992).

In germ cell-Sertoli cell cocultures from immature rats, activin-A and activin-B, but not inhibin-A, stimulate spermatogonial proliferation (Mather et al., 1990). Since ActRIIB mRNA is highly expressed in A_1 and A_2 spermatogonia at stages IX-XI (Kaipia et al., 1993), activin might act as a spermatogonial growth factor, initiating mitotic division. Interestingly, ActRIIB mRNA expression in spermatogonia is found at the same time as inhibin $ß_A$-subunit expression in Sertoli cells. Concurrently, FITC-labelled activin binds to isolated spermatogonia and additionally to late pachytene/diplotene spermatocytes and spermatids (Woodruff et al., 1992). Activin binding to spermatocytes and spermatids is to be expected, because expression of ActRII mRNA has been found in isolated mid-/late-pachytene spermatocytes and round spermatids (chapter 2). More precise, in situ hybridization demonstrated ActRII mRNA in spermatocytes and round spermatids at stages XIII-III, i.e. around the time of meiotic division (chapter 2 supplement; Kaipia et al., 1992). As a result of an increasing inhibin $ß_B$-/α-subunit mRNA ratio at stages VII-XIII (Bhasin et al., 1989; Klaij et al., 1994), larger amounts of activin-B might be produced at this time to regulate meiotic division or the beginning of spermiogenesis.

Another testicular target cell for activin is the Sertoli cell. Sertoli cells reaggregate into seminiferous tubule-like structures after addition of activin-A to germ cell-Sertoli cell cocultures (Mather et al., 1990). This process is inhibited by follistatin (Mather et al., 1993). Furthermore, activin-A inhibits FSH-stimulated aromatase activity and androgen receptor expression (chapter 4) and stimulates transferrin and inhibin secretion (chapter 5) in cultured Sertoli cells. Finally, Schneyer et al. (1991) postulated a role for the inhibin α-subunit precursors in the regulation of FSH binding to calf testis membranes. These precursors inhibit FSH binding with a potency 50-100 fold lower than that of FSH. Therefore, the biological relevance of this obsevation is obscure.

The importance of inhibin-like proteins in the testis, becomes evident from a study with mice, in which the inhibin α-subunit gene was deleted (Matzuk et al., 1992). In these mice gonadal stromal tumors developed within 4 weeks after birth, indicating that α-inhibin is a tumor suppressor gene, or alternatively that these tumors arise because of the overproduction of activin.

1.7.3 Ovary

As described in previous sections activins, inhibins and follistatins have been purified from ovarian follicular fluid, indicating that these proteins are produced within the ovary. Since a rapid loss of inhibin immunoreactivity from the blood has been found following ovariectomy of female rats, the ovary is considered as the principle source of circulating inhibin in these animals (Rivier et al., 1986). In the rat ovary a cyclic expression of the inhibin α-, $ß_A$- and $ß_B$-subunit mRNAs was found in granulosa cells of antral follicles (Meunier et al., 1988b; Woodruff et al., 1988). Granulosa cells of newly recruited follicles express inhibin α-subunit mRNA and initiate expression of inhibin $ß_A$- and $ß_B$-subunit mRNA. Expression of inhibin subunits increases during met- and di-estrus and peaks at pro-estrus. Following the primary gonadotropin surge expression decreases dramatically and as a result serum inhibin levels decline (Woodruff et al., 1989). This decrease of inhibin levels is postulated to be the reason for the secondary FSH surge at estrus, which recruits a new crop of follicles. In the corpus luteum only low expression of the

inhibin α-subunit has been found as was detected in some primordial follicles, atretic follicles, theca cells and interstial cells. In contrast, the bovine and ovine corpus luteum expresses no inhibin subunits (Rodgers et al., 1989), whereas in the *Macaca fascicularis* (Schwall et al., 1990) and the human (Davis et al., 1987; Erämaa et al., 1993) corpus luteum inhibin α- and $ß_A$-subunit have been found as well as inhibin immuno- and bioactivity (Davis et al., 1987). In bovine ovaries, preantral and small antral follicles do not express inhibin subunits. As the follicle grows inhibin α-subunit is expressed first, followed by the inhibin $ß_A$-subunit (Torney et al., 1989). In contrast to other species, the granulosa cells of healthy small antral follicles in *Macaca fascicularis* express exclusively inhibin $ß_B$-subunit mRNA (Schwall et al., 1990). This will result in the production of activin-B, which can have auto-/paracrine actions in the primate ovary or regulate pituitary FSH release. In the granulosa cells of dominant follicles, inhibin $ß_B$-subunit mRNA expression decreases to undetectable levels and only inhibin α- and $ß_A$-subunit are expressed.

Inhibin (Woodruff et al., 1990) and activin binding-sites (Sugino et al., 1988a; LaPolt et al., 1989; Woodruff et al., 1990) have been demonstrated on rat granulosa cells. Furthermore, ActRII and ActRIIB mRNAs were detected in ovaries of immature and cyclic rats (Feng et al., 1993). Two ActRII mRNA transcripts (6.0 and 3.0 kb) are present in cultured rat granulosa cells (Nakamura et al., 1993). These observations suggest auto- and paracrine effects of inhibin and activin in the ovary, as well as effects of follistatin, as partially reviewed by Findlay (1993). These effects have been summarized in table V.

Table V. Effects of inhibin and activin in the ovary

	Inhibin	Activin
Theca cells		
LH-stimulated androgen production	↑	↓
Granulosa cells		
Undifferentiated:		
FSH receptors	=	↑
LH receptors	=	↑
Inhibin secretion	?	↑
Follistatin mRNA	?	↑
Aromatase activity	↓=	↑
Progesterone production	=	=basal ↑FSH
Partially differentiated:		
Progesterone secretion	?	↑basal ↓FSH
Differentiated:		
Progesterone production	?	↓basal ↓FSH
LH-stimulated oxytocin secretion	=	↓
Spontaneous luteinization	?	↓
Oocyte		
Germinal vesicle breakdown	↓	↑

Inhibin has been described as an inhibitor of rat granulosa cell aromatase activity (Ying et al., 1986) , but this was not confirmed by others using both rat and bovine granulosa cells (Hutchinson et al., 1987; Sugino et al., 1988b; Shukovski and Findlay, 1990). Furthermore, inhibin has been described as a potent stimulator

of LH-induced androgen production by rat and human theca cells (Hsueh et al., 1987; Hillier et al., 1991a). Opposite to inhibin, activin reduces LH-stimulated androgen production in rat and human theca cells (Hsueh et al., 1987; Hillier et al., 1991b). No effect on basal androgen production has been found. In this way, inhibin enhances the supply of androgen substrate for the follicular production of estrogen. Increasing inhibin and estrogen production by dominant follicles can suppress serum FSH levels and therefore restrict the growth of non-dominant follicles, which will become atretic. The dominant follicles can survive this decrease in FSH levels by the local action of factors like activin, which has been shown to stimulate FSH receptor mRNA expression (Nakamura et al., 1993) and the number of FSH receptors (Hasegawa et al., 1988; Xiao et al., 1992b; Nakamura et al., 1993) in undifferentiated rat granulosa cells. On the other hand, FSH stimulates activin receptor number in these cells (Sugino et al., 1988a). In the presence of FSH, activin enhances aromatase activity (Hutchinson et al., 1987; Xiao et al., 1990; Miro et al., 1991; Xiao and Findlay, 1991), LH binding sites (Sugino et al., 1988b) and progesterone production (Sugino et al., 1988b; Miro et al., 1991; Xiao et al., 1990; Xiao and Findlay, 1991) in undifferentiated rat granulosa cells, without effects on these parameters in the absence of FSH. Activin also stimulates basal and FSH-stimulated inhibin α- and $ß_A$-subunit mRNA expression and inhibin production (LaPolt et al., 1989; Xiao and Findlay, 1991). Finally, activin stimulates basal and FSH-stimulated steady-state follistatin mRNA levels (Michel et al., 1992). These results indicate that activin can play a role in granulosa cell differentiation.

The effect of activin on progesterone production is dependent on the differentiation state of the granulosa cells. In partially differentiated rat granulosa cells activin stimulates basal progesterone production but inhibits FSH-stimulated progesterone production, whereas in fully differentiated rat granulosa cells both basal and FSH-stimulated progesterone production are inhibited (Miro et al., 1991). In the same way, basal and FSH- or hCG-stimulated progesterone secretion from human luteinizing granulosa cells are inhibited by activin-A (Li et al., 1992; Rabinovici et al., 1992) as are basal and FSH-stimulated aromatase activity (Rabinovici et al., 1992). Inhibin does not influence the effects of activin and has also no effect when added alone (Rabinovici et al., 1992). Progesterone production by the *Macaque* corpus luteum is also inhibited, without effect of inhibin (Brannian et al., 1992). Recombinant activin-A inhibits LH-induced production of progesterone and oxytocin (indicators of luteinization) by partially differentiated bovine granulosa cells (Shukovski et al., 1991). In fully differentiated bovine granulosa cells activin-A prevents spontaneous luteinization (Shukovski and Findlay, 1990). From these *in vitro* studies it is concluded that activin promotes differentiation in early antral stages of folliculogenesis and prevents premature luteinization in later stages of antral development. In contrast to this hypothesis, Woodruff et al. (1990) postulated that activin induces atresia, whereas inhibin stimulates follicular growth. This suggestion was based on an *in vivo* study, in which 1 μg activin or inhibin was injected into the ovarian bursa of immature rats. Less than 1% of the injected hormone was recovered in the serum resulting in unaltered serum FSH levels but ultraphysiological hormone levels in the bursal fluid. Subcutaneous administration of activin-A to intact immature female rats causes a significant increase in serum FSH, inhibin, estradiol, ovarian weight, uterine weight and ovarian FSH receptors (Doi et al., 1992). In the same study serum progesterone levels decrease, whereas the

number of follicles and their size increase. Activin-A alone has no effects on hypophysectomized immature female rats but together with PMSG ovarian weight, uterine weight and serum inhibin and estradiol levels increase (Doi et al., 1992). These data demonstrate that activin stimulates the action of gonadotropins at the level of the ovary and enhances follicular development, in agreement with the results of *in vitro* studies.

Follistatin is also present in the ovary; especially in granulosa cells and, under some circumstances, in luteal cells but not in theca cells (reviewed by DePaolo et al., 1991b). Follistatin mRNA expression is stimulated by FSH and not by LH *in vitro* and *in vivo* (DePaolo et al., 1991b). Like the expression of inhibin subunits, follistatin expression is changing during the estrous cycle of rat and cow (Nakatani et al., 1991; Shukovski et al., 1992). Follistatin mRNA first appears in secondary follicles and expression increases as folliculogenesis progresses. The strongest signal has been found in preovulatory follicles and newly formed corpora lutea. In atretic follicles no follistatin has been detected.

The effects of follistatin on rat granulosa cell function are opposite to the effects of activin. As follistatin antagonizes the actions of activin, it is concluded that follistatin favors luteinization or atresia by neutralizing the effects of activin (Findlay, 1993). However, direct actions of follistatin on granulosa cell function are not excluded. This assertion is based on the observation that both activin and follistatin enhance FSH-stimulated progesterone production by undifferentiated rat granulosa cells (Xiao et al., 1990; Xiao and Findlay, 1991), although this was not confirmed by Nakamura et al. (1992c). Furthermore, follistatin enhances forskolin-stimulated progesterone production by rat granulosa cells without effect on forskolin-stimulated aromatase activity and inhibin production (Xiao and Findlay, 1991), whereas activin enhances all three parameters (Xiao and Findlay, 1991). Finally, follistatin stimulates progesterone production by bovine theca cells, which do not express inhibin ß-subunit mRNA (Shukovski et al., 1993). This again suggests a direct effect of follistatin rather than a modulation of activin action.

Additionally, effects of activin, inhibin and follistatin on oocyte maturation have been described. In fetal life the mammalian oocyte enters meiosis, where it is arrested in the prophase of meiotic division I. After puberty resumption of meiosis occurs and is characterized by germinal vesicle breakdown (GVBD). The physiological mechanism, which keeps the oocyte in the state of meiotic arrest is not clear. O et al. (1989) postulated inhibin as an oocyte meiosis inhibitor, because it inhibits spontaneous GVBD in oocyte cultures as has been found for AMH (Takahashi et al., 1986). Activin has no effect, whereas follistatin stimulates GVBD. In contrast, Itoh et al. (1990) reported stimulation of GVBD by activin as has been found for TGF-ß. Activin-A induced stimulation of GVBD, which is blocked by follistatin, has also been reported by Sadatsuki et al. (1993).

1.7.4 Placenta

Inhibin α-, $ß_A$- and $ß_B$-subunit mRNAs have been detected in rat (Meunier et al., 1988a) and human (Petraglia et al., 1991a) placenta. In human decidua these subunits (Petraglia et al., 1990) and follistatin (Kaiser et al., 1990) are also expressed. Bioactive inhibin is present in extracts of human term placenta, with a bio- over immuno ratio different from that, found in human follicular fluid (McLachlan et al., 1986b). The involvement of inhibin in the regulation of placental function was

first suggested when the addition of an inhibin antiserum to primary cultures of human placental cells stimulated hCG production (Petraglia et al., 1987). As inhibin antiserum is effective in term but not in first trimester placenta cultures, inhibin is not regulating hCG secretion during the entire pregnancy (Mersol-Barg et al., 1990). On the other hand, activin stimulates hCG secretion from term placenta cells (Petraglia et al., 1989) and trophoblasts from first trimester human placenta (Steele et al., 1993). An effect of activin in the placenta is in accordance with the expression of ActRII and ActRIIB mRNAs found in rat placenta (Feng et al., 1993), the expression of ActRII in human first trimester and term placentae (Peng et al., 1993) and the expression of ActRI in human placenta (Attisano et al., 1993). Inhibin α- and $ß_A$-subunits are also expressed in human placental trophoblast derived chorion cells, whereas amnion cells, which originate from embryonic mesoderm, express preferentially the inhibin $ß_B$-subunit (Petraglia et al., 1993). In response to exogenous activin-A, prostaglandin E_2 release from cultured amnion cells increases (Petraglia et al., 1993), indicating a local role for activin in this tissue.

1.7.5 Adrenal gland, kidney, liver, pancreas

Inhibin α-, $ß_A$- and $ß_B$-subunit mRNA and protein have been detected in human fetal and adult **adrenal cortex** (Voutilainen et al., 1991; Spencer et al., 1992). In extracts of adult human adrenal glands and culture medium of human adrenal cells, immunoreactive inhibin α-subunit is present (Haji et al., 1991). Inhibin α- and $ß_A$-subunit mRNAs are also located in the rat adrenal gland (Meunier et al., 1988a), and inhibin α-subunit protein is demonstrated to be present the rat adrenal cortex (Merchenthaler et al., 1991). Finally, in the ovine adrenal cortex inhibin α-subunit mRNA (Crawford et al., 1987) and protein (Veeramachaneni et al., 1989) have been found.

In vivo administration of ACTH increases inhibin α-subunit mRNA in ovine (Crawford et al., 1987) and inhibin α-subunit protein in rat adrenal glands (Merchenthaler et al., 1991). ACTH also stimulates inhibin α-subunit in cultures of human adrenal cells (Haji et al., 1991; Voutilainen et al., 1991; Spencer et al., 1992), whereas epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-ß and activin-A have no effect (Spencer et al., 1992). The culture of human adrenal cells in medium containing 10% fetal calf serum induces inhibin $ß_A$-subunit mRNA expression, which is further increased by phorbol ester (Voutilainen et al., 1991).

Activin-A inhibits basal, EGF-stimulated and TGF-ß-inhibited proliferation of human fetal adrenal cells in culture, but has no effect on FGF-stimulated proliferation (Spencer et al., 1990). This effect is selective for the fetal zone adrenal cells; no effect has been found on proliferation of fetal definitive zone adrenal cells or adult adrenal cells (Spencer et al., 1992). Additionally, activin-A increases ACTH-stimulated cortisol secretion only from fetal zone cells. In contrast, inhibin-A has no mitogenic effect on basal, stimulated or inhibited proliferation of any of the adrenal cell cultures nor does it affect steroidogenesis. Based upon these results it is speculated that activin-A, in the fetal zone of the human adrenal, permits a shift from proliferation to differentiation, necessary to remodel this zone postnatally. In contrast to human adrenal cells, in cultured bovine adrenocortical cells activin-A reduces ACTH-stimulated cortisol and dehydroepiandrosterone secretion (Nishi et al., 1992). No effect on aldosterone secretion has been found, suggesting an inhibition of 17-hydroxylase activity. Activin-A does not affect cholesterol substrate

availability, since low density lipoprotein metabolism in bovine adrenocortical cells is not changed by activin-A (Hotta and Baird, 1987).

Besides the inhibin subunits, follistatin mRNA (Michel et al., 1990) and protein (Kogawa et al., 1991b) are present in the rat adrenal gland. Although effects of activin in the adrenal gland have been described, the presence of activin receptors have not been reported yet. Expression of inhibin subunits and follistatin in the adrenal gland and the reported effects of activin-A indicate a possible paracrine interaction between these factors. Disturbance of this interaction can have severe consequences as shown in knock-out mice, lacking inhibin α-subunit expression. These mice develop adrenal tumors (Matzuk, personal communication). In agreement with this finding is the absence of inhibin α-subunit immunoreactivity in human adrenal tumors (de Jong et al., 1990b).

In the rat **kidney** ActRII (Mathews and Vale, 1991) and ActRI (Attisano et al., 1993) mRNAs are expressed. Nevertheless, no reports concerning effects of activin or inhibin on renal function have been published yet. Besides activin receptor mRNAs, the presence of inhibin α-subunit (Meunier et al., 1988a; Veeramachaneni et al., 1989) and follistatin (Michel et al., 1990; Kogawa et al., 1991b) mRNAs and protein within the kidney have been reported.

In hepatocytes isolated from rat **liver**, activin-A stimulates glucogenolysis by an increase of phosphorylase activity (Mine et al., 1989). In this way, glucose output from hepatocytes is stimulated. Activin-A also increases glucagon stimulated glucose output, but has no effect on angiotensin-II stimulated glucose output. Others reported that activin induces cell death in hepatocytes *in vivo* and *in vitro*. This effect is completely blocked by addition of follistatin and partially blocked by inhibin (Schwall et al., 1993). These authors postulate that activin may be involved in normal hepatocyte turnover. Furthermore, Yasuda et al. (1993a) reported activin-A as an autocrine inhibitor of hepatocyte DNA synthesis. Although ActRII (Mathews and Vale, 1991) and ActRI (Attisano et al., 1993) mRNAs have been detected in rat liver, these effects of activin are probably not paracrine effects, since inhibin subunits (Meunier et al., 1988a) and follistatin (Michel et al., 1990) were not detected in rat liver. In accordance, no inhibin α-subunit immunoreactivity has been found in human liver tumors (de Jong et al., 1990b).

In isolated rat **pancreatic** islets, activin-A stimulates basal and glucose-induced secretion of insulin (Totsuka et al., 1988). ActRII mRNA has not been demonstrated in the pancreas (Mathews and Vale, 1991), whereas ActRIIB mRNA expression has not been examined yet. Nevertheless, ActRI mRNA has been found in the pancreas (Attisano et al., 1993). Meunier et al. (1988a) did not detect inhibin subunit mRNAs in the pancreas, but PCR analysis revealed the expression of inhibin α-, β_A- and β_B-subunit mRNAs within pancreatic islets (Ogawa et al., 1993). In another study (Yasuda et al., 1993b), only inhibin α- and β_A-subunit mRNA have been detected in these islets as well as activin-A protein. Activin-A is localized in secretory granules of pancreatic α-cells and δ-cells, together with glucagon and somatostatin, respectively. As found for pituitary cells, the sensitivity of perifused islets to exogenous activin-A is greater than in static cultures, probably because the latter cells are already stimulated by endogenous activin. Basal and activin-stimulated insulin secretion are not affected by inhibin-A (Yasuda et al., 1993b). It is possible that the effects of endogenous activin can be modulated by follistatin, since its mRNA is present in the pancreas (Michel et al., 1990; Ogawa et al., 1993).

Despite the presence of inhibin α-subunit in rat pancreatic islets, in human tumors of the pancreas, no inhibin α-subunit immunoreactivity has been found (de Jong et al., 1990b).

1.7.6 Hematopoiesis

Most mature cellular elements in the circulating blood are relatively short-lived and must be replaced constantly throughout live. The immense task of repletion of peripheral blood cells is carried out largely by the bone marrow, by a process called hematopoiesis (reviewed by Robinson and Quesenberry, 1990). A schematic overview of hematopoiesis is given in figure 8. The formation of blood elements is far more complex than the model would suggest, since the progenitor cells exist in a microenvironment consisting of stromal cells (fibroblasts, endothelial cells and macrophages). These stromal cells are essential for normal hematopoiesis; they secrete several hematopoietic regulatory factors. This was concluded from studies with anemic S1/S1d mice, which have hematopoietic progenitor cells that function normally when transplanted to normal mice but have defective stroma that will not support normal hematopoiesis. Accumulating evidence suggests that activin-A is one of the physiologically active hematopoietic factors, particularly of the erythroid lineage as outlined in table VI.

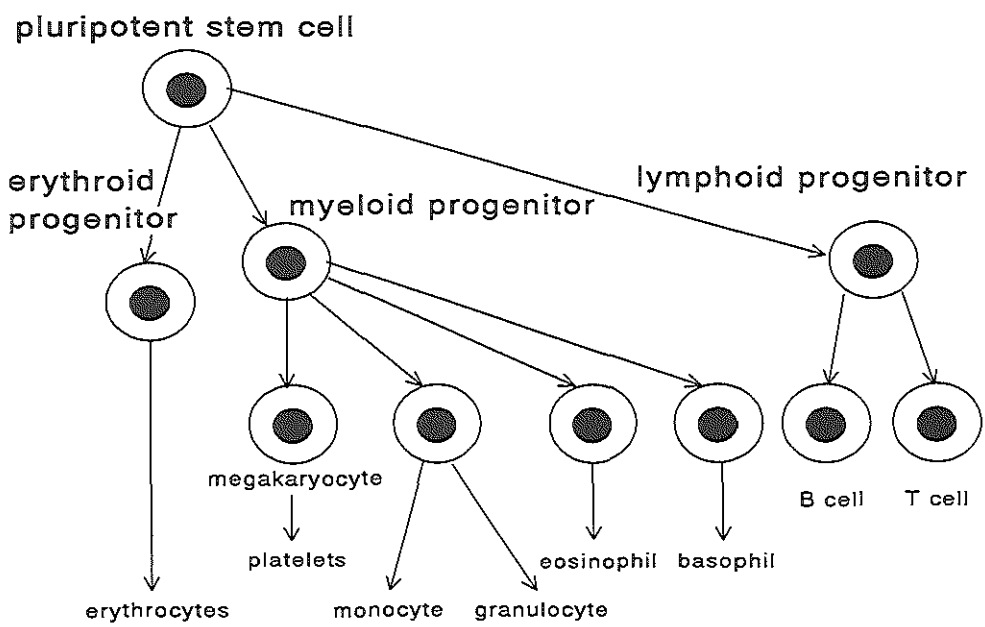

Fig.8. Overview of hematopoiesis. Hematopoietic growth factors stimulate progression of precursor cells toward different lineages.

In 1987 Eto et al. purified a protein from the culture medium of the phorbol ester-treated human monocytic leukemia cell line THP-1, that induced erythroid differentiation in murine Friend erythroleukemia cells, as evidenced by hemoglobin production and decreased colony formation. This 25 kDa protein, designated

erythroid differentiation factor (EDF), had an NH_2-terminal sequence identical to that of the inhibin β_A-subunit. Cloning of EDF cDNA revealed that EDF is completely identical to activin-A (Murata et al., 1988). The activin-A induced differentiation of Friend erythroleukemia cells is most effective at low cell density and is additive to the differentiation induced by hexamethylene bisacetamide (Yamashita et al., 1990). Compared to activin-A and activin-AB, activin-B is a less potent EDF for Friend erythroleukemia cells (Nakamura et al., 1992a; Uchiyama and Asashima, 1992). The erythroid inducing activity of activins on Friend cells is suppressed by follistatin, but not by inhibin-A or inhibin-B (Uchiyama and Asashima, 1992). An effect of activin on mouse erythroleukemia cells is not unexpected, because an activin binding site (Kd 1.5 x 10^{-10}M) on Friend erythroleukemia cells has been reported (Kondo et al., 1989). Hino et al. (1989) also reported an activin binding site (Kd 3.1 x 10^{-10}M) on these cells and demonstrated three labelled complexes (140, 76 and 64 kDa) after crosslinking of ^{125}I-activin to these cells. Furthermore, ActRIIB, but not ActRII mRNA is expressed in DS19 mouse erythroleukemia cells (Attisano et al., 1992).

Table VI. Effects of inhibin and activin on hematopoiesis

	Inhibin	Activin
General		
Erythropoietin-induced proliferation of pluripotent progenitors	=*	↑
Number of non-erythroid progenitors *in vivo*	↓	↑
Erythroid progenitors		
Differentiation of murine Friend erythroleukemia cells	=	↑
Differentiation of human K562 erythroleukemia cells	↓	↑
Erythropoietin-induced proliferation of erythroid progenitors	↓ =*	↑
Number of erythroid progenitors *in vivo*	↓	↑
Expression of globin mRNA	?	↑
Megakaryocytes		
Differentiation of murine megakaryoblastic cell line L8057	?	↑
Monocytes		
Differentiation of human promyelocytic cells into monocytes	?	↑
Chemotaxis	↑	=
γ-Interferon production	↓	=*
Interleukin 1,6 and TNF-α production	?	↑
T-lymphocytes		
PHA-stimulated [^{3}H]thymidine incorporation in rat thymocytes	↑	↓
PHA-stimulated [^{3}H]thymidine incorporation in T-lymphocytes	?	↓

*Reverses the effect of inhibin or activin

In the human erythroleukemia cell line K562, which contains activin binding sites (Kd 1 x 10^{-10}M, Campen and Vale, 1988a), activin-A also induces hemoglobin production (Yu et al., 1987). Since these cells are less sensitive for inhibin, differentiation of K562 cells can be used as a bioassay system for activin. Differentiation of these cells is accompanied by an increase in α, γ and ϵ globin transcription (Frigon et al., 1992). Additionally, activin-A potentiates the erythropoietin-stimulated proliferation of erythroid progenitor cells from human bone

marrow cultures (Yu et al., 1987; Broxmeyer et al., 1988), which is accompanied by a stimulation of α, ß and ϵ globin mRNA expression (Shao et al., 1992a). Inhibin suppresses the proliferation inducing effects of erythropoietin and activin-A, whereas inhibin and activin alone have no effect (Yu et al., 1987). In contrast, Broxmeyer et al. (1988) found that inhibin only inhibits the proliferating effect of activin. The effects of inhibin and activin are dependent on the presence of monocytes and T-lymphocytes (Broxmeyer et al., 1988), indicating that inhibin and activin effects might be mediated by these cells.

Inhibin $ß_A$-subunit mRNA but not inhibin α- or $ß_B$-subunit mRNA is present in bone marrow (Meunier et al., 1988a). This mRNA is translated, since activin bioactivity is detected in bone marrow extracts and conditioned media from primary bone marrow cultures (Shiozaki et al., 1992). The activin producing cell type within the bone marrow is probably the stromal cell; inhibin $ß_A$-subunit mRNA and activin immuno- and bioactivity have been found in cultures of normal human bone marrow stromal cells (Shao et al., 1992b) and human (Takahashi et al., 1992) and murine (Yamashita et al., 1992) bone marrow stromal cell lines. The expression of activin in bone marrow stromal cells is stimulated by phorbol ester, interleukin 1α (IL-1α), tumor necrosis factor α (TNF-α) and lipopolysaccharide (LPS), suggesting a compensatory mechanism for the suppressive effect of these inflammatory cytokines on erythropoiesis.

Activin can also be produced by monocytes activated by phorbol ester, LPS, granulocyte-macrophage colony-stimulating factor or γ-interferon (IFNγ) as it was demonstrated to be present in culture medium of the human monocytic leukemia cell line THP-1 (Eto et al., 1987), the human promyelocytic cell line HL-60 (Takahashi et al., 1990) and normal human monocytes (Erämaa et al., 1992; Shao et al., 1992b). Monocytes also can be activin target cells; HL-60 cells possess specific activin binding sites (Kd 3.4 x 10^{-10}M) and differentiate into monocyte/macrophage-like cells upon activin-A treatment (Yamada et al., 1992). This suggests that activin-A is not only an erythroid differentiation factor but also a myeloid cell differentiation factor. This is supported by the observation that activin not only acts on human bone marrow erythroid progenitor cells, but also on pluripotent progenitor cells. However, no effect on granulocyte-macrophage progenitor cells has been found (Broxmeyer et al., 1988). Furthermore, activin-A stimulates megakaryocyte differentiation (Fujimoto et al., 1991) and inhibits phytohaemagglutinin (PHA)-stimulated [^{3}H]thymidine incorporation in rat thymocytes (Hedger et al., 1989) and peripheral T-lymphocytes (Hedger and Clarke, 1993). This suggests an effect of activin on the formation of platelets and mature T-lymphocytes. In contrast, inhibin enhances PHA-stimulated [^{3}H]thymidine incorporation in rat thymocytes (Hedger et al., 1989).

Inhibin and activin may also influence the cell-mediated immune response, since inhibin stimulates human monocyte chemotaxis and decreases IFNγ production (Petraglia et al., 1991b). The latter effect is reversed by the concomitant addition of activin. In this study, no substantial effects of inhibin and activin have been observed on lymphocyte proliferation or cytotoxity, although the dose of activin employed was lower than in the studies of Hedger et al. (see above). In human peripheral blood mononuclear cell cultures activin-A induces IL-1, IL-6 and TNF-α secretion from monocytes (Yamashita et al., 1993). This increase in IL-6 secretion results in a stimulation of IL-4 induced IgE synthesis from B cells.

Modulation of hematopoiesis by inhibin and activin has also been demonstrated *in vivo*. Activin-A administration to anemic and normal mice increases the number of erythroid and non-erythroid progenitor cells (Shiozaki et al., 1989; Broxmeyer et al., 1991). Neutralization of endogenous activin by the infusion of follistatin decreases the number of erythroid progenitors in bone marrow and spleen (Shiozaki et al., 1992), confirming the participation of endogenous activin in mouse erythropoiesis. Furthermore, activin-A increases the concentration of red blood cells and hemoglobin in ovariectomized, estrogen treated immature rats, whereas no effect is observed in intact immature female rats (Schwall et al., 1989). This difference is explained by the high estradiol concentrations in the estrogen treated rats. Estradiol inhibits erythropoiesis and may allow the stimulating effect of activin to become apparent. Activin-A also induces the differentiation of murine erythroleukemia cells transplanted in mice (Shiozaki et al., 1990). In contrast, *in vivo* administration of inhibin significantly reduces the number of erythroid, pluripotent and granulocyte-macrophage progenitor cells in bone marrow and spleen of treated mice (Hangoc et al., 1992). As the inhibin α-subunit is expressed in spleen and not in bone marrow, and the inhibn β_A-subunit is expressed in both tissues (Meunier et al., 1988a), a role for inhibin in hematopoiesis in the murine spleen can be suggested.

1.7.7 Embryogenesis

1.7.7.1 *Xenopus* embryonic development

At the blastula stage of *Xenopus* embryogenesis, animal pole cells (ectoderm) at the equator of the embryo develop mesodermal tissue in response to signals from the vegetal pole (endoderm). A model for mesoderm induction is shown in figure 9. Several mesoderm inducing factors have been discovered, using isolated *Xenopus* animal caps from stage 8 embryos; among them activin-A (Asashima et al., 1990; Smith et al., 1990; van den Eijnden-van Raaij et al., 1990) and activin-B (Thomsen et al., 1990). Activin-A, activin-B and activin-AB can induce mesoderm with equal potency (Nakamura et al., 1992a; Fukui et al., 1993). Nevertheless, Slack (1991) found that the natural mesoderm inducing activity secreted by the vegetal pole is not blocked by follistatin, possibly indicating the redundancy in the factors inducing mesoderm.

Inhibin-A does not counteract the activin-A induced effects on animal caps, but rather seems to enhance them (Asashima et al., 1990). Inhibin-A alone does not induce specific morphological changes, although animal caps treated with inhibin-A are not quite the same as controls, in that some mesodermal derivatives (mesenchym and epidermis) are formed. This makes "the animal cap assay" a better bioassay than the usual pituitary assay for studing the presence of activin in biological fluids, in which inhibin is also present. This is demonstrated in chapters 3, 4 and 5.

In the animal cap assay, activin-A can induce the formation of different cell types in narrow dose ranges; low doses giving ventro-lateral structures and higher doses eliciting more dorso-anterior structures (Green and Smith, 1990; Ariizumi et al., 1991; Green et al., 1992). This defines activin as a "morphogen". Yet, the activin induced mesoderm formation is more complex; the prospective dorsal and ventral regions of the animal pole respond differently to the same concentration of

activin (Sokol and Melton, 1991). Dorsal regions develop a rounded head-like appearance with eyes, containing neural tissue, muscle and notochord (dorso-anterior structures), whereas the ventral regions develop epidermis, muscle and mesenchyme (ventro-lateral structures). This shows that dorsal and ventral regions of the animal pole are programmed to develop along different pathways. This programming takes place at early blastula stage (see below).

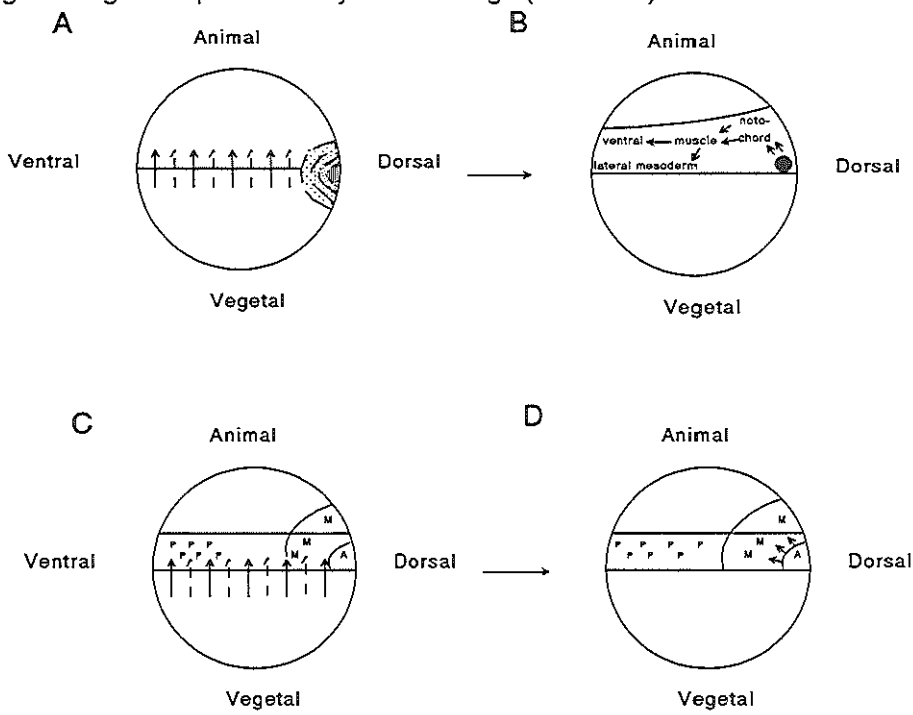

Fig.9. Model for mesoderm induction in Xenopus embryos (after Kimelman et al., 1992). ***Dorsal ventral patterning. A.*** *After fertilization, sperm mediated rotation of the cortical region of the egg activates Wnt-like activity (a factor with the same activity as Wnt-1, Xwnt-8 or noggin) with maximum activity at the dorsal midline of the equatorial region. Since at this time no zygotic transcription takes place and noggin is maternally expressed (Smith and Harland, 1992), whereas Xwnt-8 is not, noggin is the most likely candidate for this factor. This prepatterns the competence of the animal hemisphere to respond to mesoderm inducing signals; FGF and signal X (probably maternal activin), from the vegetal hemisphere such that cells on the dorsal side of the embryo form dorsal mesoderm. The combination of signal X with maximum Wnt-like activity could induce the formation of the blastula organizer.* ***B.*** *At midblastula the organizer transcribes activin-B, which induces the region above to become notochord (gastrula stage organizer). This region releases a signal, possibly activin B, which converts adjacent equatorial cells into muscles. These prospective muscle cells may also be able to release activin B, albeit at a lower level, converting their ventral neighbours to more lateral mesoderm.* ***Anterior-posterior patterning. C.*** *The same factors that produce the dorsal-ventral axis are proposed to establish the anterior-posterior axis. FGF specifies the entire marginal zone to have a posterior fate (P). The Wnt-like activity, together with signal X, specifies anterior fate (A) in the late blastula organizer. Lower amounts of Wnt-like activity in combination with FGF and signal X, might specify mid-axis positions (M).* ***D.*** *Activin-B from the late blastula organizer could instruct neigbouring regions to adopt a mid-axial fate. Although this seems a very strict model, it appears that the embryo uses overlapping and partially redundant systems for patterning the embryo.*

The mesoderm induction *in vivo* seems to be accomplished by activin-B rather than by activin-A, because inhibin $ß_B$-subunit mRNA is first detected in *Xenopus* blastulas, whereas inhibin $ß_A$-subunit transcripts appear in the late gastrula stage (Thomson et al., 1990). Activin bioactivity has been found in extracts of *Xenopus* blastulae (stage 9) and unfertilized eggs and is suppressed by follistatin (Asashima et al., 1991b). This suggests that activin is also of maternal origin, probably from follicle cells surrounding the oocyte, since expression of inhibin $ß_A$- and $ß_B$-subunit mRNA is found in these cells but not in the oocyte itself nor in the early blastula stage (Dohrmann et al., 1993). Inhibin $ß_B$-subunit mRNA is first detected in stage 8 embryos and is uniformly expressed along the animal-vegetal axis. It is also expressed uniformly along the dorso-ventral axis of stage 10 embryos. At neurula stage (stage 16) expression is mostly restricted to the dorsal site of the embryo (Dohrmann et al., 1993). Injection of activin-B mRNA into the ventral blastomere of a 32 cell embryo results in the formation of a partial second body axis (Thomsen et al., 1990; Sokol et al., 1991; Steinbeisser et al., 1993), containing notochord, muscle and melanocytes, but lacking anterior structures such as eyes. This suggests that activin alone is not sufficient to account for all properties of an endogenous dorso-anterior inducer, as has been confirmed by Bolce et al. (1993) using animal caps. Ectopic injection of activin-B mRNA also increases gap junctional permeability in 32-cell embryos, indicating that activin stimulates cell-cell communication in the early *Xenopus* embryo (Olson and Moon, 1992).

In contrast to injections of activin, injection of *Wnt* oncogene mRNA (Sokol et al., 1991; Steinbeisser et al., 1993) or *noggin* mRNA (Smith and Harland, 1992) results in a complete second body axis. Both molecules have no inherent mesoderm inducing activity but alter activin responses (Christian et al., 1992; Smith and Harland, 1992). Cells from ventral blastomere injected with *Wnt* mRNA differentiate in dorso-anterior structures including notochord and eyes upon activin-A treatment. This response is similar to the response of untreated dorsal ectoderm to activin (Sokol and Melton, 1992). Furthermore, soluble *noggin* protein added to the ventral region of the embryo results in dorsal structures (Smith et al., 1993). Therefore, *Wnt* and *noggin* are candidate factors for programming the ectoderm.

To explain activin effects, activin receptors should be expressed in the embryo. Both ActRII (Kondo et al., 1991; Nishimatsu et al., 1992a) and ActRIIB (Hemmati-Brivanlou et al., 1992; Mathews et al., 1992; Nishimatsu et al., 1992a) mRNAs have been found in unfertilized eggs. Activin receptor expression is present throughout embryogenesis, suggesting that activin plays a role in *Xenopus* embryogensis. Whole mount in situ hybridization demonstrated that ActRIIB mRNA expression is distributed uniformly in early stage embryos and becomes restricted to the central nervous sytem later on (Hemmati-Brivanlou et al., 1992).

Overexpression of ActRII by injection of ActRII mRNA into uncleaved fertilized *Xenopus* eggs generates embryos with reduced trunk structure. After injection of ActRII mRNA into the ventral blastomeres of the 16-cell stage, embryos with a secondary body axis are formed (Kondo et al., 1991), comparable with results after injection of activin-B mRNA in the ventral blastomere of early *Xenopus* embryos (Thomson et al., 1990). This shows that, in *Xenopus*, activin is important for the determination of the body axis.

Expression of a truncated ActRIIB, that contains the entire extracellular and

transmembrane domain but lacks nearly all of the cytoplasmic domain, in *Xenopus* embryos acts as a dominant negative activin receptor (Hemmati-Brivanlou and Melton, 1992). It blocks activin induced mesoderm formation in animal caps, and enhances the induction of muscle actin by basic fibroblast growth factor (bFGF), without further effect on bFGF-induced mesoderm formation. Interestingly, by blocking activin signal transduction with a truncated ActRIIB, the expression of the neural cell adhesion molecule NCAM, a neural specific marker, is enhanced. This indicates that normally neural differentiation is inhibited by activin. It has been demonstrated that activin can inhibit the retinoic acid induced differentiation of murine P19 embryonal carcinoma (EC) cells to neurons and glial cells (Hashimoto et al., 1990; van den Eijnden-van Raaij et al., 1991). A similar effect has been found in neuroblastoma cells (Hashimoto et al., 1990). This suggests that normally neural induction is possible if accompanied by an endogenous activity that blocks activin signalling through ActRIIB, e.g. follistatin or inhibin. As a matter of fact, follistatin is expressed in *Xenopus* embryos from gastrula stage onwards (Tashiro et al., 1991). Furthermore, follistatin is able to inhibit the mesoderm inducing activity of activin-A (Asashima et al., 1991a) and stimulates differentiation of the neural cell line IMR-32 (Hashimoto et al., 1992). Finally, retinoic acid induced differentiation of P19 EC cells to neural cells is accompanied by an increase in follistatin mRNA expression (van den Eijnden-van Raaij et al., 1992). These results indicate that follistatin can be involved in neural induction.

Embryos injected with the truncated ActRIIB are markedly deficient in mesodermal and axial development. In the most extreme cases there is no sign of body axis formation. The embryos appear to have retained an animal-vegetal axis, but do not have an anterior-posterior or dorsal-ventral body plan (Hemmati-Brivanlou and Melton, 1992). These results establish activin as a principal determinant of mesoderm induction and body axis formation.

Overexpression of ActRIIB in *Xenopus* embryos results, like overexpression of ActRII or activin-B, in dramatically abnormal development with perturbed axial structures; formation of ectopic dorsal mesodermal derivatives in the ventral regions of the embryo, truncation of the embryonic axis, tail and trunk protrusions and sometimes duplicated head structures (Hemmati-Brivanlou et al., 1992; Mathews et al., 1992). The same effect has been found with a C-terminally truncated ActRIIB, missing kinase domain VIII-XI (amino acids 365 to 510) (Nishimatsu et al., 1992b).

Based upon these and other results Kimelman et al. (1992) proposed a model for the early embryonic determination of dorsal-ventral and anterior-posterior body axes, which is depicted in figure 9. This model probably needs revision in the near future, as has been demonstrated recently. Two authors reported that another member of the TGF-ß family, DVR-4 (BMP-4), can induce ventral/posterior mesoderm, like FGF does. Furthermore, it can override the dorsalizing effects of activin in contrast to FGF (Dale et al., 1992; Jones et al., 1992b).

Another indication for the importance of activin in early *Xenopus* development comes from the observation that activin can induce several transcription factors in the dorsal organizer region of the embryo; homeobox containing genes: *goosecoid* (Cho et al., 1991), *Xlim* (Taira et al., 1992) and *Xnot* (von Dassow et al., 1993) and forkhead forkhead containing genes: *XFKH1* (Dirksen and Jamich, 1992) and *Pintallavis* (Ruiz i Altaba and Jessell, 1992). Fibroblast growth factor can only induce the expression of *Xnot*. Microinjection of *goosecoid*

mRNA into the ventral side of *Xenopus* embryos, from which it is normally absent leads to the formation of a second body axis (Cho et al., 1991).

1.7.7.2 Chicken embryonic development

Like in *Xenopus* embryogenesis, activin seems to play a role in the development of avian embryos. In chicken, activin can induce the formation of axial structures (full-lenght notochord, segmented somites and neural tube) when added to isolated epiblast from blastulas. Furthermore, inhibin $ß_B$-subunit mRNA is transcribed in the hypoblast, where the mesoderm inducing factor comes from, when axial mesoderm is induced in the epiblast (Mitrani et al., 1990). Cultured endoderm from stage 5 to 8 chicken embryos secretes activin-A (Kokan-Moore et al., 1991). Moreover, application of activin in a localized manner at 90° from the posterior end of the chicken central disk generates ectopic body axes, including notochord, segmental somites and a rudimentary head (Ziv et al., 1992).

Later on in chicken embryonic development activin may play a role in chondrogenesis of limb bud cells. However, results are conflicting, possibly due to differences in cell preparation (Chen et al., 1993; Jiang et al., 1993). Limb bud cell cultures from the distal one-third of the limb bud of stage 22-23 chicken embryos respond to activin treatment with increasing Alcian blue, NCAM and tenascin staining, indicating a stimulation of chondrogenesis (Jiang et al., 1993). In limb bud cell cultures from the entire limb bud of stage 24 embryos a decreased Alcian blue staining, decreased incorporation of [^{35}S]sulfate into proteoglycans and a decreased expression of the type II collagen gene have been found upon activin treatment, pointing to an inhibition of chondrogenesis (Chen et al., 1993). In the latter study, inhibin has a stimulatory effect on chondrogenesis. Effects of activin on chondrogenesis are in agreement with the expression of the inhibin $ß_A$-subunit mRNA in the developing murine skeleton (Roberts et al., 1991) and the expression of activin receptor type II mRNA in mesenchymal and ectodermal cells of the developing chicken limb buds at stage 20-21 and in the proximoventral and proximodorsal regions of limb buds at stage 23 (Ohuchi et al., 1992). Moreover, inhibin $ß_A$-subunit immunoreactivity has been detected in embryonic chicken chondrocytes, whereas inhibin α-subunit immunoreactivity was found in skeletal muscle myoblasts within the limb bud (Bläuer et al., 1992). Inhibin α-subunit immunoreactivity is also located in smooth muscle cells surrounding the gut, cardiac muscle, mesonephros and neural tube (Bläuer et al., 1992). In chicken embryos ActRII mRNA has been found in neuro-ectoderm developing to spinal cord, brain and eyes, in surface ectoderm differentiating to epidermis, and in myotomes differentiating to muscles (Ohuchi et al., 1992).

1.7.7.3 Mammalian embryonic development

In the past few years it appeared that activin can also play an important role in mammalian embryonic development. Treatment of early mouse embryos with activin results in accumulation of mRNA for the mesodermal marker *goosecoid* in the entire epiblast (Blum et al., 1992). In the early mouse embryo inhibin $ß_A$- and $ß_B$-subunit immunoreactivity is detected in fertilized eggs, 2-and 4-cell embryos, morula and compact morula (Albano et al., 1993). When, during blastocyst formation, the outer cell layer of the morula begins to differentiate as trophectoderm, ß-subunit immunoreactivity is present in the inner cell mass cells. At

4.5 days of development, the blastocyst begins to implant and immunoreactivity is localized to the trophectoderm. At these embryonic stages no inhibin α-subunit immunoreactivity is detected, so it is likely that activin is formed (Albano et al., 1993). In situ hybridization on sections of morulae and blastocysts has not detected transcripts for the inhibin α, $ß_A$ and $ß_B$ subunit (Albano et al., 1993; Manova et al. 1992). Using the more sensitive polymerase chain reaction these transcipts can be detected in unfertized eggs but not in 2-cell stage embryos (Albano et al, 1993). Only $ß_B$ transcripts have been detected in blastocysts, whereas in morula stage embryos both $ß_A$ and $ß_B$ mRNAs are detectable. The expression of $ß_B$ transcripts in the absence of $ß_A$ transcripts in 3.5 day mouse blastocytst is confirmed by van den Eijnden-van Raaij et al. (1992), who also detected ActRII and α-subunit mRNA but no follistatin transcripts at this stage. All these transcripts are expressed in 7.5-8 day and 10.5 day embryos. Besides the intra-embryonic expression of inhibin ß-subunits and follistatin, a high expression of follistatin mRNA (Kaiser et al., 1990) and $ß_A$ mRNA but not of $ß_B$ and α mRNA (Manova et al., 1992) has been found during early pregnancy in the decidua at the implantation site. Diffusion of activin and follistatin from the decidua into the embryo has been suggested.

Later in mammalian embryonic development activins may also play a role. In rat embryos from day 12 post coitum until birth, expression of inhibin $ß_A$-subunit mRNA is found in a widespread number of tissues, including heart, skeleton, brain and gonads (Roberts et al., 1991). Expression of inhibin $ß_B$-subunit mRNA is resticted to gonads, brain and salivary gland, whereas inhibin α-subunit is only detected in the gonads. It has been shown that, like in chicken, activin-A can regulate bone cell activity during rat development. It stimulates both the proliferation of osteoblast-enriched cultures from fetal-rat parietal bone and the synthesis of collagen by these cells (Centrella et al., 1991). These effects are opposed by parathyroid hormone. Furthermore, activin-A has been purified from bovine bone (Ogawa et al., 1992). Subcutaneous implantation of this purified activin-A in the ventral thoracic region of rats, promotes bone induction by bone morphogenic protein, albeit activin alone has no effect. As activin stimulates the formation of osteoclasts in mouse bone marrow cell cultures (Sakai et al., 1993), activin also seems to regulate bone resorption.

The inner cell mass (ICM) of the mammalian embryonic blastocyts contains pluripotent stem cells for all somatic cells and germ cells. They can be transformed into teratocarcinoma cells by extra-uterine transplantation (Diwan and Stevens, 1976) and also converted into cell lines by direct *in vitro* culture (Evans and Kaufman, 1981), resulting in embryonal carcinoma (EC) cells and embryonic stem (ES) cells, respectively. ES and EC cells provide an accesible *in vitro* model for the early stages of mammalian embryonic development.

In serum-free medium on surfaces of tissue culture plastic, P19 mouse EC cells need activin-A to survive (Schubert et al., 1990; Schubert and Kimura et al., 1991). In the presence of fetal calf serum (Hashimoto et al., 1990), bovine serum albumin (Atsumi et al., 1993) or on substrata of extracellular matrix proteins (Schubert and Kimura, 1991) activin induces growth of these cells. Activin dependent survival and growth has also been demonstrated for PCC3 EC cells, whereas growth of AT805, F9 and PSA-1 EC cells and ES is independent of activin-A (Atsumi et al., 1993).

Table VII. Expression of mRNAs for inhibin subunits, activin receptors and follistatin in undifferentiated and differentiated ES and EC cells.

	α	β_A	β_B	ActRII	ActRIIB	Fol	Ref
ES (8 different cell lines)	-	+	++	nd	nd	nd	1
ES-5							
undif	5.2; 3.2	-	++	++	nd	++	2
-LIF	↑ 5.2	-	↓↓	=	nd	↑	2
+RA	↑ 5.2; 3.2	-	=	=	nd	↑↑	2
P19							
undif	5.2	-	+	++	nd	++	2,3
+RA	↑ 5.2; 3.2; 1.6	-	↓	=	nd	↓↓	2,3
+DMSO/END2	nd	-	-	=	nd	↑	2
MES-1	5.2; 1.6	++	++	+	nd	++	2
EPI-7	5.2; 3.2; 1.6	++	++	+	nd	+	2
END-2	5.2	+	+	+	nd	++	2
F9							
undif	+	-	++	+	+	nd	1,4
vis	↑	-	↓	↑	↑	nd	1,4
par	↓	-	↓	nd	nd	nd	1
TERA-2							
undif	+	-	+	+	+	++	5
+RA	↓	-	↓	=	↓↓	↓↓	5

*The inhibin subunits (α, β_A and β_B), activin receptor type II (ActRII) and type IIB (ActRIIB) and follistatin (Fol) mRNA transcripts have been detected with Northern blot hybridization on total RNA or poly(A)$^+$ RNA. Normal (1.6 kb) as well as abnormal (5.2 and 3.2 kb) transcripts for the inhibin α-subunit have been detected where indicated. The presence (+) or absence (-) of transcripts is indicated. The changes in expression (↑, ↓, or =) induced by differentiation are also indicated. nd = not determined. The references (Ref) for these experiments are: **1.** Albano et al., 1993; **2.** van den Eijnden et al, 1992; **3.** Hashimoto et al., 1992; **4.** Wu et al., 1993a+b; **5.** de Jong et al., 1993. ES and EC cells cultured in the presence of leukemia inhibiting factor (LIF) remain undifferentiated (undif). Upon removal of LIF, ES cells differentiate into cells of a mesenchymal type (ES-5 -LIF), while addition of retinoic acid (RA) results in parietal endoderm-like cells (ES-5 +RA). Addition of RA to cultures of P19 EC cells in monolayer culture induces differentiation to a mixed population of fibroblast-like cells with endodermal and mesodermal characteristics (P19 +RA). Aggregation of P19 EC cells in the presence of dimethyl sulfoxide (DMSO) or a factor secreted by the visceral endoderm-like cell line END-2 results in the formation of endodermal cells and mesoderm-derived muscle tissue (P19 DMSO/END2). MES-1 is a mesodermal cell line from DMSO-treated embroid bodies. EPI-7 is a neuroepithelial derivative from RA treated embryoids. END-1 is a visceral endoderm derivative from P19 EC cells treated as aggregates with RA. Aggregation of F9 EC cells in the presence of RA results in visceral endoderm (F9 vis), whereas F9 EC cells in monolayer culture treated with RA differentiate into parietal endoderm (F9 par). TERA-2 EC cells differentiate into various celtypes.*

Effects of activin-A on P19 EC cells are in agreement with the identification of two activin binding sites (Kd 3-4 x 10^{-10}M and 1.5-3.0 x 10^{-9}M) on these cells. The same sites have been detected on PCC3 EC cells, whereas only one activin binding site (Kd 1.5 x 10^{-9}M) is present on F9 EC cells (Kondo et al., 1989). One of

these binding sites will probably represent ActRII, because mRNA encoding this receptor (6 and 3 kb) is expressed in P19 EC cells and ES cells (Hashimoto et al., 1992; van den Eijnden-van Raaij et al., 1992). Induction of differentiation in ES and P19 EC cells both in monolayer and during aggregation has no effect on ActRII mRNA expression. A similar result has been obtained with the human terato-carcinoma cell line TERA-2 (de Jong et al., 1993). In contrast, the expression of ActRIIB is strongly suppressed upon retinoic acid induced differentiation of TERA-2 cells (de Jong et al., 1993). This is different from to the murine teratocarcinoma cell line F9, where an increase in both ActRII (Wu et al., 1993a) and ActRIIB (Wu et al., 1993b) mRNA expression has been found after retinoic acid treatment. The expression of mRNAs for activin receptors, inhibin subunits and follistatin in ES and EC cells as well as the effect of differentiation on their expression is summarized in table VII. The different expression patterns upon differentiation of ES and EC cells indicate possible cell type or stage specificity.

All these results indicate that the molecules necessary for activin signal transduction and signal modulation (inhibin ß-subunits, activin receptors and follistatin) are present in amphibian, avian and mammalian embryos. The functioning of the activin signalling machinery is demonstrated by the effects induced by activin treatment. Therefore, activin(s) may play an important role in embryonic development, although they certainly are not the only factor involved.

1.8 Post-receptor events

Although the cloning of activin and TGFß receptors has shed some light on the second messenger mechanism used to translate the extracellular activin signal into a cellular response, the intracellular cascade is still a black box. Nevertheless, some literature on post-receptor events is available now.

The activin induced differentiation of Friend erythroleukemia cells is accompanied by hydrolysis of polyphosphoinositides and in consequence a rapid and transient increase of cytoplasmic free calcium (Shibata et al., 1987). A similar effect has been found in rat hepatocytes (Mine et al., 1989) and pancreatic islets (Verspohl et al., 1993). An increase of cytoplasmic free calcium concentration in response to activin-A was also described for pituitary somatotrophs (Tasaka et al., 1992). Although ActRII and ActRIIB are not directly connected to the phospholipase C dependent second messenger pathway, these results suggest an interaction with this second messenger system. It has been suggested that in the hepatocyte a pertussis toxin sensitive G protein is involved in this interaction (Mine at al., 1992). Nevertheless, the effect of activin on pituitary FSH release seems independent of protein kinase C (PKC), since activin-A can still stimulate FSH release after PKC is down-regulated by phorbol ester treatment (Katayama and Conn, 1993). It is possible that voltage-dependent Ca^{2+} channels are involved as has been suggested for the effects of activin in the rat pancreas (Shibata et al., 1993).

Modulation of the adenylate cyclase second messenger pathway by activin has also been described. In human granulosa cells (Li et al., 1992) and rat Leydig cells (Lin et al., 1989) activin-A decreases hCG-induced stimulation of intracellular cAMP production in the presence of a phosphodiesterase inhibitor. In contrast, FSH- and forskolin-stimulated cAMP production is potentiated by activin-A in rat granulosa cells (LaPolt et al., 1990; Xiao and Findlay, 1991), whereas no effect on

basal cAMP production is found (LaPolt et al., 1990; Nakamura et al., 1993).

The *Raf-1* serine/threonine protein kinase is not involved in the activin induced mesoderm induction in *Xenopus*, as concluded from experiments with a dominant negative *Raf-1* mutant (NAF). Animal caps from NAF injected embryos do not respond with muscle formation as controls do on treatment with bFGF, whereas they react normally to activin treatment (MacNicol et al., 1993). Therefore, *Raf-1* kinase seems not an obligate part of activin receptor signalling. In contrast, the proto-oncogen *Ras*, which has been suggested to act upstream of *Raf-1* in signal transduction, seems to be involved in both activin and FGF induced mesoderm formation, as a dominant negative *Ras* mutant can block elongation of animal caps upon activin or bFGF treatment (Whitman and Melton, 1992). Similarly, *Ras* is involved in growth inhibition of Mv1Lu cells by TGF-ß1 (Howe et al., 1993). Probably, TGF-ß1 interrupts cellular events leading to activation of *Ras*, resulting in growth inhibtion.

Stimulation of hemoglobin production in and differentiation of the erythroleukemia cell line K562 is preceded by a temporal inhibition of progression from G1 to S phase (Sehy et al., 1992) and an underphosphorylation of the retinoblastoma gene product (Rb). This hypophosphorylated form of Rb is involved in growth suppression, keeping cells in the G1 phase (DeCaprio et al., 1989). In Mv1Lu lung epithelial cells Rb is also retained in a hypophosphorylated state after addition of TGF-ß, resulting in inhibition of proliferation of these cells (Laiho et al., 1990). TGF-ß inhibits the formation of a stable assembly between cyclin E and cyclin dependent kinase 2 (Cdk2), resulting in the absence of cyclin E-associated kinase activity that normally accumulates in the G1 phase to phosphorylate Rb (Koff et al., 1993). This effect is probably mediated by the TGF-ß type II receptor (Chen et al., 1993), and might partially result from the TGF-ß induced inhibition of Cdk4 synthesis (Ewen et al., 1993). This cyclin dependent kinase is activated after interaction with D-type cyclins, which are synthesized prior to cyclin E. Inhibition of Cdk4 synthesis prevents Cdk2 from being activated. Moreover, TGF-ß inhibits cyclin E and cyclin A expression (Geng and Weinberger, 1993). The activin-A induced inhibition of exit from G1 phase to S phase in erythroid cells is consistent with the observed inhibition of cell growth in 3T3 fibroblasts (Kojima et al., 1989 and 1993a), gonadal cell lines (Gonzáles-Manchón and Vale, 1989), rat thymocytes (Hedger et al., 1989), GH4 cells (Ramsdell, 1991) and vascular endothelial cells (McCarthy and Bicknell, 1993). It remains to be demonstrated whether the inhibitory effect of activin on cell growth occurs via a pathway identical to that of TGF-ß. In contrast, activin-A stimulates vascular smooth muscle cell growth, probably by a stimulation of the autocrine production of IGF-1 (Kojima et al., 1993b).

The inhibition of GH biosynthesis and secretion is mediated by inhibition of the binding of the transcription factor Pit-1/GHF-1 to the GH promotor (Struthers et al., 1992). This is the result of an increased degradation of Pit-1 protein, probably by an increase of Pit-1 phosphorylation, which destabilizes the protein (Gaddy-Kurten and Vale, 1993). Effects of activin on other transcription factors e.g. *goosecoid* have also been described (see **section 1.7.7.1**).

These results indicate that activin can have its effects on several intracellular events. Nevertheless, it should be kept in mind that most of the effects described here have been found after prolonged (>1 hour) exposure to activin and could therefore be secondary effects.

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Summary

cDNA encoding the extracellular domain of the rat activin receptor was cloned using the polymerase chain reaction (PCR). This cDNA is highly homologous to cDNA encoding the extracellular domain of the mouse activin receptor, whereas at the protein level the extracellular domains of both receptors are identical. Employing this cDNA as a probe in Northern blot analysis, expression of two activin receptor mRNAs (6 kb and 4 kb) was observed, in testes of immature and mature rats. Between day 21 and 28 of postnatal development, a large increase in testicular expression of the 4 kb mRNA was found, suggesting expression of this activin receptor mRNA in germ cells. The 4 kb mRNA was indeed present in isolated pachytene spermatocytes and round spermatids, but was absent in elongating spermatids. Sertoli cells obtained from immature and mature rats expressed both the 6 kb and 4 kb mRNAs, whereas the expression of these mRNAs in Leydig cell preparations was very low. These results may imply that activin has multiple actions in the control of testicular function.

Introduction

Activins, dimers of the β-subunits of inhibin (βA or βB), were originaly isolated on basis of their ability to stimulate the release and synthesis of FSH by pituitary cells in culture (Ling et al., 1986; Vale et al., 1986). They belong to a family of structurally related proteins which includes TGFβs, Müllerian inhibiting substance, bone morphogenetic proteins, the product of decapentaplegic gene complex of *Drosophila* and Vg1 gene product of *Xenopus* (reviewed by de Jong, 1988). It is becoming more and more clear that activins can have TGFβ-like actions. Activin A induces, for instance, formation of mesoderm in *Xenopus* embryos (van den Eijnden et al., 1990) and is, like TGFβ, regarded as a cell differentation factor (Ueno et al., 1990). In the testis, activin may have a regulatory function on Leydig cell steroidogenesis (Hsueh et al., 1987; Lin et al., 1989; Mauduit et al., 1991) and spermatogenesis (Mather et al., 1990). Although a number of other biological actions were reported and binding of labelled activin to erythroleukemia cells, granulosa cells, pituitary tumor cells and embryonal carcinoma cells was demonstrated (reviewed by Ueno et al., 1990), no information was available about the structure of its receptor. However, recently Mathews and Vale (1991) described the

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cloning of cDNA encoding an activin receptor from AtT20 mouse corticotropic cells: a transmembrane serine/threonine-specific protein kinase.

In the present study, we cloned the cDNA encoding the extracellular domain of the homologous rat activin receptor and used this cDNA to study activin receptor mRNA expression in rat testis.

Materials and methods

Cloning of the cDNA encoding the extracellular domain of the rat activin receptor. All general molecular biology techniques were performed according to Sambrook et al. (1989). Based upon the recently published cDNA sequence of the mouse activin receptor (Mathews and Vale, 1991), we constructed two oligodeoxynucleotide primers (Fig. 1*A*). Both primers were changed at one nucleotide to construct a restriction site for further cloning. cDNA was synthesized from rat pituitary RNA using 4 μg total RNA, 100 ng of primer 1, 10 units of avian myeloblastosis virus reversed transcriptase (Promega, Leiden, Netherlands) and 10 units of RNase inhibitor (RNasin; Promega), according to the standard protocol obtained from the suppliers. The resulting cDNA was then amplified using the polymerase chain reaction (PCR) in 100 μl reaction mixture, containing 2% of the cDNA-synthesis reaction mixture, 400 ng of both primers and 2 units of *Thermus aquaticus* (Taq) DNA polymerase (Promega), as described by the manufacturer. Amplification was performed during 30 cycles; each cycle included denaturation for 1 min at 97°C, primer annealing for 2 min at 50°C and primer extension for 3 min at 72°C. The PCR product was inserted into the *Xba*I and *Sal*I sites of the phagemid PTZ19R (Mead et al., 1986) and sequenced by dideoxy-chain termination (Sanger et al., 1977). The *Xba*I-*Xho*I fragment was completely sequenced in two orientations, using T7 polymerase (Pharmacia, Uppsala, Sweden) with double-stranded phagemid DNA as a template.

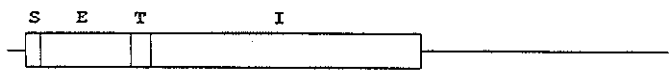
Cell preparations. An immature Leydig cell preparation, containing 30–50% Leydig cells and less then 1% Sertoli cells, was obtained from testes of 21-day-old Wistar rats as described earlier (Rommerts et al., 1985). Immediately after isolation the cells as well as the remaining tubular fraction were stored at −80°C until mRNA isolation. Highly purified immature Sertoli cells were obtained and cultured using the procedure described by Themmen et al. (1991).

Mature Leydig cells (more than 70% pure) were isolated from adult Wistar rat testes using a Ficoll centrifugation purification step (Rommerts et al., 1985). Part of the remaining tubular fraction was used to obtain a Sertoli cell enriched cell preparation, employing a method described by Oonk et al. (1985). The Leydig cell and Sertoli cell preparations and the tubular fraction were stored immediately at −80°C.

Pachytene spermatocytes and round and elongating spermatids from Wistar rats aged 32–36 days were isolated by velocity sedimentation at unit gravity (STA-PUT) as described by Grootegoed et al. (1982).

RNA isolation and Northern blot analysis. Total RNA was isolated from frozen tissue and cell preparations by LiCl/urea extraction, as described by Auffray and Rougeon (1980), followed by extraction with phenol and chloroform. Samples containing 20 μg RNA were separated by electrophoresis in a denaturing agarose gel (1% agarose; Sigma, St. Louis, MO, USA) containing ethidium bromide and blotted onto Hybond N^+ nylon membrane filters (Amersham, Amersham, UK) by diffusion. Filters were hybridized for 48 h at 42°C, with ^{32}P-labelled rat activin receptor cDNA in hybridization solution containing 50% formamide, 9% w/v dextran sulfate, 10 × Denhardt's (1 × Denhardt's contains 0.02% w/v Ficoll, 0.02% w/v polyvinyl pyrrolidone, 0.02% w/v BSA), 5 × SSC (1 × SSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 mM sodium phosphate (pH 6.8) and 100 μg/ml denaturated salmon sperm DNA. After hybridization, blots were washed to a final stringency of 0.1 × SSC/0.1% (w/v) SDS at 42°C and autoradiographed using Hyperfilm-MP (Amersham). The hybridization signals of activin receptor mRNAs were quantified using a BioRad-1D gel scanner (model 620, BioRad, Richmond, CA, USA).

A



PRIMER 1: 5′ > [611] TGGTCCTGGGTCTCGAGTAGGAACAAGTAC [581] < 3′

Xho I

PRIMER 2: 5′ > [19] CAGGGAACTGGATATCTAGAGAGAACTTCC [44] < 3′

Xba I

B

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          C
29  TCT AGA GAG AAC TTC CTA CGG CTT CTC CGG CGC CTC GGG AAA ATG GGA GCT GCT GCA AAG
                                                    Met Gly Ala Ala Ala Lys

                                                                          A
89  TTG GCG TTC GCC GTC TTT CTT ATC TCT TGC TCT TCA GGT GCT ATA CTT GGC AGA TCG GAA
    Leu Ala Phe Ala Val Phe Leu Ile Ser Cys Ser Ser Gly Ala Ile Leu Gly Arg Ser Glu

                                                                C
149 ACT CAG GAG TGT CTT TTC TTT AAT GCT AAT TGG GAA AGA GAC AGA ACC AAT CAG ACT GGT
    Thr Gln Glu Cys Leu Phe Phe Asn Ala Asn Trp Glu Arg Asp Arg Thr Asn Gln Thr Gly

        A                               T       G
209 GTT GAG CCT TGC TAT GGT GAT AAA GAC AAA CGA CGA CAT TGT TTT GCT ACC TGG AAG AAT
    Val Glu Pro Cys Tyr Gly Asp Lys Asp Lys Arg Arg His Cys Phe Ala Thr Trp Lys Asn

                                    G
269 ATT TCT GGT TCC ATT GAA ATA GTA AAG CAA GGT TGT TGG CTG GAT GAT ATC AAC TGC TAT
    Ile Ser Gly Ser Ile Glu Ile Val Lys Gln Gly Cys Trp Leu Asp Asp Ile Asn Cys Tyr

329 GAC AGG ACT GAT TGT ATA GAA AAA AAA GAC AGC CCT GAA GTG TAC TTT TGT TGC TGT GAG
    Asp Arg Thr Asp Cys Ile Glu Lys Lys Asp Ser Pro Glu Val Tyr Phe Cys Cys Cys Glu

                                                                          T
389 GGC AAT ATG TGT AAT GAA AAG TTC TCT TAT TTT CCG GAG ATG GAA GTC ACA CAG CCC ACA
    Gly Asn Met Cys Asn Glu Lys Phe Ser Tyr Phe Pro Glu Met Glu Val Thr Gln Pro Thr

                    A       G           T           T
449 TCA AAT CCT GTT ACC CCG AAA CCA CCC TAC TAC AAC ATC CTG CTG TAT TCC TTG GTA CCA
    Ser Asn Pro Val Thr Pro Lys Pro Pro Tyr Tyr Asn Ile Leu Leu Tyr Ser Leu Val Pro

      A               A   A                   A
509 CTT ATG TTA ATT GCG GGG ATT GTC ATT TGT GCG TTT TGG GTG TAC AGA CAT CAC AAG ATG
    Leu Met Leu Ile Ala Gly Ile Val Ile Cys Ala Phe Trp Val Tyr Arg His His Lys Met

                                        A
569 GCC TAC CCT CCT GTA CTT GTT CCT ACT CGA
    Ala Tyr Pro Pro Val Leu Val Pro Thr
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Fig. 1. Cloning of rat activin receptor cDNA. *A:* The cDNA sequence encoding the mouse activin receptor (Mathews and Vale, 1991) was slightly changed (asterisks) to obtain two oligodeoxynucleotide primers with the indicated restriction site. Primer 1 is located in the region encoding the intracellular domain of the receptor (I), just 3′ the region encoding the transmembrane domain (T). Primer 2 is located in the 5′ untranslated region of the mouse activin receptor cDNA. Both primers overlap the cDNA encoding signal peptide (S), extracellular domain (E) and transmembrane domain (T) of the receptor. Basepairs are numbered according to Mathews and Vale (1991). *B:* Sequence of cDNA encoding the extracellular domain of the rat activin receptor. Differences with the mouse activin receptor are indicated above the cDNA sequence. The protein encoded by this cDNA is indicated below the cDNA sequence. Primer sequence is indicated bold and underlined.

Results

Cloning of the cDNA encoding the extracellular domain of the rat activin receptor. The PCR reaction, using cDNA from rat pituitary RNA as a template, yielded a single cDNA fragment of approximately 600 bp (result not shown), which was cloned and sequenced. This cDNA showed 97% identity to basepairs 29 to 598 of the mouse activin receptor cDNA (Mathews and Vale, 1991) (Fig. 1*B*). At the predicted protein level, the mouse and rat sequences are identical. Northern blot analysis of total pituitary RNA using this cDNA as a probe showed the expression of a 6 kb mRNA (not shown).

Expression of activin receptor mRNA in testes from immature and mature rats. Northern blot analysis of total RNA from testes of immature and mature rats showed the expression of two activin receptor mRNAs (Fig. 2*A*), both hybridization signals appeared to be double bands. The expression of the smallest messenger (4 kb) was low in testes of young rats, increased between day 21 and 28 of postnatal life to high

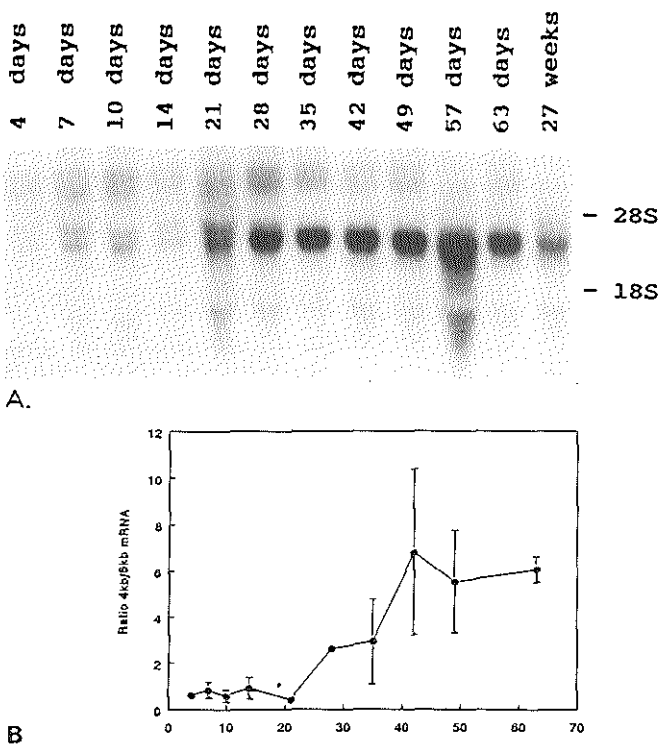

Fig. 2. Activin receptor mRNA expression in testes of rats of various ages. *A:* Northern blot analysis of the expression of activin receptor mRNAs in testes from immature and mature rats, using the cloned cDNA as a probe. The ages of the rats from which the testes were obtained are indicated at the top. The positions of ribosomal RNA bands (28S and 18S) are indicated on the right. *B:* Ratios between the testicular 4 kb and 6 kb activin receptor mRNAs in testis from rats of various ages. Values are expressed as means with standard deviation ($n = 3$).

levels in testes of older rats, but decreased slightly in testis of 27-week-old rats. The expression of the large messenger (6 kb) decreased with increasing age. This change was found in three separate Northern blots and is expressed as the ratio between the amounts of the 4 kb and the 6 kb messenger in Fig. 2*B*.

In testes of vitamin A-deficient adult rats, which have a deficient spermatogenesis, the expression of the 4 kb mRNA was decreased, compared to that in normal adult rat testis (Fig. 3).

Expression of activin receptor mRNA in different testicular cell types. The localization of the expression of the two activin receptor mRNAs in rat testes was studied by Northern blot analysis of mRNA obtained from preparations of different testicular cell types (Fig. 4). In preparations of Leydig cells from immature and mature rat testes, expression of small amounts of both messengers was detected. The mRNAs were also present in tubular preparations. In Sertoli cells, obtained from immature rat testes, a relatively high expression of both messengers was detected. This was not only demonstrated in Sertoli cels immediately after isolation, but also in Sertoli cells, cultured

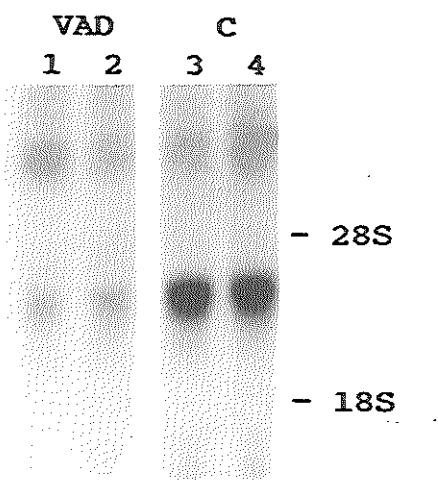

Fig. 3. Activin receptor mRNA expression in testes of vitamin A-deficient rats (lanes 1 and 2) and control rats (lanes 3 and 4).

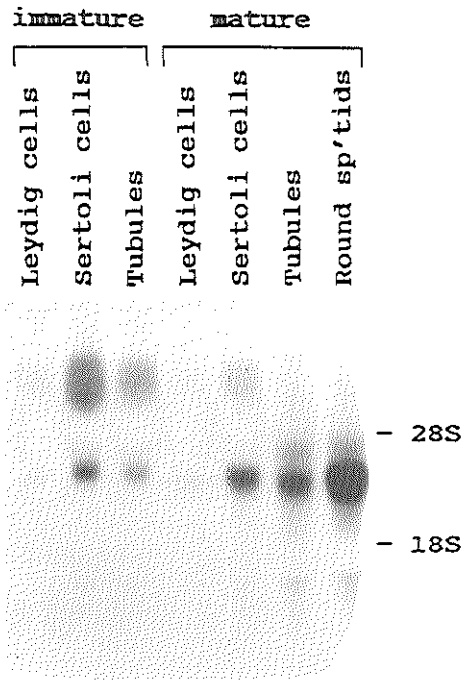

Fig. 4. Activin receptor mRNA expression in Leydig cell preparations, Sertoli cells and tubules obtained from immature and mature rats, and in round spermatids, immediately after isolation of the cells.

for several days and osmotically shocked to remove germ cells (result not shown). Total RNA from an adult Sertoli cell-enriched preparation also contained both activin receptor mRNAs; the 4 kb mRNA was most abundant, possibly because of contaminating germ cells. Finally, mid-/late-pachytene spermatocytes and round spermatids showed a very high expression of the 4 kb mRNA, whereas elongating spermatids did not express any of the activin receptor mRNAs (Fig. 5).

Discussion

The results of this study demonstrate that the cDNAs encoding the extracellular domain of the activin receptor of mouse and rat are higly ho-

mologous. At the protein level, the extracellular domains of mouse and rat activin receptor are identical. This is in agreement with the fact that mature porcine, human and rat activin βB-chains are very conserved, whereas the mature βA-chains are completely identical (Esch et al., 1987). The strict conservation of both receptor and ligand suggests the importance of activin as a physiological regulator.

Two transcripts of 4 kb and 6 kb were detected in total testis RNA, as was earlier reported by Mathews and Vale (1991) for the mouse testis. The sharp increase in relative expression of the 4 kb transcript in testis of rats between day 21 and 28 of postnatal development, coincides with an increase in the number of pachytene spermatocytes and the appearance of the first round spermatids (de Jong and Sharpe, 1977). Indeed, the 4 kb activin receptor mRNA was detected in isolated pachytene spermatocytes and round spermatids, whereas the 6 kb mRNA was not detected in these cell types. This explains the relative decrease in the expression of the 6 kb messenger with increasing age. Activin receptor mRNA was absent in elongating spermatids, although other genes are transcribed in these germ cells (reviewed by Erickson, 1990). This may indicate translation and/or breakdown of activin receptor mRNA around the period of progression of round spermatids into elongating spermatids.

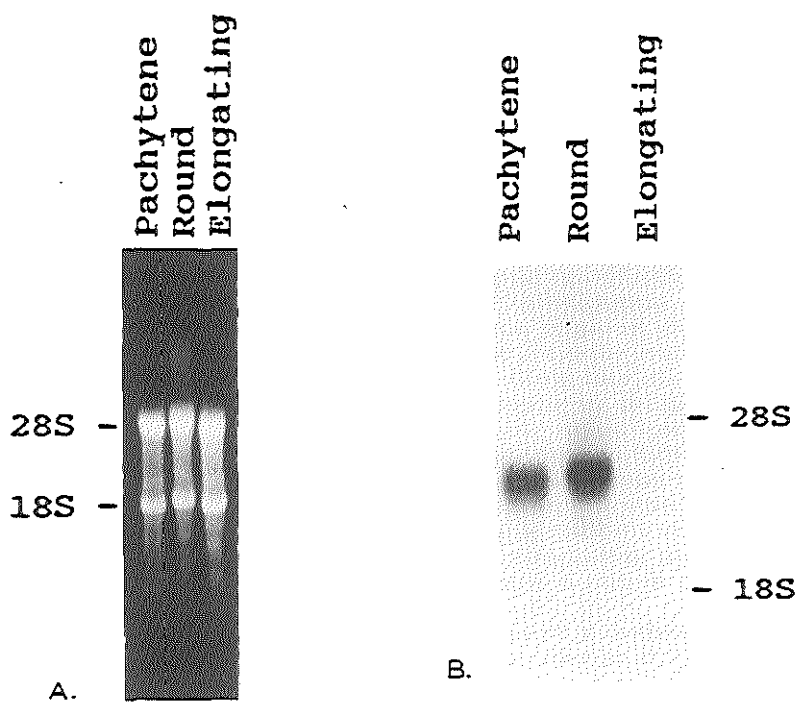

Fig. 5. Activin receptor mRNA expression in germ cells. *A:* Ethidium bromide staining of 20 μg total RNA obtained from mid-/late-pachytene spermatocytes, round spermatids and elongating spermatids. *B:* Northern blot analysis of the expression of activin receptor mRNA in germ cells after blotting of the RNA from *A*.

Functional expression of activin receptors in spermatocytes and spermatids will be the subject of further study.

The involvement of germ cells in the expression of the small activin receptor mRNA was further demonstrated by Northern blot analysis of RNA from testes of vitamin A-deficient rats. Tubules of these rats only contain Sertoli cells, A-spermatogonia and a few spermatocytes (van Pelt and de Rooij, 1991). A low expression of the 4 kb activin receptor mRNA in testes of these rats was found.

Germ cells are embedded in Sertoli cells which express inhibin βB subunit mRNA (Toebosch et al., 1988) and secrete bioactive activin (Grootenhuis et al., 1989). Here we show that pachytene spermatocytes and round spermatids express activin receptor mRNA. Therefore activin may act as a Sertoli cell secreted factor controlling spermatogenesis at different stages of the spermatogenic cycle. Indeed, Mather et al. (1990) showed that activin can stimulate spermatogonial proliferation. We have not isolated spermatogonia, but the activin receptor mRNA detected in immature rat testes could partially be of spermatogonial origin.

There is little information concerning growth factor mediated interaction between Sertoli cells and germ cells during spermatogenesis. One interesting other candidate for such an interaction is the peptide growth factor encoded by the *Steel* locus in the mouse (mast-cell growth factor: MGF), which is the ligand for the tyrosine kinase receptor protein encoded by the c-*kit* proto-oncogene. Effects of MGF on primordial germ cells have recently been shown (Dolci et al., 1991; Godin et al.,1991). In addition, Sorrentino et al. (1991) demonstrated the expression of the c-*kit* proto-oncogene in mouse spermatogonia and the mRNA for MGF was detected in Sertoli cells (Rossi et al., 1991).

Sertoli cells also contribute to the amount of activin receptor mRNA in rat testis, expressing both activin receptor mRNAs. Expression of several transcripts of receptor mRNA by somatic cells has been demonstrated for other hormone receptors including the growth hormone receptor (Smith et al., 1989). The relatively high expression of activin receptor mRNAs in Sertoli cells suggests that activin may affect Sertoli cell function. This would parallel the effects of activin on aspects of granulosa cell function: Hutchinson et al. (1987) reported stimulation of aromatase activity in granulosa cells, whereas Hasegawa et al. (1988) described induction of FSH receptors on granulosa cells, and Sugino et al. (1988) reported stimulation of the secretion of inhibin-like immunoreactivity by granulosa cells.

Expression of activin receptor mRNAs was also found in Leydig cell preparations. Since these preparations of Leydig cells were contaminated with other cell types from the interstitial compartment and with small amounts of tubular cells, it is possible that these activin receptor mRNAs are not localized in the Leydig cells. However, the observed inhibition of Leydig cell steroidogenesis by activin (Hsueh et al., 1987; Lin et al., 1989; Mauduit et al., 1991), suggests that activin receptors may play a role in the regulation of Leydig cell function.

In summary, the extracellular domain of the rat activin receptor is identical to that of the mouse activin receptor. In the rat testis, this receptor is expressed as two differentially sized mRNAs. Both messengers are expressed in Sertoli cells and at low levels in Leydig cell preparations. Mid-/late-pachytene spermatocytes and round spermatids only express the smaller mRNA, whereas elongating spermatids do not express activin receptor mRNA at all. These results may indicate multiple actions for activin in control of testicular function.

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Supplement* to chapter 2

To study the expression of activin receptor type II (ActRII) in more detail, in situ hybridization on sections of mature rat testes was performed. A low level of ActRII mRNA expression in all stages of the spermatogenic cycle was found. Expression of ActRII mRNA increased in spermatocytes at stage XIII and during meiotic division. A very high expression was detected in early round spermatids in stages I-III (Fig.6). These results indicate that the 4 kb activin receptor mRNA found in isolated germ cells is highly expressed in germ cells around the time of meiotic division. This was confirmed by Kaipia et al. (1992).

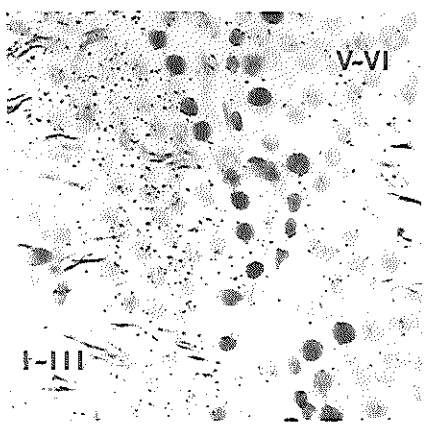

Fig.6 In situ hybridization of a testis of an adult rat with a probe for activin receptor type II. Stages of the spermatogenic cycle have been indicated.

To investigate whether the 4 kb ActRII mRNA was actively translated, polysome distribution analysis was performed on total testes of 34 day old rats and isolated spermatids, as described by Klaij et al. (1992). Actively translated messengers form heavy complexes with ribosomes and will sediment in the high molecular weight fraction (polysomal fraction) on sucrose gradients. As shown in figure 7 part of the 4 kb ActRII mRNA in total testes (Fig.7A, HKM) and isolated spermatids (Fig.7B, HKM) was present in the polysomal fraction of the sucrose gradient, and could be dissociated from the ribosomes by addition of EDTA (Fig.7A+B, HKE), indicating that a part of this mRNA population is translated.

For the detection of ActRII protein we injected rabbits with synthetic peptides to raise antibodies to amino acid residues 37-56 (WERDRTNQTGVEPCYGDKDK) and amino acid residues 80-99 (LDDINCYDRTDCIEKKDSPE) of the rat ActRII. ELISA demonstrated that antibodies against both peptides (AS 778 and AS 624, respectively) were generated (result not shown).

* These results have been published partly as a miniposter at the 7th European Workshop on Molecular and Cellular Endocrinology of the Testis, Castle Elmau, Germany, May 1992 (de Winter JP, van de Kant HJG, Hoogerbrugge JW, de Rooij DG, Themmen APN, Grootegoed JA and de Jong FH (1992). Activin receptor mRNA expression in germ cells of the male rat. Miniposter 75)

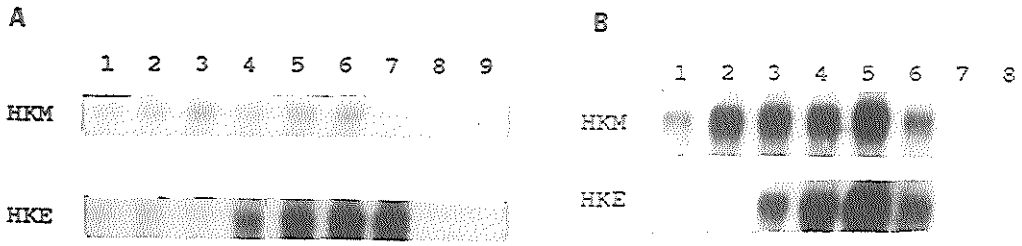

Fig.7 Polysome distribution analysis of the 4 kb activin receptor type II mRNA on total testes of 34 day old rats (A) and isolated spermatids (B) in buffer without EDTA (HKM) or with EDTA (HKE). Fraction 1 is the high molecular weight fraction, fraction 9 is the low molecular weight fraction.

A fusion protein of the extracellular domain of ActRII and gluthathione S-transferase was constructed using the vector pGEX-2T (Smith and Johnson, 1988) to see whether ActRII could be detected by AS 778 and AS 624. As shown in figure 8 both antisera recognized the fusion protein on Western blot, whereas overnight preincubation of the antisera with the appropriate peptides prevented the detection. The gluthatione S-transferase alone was not recognized. Although these results indicate that ActRII is recognized by both antisera, we obtained inconclusive results with Western blotting of crude membrane preparations from different testicular cell types, immunoprecipitation and immunocytochemistry. Therefore, we can not draw a reliable conclusion about the presence of ActRII protein on germ cells. However, Woodruff et al. (1992) detected binding of FITC-labeled activin A to late pachytene spermatocytes and spermatids, indicating that activin binding proteins are present on these cells.

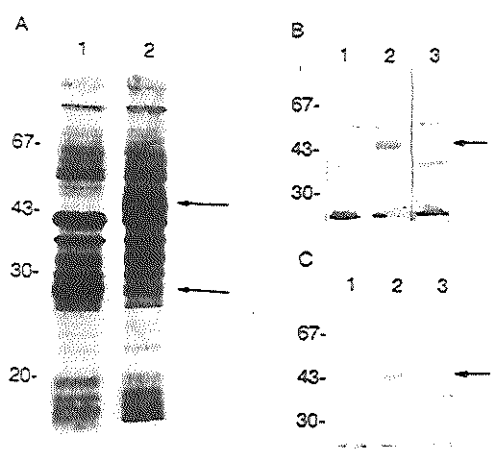

Fig.8 Detection of a fusion protein of the extracellular domain of activin receptor type II (ActRII) and gluthathione S-transferase (pGEX-2T) by AS 778 and AS 624. A Coomasie staining of proteins from E.coli transformed with pGEX-2T (lane 1) or pGEX-2T/ActRII (lane 2). B Western blot of proteins from E.coli transformed with pGEX-2T (lane 1) or pGEX-2T/ActRII (lanes 2 and 3) with AS 778 with (lane 3) or without (lanes 1 and 2) preincubation with the appropriate peptide. C Western blot of protein from E.coli transformed with pGEX-2T (lane 1) or pGex-2T/ActRII (lanes 2 and 3) with AS 624 with (lane 3) or without (lanes 1 and 2) preincubation with the appropriate peptide. Arrows indicate gluthatione S-transferase (lower arrow) and ActRII fusion protein (upper arrow).

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Testicular Leydig cells in vitro secrete only inhibin α-subunits, whereas Leydig cells tumors can secrete bioactive inhibin

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Testicular Leydig cells in vitro secrete only inhibin α-subunits, whereas Leydig cell tumors can secrete bioactive inhibin

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Summary

The secretion of inhibin and inhibin-related proteins by testicular Leydig cells was studied by estimation of inhibin immunoreactivity and bioactivity in spent media of preparations of immature and mature rat Leydig cells and of tumor Leydig cells. Immature and mature rat Leydig cells expressed inhibin α-subunit mRNA and secreted immunoreactive inhibin. The immunoreactive material did not contain inhibin bioactivity as measured by an in vitro rat pituitary bioassay system. Results of pulse labeling with [^{35}S]methionine followed by immunoprecipitation indicated that the inhibin-related proteins secreted by the immature Leydig cell preparations are 26 kDa and 44 kDa molecules. Mature rat Leydig cells only secreted the 44 kDa inhibin-related protein.

Tumor Leydig cells (rat H540 and mouse MA10) secreted immunoreactive and bioactive inhibin, which could be immunoneutralized by an antibody against inhibin. In the culture medium of some H540 tumor Leydig cells 26 kDa and 42 kDa inhibin-related proteins and 30 kDa inhibin were detected. In culture medium of other H540 tumor Leydig cells, not secreting bioactive inhibin, only 26 kDa and 42 kDa inhibin-related proteins were found. No activin bioactivity was detected in culture media of immature rat Leydig cells, H540 and MA10 tumor Leydig cells.

It is concluded that normal Leydig cells secrete inhibin α-subunits, while Leydig cell tumors can also secrete bioactive inhibin. Neither normal Leydig cells nor Leydig cell tumors produce activin.

Introduction

Inhibin is a glycoprotein hormone composed of an α-subunit which is disulphide-linked to either of two β-subunits, βA or βB. Inhibin suppresses follicle-stimulating hormone (FSH) secretion by the pituitary gland (for reviews see de Jong, 1988; Ying, 1988; de Kretser and Robertson, 1989). Dimers of inhibin β subunits (βAβA or βAβB) possess FSH-stimulating activity (Ling et al., 1986; Vale et al., 1986) and were named activins. Finally monomeric inhibin α-subunits (18 kDa, 26 kDa and 44 kDa) have also been described (Knight et al., 1989; Robertson et al., 1989; Sugino et al., 1989).

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In the testis, bioactive and immunoreactive inhibin is produced by Sertoli cells (Grootenhuis et al., 1990). Release of immunoreactive, but not bioactive inhibin, by Sertoli cells in vitro is stimulated by FSH (Grootenhuis et al., 1990), whereas human chorionic gonadotrophin (hCG) does not affect the secretion of immunoreactive inhibin by Sertoli cells in vitro (Bicsak et al., 1987). However, in vivo administration of hCG or luteinizing hormone (LH) increased levels of immunoreactive inhibin in plasma of rats (Sharpe et al., 1988; Drummond et al., 1989) and men (McLachlan et al., 1988; Burger et al., 1990). These results suggest a role for Leydig cells in inhibin secretion.

Indeed results of immunocytochemical studies (Bergh and Cajander, 1990; Teerds et al., 1991) and in situ hybridization (Roberts et al., 1989) indicated the presence of inhibin-like material and mRNA for inhibin subunits in Leydig cells. Furthermore, Risbridger et al. (1989) detected immunoreactive and bioactive inhibin in culture medium of Leydig cells from adult rats, whereas Lee et al. (1989) reported that immature pig and rat Leydig cells synthesize activin. The aim of the present study was to characterize the inhibin-related proteins secreted by Leydig cells using radioimmunoassay, in vitro bioassay, Western blotting and immunoprecipitation of [^{35}S]methionine-labeled proteins.

Materials and methods

Cell preparations

Immature Leydig cells were isolated from 21–22-day-old Wistar rat testes as described by Rommerts et al. (1985). Cells were attached to the surface of plastic 150 cm^2 flasks (Costar, Cambridge, MA, USA) or 6-well plates (Costar) during 1 h in RPMI (Seromed, Biochrom, Berlin, Germany) containing 0.1% bovine serum albumin (Sigma, St. Louis, MO, USA). After washing, the cell preparation contained approximately 30–50% Leydig cells and less than 1% Sertoli cells. The cells were cultured for 24 h in the same medium with or without ovine LH (oLH; 1 or 100 ng/ml culture medium, NIH S-20) or human FSH (hFSH; 25 mU or 250 mU/ml culture medium; Metrodin, Serono, Geneva, Switzerland). Media were collected and assayed for inhibin immunoreactivity and cells were lysed in 1 N NaOH. Further batches of medium were pooled, concentrated and exchanged against 0.01 M phosphate-buffered saline (pH 7.0), using Centriprep 10 filters, which have a molecular weight cut-off at 10 kDa (Amicon, Danvers, MA, USA). Inhibin immunoreactivity and bioactivity were measured in the concentrated and exchanged media. Cells were used to isolate mRNA after an overnight culture period. In separate experiments cells from 6-well plates were labeled with [^{35}S]methionine after overnight culture in RPMI containing 0.1% bovine serum albumin.

Mature Leydig cells were prepared from adult (at least 12 weeks old) Wistar rat testes using a Ficoll centrifugation purification step (Rommerts et al., 1985). Cells were treated like immature Leydig cells, but were attached during 1 h in RPMI containing 1% (v/v) fetal calf serum (Gibco, Paisley, Scotland, UK). These cell preparations contained approximately 90% Leydig cells.

H540 rat tumor Leydig cells, grown subcutaneously in rats were isolated as previously described to obtain a preparation of more than 99% H540 tumor cells (Rommerts et al., 1985). The cells were treated like immature Leydig cells. Cells were stimulated with 1000 ng oLH/ml culture medium. Two types of responses were noted. Some H540 tumor Leydig cell preparations produced bioactive and immunoreactive inhibin (A), whereas other preparations only secreted immunoreactive inhibin (B). The mechanism that caused this difference is not clear.

MA10 mouse tumor Leydig cells (100% pure, Ascoli, 1981), were cultured in plastic 75 cm^2 flasks (Costar) or 6-well plates (Costar), containing RPMI with 10% horse serum (Gibco) until confluency. Medium was then replaced by RPMI containing 0.1% bovine serum albumin and the cells were treated like immature Leydig cells.

Sertoli cells were obtained from testes of 21–22-day-old Wistar rats (Oonk et al., 1985) and cultured in 6-well plates (Costar) as described by Grootenhuis et al. (1989). After 1 day of culture, germ cells were removed by replacing the culture medium with 10% Eagle's minimal essential medium (MEM, Gibco) for 2 min as described by Toebosch et al. (1989). After the hypotonic shock treatment the medium was replaced twice with

MEM. The cells were cultured for another 2 days with or without 500 ng ovine FSH (oFSH, NIH S-16)/ml culture medium, followed by labeling with [^{35}S]methionine. Alternatively, Sertoli cells were cultured for 24 h with or without 500 ng oFSH/ml culture medium and lysed in 1 N NaOH. Culture media were assayed for inhibin immunoreactivity.

RNA isolation and Northern blot analysis

Total RNA was isolated from the different Leydig cell preparations by an extraction with 3 M LiCl and 6 M urea, as described by Auffray and Rougeon (1980), followed by extraction with phenol and chloroform. Samples containing 40 μg RNA were separated by electrophoresis in a denaturing agarose gel (0.7% agarose; Sigma, St. Louis, MO, USA) containing ethidium bromide and blotted on GeneScreen membranes (NEN, Boston, MA, USA) by diffusion. Blots were hybridized with probes for the inhibin subunits and actin as described earlier (Klaij et al., 1990).

Inhibin radioimmunoassay

Inhibin-like immunoreactivity present in the culture media of the different cell preparations was measured using an antiserum (No. 1989) against purified 32 kDa bFF inhibin and iodinated 32 kDa bFF inhibin as described by Robertson et al. (1988). These materials were kindly provided by Dr. G. Bialy (NICHHD, Bethesda, MD, USA). Bovine follicular fluid with an arbitrary potency of 1 U/μg protein (Grootenhuis et al., 1989) was used as an inhibin standard.

Inhibin bioassay

The amount of bioactive inhibin in the culture medium was determined by an in vitro rat pituitary bioassay system, measuring the suppression of basal FSH release (Grootenhuis et al., 1989). Results of the FSH assay have been expressed in terms of NIADDK-rat FSH-RP2. Charcoal-treated bovine follicular fluid was used as inhibin standard. In this bioassay system activin stimulates FSH release as demonstrated using recombinant activin A (see below). Inhibin bioactivity was immunoneutralized with a polyclonal antiserum against partially purified bovine inhibin (van Dijk et al., 1986).

Activin bioassay

Activin bioactivity was measured using the 'animal cap bioassay', in which culture media of different Leydig cell preparations were tested on their ability to induce mesodermal tissue in animal cap explants from *Xenopus* blastula (modified method of Symes and Smith, 1987). Spawning was induced in *Xenopus laevis* by injection of 300 IU human chorionic gonadotropin (Pregnyl, Organon, Oss, Netherlands) in the dorsal lymph sac of the females. The animals were left at room temperature. Eggs were laid about 10 h after the injection. Embryos were obtained by artificial fertilization in 5% De Boers solution (110 mM NaCl; 1.3 mM KCl; 0.44 mM $CaCl_2$, pH 7.2) and allowed to develop at room temperature to the blastula stage. Stage 8 embryos were chemically dejellied using 2% cysteine-hydrochloride (Janssen, Antwerp, Belgium; pH 7.8–8.1) and transferred to Petri dishes coated with 1% Noble agar (Difco, Detroit, MI, USA) and containing 0.05% normal amphibian medium (NAM). A disc of animal pole 'test tissue' from the center of the pigmented animal hemisphere was dissected out using electrolytically sharpened tungsten needles. After excision, the animal cap was immediately transferred with the blastocoel facing upwards in 0.75% NAM, containing 0.1% bovine serum albumin (BSA) (fraction V, Sigma) and the test factor. The conditioned media from Leydig cells were tested both before and after heat treatment (10 min at 95°C). Recombinant activin A (Huylebroek et al., 1990) was used as positive control. Both experimental and control caps were cultured at 22°C for 3 days. Six to ten explants were tested for each sample in each of four independent experiments.

Labeling of secreted proteins with [^{35}S]methionine

Leydig cell and Sertoli cell preparations in 6-well plates were incubated for 30 min in methionine-free RPMI or MEM, respectively. Subsequently the media were replaced by 600 μl methionine-free RPMI or MEM per well containing 45 μCi [^{35}S]methionine (Amersham, Buckinghamshire, UK) per ml. Culture was performed with or without 100 ng oLH/ml (1000 ng oLH/ml in case of H540 tumor Leydig cells) or 500 ng oFSH/ml. After a 6 h labeling period media

were collected and centrifuged at 8000 × *g* for 5 min. Supernatants were used for immunoprecipitation and cells were lysed in 1 N NaOH. Inhibin-like proteins were immunoprecipitated by addition of one of two different rabbit polyclonal antisera against synthetic peptides. One (anti-αc-antiserum) was directed against the 22 N-terminal amino acid residues of the α-subunit of 32 kDa bovine inhibin (Grootenhuis et al., 1989). The other (anti-pro-antiserum) was directed against the 20 C-terminal amino acid residues of the pro-part of the rat inhibin α-subunit precursor. After overnight incubation with 5 μl of antiserum per ml culture medium at 4°C, 50 μl IgSorb (The Enzyme Center, Maiden, MA, USA)/ml culture medium was added. 30 min later tubes were centrifuged for 20 min at 8000 × *g*. Pellets were washed 3 times with 1 ml 0.01 M phosphate-buffered saline (pH 7.0) containing 1 mM EDTA, 0.05% (w/v) sodium dodecyl sulfate (SDS) and 1% (v/v) Triton X-100 and twice with 0.001 M phosphate-buffered saline (pH 7.0). Pellets were taken up in 20 μl sample buffer, boiled for 3 min, centrifuged at 8000 × *g* and loaded on SDS polyacrylamide gels. Immunoprecipitations were also performed with sera obtained from the same rabbits before immunization (pre-immune sera) to evaluate the specificity of the immunoprecipitation.

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of ^{35}S-labeled proteins was performed on 1.5 mm thick, 8–15% gradient gels as described by Laemmli (1970) with or without prior reduction (1% (v/v) β-mercaptoethanol) of the samples. In parallel lanes the Rainbow ^{14}C-labeled molecular weight markers (Amersham) were separated for molecular weight determination. Gels were fixed in water/acetic acid/methanol (3:2:5, v/v/v), stained with Coomassie brilliant blue, destained in water/acetic acid/methanol (6.3:0.7:3, v/v/v) and dried on a BioRad (Richmond, CA, USA) gel dryer. Subsequently, Hyperfilm MP (Amersham) was exposed to the dried gel at −80°C, using an intensifying screen.

Samples used for Western blotting and molecular weight markers (Pharmacia, Uppsala, Sweden) were separated on 0.75 mm thick, 15% gels with the Mini-Protean II system (BioRad) as described by Laemmli (1970). The same system was used to blot the separated proteins onto nitrocellulose in 1 h at 100 V. Western blotting was performed as described by van Laar et al. (1989), using the antisera also used in the immunoprecipitations.

Estimation of DNA

The DNA content of the cell lysates was determined by a fluorometric assay using 3,5-diaminobenzoic acid dihydrochloride (DABA) as a fluorescent dye (Aldrich-Chemie, Steinheim, Germany). Samples were neutralized with 1 N HCl. A 50 μl portion of the neutralized sample was mixed with 50 μl 1.5 M DABA and incubated for 45 min at 60°C. Subsequently 2 ml 1 N HCl was added and the fluorescence of the samples was measured using a Perkin-Elmer fluorimeter at wavelengths of 415 nm (excitation) and 500 nm (emission). Calf thymus DNA was used as a standard.

Results

Expression of inhibin-subunit mRNA

Inhibin α-subunit mRNA of 1.6 kb, as reported previously by Klaij et al. (1990), was expressed in all Leydig cell preparations examined after overnight culture (Fig. 1). The amount of inhibin α-subunit mRNA found in the mature Leydig cell preparation was very small (Fig. 1, lane 9). However, immediately after isolation of the cells more α-subunit mRNA was detected (Fig. 1, lane 4). Inhibin βB-subunit mRNAs (4.2 kb and 3.5 kb respectively) were only detected in total testis RNA and Sertoli cell RNA of 21-day-old rats and in RNA isolated from tubuli of mature rats, but not in RNA from the examined Leydig cell preparations (Fig. 1). Inhibin βA-subunit mRNA was not detected in any of the examined preparations (result not shown).

Inhibin immunoreactivity

Culture medium derived from Leydig cell preparations and Sertoli cells was assayed for inhibin immunoreactivity by radioimmunoassay (RIA). Results of one experiment have been sum-

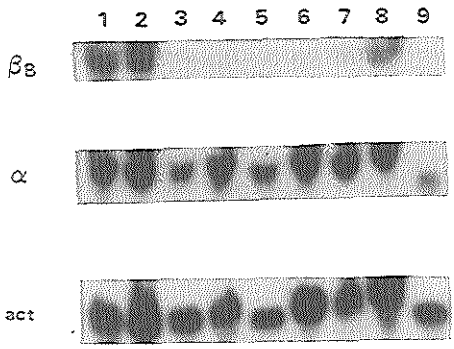

Fig. 1. Northern blot analysis of the expression of inhibin βB-subunit mRNA, inhibin α-subunit mRNA and actin mRNA in 21-day-old rat testes (1), and preparations of immature rat Sertoli cells (2), immature rat Leydig cells (3), mature rat Leydig cells immediately after isolation (4), H540 (A) tumor cells (5), H540 (B) tumor cells (6), MA10 tumor cells (7), mature rat tubuli (8) and mature rat Leydig cells after overnight culture (9).

TABLE 1

IMMUNOREACTIVE INHIBIN IN CULTURE MEDIA OF LEYDIG AND SERTOLI CELL PREPARATIONS AFTER 24 h OF CULTURE (MEANS ± SEM)

Values have not been corrected for the purity of the cell preparations.

Cell type and additions	n	Inhibin (U/μg DNA/24 h)
Sertoli cells		
− FSH	6	0.48 ± 0.03
+ FSH (500 ng/ml)	6	1.83 ± 0.15 **
Immature Leydig cells		
− LH	6	0.25 ± 0.02
+ LH (100 ng/ml)	6	0.41 ± 0.03 **
Mature Leydig cells		
− LH	3	0.14 ± 0.02
+ LH (100 ng/ml)	3	0.24 ± 0.01 **
H540 (A) tumor cell		
− LH	6	4.10 ± 0.23
+ LH (1000 ng/ml)	6	4.06 ± 0.28
MA10 tumor cells		
− LH	6	0.64 ± 0.05
+ LH (100 ng/ml)	6	0.47 ± 0.05 *

* Significantly different ($P < 0.05$) from appropriate control (Student's t-test).

** Significantly different ($P < 0.01$) from appropriate control (Student's t-test).

TABLE 2

EFFECT OF DIFFERENT CONCENTRATIONS OF OVINE LH (oLH) AND HUMAN FSH (hFSH) ON SECRETION OF IMMUNOREACTIVE INHIBIN BY IMMATURE RAT LEYDIG CELL PREPARATIONS (MEANS ± SEM, $n = 3$)

Addition	Inhibin (U/ml culture medium)
Control	0.24 ± 0.03
1 ng oLH/ml	0.52 ± 0.06 *
100 ng oLH/ml	0.56 ± 0.02 *
25 mU hFSH/ml	0.22 ± 0.04
250 mU hFSH/ml	0.33 ± 0.10

* Significantly different ($P < 0.02$) from control (Student's t-test).

marized in Table 1; two similar experiments yielded essentially the same results. Immature and mature rat Leydig cell preparations and Sertoli cells secreted immunoreactive inhibin. Secretion of this immunoreactivity was significantly stimulated by LH (Leydig cells) or FSH (Sertoli cells), suggesting that at least part of the inhibin-like material is secreted by Leydig and Sertoli cells. In a separate experiment human FSH (Metrodin) had no significant effect on secretion of immunoreactive inhibin by immature rat Leydig cells (Table 2). H540 and MA10 tumor Leydig cells secreted a relatively large amount of inhibin-like material, but LH did not stimulate its secretion. In MA10 cells LH even appeared to inhibit release of inhibin-like material.

Inhibin bioactivity

Immature rat Leydig cells. Addition of culture medium obtained from immature rat Leydig cell preparations caused a stimulation of FSH release in the rat pituitary cell culture (Fig. 2). The same medium did also inhibit basal LH release (data not shown). After exchange of the medium against phosphate-buffered saline, FSH-release-stimulating and LH-release-inhibiting activity disappeared from the culture medium. This is in contrast with the effects of culture medium containing recombinant activin A, which stimulated basal FSH release both before and after exchange (Fig. 2), without effect on basal LH release. In order to avoid these effects further experiments were per-

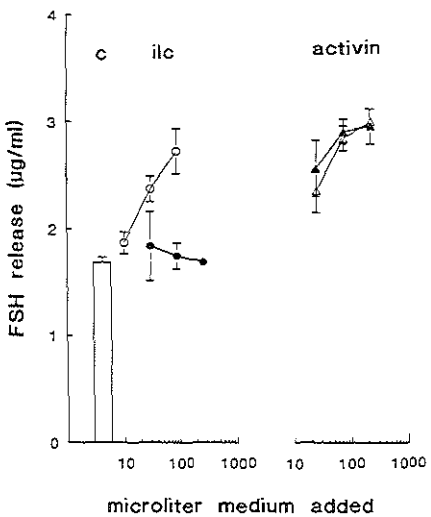

Fig. 2. Effect of culture medium from an immature rat Leydig cell preparation (ilc), culture medium containing recombinant activin A (activin) and control medium (c) on basal FSH release by rat pituitary cells, before (open symbols) and after (closed symbols) exchange of the culture medium.

formed with exchanged media. Results of one of these experiments are shown in Fig. 3; in five parallel experiments, similar data were obtained. Addition of an immunoneutralizing antiserum

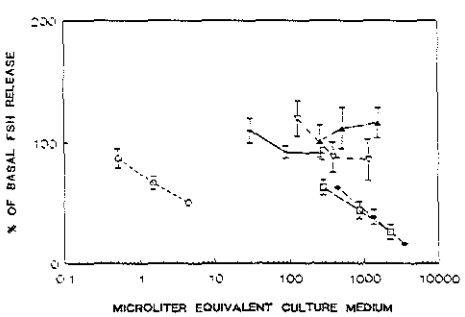

Fig. 3. Effect of exchanged culture media from different Leydig cell and Sertoli cell preparations on basal FSH release by rat pituitary cells ((○) rat Sertoli cell culture medium, (▲) immature rat Leydig cell culture medium, (▽) mature rat Leydig cell culture medium, (□) H540 (A) tumor cell medium, (+) H540 (B) tumor cell medium, (◆) MA10 tumor cell medium).

TABLE 3

EFFECT OF INHIBIN IMMUNONEUTRALIZATION ON FSH RELEASE BY RAT PITUITARY CELLS, AFTER ADDITION OF CULTURE MEDIA OBTAINED FROM PREPARATIONS OF IMMATURE RAT LEYDIG CELLS AND DIFFERENT TUMOR LEYDIG CELLS (MEANS ± SEM, $n = 3$)

Addition	FSH release (ng/ml)
Control	19.9 ± 2.2
Immature Leydig cell medium	
without neutralization	20.5 ± 4.0
with neutralization	21.0 ± 1.7
H540 (A) tumor cell medium	
without neutralization	6.8 ± 1.3
with neutralization	20.1 ± 3.8
MA10 tumor cell medium	
without neutralization	4.7 ± 1.0
with neutralization	14.2 ± 0.6

against inhibin did not affect FSH release in the presence of immature Leydig cell culture medium (Table 3) or culture medium containing recombinant activin A (not shown).

Mature Leydig cells. Exchanged culture medium from mature rat Leydig cell preparations, which contained immunoreactive inhibin, did not affect basal FSH release significantly. Results of one experiment have been included in Fig. 3; in four parallel experiments also no specific suppression of FSH release was observed.

Tumor Leydig cells. Fig. 3 also shows the effect of addition of exchanged culture media, obtained from Sertoli cells and different tumor Leydig cell preparations, on basal FSH release by rat pituitary cells. Media from Sertoli cells, H540 (A) tumor Leydig cells and MA10 tumor Leydig cells suppressed basal FSH release. Suppression was parallel to that caused by addition of bovine follicular fluid. In contrast, the culture medium from the H540 (B) tumor cells did not suppress basal FSH release (Fig. 3). Addition of the immunoneutralizing inhibin antiserum abolished the effect of culture medium derived from H540 (A) tumor cells and MA10 tumor cells (Table 3).

Activin bioactivity. Analysis of conditioned media from immature rat Leydig cell preparations, H540 (A) and MA10 tumor Leydig cells in the animal cap bioassay did not show any mesoderm inducing capacity, indicating that less than

0.2 ng activin/ml culture medium was present (Smith et al., 1990). The same results were obtained after heating of the samples (data not shown).

Immunoprecipitation of [^{35}S]methionine-labeled proteins

In order to visualize inhibin-like proteins, secreted by the various testicular cell types, proteins were labeled with [^{35}S]methionine, immunoprecipitated and separated by SDS-PAGE (Fig. 4). With a polyclonal antiserum against the first 22 N-terminal amino acid residues of the α-subunit of bovine 32 kDa inhibin, Sertoli cell secreted proteins of 44 kDa, 30 kDa and 26 kDa were specifically precipitated (Fig. 4*a*, lane B2). There were also proteins aspecifically precipitated (Fig. 4*a*, lane B1), due to binding to IgSorb used to precipitate the antibodies (data not shown). The inhibin-like proteins of 44 kDa and 26 kDa were also specifically precipitated with a polyclonal antiserum against the 20 C-terminal amino acid residues of the pro-sequence of the rat inhibin α-subunit (Fig. 4*c*, lane B2). Their secretion was stimulated by FSH (Fig. 4*a*, lane B3). Similar 44 kDa and 26 kDa inhibin-related proteins were present after immunoprecipitation of [^{35}S]methionine-labeled proteins secreted by an immature rat Leydig cell preparation (Fig. 4*a*, lane A2) and their secretion was stimulated by

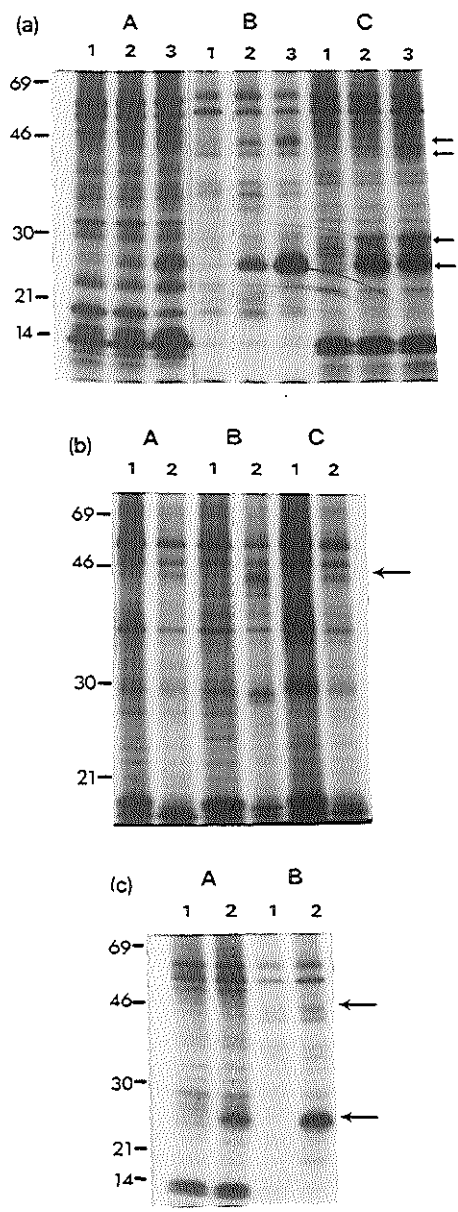

Fig. 4. Immunoprecipitation of [^{35}S]methionine-labeled proteins secreted from Sertoli and Leydig cell preparations. For description of the antisera see Materials and methods. Panel *a*: Precipitation of proteins secreted by immature Leydig cells (A), immature Sertoli cells (B) and H540 (A) tumor cells (C) with pre-immune serum (lanes 1) or with the anti-αc-antibody (lanes 2 and 3). Cells were not stimulated (lanes 2) or stimulated with ovine LH or ovine FSH (lanes 3). Panel *b*: Precipitation of proteins secreted by mature Leydig cells with pre-immune sera (lanes 1) or specific antisera (lanes 2). Precipitation A represents proteins secreted without stimulation and precipitated with the anti-αc-antibody. Precipitation B represents proteins secreted after stimulation with LH and precipitation with the same antibody. Precipitation C shows proteins secreted without stimulation after precipitation with the anti-pro-antibody. Panel *c*: Precipitation of proteins secreted by H540 (A) tumor cells (A) and immature Sertoli cells stimulated with oFSH (B) with pre-immune serum (lanes 1) or the anti-pro-antibody (lanes 2).

LH (Fig. 4*a*, lane A3). Only the 44 kDa inhibin-like protein was detected in culture medium of a mature rat Leydig cell preparation after precipitation with both antisera (Fig. 4*b*, lanes A2 and C2); its secretion was stimulated by the addition of LH (Fig. 4*b*, lane B2).

H540 (A) tumor Leydig cells secreted inhibin-like proteins of 42 kDa, 30 kDa and 26 kDa (Fig. 4*a*, lane C2) but in culture medium of H540 (B) tumor Leydig cells only the 42 kDa and 26 kDa proteins were detected (results not shown). This 26 kDa protein was also precipitated with the antiserum against the 20 C-terminal amino acid residues of the pro-sequence (Fig. 4*c*, lane A2). Addition of LH did not affect the secretion of these proteins (Fig. 4*a*, lane C3).

After reduction of the samples the 26 kDa protein disappeared, while a 18 kDa protein was detected (results not shown).

Western blotting

Western blotting was not sensitive enough to detect inhibin-like proteins in culture media of immature and mature rat Leydig cell preparations. However, this technique showed the 26 kDa inhibin-related protein in culture media of H540 (A) tumor Leydig cells and Sertoli cells with both antisera used (Fig. 5). This 26 kDa protein was reduced with 1% β-mercaptoethanol to give an 18 kDa protein, which was immunoreactive with the antiserum against the first 22 N-terminal amino acid residues of the α-subunit of bovine 32 kDa inhibin (result not shown). Also 30 kDa inhibin was detected in culture medium of both cell types (Fig. 5) with the antiserum against the αc-part of the inhibin molecule.

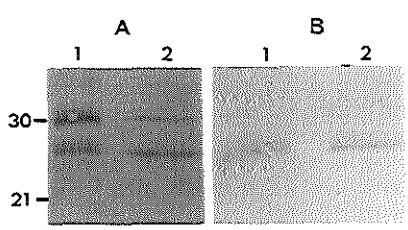

Fig. 5. Western blot of Sertoli cell culture medium (1) and H540 (A) tumor Leydig cell medium (2) with an antibody against the αc part (A) or against the pro-part (B) of the inhibin α-subunit.

Discussion

There is growing evidence that production of inhibin and inhibin-related proteins is not restricted to Sertoli cells and granulosa cells. Meunier et al. (1988a) showed expression of inhibin α, βA and βB subunits in various extragonadal tissues. These authors also detected inhibin α-subunit mRNA in different rat ovarian cell types, including interstitial cells (Meunier et al., 1988b). The results of the present study indicate that interstitial cells of immature and mature rat testes also can produce inhibin subunits, in agreement with the observations of Roberts et al. (1989). In immature rat Leydig cell preparations expression of inhibin α-subunit mRNA was detected, resulting in inhibin immunoreactivity in culture medium of these cells. The measured immunoreactive material is not likely to be produced by contaminating Sertoli cells, because human FSH did not affect secretion of immunoreactive material in this Leydig cell preparation (Table 2).

The immunoreactive material did not cause FSH suppression in the in vitro bioassay for inhibin. The results of the immunoneutralization experiment (Table 3) argue against the possibility that similar amounts of bioactive inhibin and activin are produced by immature Leydig cells, and contrast with the data of Lee et al. (1989), who detected FSH-release-stimulating activity in the culture medium of immature pig and rat Leydig cells. However, these authors did not exchange the culture medium of the Leydig cells, in order to exclude effects of small molecular weight substances present in these media. In the present study the FSH-release-stimulating activity found before exchange disappeared after exchange of the media, whereas the activity of recombinant activin A was not affected by this exchange. Furthermore the results of the animal cap bioassay and the absence of inhibin β-subunit mRNAs make it unlikely that immature rat Leydig cells produce activin.

The immunoreactive material present in immature rat Leydig cell culture medium corre-

sponds to inhibin-related proteins of 44 kDa (presumably proαNαC) and 26 kDa (presumably proαC), which are both encoded by the inhibin α-subunit precursor mRNA. Those proteins do not possess inhibin bioactivity as shown earlier by Grootenhuis et al. (1990) for the 26 kDa protein. Furthermore, in culture medium of H540 (B) tumor Leydig cells, which only secreted similar 42 kDa and 26 kDa inhibin-related proteins, no inhibin bioactivity was detected.

Mature rat Leydig cells express inhibin α-subunit mRNA and immunoreactive inhibin is measured in culture medium of these cells. This immunoreactive material is devoid of inhibin bioactivity since specific suppression of FSH release was not found after addition of this medium to cultured pituitary cells. The absence of βA and βB mRNA expression correlates with this observation. However, Risbridger et al. (1989) detected bioactive inhibin in mature rat Leydig cell culture medium, and more recently described the presence of immunoreactive follistatin in mature Leydig cell culture medium (Risbridger et al., 1991). The reason for the differences between the present observations and those of Risbridger et al. (1989, 1991) are not clear.

Precipitation of [^{35}S]methionine-labeled proteins revealed the immunoreactive protein, secreted by mature Leydig cell preparations to be a 44 kDa inhibin-related protein (presumably proαNαC). It seems that Leydig cells lose their ability to process the inhibin α-subunit precursor with increasing age, because no 26 kDa inhibin-related protein was detected in mature rat Leydig cell culture medium, different from the situation in the culture medium immature rat Leydig cell preparations.

In contrast to normal Leydig cells, two out of three tumor Leydig cells did produce bioactive inhibin. These cells expressed inhibin α-subunit mRNA but no inhibin β-subunit mRNAs were detected; the expression of inhibin β-subunit mRNAs was probably below the detection limit of the Northern blot technique. The presence of bioactive inhibin, which could be immunoneutralized by an inhibin antiserum, indicates that inhibin β-subunit must be present. Expression of inhibin α- and βB-subunit mRNAs in cultured MA10 cells, without expression of βA-subunit mRNA was reported earlier by Feng et al. (1990). H540 tumor Leydig cells (A) secreted 30 kDa inhibin and 42 kDa and 26 kDa inhibin-related peptides. In culture medium of H540 (B) 30 kDa inhibin was not present. LH had no effect on the release of inhibin-related material from H540 tumor Leydig cells, perhaps because the level of LH receptors is only about 1% of that present in normal Leydig cells from adult rats (Erichsen et al., 1984). In these cells steroid production can also hardly be stimulated by LH. Finally, no activin bioactivity was detected in the medium of H540 and MA10 tumor Leydig cells. This contrasts with the results of Lee et al. (1989) who observed FSH-stimulating activity in the medium of the immortalized mouse Leydig cell line TM3. However, using the animal cap assay, which has a higher specificity for activin bioactivity, mesoderm inducing activity could not be detected in culture media from TM3, R2C or I-10 Leydig cell lines (H.M.J. Vanderstichele, non-published results).

Inhibin-like material was detected by immunocytochemistry in human (Bergh and Cajander, 1990) and rat (Teerds et al., 1991) normal and tumor Leydig cells. Furthermore, Roberts et al. (1989) detected positive staining in normal rat Leydig cells and de Jong et al. (1990) found inhibin immunoreactivity in homogenates of human Leydig cell tumors. In contrast several authors (Merchenthaler et al., 1987; Rivier et al., 1988; Saito et al., 1989; Shaha et al., 1989) did not detect immunocytochemical staining for inhibin subunits in normal Leydig cells, probably because of the relatively low amounts of inhibin-like material produced by these cells.

Several reports have demonstrated that in vivo administration of hCG or LH increases serum immunoreactive inhibin levels in adult rats (Sharpe et al., 1988; Drummond et al., 1989) and men (McLachlan et al., 1988). In the adult rat this seems to be the result of a change in the polarity of inhibin-α secretion by Sertoli cells (Maddocks and Sharpe, 1990), rather than a contribution of Leydig cells. Destruction of Leydig cells by ethylene-1,2-dimethanesulfonate (EDS) did not lead to any decrease in the levels of immunoactive inhibin in blood and testis (Maddocks and Sharpe, 1989). The possible role of

inhibin-related proteins produced by Leydig cells needs to be determined by further studies.

Nothing is known about the contribution of inhibin α-subunits from immature rat Leydig cells to serum immunoreactive inhibin levels. Part of the serum inhibin immunoreactivity in immature rats could be of Leydig cell origin.

We conclude that immature and mature Leydig cells and Leydig cell tumors can secrete inhibin-like material. In contrast to Sertoli cells and Leydig cell tumors, immature and mature Leydig cells do not secrete inhibin bioactivity, but only α-subunits. Immature Leydig cells secrete inhibin-like proteins of 44 kDa (presumably proαNαC) and 26 kDa (presumably proαC), while mature Leydig cells only secrete the former inhibin-related protein. Neither normal Leydig cells nor Leydig cell tumors secrete activin.

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Activin is produced by rat Sertoli cells in vitro and can act as an autocrine regulator of Sertoli cell function

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Activin Is Produced by Rat Sertoli Cells *in Vitro* and Can Act as an Autocrine Regulator of Sertoli Cell Function*

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ABSTRACT

The production of activin by Sertoli cells isolated from 21-day-old rats was studied using the mesoderm-inducing activity of activin on *Xenopus laevis* animal cap explants, immunoprecipitation and Western blotting. Furthermore, the effects of recombinant bovine activin-A on rat Sertoli cell aromatase activity and FSH and androgen receptor gene expression were examined.

Animal cap explants from *Xenopus laevis* blastulas elongated after culture in conditioned medium of Sertoli cells cultured with or without ovine FSH or conditioned medium of the mouse Sertoli cell-derived TM4 cell line. Animal cap explants cultured in control medium remained spherical. This elongation was also found in the more than 10-kilodalton fraction of the conditioned medium and after heating for 10 min at 95 C, indicating that heat-stable activin-like bioactivity is present in the culture medium. Immunoprecipitation of [^{35}S]methionine-labeled proteins and Western blotting of Sertoli cell-conditioned medium with polyclonal antisera against the inhibin β-subunits indicated the presence of 24- to 25-kilodalton activin-like immunoreactive material.

Sertoli cell aromatase activity was dose-dependently stimulated by ovine FSH after 72 h of culture. Recombinant bovine activin-A partly inhibited this stimulation in a dose-dependent way. This inhibition was also found after 24 h of culture. Furthermore, basal and FSH-stimulated androgen receptor mRNA expression in Sertoli cells and binding of the synthetic androgen R1881 to Sertoli cells were decreased after 24 h of culture in the presence of recombinant bovine activin-A. In the same experiments, FSH receptor mRNA expression was not significantly affected. These results indicate that activin can act as an autocrine regulator of Sertoli cell function. (*Endocrinology* 132: 975–982, 1993)

ACTIVINS, which are homo- or heterodimers of the βA- and βB-subunits of inhibin, were originally recognized in and isolated from ovarian follicular fluid by virtue of their ability to stimulate the synthesis and release of FSH from pituitary cells in culture (1, 2). The activins are members of a family of structurally related proteins, including transforming growth factors-β (TGFβs), Müllerian-inhibiting substance, bone morphogenetic proteins, the product of the decapentaplegic gene complex of *Drosophila* and the Vg1 gene product of *Xenopus* (for review, see Ref. 3). It is now clear that activins exert many biological functions and can be regarded as cell differentiation factors (4). In the testis, activins may play a role in the regulation of both Leydig cell steroidogenesis (5–7) and spermatogenesis (8).

Shintani *et al.* (9) reported high levels of immunoreactive activin in testicular homogenates, but it is not clear by which testicular cell type activin is produced. Lee *et al.* (10) indicated that Leydig cells might be the source of activin in the testis. In contrast, de Winter *et al.* (11) demonstrated that the β-subunits of inhibin are not expressed in Leydig cells, resulting in the secretion of inhibin α-subunits only. Furthermore, Grootenhuis *et al.* (12) showed that a 25-kilodalton (kDa) fraction of Sertoli cell-conditioned medium (SCCM) could stimulate the release of FSH from pituitary cells in culture. For these reasons, we studied the presence of activin in SCCM by bioassay, immunoprecipitation, and Western blotting.

Recently, we reported that activin receptor type II mRNA is expressed in rat Sertoli cells (13). So far, effects of activin on Sertoli cells have not been described. Since the main regulators of Sertoli cell function are FSH and androgens, we investigated whether recombinant bovine activin-A can influence the actions of these hormones, using the induction of aromatase and the expression of FSH and androgen receptor genes as response parameters.

Materials and Methods

Isolation and culture of Sertoli cells

Highly purified Sertoli cells from testes of immature (17- to 21-day-old) Wistar rats were isolated and cultured using the procedure described by Themmen *et al.* (14). In short, decapsulated testes were incubated for 20 min at 37 C in PBS, containing DNase-I (5 μg/ml; DN25, Sigma, St. Louis, MO), collagenase (1 mg/ml; CLS, Worthington, Freehold, NJ), trypsin (1 mg/ml; TRL, Worthington), and hyaluronidase (1 mg/ml; I-S, Sigma). After four successive washes by sedimentation at unit gravity in PBS-DNase, the tubular fragments were incubated in PBS-DNase containing 1 mg/ml collagenase and 1 mg/ml hyaluronidase for 20 min at 37 C. The fragments were washed four times, as previously described, and dispersed using a Dounce homogenizer. The cells were then washed four times with PBS-DNase and once with Eagle's Minimum Essential

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Medium (MEM: Gibco, Grand Island, NY) by sedimentation at 100 × *g* for 2 min. The cell preparation was plated in 75-cm² culture flasks (Costar, Cambridge, MA) for RNA extraction, in 6-well plates (Costar) for [^{35}S]methionine labeling of Sertoli cell-secreted proteins, in 24-well plates (Costar) for estimation of [^{3}H]R1881 binding, and in 96-well plates (Costar) for determination of aromatase activity at a density of 0.5 × 10^{6} cells/cm². The cells were cultured for 48 h at 37 C in MEM supplemented with nonessential amino acids, glutamine, antibiotics, and 1% fetal calf serum (FCS; Sebak, Aidenback, Germany). Then, they were shocked hypotonically for 2 min in 0.1-fold concentrated MEM to remove germ cells. The culture was continued for 24 h in MEM containing 0.1% BSA (fraction V; Sigma). Subsequently, medium was replaced with MEM containing 0.1% BSA and hormones.

To obtain large amounts of SCCM, Sertoli cells isolated from testes of 21-day-old rats were cultured in 150-cm² flasks for 4–5 weeks in MEM with or without 500 ng ovine FSH/ml (oFSH: NIH S-16). Medium was collected every 3 or 4 days. This SCCM was used in the animal cap bioassay directly or after concentration and exchange against 20 mM Tris-HCl (pH 7.9) using a filtration unit (Amicon, Lexington, MA) with a hollow fiber (HIP 10–43, Amicon, mol wt cut-off, 10 kDa).

Culture of the Sertoli cell-derived cell line TM4 (CRL 1715)

For the preparation of conditioned medium from mouse TM4 cells (15), cells were grown to confluency in 150-cm² culture flasks (Costar) containing Ham's F-12 (Gibco)-Dulbecco's Modified Eagle's Medium (DMEM: Gibco; 1:1) supplemented with 2.5% FCS (Integro, Zaandam, The Netherlands) and 5% horse serum (Gibco). The cells were then cultured for 1 day in the same medium without serum and finally for 2 additional days in 30 ml serum-free medium. The latter medium was collected, filtered using a 0.22-μm filter (Costar), and stored at −20 C until use in the animal cap or TGFβ bioassay.

Animal cap bioassay

Activin bioactivity was studied using the animal cap bioassay, in which conditioned medium from Sertoli cells, cultured with or without FSH, and conditioned medium from TM4 cells were tested for their ability to induce an elongation of animal cap explants from *Xenopus laevis* blastulas (stage 8). The assay was performed according to a modification of the method of Symes and Smith (16), as described by de Winter *et al.* (11). Briefly, stage 8 embryos were chemically dejellied using 2% cystein-hydrochloride (pH 7.8–8.1). A disc of animal pole test tissue from the center of the pigmented animal hemisphere was dissected and transferred with the blastocoel facing upward in 0.75% normal amphibian medium containing 0.1% BSA and the test factor. The animal cap explants were cultured at 22 C for 3 days.

TGFβ bioassay

The secretion of bioactive TGFβ by Sertoli cells was measured using the mink lung epithelial cell assay modified according to Cone *et al.* (17). This assay is based on growth inhibition of the Mv1L4 cell line TCC CCL64 by TGFβ. Briefly, cells were grown in DMEM supplemented with 10% FCS and 1% nonessential amino acids at 37 C. At subconfluency, cells were trypsinized and seeded at a 1:8 dilution. After 24 h, medium was replaced by DMEM containing 1% FCS and 1% nonessential amino acids, and cells were cultured for another 24 h. Then, 10^{4} cells were incubated in 96-well microtiter plates with serial dilutions of test samples in DMEM-1% FCS-1% nonessential amino acids. After 48 h, [^{3}H]thymidine (0.5 μCi/well) was added, and incubation was continued for 6 h. Cell-associated counts were determined. Pure porcine TGFβ1 or porcine TGFβ2 (British Biotechnology Ltd., Abingdon, Berkshire, United Kingdom) were used as positive controls. Each sample was tested in duplicate.

Labeling and immunoprecipitation of Sertoli cell proteins

After preculture for 3 days and hypotonic shock treatment, Sertoli cells from 21-day-old rats were incubated for 30 min in methionine-free MEM (Gibco). Subsequently, the medium was replaced with 600 μl methionine-free MEM/well containing 45 μCi [^{35}S]methionine (Amersham, Aylesbury, Buckinghamshire, United Kingdom). After 8 h of labeling, media from two wells were pooled and centrifuged at 8000 × *g* for 5 min. Supernatants were used for immunoprecipitation with polyclonal antiserum raised against a prokaryotic fusion protein containing the entire mature bovine inhibin βA-chain (AS 065, Innogenetics, Ghent, Belgium), against C-terminal amino acid residues 94–113 of the rat inhibin βB-chain, or against N-terminal amino acid residues 1–22 of the αC-subunit of 32-kDa bovine inhibin. After overnight incubation with 5 μl antiserum at 4 C, 50 μl protein-A-Sepharose CL-4B suspension (Pharmacia, Uppsala, Sweden) were added, and tubes were rotated for 30 min at 4 C. The Sepharose beads were spun down and washed three times with 1 ml 0.01 M PBS (pH 7.0) containing 1 mM EDTA, 0.05% (wt/vol) sodium dodecyl sulfate (SDS), and 1% (vol/vol) Triton X-100 and twice with 0.001 M PBS (pH 7.0). Beads were taken up in 40 μl sample buffer (50 mM Tris, 2 mM EDTA, 10% glycerol, 2% SDS, and 0.001% bromophenol blue, pH 6.8) with or without 2% β-mercaptoethanol, boiled for 5 min, and centrifuged. The resulting supernatants were loaded on SDS-polyacrylamide gels.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE and Western blotting were carried out as described by de Winter *et al.* (11). Western blotting was performed with concentrated conditioned medium from control Sertoli cells and the antisera against inhibin βB- and α-subunits, which were also used for immunoprecipitation. Goat antirabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma), naphtol AS-MX phosphate (Sigma), and 4-aminodiphenylamine diazonium sulfate (Sigma) were used to visualize the proteins to which the antisera bound.

Aromatase assay

Sertoli cells were incubated for 24 or 72 h at 37 C under an atmosphere of 5% CO_2 in MEM containing 0.1% BSA and 0.5 μM testosterone. Incubation was performed in the presence or absence of oFSH (NIH S-17) and recombinant bovine activin-A (Innogenetics, Ghent, Belgium). After incubation, estradiol production was measured using a commercially obtained RIA (DPC Coat-a-Count, Los Angeles, CA). Cells were lysed in 1 N NaOH, and the DNA content of the cell lysates was determined by a fluorometric assay using 3,5-diaminobenzoic acid dihydrochloride (Aldrich-Chemie, Steinheim, Germany) as a fluorescent dye (11). Estradiol production was expressed per μg DNA.

RNA isolation and Northern blot analysis

Total RNA was isolated from Sertoli cells by extraction with 3 M LiCl and 6 M urea (18), followed by extraction with phenol and chloroform. Samples containing 30 μg total RNA were separated by electrophoresis in a denaturing formaldehyde-agarose gel (1% agarose; Sigma) containing ethidium bromide and blotted onto Hybond N+ nylon membrane filters (Amersham) by diffusion. To detect androgen receptor mRNA, filters were hybridized with a ^{32}P-labeled human androgen receptor cDNA probe [0.5-kilobase (kb) *Eco*RI-*Eco*RI fragment, corresponding to part of the steroid-binding domain and the 3′-untranslated region of the receptor mRNA] (19). For detection of FSH receptor mRNA, the same filters were hybridized with ^{32}P-labeled rat FSH receptor cDNA probes (20); pRK-FSHR-NH2 (0.86-kb *Eco*RI-*Eco*NI fragment, corresponding to amino acid residues 1–265 of the receptor) and pRK-FSHR-COOH (1.3-kb *Eco*NI-*Bam*HI fragment, corresponding to amino acid residues 266–692 of the receptor). Finally, filters were hybridized with a hamster actin probe. All hybridizations were performed for 48 h at 42 C in hybridization solution containing 50% formamide, 9% (wt/vol) dextran sulfate, 10 × Denhardt's [1 × Denhardt's contains 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinyl pyrrolidone, and 0.02% (wt/vol) BSA], 5 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 10 mM sodium phosphate (pH 6.8), and 100 μg/ml denaturated salmon sperm DNA. After hybridization, blots were washed to a final stringency of 0.1 × SSC-0.1% (wt/vol) SDS at 42 C and autoradiographed using Hyperfilm-MP (Amersham). The hybridization

signals were quantified using a Bio-Rad-1D gel scanner (model 620, Bio-Rad, Richmond, CA). Values were normalized using the signals obtained after hybridization with hamster actin cDNA.

Androgen binding assay

The androgen binding assay was carried out as described by Blok *et al.* (21) using the synthetic androgen [^{3}H]R1881 (New England Nuclear Products, Stevenage, Hertfordshire, United Kingdom) as a specific ligand for the androgen receptor. After 3 days of preculture, Sertoli cells isolated from testes of 17-day-old rats were incubated for 24 h in MEM containing 0.1% BSA, recombinant bovine activin-A (50 ng/ml), and/or oFSH (50 ng/ml). After the incubation, Sertoli cells were washed four times with MEM containing 0.1% BSA. The cells were then incubated with triamcinolone acetonide (4 μM) to occupy progesterone receptors and [^{3}H]R1881 (7 nM) with or without excess unlabeled R1881 (10 μM) for 2 h at 37 C. Subsequently, the cells were placed on ice and washed four times with MEM to remove free [^{3}H]R1881. The cells were then lysed in 1 N NaOH, and the bound [^{3}H]R1881 was counted. Specific binding was calculated and expressed as a percentage of R1881 binding in control Sertoli cells.

Experimental animals

All animal experimentation described in this manuscript was conducted in accordance with the highest standards of humane animal care, as outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Results

Production of activin by immature rat Sertoli cells in vitro

Animal cap explants of *Xenopus laevis* embryos were cultured in the presence of conditioned medium (nonconcentrated or concentrated and exchanged against 20 mM Tris-HCl, pH 7.9) from Sertoli cells cultured with or without FSH or in nonconcentrated conditioned medium from TM4 cells. After 1 day of culture, elongation of the explants was observed with all media tested, whereas explants cultured in control medium remained spherical (Fig. 1). After several days of culture, when control embryos were at tailbud stages, the animal caps treated with conditioned medium from the Sertoli cells were swollen by fluid intake and became vesicles several times their original volume. Some of them expressed melanophores. The control caps still remained spherical (not shown). These results indicate that mesoderm-inducing products were released by the Sertoli and TM4 cells. These products are heat stable, because elongation of the explants was also observed after heating the conditioned media from Sertoli and TM4 cells for 10 min at 95 C (not shown). Since TGFβ is heat stable and can induce mesoderm, the amount of TGFβ in the tested samples was verified using the epithelial mink lung cell bioassay. From the results presented in Table 1, it is clear that Sertoli cells do secrete some bioactive TGFβ, but not enough to cause elongation of the animal caps after the addition of nonconcentrated conditioned media from Sertoli and TM4 cells. These results indicate that activin, which is also heat stable, may be the mesoderm-inducing product secreted by Sertoli cells. To consolidate this, Sertoli cells were labeled with [^{35}S]methionine, and secreted activin was precipitated from the conditioned medium with two different rabbit polyclonal antisera against the inhibin β-subunits (Fig. 2A). Both antisera precipitated a protein of 24–25 kDa. Preincubation of the anti-βA antiserum with 50 ng recombinant bovine activin-A decreased the precipitation of labeled protein. Precipitation with preimmune serum was also performed as a control. A faint band was still present after precipitation with preimmune serum and after preincubation with activin-A. Less stringent washing of the Sepharose beads with 50 mM Tris, 0.1 M EDTA, and 1 M NaCl (pH 8.0) resulted in precipitation of equal amounts of the 24- to 25-kDa protein by preimmune and immune serum (not shown), indicating nonspecific binding of this band of radioactivity to the beads. In support of this nonspecificity of the remaining 24- to 25-kDa radioactivity, this protein was not detected after Western blotting with preimmune serum (see below). The α-antiserum precipitated 26-kDa pro-αC and 30-kDa inhibin, as described previously (11). Reduction of the samples after immunoprecipitation with the α-antiserum showed a large amount of 18-kDa free α-subunit (from inhibin and pro-αC) and some 14-kDa free β-subunit (from inhibin), whereas reduction of samples immunoprecipitated with the βB-antiserum resulted in a smaller amount of 18-kDa free α-subunit (from inhibin) and more 14-kDa free β-subunit (from inhibin and activin; Fig. 2B).

On Western blots, the antiserum against the inhibin βB-subunit detected a 24- to 25-kDa protein in SCCM, whereas preimmune serum did not. This antiserum also stained 30-kDa inhibin. Figure 3 shows the results of a representative experiment of four experiments performed. After reduction using β-mercaptoethanol, the 24- to 25-kDa protein disappeared, whereas free β-subunit appeared (Fig. 3). The α-antiserum detected 26-kDa pro-αC and 30-kDa inhibin, as described previously (11). After reduction, free α-subunit was detected.

Effect of recombinant bovine activin-A on FSH-stimulated aromatase activity

FSH stimulated estradiol levels dose-dependently in culture medium of Sertoli cells obtained from 21-day-old rats after 72 h of culture (not shown). Recombinant bovine activin-A had an inhibitory effect on the aromatase activity induced by 20 ng ovine FSH/ml (Fig. 4), without a significant effect on the amount of Sertoli cell DNA. At 50 ng activin-A/ml, a significant inhibition of 25.4 ± 3.3% (mean ± SEM; n = 4 incubations; $P = 0.01$, by Tukey's test) was found. Basal aromatase activity was not affected (not shown). In two similar experiments with Sertoli cells from 17-day-old rats, 50 ng activin-A/ml also produced a significant inhibition of aromatase activity stimulated with 50 ng oFSH/ml: estradiol levels decreased to 82.4 ± 1.3% and 74.1 ± 6.6% (mean ± SEM; n = 4 incubations; $P < 0.05$, by Student's t test) of control values after 72 h of culture. Finally, in a short term (24-h) experiment with Sertoli cells obtained from 17-day-old rats, recombinant bovine activin-A suppressed FSH-stimulated (50 ng/ml) aromatase activity significantly to 54.8 ± 2.6% of control values (mean ± SEM; n = 4 incubations; $P < 0.01$, by Student's t test).

Effect of recombinant bovine activin-A on FSH and androgen receptor expression

Culture of Sertoli cells obtained from 17- to 21-day-old rats for 24 h in the presence of oFSH resulted in increased

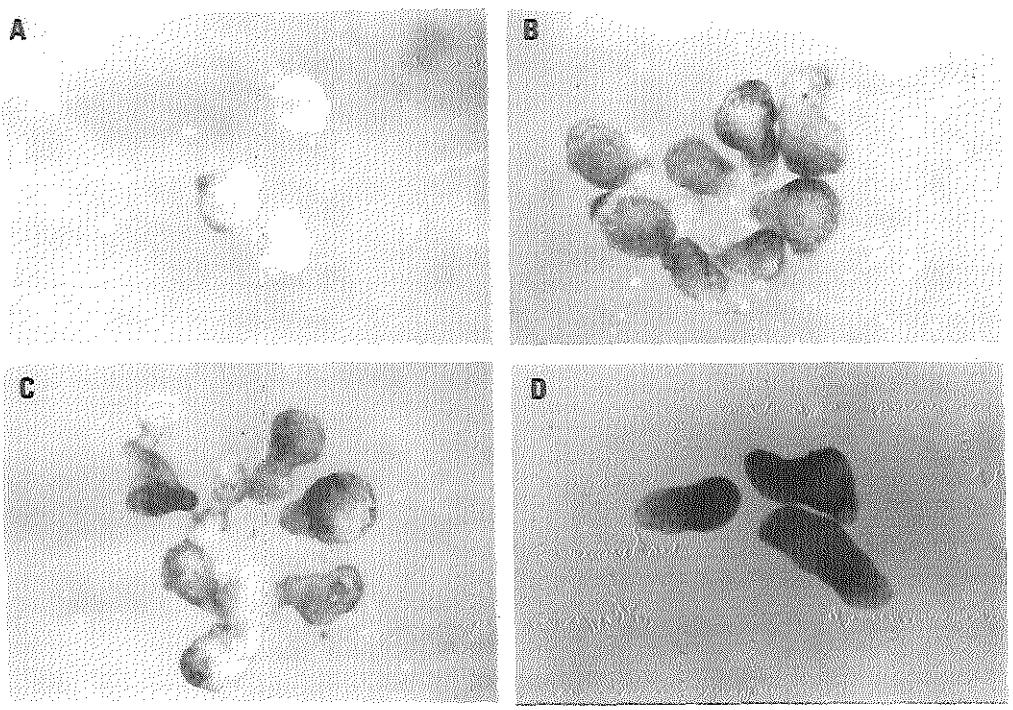

FIG. 1. Effect of conditioned media from Sertoli cells and TM4 cells on elongation of animal cap explants isolated from stage 8 *Xenopus laevis* embryos. Explants were cultured for 1 day in 0.75% normal amphibian medium (NAM) containing 0.1% BSA (A), in the same medium diluted 1:2 with concentrated conditioned medium from control Sertoli cells (B) or oFSH-stimulated Sertoli cells (C), and with a 1:6 dilution of nonconcentrated medium of TM4 cells (D). The experiments were performed three or four times with animal caps from different embryos. The figure is a representative sample from one experiment.

TABLE 1. Concentration of bioactive TGFβ present in conditioned medium used in the animal cap bioassay

Conditioned medium	TGFβ (ng/ml)
Control Sertoli cell (nonconcentrated)	0.83
FSH-stimulated Sertoli cell (nonconcentrated)	0.66
Control Sertoli cell (50-fold concentrated)	87.8
FSH-stimulated Sertoli cell (25-fold concentrated)	20.0
TM4 cell (nonconcentrated)	1.20

Mink lung epithelial cells were cultured in 2-fold serial dilutions of conditioned medium or TGFβ standard (TGFβ1 or TGFβ2), as described in *Materials and Methods*. Each sample was tested in duplicate. The concentration of TGFβ in the conditioned medium was calculated by comparing the growth inhibition induced by the conditioned medium with the inhibition obtained using the TGFβ standard.

androgen receptor mRNA expression (182.3 ± 36.0% of control values; mean ± SEM; n = 3 experiments) and decreased FSH receptor mRNA expression (67.9 ± 12.6% of control values; n = 4 experiments), as reported previously (14, 21). Recombinant bovine activin-A reduced both basal and FSH-stimulated androgen receptor mRNA expression to 53.1 ± 6.3% (n = 3 experiments) and 73.2 ± 7.5% (n = 3 experiments) of values without activin, respectively. The addition of activin-A had no significant effect on basal or FSH-inhibited FSH receptor mRNA expression, reaching 81.4 ± 16.6% (n = 4 experiments) and 86.8 ± 15.9% (n = 4 experiments) of the value obtained without activin. The results of one representative experiment are displayed in Fig. 5A. The decrease in androgen receptor mRNA expression was reflected in a significant decrease in specific [^{3}H]R1881 binding (Table 2).

In short term experiments (4–5 h), FSH has been shown to inhibit androgen receptor mRNA expression (21a). In a single experiment, we also studied whether recombinant bovine activin-A might have an effect on this short term down-regulation and found that after 4 h of culture, basal and FSH-inhibited androgen receptor mRNA expressions were reduced to 62% and 68% (corrected for actin hybridization) of values without activin, respectively (Fig. 5B).

Discussion

An early event in the embryogenesis of vertebrates is the induction of mesodermal tissue from cells of the animal pole

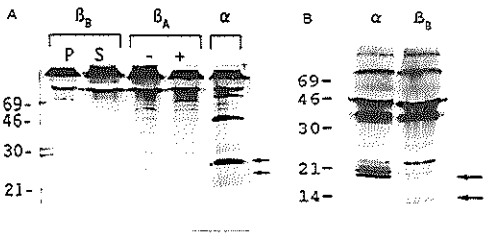

FIG. 2. A. Autoradiogram of immunoprecipitated [^{35}S]methionine-labeled proteins secreted by Sertoli cells. Proteins secreted by Sertoli cells obtained from 21-day-old rats were precipitated with polyclonal antisera against the inhibin βB-subunit (P, preimmune serum; S, antiserum), the inhibin βA-subunit (−, without competition with recombinant bovine activin-A; +, with competition), and the inhibin α-subunit and separated by SDS-PAGE. For description of the antisera, see *Materials and Methods*. *Arrows* indicate 26-kDa pro-αC (*upper arrow*) and 25-kDa activin (*lower arrow*). B. Autoradiogram of immunoprecipitated and reduced [^{35}S]methionine-labeled proteins secreted by Sertoli cells. Proteins were first precipitated with polyclonal antisera against the inhibin α- and βB-subunits and then reduced using 2% β-mercaptoethanol. *Arrows* indicate 18-kDa free α-subunit (*upper arrow*) and 14-kDa free β-subunit (*lower arrow*).

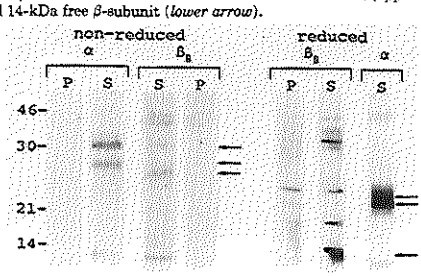

FIG. 3. Western blot of SCCM. Proteins secreted by Sertoli cells obtained from 21-day-old rats were not reduced (*left panel*) or were reduced with 2% β-mercaptoethanol (*right panel*) and separated by SDS-PAGE. Polyclonal antisera against the inhibin βB-subunit and the inhibin α-subunit (P, preimmune serum; S, antiserum) were used to detect inhibin-like proteins. *Arrows in the left panel* indicate 30-kDa inhibin (*upper arrow*), 26-kDa pro-αC (*middle arrow*), and 24- to 25-kDa activin (*lower arrow*). *Arrows in the right panel* indicate free inhibin α-subunit doublet (*upper and middle arrows*) and free inhibin β-subunit (*lower arrow*).

by signals coming from the vegetal pole. This process can be mimicked *in vitro* by members of the heparin-binding growth factor family, acidic and basic fibroblast growth factor (22, 23) and the products of the protooncogenes *int*-2 and *hst* (kfgf) (24), and by members of the TGFβ family, TGFβ2 (25), TGFβ3 (26), activin-A (27–29), and activin-B (30). Recently, the product of protooncogene *int*-1 was found to be involved in mesoderm induction (31). The usual test for mesoderm-inducing activity involves the culture of isolated animal pole explants in the presence of the test factor. In the absence of a mesoderm-inducing component, the test tissue differentiates as epidermis and remains spherical. Induced tissue elongates and mesodermal cell types (mesenchyme, muscle, notochord, and neural tissue) arise. This change in shape can be used as a reliable marker for mesoderm induction (16). In

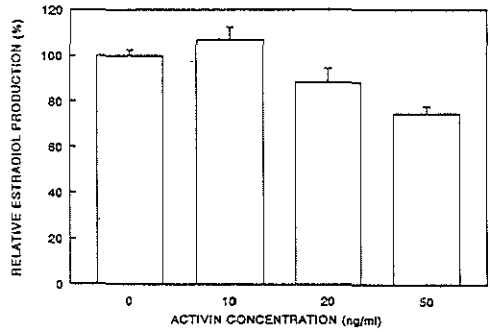

FIG. 4. Effects of different doses of recombinant bovine activin-A on oFSH-stimulated (20 ng/ml) aromatase activity after 72 h of culture. Values are expressed as percentages of oFSH-stimulated estradiol production per μg Sertoli cell DNA. The results represent the mean ± SEM of one experiment (n = 4 incubations).

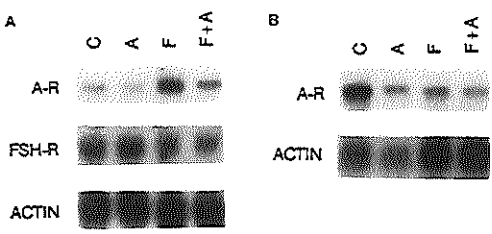

FIG. 5. A. Effects of recombinant bovine activin-A (50 ng/ml) and oFSH (50 ng/ml) on androgen receptor (A-R) and FSH receptor (FSH-R) mRNA expression in rat Sertoli cells isolated from 21-day-old rats after 24 h of culture. B, Effects of recombinant bovine activin-A (50 ng/ml) and oFSH (50 ng/ml) on androgen receptor (A-R) mRNA expression in rat Sertoli cells isolated from 17-day-old rats after 4 h of culture.

a recent study we used this assay to show that Leydig cells and Leydig cell tumors do not produce activin (11).

Culture of *Xenopus* animal pole tissue in diluted conditioned medium from immature rat Sertoli cells and mouse TM4 cells revealed the secretion of a heat-stable mesoderm-inducing factor, which was retained by a filter with a mol wt cut-off at 10 kDa. This excludes the mesoderm-inducing effects of a number of the above-mentioned factors. Basic fibroblast growth factor, which is secreted by Sertoli cells (32), is heat labile (33). TGFβ was detected in SCCM, using the mink lung epithelial cell bioassay, which is sensitive for TGFβ1, TGFβ2, TGFβ3, and TGFβ5 (26, 34). However, the amount of TGFβ present in nonconcentrated SCCM and conditioned medium of TM4 cells was below the concentration necessary for mesoderm induction [TGFβ2, 3–12 ng/ml (25); TGFβ3, 0.8–1.4 ng/ml (26)]. Furthermore, Sertoli cells from immature rat testes only secrete TGFβ1 (35, 36), which has no mesoderm-inducing activity (26). *int*-1 encodes a secretory glycoprotein associated with the cell surface, probably functioning locally in cell to cell signalling (for review, see Ref. 37). In the mouse, *int*-1 mRNA is only expressed in

TABLE 2. Effects of recombinant bovine activin-A and FSH on specific [^{3}H]R1881 binding to rat Sertoli cells after 24 h of culture

Addition	[^{3}H]R1881 binding (% of control)
Control	100.0 ± 1.1
Activin (50 ng/ml)	87.6 ± 2.0[a]
FSH (50 ng/ml)	111.3 ± 1.4[b]
FSH (50 ng/ml) + activin (50 ng/ml)	102.7 ± 3.0[c]

Sertoli cells were isolated from 17-day-old rats and incubated with the indicated additions for 24 h. Subsequently, R1881 binding was estimated, as described in *Materials and Methods*. Values are calculated as percentages of R1881 binding in control cells; the mean absolute number of receptors was 2156/cell. The results represent the mean ± SEM of two experiments, with triplicate incubations.

[a] Significantly different from binding to control cells ($P = 0.002$, by Tukey's test).

[b] Significantly different from binding to control cells ($P = 0.004$, by Tukey's test).

[c] Significantly different from binding to FSH-stimulated cells ($P = 0.034$, by Tukey's test).

postmeiotic male germ cells and midgestational embryos (38). It is, therefore, not likely that the product of *int*-1 is present in conditioned medium of Sertoli cells obtained from immature rat testes. Activin, in contrast, is heat and acid stable (28), is retained by filters with a cut-off at 10 kDa, and can be secreted by Sertoli cells, which express inhibin βB-subunit mRNA (39). Immunoprecipitation of [^{35}S]methionine-labeled Sertoli cell-secreted proteins and Western blotting of SCCM showed the secretion of a 24- to 25-kDa protein, which is recognized by antisera against the inhibin β-subunits. This protein can be reduced to a 14-kDa protein, which is also recognized by the inhibin-βB antiserum. Finally, Grootenhuis *et al.* (12) described a 25-kDa protein in SCCM that can stimulate FSH release from dispersed pituitary cells. This mol wt correlates well with the mol wt of 25.9 kDa described for recombinant human activin-B (40) and 25 kDa described for native porcine activin-B (41). Taking these results together, we conclude that activin is secreted by Sertoli cells from 21-day-old rats *in vitro*.

Sertoli cells isolated from immature rat testes express activin receptor type II mRNA (13). Here we demonstrate that recombinant bovine activin-A can inhibit the FSH-stimulated conversion of testosterone to estradiol. The relative effect after long term (72-h) culture is smaller than after 24 h of culture, possibly because of the endogenous production of activin, the breakdown of the added activin, and the secretion of activin-binding proteins. This can also be the explanation for the relatively high dose of activin needed for a significant response. A similar inhibition of aromatase activity was shown in immature porcine Sertoli cells for TGFβ1 (42). These researchers suggested enhancement of cAMP-phosphodiesterase activity by TGFβ1. In contrast, activin and TGFβ stimulate aromatase activity in granulosa cells (43, 44).

FSH administration to Sertoli cells obtained from immature rats results in a transient down-regulation of androgen receptor mRNA expression at 5 h, followed by an up-regulation of androgen receptor mRNA expression and androgen binding at 24–72 h (21a). Here, we show an inhibitory effect of recombinant bovine activin-A on basal, short term down-regulated and long term up-regulated androgen receptor mRNA expression. In the long term experiments, a similar, but less pronounced, suppressive effect was found on the amount of binding of the synthetic androgen R1881. The smaller effect on androgen binding might be explained by a shorter half-life of the mRNA compared to the half-life of the protein, as described by Blok *et al.* (21a). In contrast, no significant effect was found on FSH receptor mRNA expression.

As discussed above, the effects of activin on the investigated parameters of Sertoli cell function were relatively small, possibly because of endogenous production of activin by the Sertoli cells. However, *in vivo* the ratio of the expression of inhibin α- and βB-subunits varies during the cycle of the seminiferous epithelium (45). This might result in changing activin/inhibin ratios, which, in turn, could affect androgen receptor mRNA expression. Variations in androgen receptor mRNA expression during the cycle of the seminiferous epithelium of the rat testis have actually been described (46). This suggests that Sertoli cells may regulate their sensitivity to androgen, one of the main regulators of spermatogenesis, by changing the ratio between inhibin α- and β-subunits.

The ratio of the expression of inhibin α- and βB-subunit mRNAs in total testes of rats of various ages is constant from day 21 onward (47). This suggests that activin can also be produced by Sertoli cells in the adult testis, provided that the translation efficiencies of these mRNAs do not change. Indeed, Shintani *et al.* (9) detected relatively high levels of immunoreactive activin in rat testicular extract, which are unlikely to be produced by Leydig cells (11).

Apart from the autocrine effects of Sertoli cell-secreted activin, it may also have a paracrine effect on spermatogenesis: Mather *et al.* (8) showed activin-A binding to spermatogonia and stimulation of spermatogonial proliferation by activin-A and -B, de Winter *et al.* (48) detected a high expression of activin receptor type II mRNA in pachytene spermatocytes and round spermatids around meiosis, and Woodruff *et al.* (49) reported binding of fluorescein isothiocyanate-labeled activin-A to spermatogonia, late pachytene-diplotene spermatocytes, and spermatids.

In conclusion, production of activin by immature rat Sertoli cells was demonstrated. Activin can act as a specific autocrine regulator of Sertoli cell function, inhibiting FSH-stimulated aromatase activity, androgen receptor mRNA expression, and androgen binding, without significantly affecting FSH receptor mRNA expression. To our knowledge, activin is the first Sertoli cell-secreted product shown to regulate androgen receptor expression in Sertoli cells.

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Peritubular myoid cells from immature rat testes secrete activin-A and express activin receptor type II in vitro.

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ABSTRACT

The expression of activin type II and IIB receptors, inhibin α-, $ß_A$- and $ß_B$-subunit mRNAs and the secretion of immunoreactive and bioactive activin during culture of testicular peritubular myoid cells and peritubular myoid cell lines were studied.

Cultured peritubular myoid cells and cell lines expressed high levels of inhibin $ß_A$-subunit mRNA and some inhibin α- and $ß_B$-subunit mRNA. Activin receptor type II mRNA was also detected, whereas activin receptor type IIB mRNA expression was not found. Expression of the $ß_A$-subunit mRNA was present immediately after isolation of the cells and increased during culture in Eagle's Minimal Essential Medium containing 10% fetal calf serum. $ß_A$-subunit mRNA expression was not regulated by the synthetic androgen R1881. Western blotting of peritubular myoid cell and peritubular cell line conditioned media with a polyclonal antiserum against recombinant activin-A revealed the presence of 25 kDa activin-A, whereas activin bioactivity was detected using the animal cap assay.

Because of the secretion of activin-A by peritubular myoid cells, the effects of recombinant activin-A on Sertoli cell inhibin and transferrin secretion were examined. Activin-A stimulated both basal and FSH-stimulated inhibin and transferrin production by Sertoli cells after 72 h of culture. These effects resemble the effects of the testicular paracrine factor PmodS on Sertoli cell function.

It is concluded that activin-A is secreted by peritubular cells in vitro and that activin-A shares a number of effects on Sertoli cell function with PmodS.

INTRODUCTION

Activins, homo- or hetero-dimers of the inhibin $ß_A$- and $ß_B$-subunit, were originally purified from ovarian follicular fluid on the basis of their ability to stimulate the synthesis and release of FSH from pituitary cells in culture (1,2). It is currently accepted that activins also exert many other biological functions, and can be regarded as cell differentiation factors (3). Recently, activin receptors (type II and IIB), with homology to serine/threonine kinases, have been cloned (4,5). Of these receptors, activin receptor type II (ActRII) is expressed in several testicular cell types, including Sertoli cells, pachytene spermatocytes and round spermatids (6,7). Activin receptor type IIB is mainly expressed in Sertoli cells and A-spermatogonia (8). Therefore, activins may play an important role as para- or autocrine factors in the testis. Actually, effects of recombinant activin-A on Sertoli cell function have been demonstrated (9,10) and regulatory roles of activins in Leydig cell steroidogenesis (11,12,13) and spermatogenesis (10) have been suggested. Furthermore, the production of activin-B by Sertoli cells from testes of immature rats has been described (9), whereas secretion of activin-A in the immature rat testis has been ascribed to Leydig cells (14).

In this study, the production of activin and the expression of activin receptors in cultured peritubular myoid cells and newly established peritubular cell lines were examined. The effects of activin-A on Sertoli cell function were also studied in more detail and compared with effects described for the peritubular myoid cell product PmodS (15), another testicular paracrine factor, which stimulates the secretion of androgen binding protein (ABP), transferrin and inhibin (16,17) and inhibits FSH-stimulated aromatase activity (18) in rat Sertoli cells.

MATERIALS AND METHODS

Isolation and culture of peritubular myoid cells

All animal experimentation described in this manuscript was conducted in accordance with the highest standards of humane animal care, as outlined in the NIH guide for the care and use of laboratory animals. Peritubular myoid cells were isolated from testes of 21-23-day-old Wistar rats as described by Blok et al. (19). In short, decapsulated testes were incubated 20 minutes at 37°C (120 cycles/min) in phosphate buffered saline (PBS) containing DNase I (5 μg/ml; type DN25, Sigma, St Louis, MO, USA), collagenase (1 mg/ml; type CLS, Worthington, Freehold, NJ, USA), trypsin (1 mg/ml; type TRL, Worthington) and hyaluronidase (1 mg/ml; type I-S, Sigma). Tubular fragments were separated from the peritubular cell fraction by sedimentation at unit gravity. The supernatant was filtered through a 60 μm nylon filter and cells were precipitated by centrifugation at 250xg for 2 min. Cells were plated at a density of approximately 5×10^4 cells/cm^2 in 175 cm^2 culture flasks (Costar, Cambridge, MA, USA) and cultured in Eagle's Minimal Essential Medium (MEM; Gibco, Grand Island, NY, USA) supplemented with antibiotics and non-essential amino acids containing 10% fetal calf serum (FCS, Sebak, Aidenback, Germany). After 2 days of culture, the cells were trypsinized, seeded 1:10 and cultured for another 3 days in MEM/10% FCS. Total RNA was then isolated or the cells were cultured for a final 3 day period in serum-free MEM to obtain conditioned medium. This isolation method is refered to as METHOD 1.

Peritubular myoid cells were also isolated from testes of 19-day-old Wistar rats using an alternative method (METHOD 2). Decapsulated testes of 20 rats were incubated for 70 min at 32°C (140 cycles/min) in 20 ml PBS containing DNase (10 μg/ml; Boehringer Mannheim, Mannheim, Germany) and trypsin (2.5 mg/ml; Boehringer Mannheim) to remove Leydig cells. Trypsinization was stopped by the addition of 0.5 % soybean trypsin inhibitor (Boehringer Mannheim). The tubular fragments were washed three times with PBS and digested for 20 min at 32°C (140 cycles/min) in PBS/DNase supplemented with collagenase (1 mg/ml; Boehringer Mannheim) and hyaluronidase (1 mg/ml; Merck, Darmstadt, Germany). The supernatant was filtered through a nylon screen (Nytal; 100 μm), peritubular cells were seeded in two 175 cm^2 culture flasks (Costar) and cultured for 5h in RPMI (Seromed, Biochrom, Berlin, Germany) containing 10% FCS. Then cells were washed twice with culture medium to remove Sertoli cells and fresh RPMI/10% FCS was added. After 3 days of culture cells were trypsinized, seeded 1:2 and cultured for another 3 days in RPMI/10% FCS. Cells were trypsinized again, seeded 1:2 and cultured for 5 days in RPMI/10% FCS. Total RNA was isolated after this culture period.

Isolation and culture of Sertoli cells

Highly purified Sertoli cells (with less than 0.1 % peritubular myoid cells) from testes of immature (21-day-old) Wistar rats were isolated and cultured using the procedure described by Themmen et al. (20). Briefly, decapsulated testes were incubated for 20 min at 37°C in PBS, containing DNase I (5 μg/ml; type DN25, Sigma), collagenase (1 mg/ml; type CLS, Worthington), trypsin (1 mg/ml; type TRL, Worthington) and hyaluronidase (1 mg/ml; type I-S, Sigma). After four successive washes by sedimentation at unit gravity in PBS/DNase, the tubular fragments were incubated in PBS/DNase containing 1 mg/ml collagenase and 1 mg/ml hyaluronidase for 20 min at 37°C. The fragments were washed 4 times as described above, and dispersed using a Dounce homogenizer. The cells were then washed four times with PBS/DNase and once with MEM by sedimentation at 100xg for 2 min. The cell preparation was plated at a density of $0.5.10^6$ cells/cm^2 in 75 cm^2 culture flasks (Costar) for RNA extraction or in 48 well-plates (Costar) for determination of the effect of recombinant activin-A on Sertoli cell inhibin and transferrin production. The cells were cultured for 48 h at 37°C in MEM supplemented with non-essential amino acids, glutamine, antibiotics and 1 % FCS. Then they were shocked hypotonically for 2 min in 0.1x MEM to remove germ cells. The culture was continued for 24 h in MEM containing 0.1 % bovine serum albumin (BSA, fraction V, Sigma). Subsequently, total RNA was isolated or medium was replaced by MEM containing 0.1 % BSA and hormones (oFSH (S-17), NIH Bethesda, MD, USA; recombinant bovine activin-A, Innogenetics, Ghent, Belgium). After 72 h of culture, medium was collected and frozen until measurement of immunoreactive inhibin and transferrin. Cells were lysed in 1 N NaOH and the DNA content of the cell lysates was determined.

Alkaline phosphatase staining

Peritubular myoid cell preparations were stained for alkaline phosphatase to determine the relative amount of peritubular cells (21). Cells were fixed for 25 min in 70% ethanol and subsequently stained for approximately 20 min in 0.2 M Tris-buffer (pH 9.1) containing 10 mM $MgCl_2$, 2 mM 4-aminodiphenylamine diazonium sulphate (Sigma) and 1.4 mM naphthol AS-MX phosphate (Sigma). Cells were washed with 70% ethanol and examined by microscopy.

Pregnenolone measurement

To determine the presence of Leydig cells in the preparation obtained by METHOD 1, pregnenolone production was measured. Cells were isolated, cultured for 2 days, trypisinized, seeded 1:10 and cultured for an additional 3 days as described in METHOD 1. After a total culture period of 5 days, medium was replaced by MEM containing 0.1% BSA, 5 μM SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphthalenone; Ciba-Geigy, Basel, Switzerland) and 5 μM epostane (4α,5α-epoxy-17ß-hydroxy-4ß,17α-dimethyl-3-oxo-androstane-2α-carbonitrile; Sterling-Wintrop, New York, NY, USA) as inhibitors of pregnenolone metabolism. Cells were cultured for 3 h in the presence or absence of 100 ng ovine LH (NIH-oLH S23)/ml culture medium after which medium was collected and pregnenolone was measured by radioimmunoassay (22).

Establishment of peritubular myoid cell lines

The establishment and the characterization of these cell lines will be published elsewhere in more detail (Briers et al., manuscript in preparation). Briefly, testes from 10-day-old Wistar rats were decapsulated and digested successively at 32°C with collagenase (0.5 mg/ml for 60 min), collagenase (1 mg/ml for 30 min) and hyaluronidase (1 mg/ml for 45 min). After dispersion of the remaining tubular fragments, testicular cells were seeded at $1.3x10^6$ cells/cm^2 in 75 cm^2 culture flasks (Costar) and cultured in RPMI/10% FCS till 75% confluency. Then cells were transfected by lipotransfection (3 μg DNA/10 μl lipofectin/1 ml optimem (Gibco)) with pSVv-myc (ATTC Rockville, MD, USA). This resulted in four rat testicular cell lines (RTC 8T1, RTC 8T3, RTC 8T6 and RTC 8T12). Another peritubular myoid cell line (RTC 8C) originated spontaneously. All cell lines, with the exception of RTC 8T1, were characterized as peritubular myoid-like cells based upon morphology, immunocyto-chemical markers, growth factor secretion and secretion of PModS like activity (23). The nature of RTC 8T1 is still indistinct. The same procedure was used to obtain an immortalized stromal prostate cell line (RSPC 2T).

RNA isolation and Northern blot analysis

Total RNA was isolated by extraction with 3M LiCl and 6 M urea (24), followed by phenol/chloroform extraction. Samples containing 20 μg total RNA were separated by electrophoresis in a denaturing formaldehyde-agarose gel (1% agarose; Sigma) containing ethidium bromide and blotted onto Hybond N+ nylon membrane filters (Amersham, Aylesbury, Buckinghamshire, United Kingdom) by diffusion. Filters were hybridized with ^{32}P-labeled cDNA probes encoding rat inhibin α-subunit (1.25 kb EcoRI fragment, corresponding to the entire α-subunit; α7/pUC18 (25)), rat inhibin $ß_A$-subunit (1.4 kb EcoRI fragment, corresponding to the entire $ß_A$-subunit; ßA30/pUC18 (25)), and rat inhibin $ß_B$-subunit (1.5 kb EcoRI fragment, corresponding to the entire mature part of the inhibin $ß_B$-subunit and parts of the N-terminal domain and 3' untranslated sequences; ßB11/pUC18 (25)); rat activin receptor type II (0.6 kb BamHI/HindIII fragment, corresponding to amino acids 1 to 175 of activin receptor type II (6)); rat activin receptor type IIB (0.5 kb Sac I fragment, corresponding to subdomains II to VIII of the kinase domain of activin receptor type IIB (Wesseling et al., unpublished results)); and hamster actin. All hybridizations were performed for 48 h at 42°C, in hybridization solution containing 50% formamide, 9% (w/v) dextran sulfate, 10xDenhardt's (1x Denhardt's contains 0.02 % (w/v) Ficoll, 0.02 % (w/v) polyvinyl pyrrolidone, 0.02 % (w/v) BSA), 5xSSC (1xSSC contains 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 mM sodium phosphate (pH 6.8) and 100 μg/ml denaturated salmon sperm DNA. After hybridization, blots were washed to a final stringency of 0.1x SSC/0.1 % (w/v) SDS at 42°C and autoradiographed using Hyperfilm-MP (Amersham). The hybridization signals were quantified using a BioRad-1D gel scanner (model 620, BioRad, Richmond, CA, USA). Values were normalized using the signals obtained after hybridization with hamster actin cDNA.

SDS-polyacrylamide gel electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis and Western blotting were carried out as described by de Winter et al. (26). Western blotting was performed with conditioned media from peritubular myoid cells and cell lines, which were concentrated using Centriprep 10 filters, which have a molecular weight cut-off at 10 kDa (Amicon, Danvers, MA, USA). The antisera used were raised against a prokaryotic fusion protein containing the entire mature bovine inhibin β_A-chain (AS 227, Innogenetics, Ghent, Belgium) or against N-terminal amino acid residues 1 to 22 of the α_c-subunit of 32 kDa bovine inhibin (27). The latter antiserum recognizes 30 kDa inhibin, 26 kDa proαc and the 20 kDa α-subunit monomer (9). Goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma), naphtol AS-MX phosphate (Sigma) and 4-aminodiphenylamine diazonium sulfate (Sigma) were used to visualize immunoreactive proteins.

Rat pituitary bioassay

The amount of bioactive activin in the culture medium of peritubular myoid cells and cell lines was determined by an in vitro rat pituitary bioassay system, measuring the stimulation of basal FSH release (27). In this bioassay system activin stimulates FSH release at concentrations above approximately 2.2 ng/ml. Results of the FSH assay have been expressed in terms of NIADDK-rat FSH-RP2. Charcoal-treated bovine follicular fluid (bFF) was used as an inhibin standard. Inhibin bioactivity was immunoneutralized with 5 μl polyclonal antiserum against partially purified bovine inhibin (28).

Animal cap bioassay

Activin bioactivity was also studied using the animal cap bioassay, in which conditioned media from peritubular myoid cells and cell lines were tested for their ability to induce mesoderm in animal cap explants from Xenopus laevis blastulas (stage 8). In this bioassay activin is active at concentrations higher than approximately 0.2 ng/ml. The assay was performed according to a modification of the method of Symes and Smith (29), as described by de Winter et al. (26).

Erythrodifferentiation assay

Another bioassay used to study activin bioactivity is the erythrodifferentiation assay, which is based on the activin-dependent differentiation of human erythro-leukemia cells (K652, CCL 243), visualized by hemoglobin accumulation as described by Yu et al. (30).

Inhibin radioimmunoassay

Inhibin-like immunoreactivity present in the culture media of the different cell preparations was measured using an antiserum against purified 32 kDa bFF inhibin (As 1989) and iodinated 32 kDa bFF inhibin as described by Robertson et al. (31). These materials were kindly donated by the NICHHD. Bovine follicular fluid with an arbitrary potency of 1 U/μg protein (27) was used as an inhibin standard.

Transferrin radioimmunoassay

Sertoli cell transferrin production was measured by radioimmunoassay using a rabbit antiserum against rat transferrin as described by Swinnen et al. (32).

Estimation of DNA

The DNA content of the cell lysates was determined by a fluorometric assay using 3,5-diaminobenzoic acid dihydrochloride (DABA) as a fluorescent dye (Aldrich-Chemie, Steinheim, Germany). Samples were neutralized with 1 N HCl. A 50 μl portion of the neutralized sample was mixed with 50 μl 1.5 M DABA and incubated for 45 min at 60°C. Subsequently 2 ml 1 N HCl was added and the fluorescence of the samples was measured using a Perkin Elmer fluorimeter at wavelengths of 415 nm (excitation) and 500 nm (emission). Calf thymus DNA was used as a standard.

RESULTS

Expression of mRNA for the inhibin subunits and activin receptors.

Peritubular myoid cells were isolated by METHOD 1 and stained for alkaline phosphatase after 1, 2 and 5 days of culture in MEM/10% FCS (Fig.1).

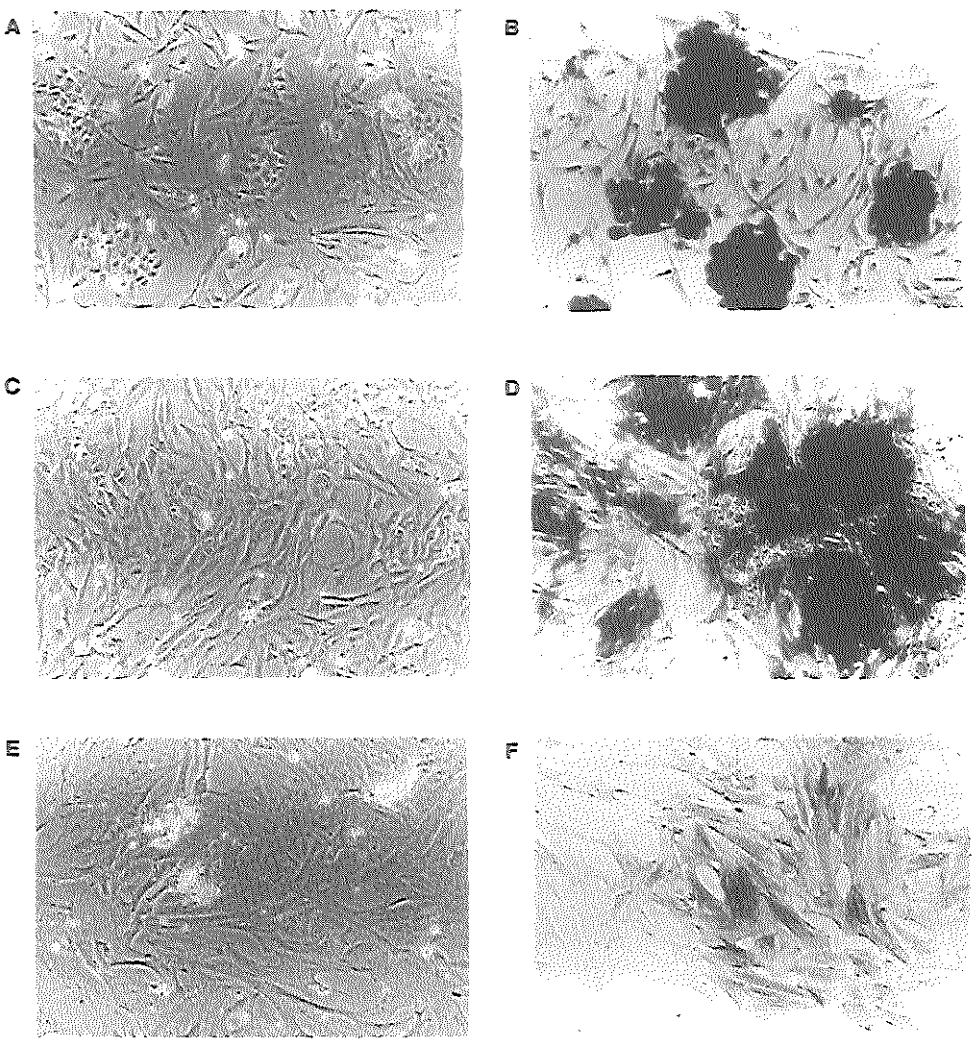

Fig. 1. Alkaline phosphatase staining of peritubular myoid cell preparations cultured in MEM/10% FCS. Peritubular myoid cells were isolated by METHOD 1. Cells were stained after 1 (B), 2 (D) and 5 (F) days of culture. Unstained cells are shown in A (day 1), C (day 2) and E (day 5).

After 1 day of culture approximately 40% of the cells were alkaline phosphatase positive. This increased to approximately 70% after 2 days of culture. After trypsinization at day 5 of culture alkaline phosphatase staining was weaker, but approximately 90% of the cells were positive. In this final culture (5 days after isolation) pregnenolone was detected in the culture medium (5.4 ± 0.43 pmol/3h (mean ± SEM, n=6)), but its level was not stimulated by the addition of LH (5.0 ± 0.39 pmol/3h (mean ± SEM, n=6)). These data indicate that some Leydig cells might still be present in this preparation, but the Leydig cell contamination is below 10%.

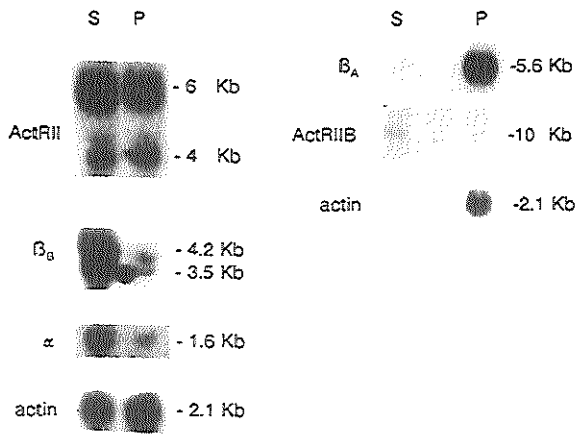

Fig. 2. Expression of mRNA for the inhibin α-, $ß_A$- and $ß_B$- subunit and activin receptor type II (ActRII) and type IIB (ActRIIB) in cultured Sertoli cells (S) and peritubular myoid cells (P). Peritubular myoid cells were isolated by METHOD 1, cultured for 2 days, split 1:10 and than cultured for an additional 3 days. Sertoli cells were cultured for 2 days, hypotonically shocked and cultured for an extra day. Results of two separate blots are shown.

High expression of inhibin $ß_A$-subunit mRNA was found in peritubular myoid cells isolated by METHOD 1 but not in Sertoli cells (Fig.2). Cultured peritubular myoid cells showed a low expression of inhibin α- and $ß_B$-subunit mRNA compared to that in Sertoli cells (Fig.2). Furthermore, two ActRII transcripts (6 and 4 kb) were expressed, whereas no ActRIIB mRNA was detected in the peritubular myoid cells. ActRIIB expression was low in Sertoli cells.

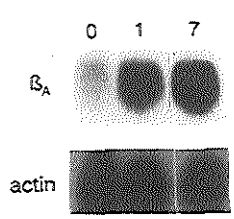

Fig. 3. Expression of inhibin $ß_A$-subunit mRNA in peritubular myoid cells immediatly after isolation (0), after 1 day (1) and after 7 days (7) of culture in MEM containing 10% FCS. Cells were isolated by METHOD 1.

In freshly isolated peritubular myoid cell preparations, expression of the inhibin $ß_A$-subunit mRNA was also found (Fig.3). The expression of this messenger increased after culture in MEM containing 10% fetal calf serum.

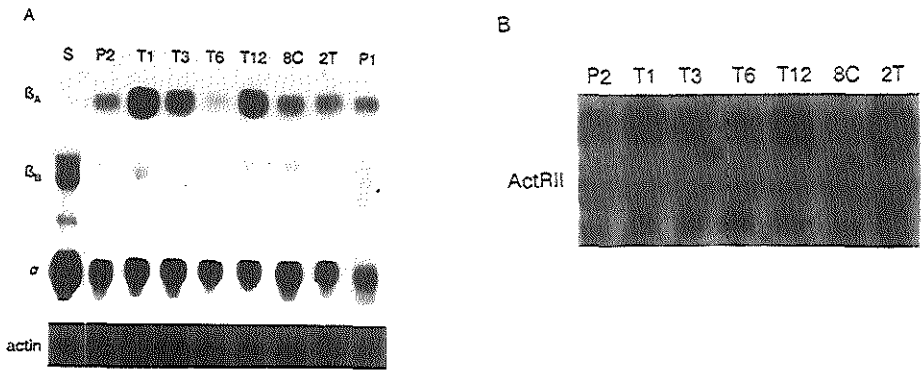

*Fig. 4. **A.** Expression of mRNA for the inhibin α-, $ß_A$- and $ß_B$-subunit in Sertoli cells (S), peritubular myoid cells isolated following METHOD 2 (P2), immortalized peritubular cells (T1, T3, T6, T12 and 8C), immortalized stromal prostate cells (2T) and peritubular myoid cells isolated following METHOD 1 (P1). **B.** Expression of ActRII in P2, T1, T3, T6, T12, 8C and 2T. Both the 4 and 6 kb activin receptor type II transcripts are present.*

In the four different rat peritubular myoid cell lines expression of ActRII and inhibin α-, $ß_A$- and $ß_B$-subunit mRNA was found, like in cultured peritubular cells isolated by METHOD 1 and 2 (Fig.4). In cell line RTC 8T6 the expression of inhibin $ß_A$-subunit mRNA was low, whereas expression of $ß_B$-subunit mRNA was not detected. Immortalized rat stromal prostate cells also expressed ActRII, inhibin α-, $ß_A$- and $ß_B$-subunit mRNA.

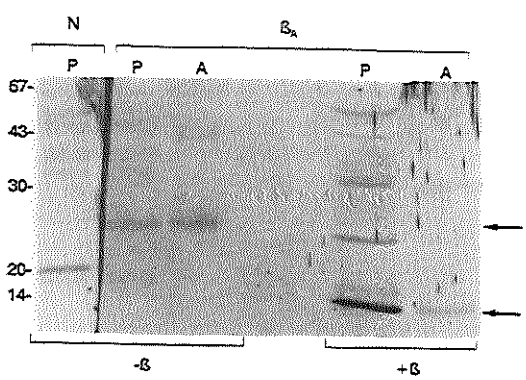

Fig. 5. Activin secretion by peritubular myoid cells. Western blot of peritubular myoid cell conditioned medium (P) and recombinant activin-A (A) was performed with an antiserum against recombinant activin-A ($ß_A$) or normal rabbit serum (N). Samples were not reduced (-ß) or reduced with 2% ß-mercaptoethanol (+ß). Cells were isolated by METHOD 1. Arrows indicate activin (upper arrow) and inhibin $ß_A$-subunit (lower arrow).

Activin production by peritubular myoid cells.

By Western blotting, a 25 kDa protein, comigrating with recombinant activin-A, was detected in peritubular myoid cell conditioned medium, using an antiserum raised against recombinant activin-A (cells prepared by METHOD 1: Fig.5, by METHOD 2: not shown). Using normal rabbit serum, this protein was not stained. Upon reduction an immunoreactive protein of 14 kDa appeared, again indicating the presence of immunoreactive activin-A. The same protein was secreted by the immortalized peritubular and prostate cells (Fig.6). The amount of activin-A was relatively high in those lines, where high expression of $ß_A$-subunit mRNA was found (RTC 8T1, 8T3 and 8T12), whereas low amounts of protein were found in media from RTC 8T6 and RSPC 2T. In the latter cell lines the expression of the $ß_A$-subunit was also low. The secretion of immunoreactive activin by the peritubular cell line RTC 8C was not investigated.

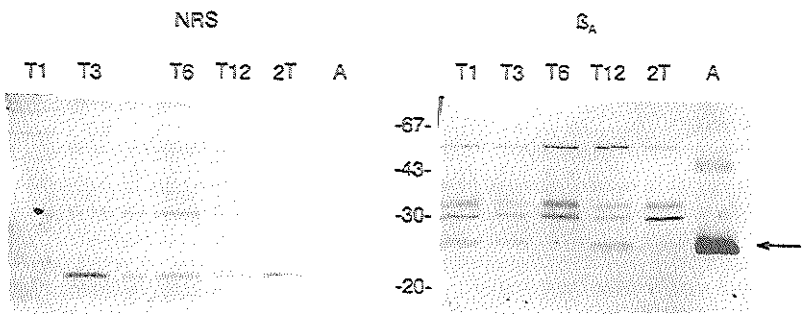

Fig. 6. Activin secretion by peritubular myoid cell lines. Western blot of conditioned media from immortalized peritubular cells (T1, T3, T6 and T12), immortalized stromal prostate cells (2T) and recombinant activin-A (A) was performed with an antiserum against recombinant activin-A ($ß_A$) or normal rabbit serum (NRS). Arrow indicates activin.

Secretion of bioactive activin.

In an in vitro rat pituitary bioassay system we found no significant increase in FSH release with media from different peritubular cultures and peritubular cell lines. To exclude that this was due to the presence of bioactive inhibin, interfering with activin, inhibin bioactivity was immunoneutralized. In these experiments again no increase in FSH release was found with the media tested, although in the same experiment the inhibiting effect of follicular fluid on FSH release was blocked. This leads to the conclusion that the activin concentration in these media is below the detection limit of the assay.

In the "animal cap bioassay" activin-like material was demonstrated to be present in media of peritubular cells and cell lines. The strongest mesoderm induction was found with medium from the RTC 8T1 cell line. This medium also stimulated hemoglobin production in K562 cells before and after heat treatment (5 min, 95°C). The results of these bioassays have been summarized in TABLE 1.

Secretion of other inhibin-like proteins

As shown in figure 4, peritubular cells expressed inhibin α-subunit mRNA after culture. Therefore, the amount of immunoreactive inhibin secreted by the cells was

measured. No immunoreactive inhibin was detected in the culture medium. Western blotting with an antibody against N-terminal amino acid residues 1 to 22 of the αc-subunit of 32 kDa bovine inhibin revealed the presence of a 20 kDa protein in culture medium of RTC 8T1 both before and after reduction with 2% ß-mercaptoethanol (Fig.7). This 20 kDa protein was not detected by pre-immune serum from the rabbit in which the antiserum was raised. The same result was obtained with media from RTC 8T3 and RTC 8T6, whereas in medium from RTC 8T12 a small amount of the 20 kDa protein was detected after reduction only (not shown).

TABLE 1. *Secretion of biologically active activin by peritubular myoid cells and peritubular cell lines determined by different bioassays.*

	P1	P2	8T1	8T3	8T6	8T12	8C
Pit -	-	-	-	-	-	-	-
+	-	-	-	-	-	-	-
Cap	+	+	++	+	+	+	nd
K562	nd	nd	+	nd	nd	nd	nd

Culture media of peritubular myoid cells isolated by METHOD 1 (P1) or METHOD 2 (P2) and peritubular myoid cell lines were tested for the presence of bioactive activin in a rat pituitary bioassay (Pit), without (-) or with (+) inhibin immunoneutralization; in the animal cap bioassay (Cap) or in the K562 cell assay (K562). The following symbols are used: - no response, + response, ++ strong response, nd not determined. Data of 2 or 3 separate experiments, which all yielded similar results, have been summarized.

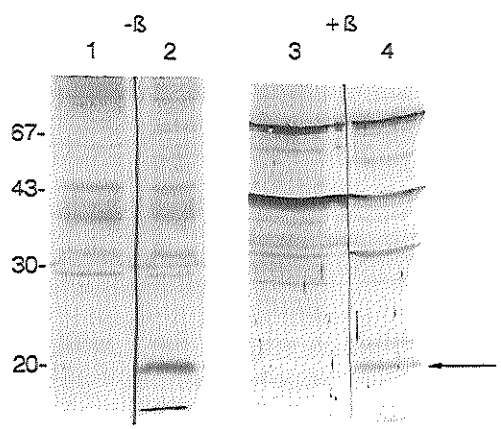

Fig. 7. Secretion of inhibin α-subunit by peritubular cells. Western blot of conditioned medium from RTC 8T1, before (-ß) and after reduction (+ß) with 2% ß-mercaptoethanol. Lanes 2 and 4 were stained with antiserum against N-terminal amino acid residues 1 to 22 of the αc-subunit of 32 kDa bovine inhibin, lanes 1 and 3 were stained with serum from the same rabbit obtained before immunization (preimmune serum). Arrow indicates the 20 kDa α_c-subunit.

Regulation of $ß_A$-subunit mRNA expression in peritubular cells.

Figure 8 shows a representive experiment, in which the peritubular myoid cells

were cultured for 24h in the presence of Sertoli cell conditioned medium (SCCM), the synthetic androgen R1881 or dibutyryl cAMP (dbcAMP), starting two days after isolation. Compared to untreated peritubular myoid cells, SCCM stimulated the expression of $ß_A$-subunit mRNA (200 ± 38.9% of control values; mean ± sd; n = 2). Despite the presence of androgen receptors (not shown) R1881 had no significant effect (76.3 ± 21.7% of control values; mean ± sd; n = 3), whereas dbcAMP inhibited the expression of $ß_A$-subunit mRNA (46.0 ± 35.8% of control values; mean ± sd; n = 3).

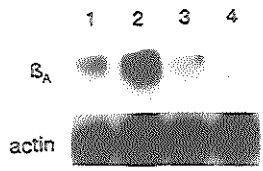

Fig. 8. Effects of various factors on inhibin $ß_A$-subunit mRNA expression in peritubular myoid cells. Two days after isolation by METHOD 1 peritubular myoid cells were cultured for 24h in MEM (lane 1) or MEM supplemented with Sertoli cell conditioned medium (lane 2), 1.10^{-9} M R1881 (lane 3) or 5.10^{-4} M dibutyryl cAMP (lane 4).

Effects of recombinant activin-A on Sertoli function.

Recombinant activin-A significantly stimulated the basal and FSH stimulated secretion of immunoreactive inhibin and transferrin by Sertoli cells obtained from testes of 21-day-old rats. Results of one representative experiment are shown in Table 2. Similar results were obtained in two independent experiments. Combination of the data from these three experiments gave rise to the same significant differences as indicated in Table 2, although for transferrin the differences were not always significant in the separate comparisons. As overall effect inhibin secretion was stimulated to 157 ± 14% of control values (mean ± SEM, $P<0.01$, by Student's *t* test), whereas transferrin secretion was stimulated to 113.8 ± 3.8% of control values (mean ± SEM, $0.02<P<0.05$, by Student's *t* test). There was no effect of activin-A on the amount of Sertoli cell DNA (not shown).

***TABLE 2.** Effects of recombinant activin-A on secretion of immunoreactive inhibin and transferrin by rat Sertoli cells after 72 h of culture.*

Addition	Inhibin secretion (U/µg Sertoli cell DNA)	Transferrin secretion (ng/µg Sertoli cell DNA)
Control	17.0 ± 1.3	184.2 ± 3.6
Activin (50 ng/ml)	27.8 ± 1.2[a]	209.2 ± 7.9[b]
FSH (50 ng/ml)	63.9 ± 5.1	274.1 ± 14.2
FSH (50 ng/ml) + Activin (50 ng/ml)	85.7 ± 3.8[a]	319.6 ± 8.9[b]

Sertoli cells were isolated from 21-day-old rats and incubated with the indicated hormones for 72 h. Subsequently, immunoreactive inhibin and transferrin were measured in the culture medium. Results represent the mean ± SEM of one experiment with quintuplate incubations.

[a]Significantly different from values without activin ($P<0.01$, by Student's t test).

[b]Significantly different from values without activin ($0.02<P<0.05$, by Student's t test).

DISCUSSION

Cultured peritubular myoid cells and peritubular cell lines express inhibin $ß_A$-subunit mRNA and secrete immunoreactive and bioactive activin-A. Since peritubular myoid cells and stromal prostate cells show morphological and functional similarities (32,33,34), it is interesting to note that activin is also secreted by immortalized stromal prostate cells, especially since activin-A has been reported as an inhibitor of the growth of the human prostatic carcinoma cell line LNCaP (35).

Expression of inhibin $ß_A$-subunit mRNA was found in peritubular cell preparations immediately after isolation. Nevertheless, it remains unclear if peritubular myoid cells *in vivo* do secrete activin. Several authors investigated the expression of inhibin subunit mRNAs (7,36) and inhibin subunit proteins (36,37,38) in rat testes using in situ hybridization and immunocytochemistry, but no one reported the presence of $ß_A$-subunits in peritubular myoid cells. This could be due to the flat nature of these cells and the small amount of cytoplasm in which the mRNA and protein should be localized. The low initial expression of $ß_A$-subunit mRNA increased during culture in MEM/10% FCS. This suggests that the activin production *in vitro* may partially be a culture artifact. This raises the interesting question whether purification of candidate testicular paracrine factors, like PmodS, from culture medium of testicular cells provides a good model system for the interactions between testicular cells *in vivo*. As for activin, the secretion of PmodS by peritibular myoid cells *in vivo* has not been demonstrated yet. The expression of the inhibin $ß_A$-subunit is inhibited by dbcAMP, which could indicate that activation of this second messenger system *in vivo* is responsable for the low initial $ß_A$-subunit expression. A factor that could possibly increase the expression of the inhibin $ß_A$-subunit in peritubular myoid cells is TGF-ß as has been shown in vascular endothelial cells (39) and differentiated cell lines derived from P19 embryonal carcinoma cells (40). TGF-ß is present in serum (41), fetal calf serum (Vanderstichele, non-published results) and Sertoli cell culture medium (42), of which the latter two increase the expression of the inhibin $ß_A$-subunit. Moreover, TGFß1 increases the production of a number of radiolabeled proteins secreted by peritubular myoid cells (42).

Activin secretion by Leydig cells from immature rat testis has also been reported (14), but seems to be restricted to the first 19 days postnatally (43). In Leydig cells from 21-day-old and adult rat testes no activin was detected (26). Therefore, it is unlikely that the activin found in the peritubular myoid cell cultures obtained from testes of 21 to 23-day-old rats using METHOD 1, is of Leydig cell origin, although some Leydig cells might be present. Moreover, Leydig cells were removed by trypsin treatment in METHOD 2, which was demonstrated by the absence of C_{19} and C_{21} steroids in the culture medium of these cells and their insensitivity to LH (44). In these cells and in the peritubular myoid cell lines $ß_A$-subunit expression and activin-A secretion were also found.

Despite the expression of inhibin α-subunit mRNA in peritubular myoid cells and cell lines, neither inhibin immunoreactivity nor inhibin bioactivity was detected in the culture medium. This surprising result can be explained on the basis of the results of Western blotting of peritubular myoid cell line culture medium with an antibody against amino acid residues 1-22 of the αc-subunit of bovine inhibin, in which the 20 kDa inhibin α-subunit monomer was detected. This inhibin α-subunit monomer is not recognized by the radioimmunoassay used (31). In culture medium of Sertoli cells the

20 kDa monomer is only found after reduction of the secreted proteins and is derived from 32 kDa inhibin and 29 kDa proαc (45). This indicates that the processing of the inhibin α-subunit in peritubular myoid cells is different from that in Sertoli cells and Leydig cells as in the latter cells proαc is also found (26).

Peritubular myoid cells and peritubular myoid cell lines also express activin receptor type II. Therefore auto- and paracrine effects of activin on these cells can be expected. The expression of activin receptor type II in peritubular cells is comparable to that in Sertoli cells, but is much higher than the expression found in Leydig cell preparations (6).

Mesenchyme-derived peritubular myoid cells surround the seminiferous tubules and form a basement membrane in cooperation with epithelium-derived Sertoli cells (46). The close proximity between peritubular myoid cells and Sertoli cells suggests a mutual effect on the function of both cell types. In fact, conditioned medium from Sertoli cells of 20-day-old rats contains heparinoids that inhibit the incorporation of [^{3}H]-thymidine and the proliferation of peritubular myoid cells (47). On the other hand, several authors reported effects of peritubular myoid cells on Sertoli cell function. Coculture of peritubular myoid cells and Sertoli cells causes the formation of seminiferous tubule-like structures (48,49). Activin secretion by peritubular myoid cells might be an explanation for this phenomenon, since Sertoli cell monolayers reaggregate into tubule-like structures upon activin treatment (10). Furthermore, the secretion of androgen binding protein (33,50,51,52,53), transferrin (33,51,52,54), α_2-macroglobulin and clusterin (55) from Sertoli cells is stimulated by coculture with or conditioned medium from peritubular myoid cells, whereas Sertoli cell aromatase activity (33,44) and production of plasminogen activator (56) are inhibited. Most of these effects are attributed to the androgen regulated peritubular myoid cell product PmodS (15), which stimulates the secretion of androgen binding protein (ABP), transferrin and inhibin by Sertoli cells (16,17) and inhibits FSH-stimulated aromatase activity in Sertoli cells from 20-day-old rats (18). PmodS has no effect on plasminogen activator activity (16), while effects on α_2-macroglobulin and clusterin have not been documented yet.

Recently, we demonstrated that activin inhibits FSH-stimulated aromatase activity in Sertoli cells (9). Here we show that activin-A stimulates Sertoli cell production of immunoreactive inhibin and transferrin. Stimulation of inhibin secretion by activin-A has also been demonstrated in cultured granulosa cells (57) and may be an autocrine protection mechanism against overexposure to activin.

Comparing activin-A and PmodS, it is likely that we deal with different factors. The molecular weights of PmodS A (56 kDa, reducing conditions (15)) and PmodS B (59 kDa, reducing conditions (15)) are higher than the molecular weight of activin-A (14 kDa, reducing conditions). Another difference is the effect on Leydig cells; activin can inhibit Leydig cell steroidogenesis (11,12,13), whereas PmodS has no effect (58). Furthermore, the secretion of PmodS is stimulated by androgens (52), while we found no effect of the synthetic androgen R1881 on peritubular expression of the inhibin β_A-subunit mRNA. Finally, the effects of activin-A on Sertoli cell transferrin production are less pronounced than the effects of PmodS. However, the present results indicate that a number of effects of peritubular myoid cell conditioned medium, which have been ascribed to PmodS, may also be partially due to the presence of activin-A in this medium. From this point of view, it would be interesting to study the effects of activin-A on the secretion of ABP, plasminogen activator, α_2-macroglobulin and clusterin by

Sertoli cells, as peritubular myoid cells affect these parameters.

In conclusion, activin-A secretion by peritubular myoid cells and immortalized peritubular and stromal prostate cells *in vitro* was demonstrated in this study. Activin-A shares some effects on Sertoli cell function with the testicular paracrine factor PmodS: it inhibits FSH-stimulated aromatase activity and stimulates inhibin and transferrin secretion. However, activin secretion seems not to be regulated by androgens and the characteristics of both substances are dissimilar. We therefore conclude that peritubular myoid cell derived activin-A has PmodS-like effects on Sertoli cells, but is not identical with PmodS.

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CHAPTER 6

DISCUSSION

Since the introduction of the inhibin concept by Mottram and Cramer (1923) and McCullagh (1932), inhibin was considered as a gonadal hormone exerting a negative feedback action on pituitary FSH-release. However, the purification of inhibin and activin, and the cloning of the inhibin subunit cDNAs in the mid-eighties (reviewed by de Jong et al., 1990) heralded a new era of inhibin research. Using the inhibin cDNAs, expression of the inhibin subunits was not only found in the gonads, where it was to be expected, but also in extra-gonadal tissues (Meunier et al., 1988). Surprisingly, inhibin subunits were also present in the pituitary, which brought about the concept of inhibin and activin as para- and autocrine factors. Recombinant expression of the inhibin cDNAs yielded recombinant inhibin and activin, which were fruitfully used to demonstrate para- and autocrine effects of inhibin and activin in gonadal and extra-gonadal tissues, as has been described in **section 1.7**. The bioactivity of activin can be neutralized by the activin binding protein follistatin, which can also bind inhibin (**section 1.6.1**). With the cloning of several activin receptors (Mathews and Vale, 1991; Attisano et al., 1992; Attisano et al., 1993) a new phase of inhibin and activin biology has been entered, in which the mechanisms of inhibin and activin action will be unraveled.

6.1 Inhibin and activin receptors

Although activin receptor type II was the first TGF-ß superfamily receptor to be cloned, much more is known about the TGF-ß receptors than about the activin receptors at the moment. As has been discussed in **section 1.5**, type I, II and III receptors for TGF-ß exist, which cooperate to transduce the TGF-ß signal (López-Casillas et al., 1993). The type III receptor is a ligand presenting receptor, which eliminates biological differences between TGF-ß isoforms. The interaction of the type I and type II receptor generates an active signalling complex which translates a TGF-ß stimulus into an intracellular signal (Franzén et al., 1993). From these results it is clear that the combined action of the different TGF-ß receptors determines the cellular response to a TGF-ß stimulus. Therefore, the expression and regulation of the different receptor types will be important factors in determining sensitivity to TGF-ß.

As shown in table I, cross-linking of radiolabelled activin has revealed activin binding sites on several activin responsive cells. The cross-linked complexes resemble the TGF-ß type I, II and III receptor complexes and vary depending on the cell type examined. Two different activin type II receptors (ActRII and ActRIIB) have been cloned (Mathews and Vale, 1991; Attisano et al., 1992). Furthermore, an activin type I receptor has been cloned (Ebner et al., 1993a), which generates an active signalling complex with ActRII and ActRIIB (Attisano et al., 1993). For this reason, the activin signalling system appears to be similar to that of the TGF-ßs. The activin type II receptors seem to have similar affinity for activin-A, -AB and -B, whereas the affinity for inhibin is 10-fold lower. The intriguing question arises whether a type III receptor can increase the binding of inhibin to the activin receptors, as was found for the binding of different TGF-ß isoforms to the TGF-ß type II receptor. This is a relevant suggestion, because inhibin and activin share a common ß-subunit, which could bind to the same type II receptors. In this model, the specificity for the different inhibin and activin molecules will depend on inhibin and activin specific type I receptors. Another possiblity is that the inhibin receptor is a combination of an α-subunit specific type II receptor and an ß-subunit specific

type I receptor. In this view, the combined expression of the different types of receptors will determine whether cells are sensitive to inhibin, activin or both.

The proposed model resembles the interaction of platelet-derived growth factor (PDGF) and its receptors (reviewed by Heldin and Westermark, 1990). PDGF is a dimeric 30 kDa protein composed of disulfide-linked A and B chains, which are synthesized as large precursor molecules. The three possible dimers AA, AB and BB have been purified. The availability of these PDGF isoforms has led to the identification of two distinct but structurally similar receptor types (α and ß), with tyrosine kinase activity. The α-receptor binds all PDGF isoforms, whereas the ß-receptor binds PDGF-BB with high affinity, PDGF-AB with lower affinity and does not bind PDGF-AA. Dimerization of two receptor molecules is essential for the activation of the kinase activity of the receptor complex, which can be a hetero- or homodimer. In contrast to the PDGF receptor system, only heterodimers of activin type I and type II receptors appear to yield a biological response. In accordance with this hypothesis, no ligand-induced autophosphorylation of ActRIIB has been observed (Nakamura et al., 1992b), whereas the ligand-induced autophosphorylation of ActRII is very weak compared to that of the PDGF receptor (Ichijo et al., 1993). This could indicate that the type I receptor phosphorylates the type II receptor after ligand induced dimerization. This possibility can be studied by co-transfection experiments of receptors with normal and inactive kinase domains, coupled to metabolic phosphate labelling and immunoprecipitation. These and other experiments will resolve the many questions that surround inhibin and activin signal transduction. One of these questions concerns the specificity of type I receptors. Neither activin nor TGF-ß type I receptors bind exclusively activin or TGF-ß (Attisano et al., 1993; Ebner et al., 1993b), although they signal only in the presence of a specific ligand and a specific type II receptor. This indicates that activin and TGF-ß can have antagonistic effects on cells were activin and TGF-ß type II receptors are present, which compete for the same type I receptor that only signals in a complex with one of these type II receptors. On the other hand it is possible that some promiscuity exists in that activin and TGF-ß share some of the type I receptors to give the same signal. In this respect the signal transduction of the TGF-ß superfamily members resembles that of the cytokine receptors. The receptors for interleukin-3, interleukin-5 and granulocyte-macrophage colony stimulating factor are composed of a ligand specific α-chain and a common ß-chain (reviewed by Miyajima et al., 1992). These cytokines can induce similar signals on their respective target cells, which indicates that this common ß-subunit is likely to be the key signal transducer. The same has been found for the interleukin-6, leukemia inhibitory factor, oncostatin M and ciliary neurotrophic factor receptors, which share a 130 kDa signal transducer, designated gp130 (reviewed by Kishimoto et al., 1992). Furthermore, the interleukin-2 receptor exists of an α-, ß- and γ-chain (reviewed by Taniguchi and Minami, 1993) of which the γ-chain is shared between the receptors for interleukin-2, interleukin-4 and interleukin-7 (Kondo et al., 1993; Noguchi et al., 1993). However, these cytokines can induce different signals. A difference between growth factor receptors and these cytokine receptors is the lack of a kinase domain in the latter group of receptors. Therefore, these receptors use their interaction with the Src family of nonreceptor tyrosine kinases for signal transduction.

The knowledge and techniques from growth factor and cytokine receptors

will be helpfull to understand the mechanisms of TGF-ß superfamily signal transduction.

6.2 Activin receptors in the testis

Two ActRII transcripts of 4 and 6 kb are present in total testis RNA of mouse (Mathews and Vale, 1991) and rat (**chapter 2**; Feng et al., 1993). Between day 21 and 28 of postnatal development in the rat, the relative expression of the 4 kb mRNA strongly increases due to an increase in the number of pachytene spermatocytes and the appearance of round spermatids, which both express this mRNA (**chapter 2**). More precisely, ActRII mRNA is expressed in spermatocytes and spermatids around the time of meiotic division at stages XIII-III of the seminiferous epithelial cycle (**chapter 2 supplement**; Kaipia et al., 1992). The expression of the 6 kb ActRII transcript decreases with increasing age, because of a relative rise in the numbers of cells lacking this mRNA. The 6 kb mRNA (and the 4 kb mRNA) are expressed in Sertoli cells (**chapter 2**) and peritubular myoid cells (**chapter 5**). The expression of ActRII mRNA in Leydig cells is doubtful, since the low expression found in a partially pure Leydig cell preparation (**chapter 2**) could result from contaminating peritubular myoid cells. The expression pattern of ActRIIB in total testis RNA is comparable to that of the 6 kb ActRII transcript (Wesseling et al., non-published results; Feng et al., 1993). ActRIIB mRNA is expressed in Sertoli cells (**chapter 5**) and A_1/A_2 spermatogonia (Kaipia et al., 1993). Since activin-A is a potential regulator of Leydig cell steroidogenesis (Hsueh et al., 1987; Lin et al., 1989; Mauduit et al., 1991) and the expression of ActRII mRNA in Leydig cells is doubtful, ActRIIB mRNA is possibly also expressed in Leydig cells, provided that no other activin type II receptors exist.

With the knowledge of the expression pattern of ActRII and ActRIIB mRNA, it is important to investigate the receptor proteins in the testis. For this purpose, specific receptor antibodies are necessary to perform immunocytochemistry, immunoprecipitation and Western blotting. A first attempt to obtain and characterize antibodies against ActRII has been described in **chapter 2 supplement**. Another way to investigate the receptor protein should be binding studies with labelled activins (AA, AB and BB) to obtain more information about receptor affinity and number. Binding of FITC-labelled activin to isolated spermatogonia, late pachytene/diplotene spermatocytes and spermatids has been demonstrated (Woodruff et al., 1992), but nothing is known about receptor affinity and number. Cross-linking of cells with labelled activin and resolving of the labelled hormone-receptor complexes by SDS-PAGE will probably reveal other activin binding proteins including type I and III receptors. Studies related to the expression of activin type II receptors should be extended with investigations on the expression of type I and type III receptors. Concerning the type I receptor, special attention should be given to **R2** (**ALK-4**), which is expressed in the testis (He et al., 1993), because the activin specific type I receptor **TSK 7L** (**ALK-2**, **SKR1**, **R1**, **ActXR1**) is expressed at very low levels in the testis (Tsuchida et al., 1993). Furthermore, **ALK-1** (**R3**, **TSR-1**) might be involved in transmission of the activin signal, as it has been shown to bind activin (Attisano et al., 1993).

6.3 Production of activins in the testis

High levels of immunoreactive activin have been demonstrated to be present in testis homogenates (Shintani et al., 1991), suggesting a testicular source of activin. First, Leydig cells were considered as an activin secreting cell type within the rat testis (Lee et al., 1989). Nevertheless, activin secretion by rat Leydig cells becomes undetectable from 19 days of age onwards (Mather et al., 1992). In Leydig cells from 21-day-old and adult rats only the inhibin α-subunit mRNA is expressed and only inhibin α-subunit protein, the function of which is not understood yet, is secreted (**chapter 3**). In contrast, Risbridger et al. (1989) reported the secretion of immunoreactive and bioactive inhibin by Leydig cells from adult rats. The reason for these diverging observations could be the presence of follistatin in Leydig cell culture medium. Follistatin has been detected in Leydig cells from the adult rat testis by immunocytochemistry (Kogawa et al., 1991b), whereas immunoreactive follistatin was also found in culture medium of Leydig cells from mature rats (Risbridger et al., 1991). In the study of Risbridger et al. (1989), inhibin bioactivity was measured using pituitary cells from immature female rats, while in **chapter 3** pituitary cells from adult male rats were used. As described in **section 1.7** the pituitaries of immature female rats and adult male rats show different responses to inhibin and activin. *In vivo* administration of activin-A stimulates FSH secretion in immature female rats, but has no effect in adult male rats (Carroll et al., 1991b). Therefore, the pituitaries of immature female rats might be more sensitive to follistatin, which can explain the observed difference.

Peritubular myoid cells from immature rats secrete activin-A *in vitro* (**chapter 5**). The involvement of these cells in the *in vivo* secretion of activin-A needs further study, but seems relevant as in preparations of peritubular myoid cells inhibin β_A-subunit mRNA is detected immediately after isolation. Since the origins of peritubular myoid cells and Leydig cells are closely associated (Skinner et al., 1991) it is interesting that both cell types can secrete activin at certain stages of their development. Results of future research should indicate why the immature Leydig cell stops producing activin and processes the inhibin α-subunit precursor in a different way (**chapter 3**). Apparently, the differentiation to peritubular myoid cells does not affect activin secretion, whereas the peritubular myoid cell is the only cell described so far, which produces the 20 kDa α-subunit of inhibin (**chapter 5**).

In vitro, activin is also secreted by Sertoli cells from immature rats (**chapter 4**). Since these Sertoli cells only express inhibin β_B-subunit mRNA (**chapter 5**), most likely activin-B is produced. From day 21 onwards the ratio of the expression of the inhibin α- and β_B-subunit mRNAs in total testes of rats of various ages is constant (Klaij et al., 1992). Assuming that the translation efficiencies of these mRNAs do not change, this suggests that activin-B can also be produced by Sertoli cells in the adult testis. The synthesis of activin-A seems to be restricted to Sertoli cells associated with a small part of the seminiferous epithelial cycle, since inhibin β_A-subunit mRNA has only been found in Sertoli cells at stages VIII-XI (Kaipia et al., 1992). The independent cyclic expression of the inhibin subunit mRNAs found throughout the seminiferous cycle (Bhasin et al., 1989; Kaipia et al., 1991, 1992; Klaij et al., 1994), suggests that the relative secretion of inhibin and activin depends on the stage of the seminiferous epithelium. Immunocytochemistry has demonstrated the presence of both inhibin α- and β-subunit proteins within the

seminiferous tubules (Roberts et al., 1989b; Saito et al., 1989; Shaha et al., 1989), but it is not clear whether the immunoreactive material represents inhibin, activin or loose subunits. To distinguish between these possibilities it is important to raise antibodies to recombinant inhibins and activins, which recognize only the whole molecule.

Besides the expression of inhibins, activins and their receptors it is important to study the localization of follistatins as they can neutralize activin action (**section 1.6.1**). Follistatins can play a role as auto- or paracrine regulators in the testis by modulating activin bioactivity or by direct effects and should be included in studies concerning activin action. Follistatins have been localized in Leydig cells (Kogawa et al., 1991b; Risbridger et al., 1991), in spermatocytes and spermatids (Kogawa et al., 1991b) and in Sertoli cells during stages IX-XI of the seminiferous cycle (Kaipia et al., 1992).

6.4 Activins and testicular function

The expression of activin receptors in the rat testis and the secretion of activins by several testicular cell types suggest auto- and paracrine effects of activins on testicular function as has been described in **section 1.7.2.2**. Activin-A has been postulated as a regulator of Leydig cell steroidogenesis in a species dependent way. In the neonatal and adult rat activin-A appears to inhibit LH/hCG-stimulated testosterone secretion (Hsueh et al., 1987; Lin et al., 1989), whereas in the immature pig LH-stimulated testosterone secretion is slightly stimulated (Mauduit et al., 1991). The effects of activin on steroidogenesis can be autocrine or paracrine depending on the developmental stage of the animal, since the localization of activin secretion varies during development as mentioned above.

Activins appear to have a direct regulatory role in spermatogenesis. Activin-A binding sites have been demonstrated on isolated spermatogonia (Woodruff et al., 1992). This binding represents ActRIIB, since its mRNA is highly expressed in A_1 and A_2 spermatogonia at stages IX-XI of the spermatogenic cycle (Kaipia et al., 1993). At stages VIII-XI, inhibin $ß_A$-subunit and follistatin are expressed in Sertoli cells (Kaipia et al., 1992). Therefore, a possible role for activin is the initiation of mitotic division of A_1 spermatogonia, which is confirmed by the observation that both activin-A and activin-B can stimulate spermatogonial proliferation in germ cell-Sertoli cell cocultures (Mather et al., 1990). This effect is not inhibited by follistatin (Mather et al., 1993), so that the coexpression of the inhibin $ß_A$-subunit and follistatin probably does not hamper this reponse *in vivo*. In the same cocultures, follistatin completely inhibits the activin induced reaggregation of Sertoli cells (Mather et al., 1993), which express both ActRII (**chapter 2**) and ActRIIB mRNA (**chapter 5**). This difference is probably due to different affinities of the receptors which mediate the activin effect and the amount of receptors present. ActRIIB is expressed as several alternatively spliced isoforms of which ActRIIB2 has a higher affinity for activin-A (K_d 1.0×10^{-10} M) than ActRIIB4 (K_d 3.8×10^{-10} M) or ActRII (K_d 3.1×10^{-10} M) (Attisano et al., 1992). The dissociation constant of follistatin for activin-A binding is estimated between 1.3×10^{-10} M (Xiao et al., 1992) and 9.1×10^{-10} M (Kogawa et al., 1991a), with other authors reporting 5.9×10^{-10} M (Nakamura et al., 1990) and $5.4\text{-}6.8 \times 10^{-10}$ M (Sugino et al., 1993). The latter authors found no differences in affinity for the different follistatin molecules that can

be generated (FS288, FS300 and FS315 in the glycosylated or non-glycosylated state). Another possibility is that the binding of activin-A to some activin receptors is not disturbed by follistatin binding, whereas the binding to others is hindered by follistatin binding. Transfection of the activin receptor cDNAs to perform binding studies in the presence and absence of follistatin should solve these questions.

Isolated late pachytene/diplotene spermatocytes and early round spermatids express ActRII mRNA (**chapter 2**) and bind activin-A (Woodruff et al., 1992). In situ hybridization located ActRII mRNA in spermatocytes and spermatids around the time of meiotic division at stages XIII-III of the seminiferous epithelial cycle (**chapter 2**; Kaipia et al., 1992). This is preceded by an increase in the ratio inhibin β_B-/α-subunit mRNA in Sertoli cells at stages VII-XIII (Bhasin et al., 1989; Klaij et al., 1994), which could result in the secretion of activin-B. Activin-B might play a role in the regulation of the meiotic division or in the onset of spermiogenesis. To study the possible effects of activins on these processes isolated spermatocytes and spermatids could be cultured in the presence or absence of activins and ^{32}P-labelled ortho-phosphate or ^{35}S-labelled methionine, which will hopefully reveal effects on morphology, phosphorylation and protein synthesis, respectively. This might be combined with immunoprecipitation of ActRII to study receptor activation or immunoprecipitation with phosphoserine and phosphothreonine antibodies to find out whether there is specific phosphorylation on serine and threonine in response to activin. Although these phosphorylation studies seem useful, it should be stated that the incorporation of radiolabelled phosphate in germ cells is not very high (Grootegoed, personal communication). To overcome this problem membrane preparations might possibly be used.

Activins can also have indirect effects on spermatogenesis, since activin-A can suppress androgen receptor expression in Sertoli cells from immature rats in vitro (**chapter 4**). This effect of activin may change the sensitivity of Sertoli cells to testosterone, a main regulator of spermatogenesis. Although changes in androgen receptor mRNA expression during the seminiferous epithelial cycle have been described (Linder et al., 1991), it remains to be determined if activins regulate androgen receptor expression *in vivo*. Activin-A also inhibits aromatase activity (**chapter 4**), but the resulting decrease in testosterone conversion cannot compensate for the decrease in androgen receptor expression, because the testicular testosterone levels are 50- to 150-fold higher than necessary for androgen receptor saturation (Buzek and Sandborn, 1990). Moreover, it has been shown that testicular testosterone levels are considerably higher than needed for ongoing spermatogenesis (Huang and Boccabella, 1988; Awoniyi et al., 1989; Sun et al., 1989). Furthermore, activin-A stimulates immunoreactive transferrin secretion from Sertoli cells of immature rats (**chapter 5**). Since Sertoli cell transferrin production increases (Anthony et al., 1991) and aromatase activity decreases (Rosseli and Skinner, 1992) during rat puberty, the effects of activin on transferrin production and aromatase activity may indicate a role of activin in the differentiation of Sertoli cells. Finally, activin-A stimulates the secretion of immunoreactive inhibin from Sertoli cells of immature rats (**chapter 5**). The inhibin immunoreactivity is measured using a radioimmunoassay recognizing the inhibin α-subunit, so one can speculate that activin-A increases the synthesis of inhibin α-subunit to prevent activin overproduction. This compensating mechanism is absent in inhibin α-subunit knockout mice, which may explain the formation of testicular stromal tumors in

these animals (Matzuk et al., 1992).

Despite the secretion of activin-B by Sertoli cells in culture, the effects of recombinant activin-A on Sertoli cell function have been described as autocrine effects in **chapter 4**. Now it is clear that these effects may be paracrine effects of activin-A secreted by peritubular myoid cells (**chapter 5**). It is important to determine whether activin-B can have the same effects on Sertoli cell function as activin-A, since activin-B appears to be less potent than activin-A in stimulating pituitary FSH release and erythroid differentiation (Nakamura et al., 1992a). Nevertheless, both activins can induce mesoderm (Nakamura et al., 1992a; Sugino et al., 1993) and stimulate spermatogonial proliferation (Mather et al., 1990) with equal potency.

As discussed in this chapter activins may play a role in cell-cell interaction in the testis of immature and mature rats. Future studies have to prove the importance of activins in the *in vivo* situation e.g. by intratesticular injection of activins, follistatins or antibodies which block activin bioactivity.

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SUMMARY

In males, the testis is a crucial organ for reproduction. As outlined in **section 1.2**, the testis produces sperm in the seminiferous tubules and androgens in the interstitium. To achieve this, the cooperation between the different testicular cell types is necessary. Several regulatory factors have been implicated in this interaction, among them activins and inhibins. The involvement of activins and inhibins in mammalian reproduction has been known for some time. Already in 1932 McCullagh suggested that gonadotropin release from the pituitary is suppressed by a gonadal factor named "inhibin". During the purification of inhibin from follicular fluid also activin was discovered as a gonadal hormone that stimulates FSH release from pituitary cells. In this thesis studies are reported on the production of activins in the rat testis as well as the expression of activin receptors and intratesticular effects of activin.

Chapter 1 is a review of recent literature on the inhibin and activin proteins. The structure of the inhibin subunits is shown in **section 1.3**. The TGF-ß superfamily, to which inhibins and activins belong, and the receptors for members of this family are discussed in **section 1.4** and **1.5**. Literature on other inhibin and activin binding proteins, follistatin and α_2-macroglobulin, is summarized in **section 1.6**. In **section 1.7** the expression of inhibin subunits and activin receptors in different organs and the effects of inhibins and activins in these organs are discussed.

In testes of immature and mature rats two activin receptor type II mRNA transcripts (6 kb and 4 kb) are expressed (**chapter 2**). In cultured peritubular myoid cells (**chapter 5**), Sertoli cells and Leydig cells (**chapter 2**) both mRNAs are present, whereas in germ cells around the time of meiotic division only the 4 kb mRNA is found (**chapter 2**). A part of the 4 kb mRNA population is translated on ribosomes. To visualize this protein by Western blotting, antibodies against the extracellular domain of the receptor were raised. However, the specificity of these antibodies still remains doubtful (**chapter 2 supplement**). Sertoli cells also express a 10 kb activin receptor type IIB mRNA, which is absent in peritubular myoid cells (**chapter 5**).

The presence of activin receptor mRNAs in Sertoli cells predicts effects of activin on Sertoli cell function. In **chapter 4** it is shown that recombinant activin-A inhibits FSH-stimulated aromatase activity and androgen receptor expression in Sertoli cells from immature rats, without affecting FSH receptor mRNA expression. Also basal androgen receptor expression is inhibited in these cells. On the other hand, recombinant activin-A stimulates immunoreactive inhibin and transferrin secretion (**chapter 5**). To find out whether these effects are physiologically relevant, the secretion of activin by different testicular cell types was investigated.

Chapter 3 deals with the production of inhibin and activin by Leydig cells from immature and adult rats. This study was performed because seemingly conflicting results on the production of inhibin and activin by Leydig cells were described. It is shown that Leydig cells from both immature and adult rats express inhibin α-subunit mRNA and secrete inhibin α-subunit protein. Strikingly, Leydig cells from adult rats secrete exclusively proαNαC, whereas Leydig cells from

immature rats secrete proαNαC and its processed form proαC. Biologically active inhibin or activin is not detected in culture medium from normal Leydig cells, but biologically active inhibin is secreted by some Leydig cell tumors from rat and mouse.

Activin-B is secreted by Sertoli cells from immature rats in culture as concluded from several lines of evidence (**chapter 4**). This could indicate that the effects on Sertoli cell function found with recombinant activin-A are representative for autocrine effects of activin-B as suggested by the title of this chapter. Since activin-A and activin-B have different potencies in several biological systems this needs further investigation. Activin secretion by Sertoli cells is essential to postulate a biological relevance for the expression of the activin type II receptor in germ cells around the time of meiotic division. These germ cells are surrounded by an unique microenvironment, because of the presence of the blood-testis barrier, which can only be influenced by the Sertoli cell.

Activin-A is secreted by peritubular myoid cells in culture and peritubular myoid-like cell lines as presented in **chapter 5**. Expression of the inhibin β_A-subunit mRNA increases during culture, but is also found immediately after isolation of the cells. This indicates that activin-A may also be secreted by peritubular myoid cells *in vivo*. Although activin-A has some properties in common with the peritubular myoid cell factor PModS, these factors are probably not identical as discussed in **chapter 5**. One of the differences between activin-A and PmodS is that the secretion of PModS is stimulated by androgens, whereas the expression of the inhibin β_A-subunit is not.

The results presented in this thesis and some questions surrounding inhibin and activin signal transduction are discussed in **chapter 6**. From this discussion it is clear that activins may play a role in the regulation of testicular function. Nevertheless, several findings should be clarified by future research, for which some possiblities are presented.

SAMENVATTING

De testis (testikel, zaadbal) is ongetwijfeld het centrale voortplantingsorgaan van de man. Het bestaat uit een aantal kluwens van zaadbuisjes omgeven door interstitieel weefsel. In de zaadbuisjes ontwikkelen zich de zaadcellen (spermatozoën), terwijl in het interstitiële weefsel het mannelijke geslachtshormoon testosteron wordt geproduceerd. De wanden van de zaadbuisjes worden gevormd door de basaalmembraan, die aan de binnenzijde is bekleed met Sertoli cellen, die zorg dragen voor een geschikt milieu voor de zich ontwikkelende zaadcellen, en aan de buitenzijde met peritubulaire myoid cellen, die de zaadbuisjes stevigheid en contractiele eigenschappen geven. In het interstitium bevinden zich de testosteron-producerende Leydig cellen. Voor een goed functioneren van de testis is samenwerking tussen de verschillende cellen in de testis noodzakelijk. Van verscheidene factoren, waaronder inhibines en activines, is gepostuleerd dat ze een regulerende rol spelen in deze interactie. Suggesties voor een rol voor inhibine en activine in de voortplanting van zoogdieren zijn niet nieuw. Al in 1932 postuleerde McCullagh dat de afgifte van gonadotrope hormonen in de hypofyse geremd wordt door een factor uit de gonaden, die "inhibine" genoemd werd. Tijdens de zuivering van inhibine uit ovariëel follikelvocht werd ook een hormoon ontdekt, dat de afgifte van FSH kan stimuleren en dat daarom de naam "activine" kreeg. In het onderzoek beschreven in dit proefschrift, wordt de productie van activines in de testis van de rat bestudeerd. Verder worden de verdeling van receptoren voor dit hormoon over de verschillende cellen in de testis en de effecten van dit hormoon in de testis onderzocht.

Hoofdstuk 1 is een overzicht van recente literatuur betreffende inhibine en activine. De structuur van de verschillende inhibine subunits is beschreven in **paragraaf 1.3.** De TGF-ß superfamilie, waartoe inhibine en activine behoren, en de receptoren voor de leden van deze familie worden besproken in de **paragrafen 1.4** en **1.5.** Literatuur over follistatine en α_2-macroglobuline, twee andere inhibine/activine bindende eiwitten is samengevat in **paragraaf 1.6.** In **paragraaf 1.7** worden het voorkomen van inhibine subunits en activine receptoren, alsmede de effecten van inhibine en activine in verschillende organen bediscusieerd.

In testes van jonge en volwassen ratten worden twee verschillende mRNAs (6 kb en 4 kb) van de activine type II receptor gevonden (**hoofdstuk 2**). Beide mRNAs zijn ook aanwezig in gekweekte peritubulaire myoid cellen (**hoofdstuk 5**), Sertoli cellen en Leydig cellen (**hoofdstuk 2**). In voorlopers van zaadcellen, die zich in hun ontwikkeling rond de meiotische deling bevinden, wordt alleen het 4 kb mRNA aangetroffen (**hoofdstuk 2**). Een gedeelte van de 4 kb mRNA populatie wordt in eiwit vertaald op ribosomen. Om dit eiwit aan te tonen door middel van Western blots werden antilichamen tegen het extracellulaire deel van de activine type II receptor opgewekt. De specificiteit van deze antilichamen is echter nog twijfelachtig zodat de aanwezigheid van activine type II receptor eiwit vooralsnog onduidelijk is (**hoofdstuk 2 supplement**). Naast mRNA voor de activine type II receptor wordt in Sertoli cellen ook een 10 kb mRNA voor de activine type IIB receptor gevonden, dat afwezig is in peritubulaire myoid cellen (**hoofdstuk 5**).

De aanwezigheid van activine receptor mRNAs in Sertoli cellen voorspelt dat

activine invloed kan hebben op het functioneren van deze cellen. Dit blijkt inderdaad het geval. Recombinant activine-A remt de FSH-gestimuleerde vorming van oestradiol uit testosteron (aromatase activiteit) in Sertoli cellen uit jonge ratten, zonder een effect te hebben op FSH receptor mRNA expressie (**hoofdstuk 4**). Verder worden zowel de basale als de FSH-gestimuleerde expressie van de androgeen receptor geremd (**hoofdstuk 4**). Tot slot stimuleert recombinant activine-A de afgifte van immunoreactief inhibine en transferrine uit deze cellen (**hoofdstuk 5**). Om er achter te komen of deze waarnemingen enige biologische relevantie hebben, werd onderzocht of de verschillende cellen in de testis ook activine maken.

Hoofdstuk 3 gaat over de productie van inhibine en activine door Leydig cellen uit jonge en volwassen ratten. Dit onderzoek werd verricht naar aanleiding van schijnbaar tegenstrijdige literatuur over dit onderwerp. Het hoofdstuk laat zien dat Leydig cellen van jonge en volwassen ratten de inhibine α-subunit tot expressie brengen en inhibine α-subunit eiwit uitscheiden. Opmerkelijk is dat Leydig cellen uit volwassen ratten alleen de inhibine α-subunit precursor proαNαC maken, terwijl Leydig cellen uit jonge ratten ook een proteolytisch splitsingsproduct van deze precursor, proαC, uitscheiden. Biologisch actief inhibine of activine wordt niet gevonden in kweekmedium van normale Leydig cellen. Echter in kweekmedium van sommige Leydig cel tumoren uit rat en muis is biologisch actief inhibine aanwezig.

Er zijn verschillende aanwijzingen dat Sertoli cellen uit jonge ratten activine-B maken (**hoofdstuk 4**). Dit zou kunnen betekenen dat de effecten van recombinant activine-A op Sertoli cel functie ook door activine-B van de Sertoli cel kunnen worden bewerkstelligd, zoals gesuggereerd wordt door de titel van dit hoofdstuk. Dit behoeft echter nader onderzoek, omdat gebleken is dat activine-A en activine-B verschillende potenties hebben in verschillende biologische systemen. Wil de expressie van de activine type II receptor in voorlopers van zaadcellen een biologische relevantie hebben, dan is de uitscheiding van activine door Sertoli cellen essentieel. Door de aanwezigheid van de bloed-testis barrière worden deze voorlopers namelijk omgeven door een uniek micro-milieu, dat alleen via Sertoli cellen beïnvloed kan worden.

Uit **hoofdstuk 5** blijkt dat gekweekte peritubulaire myoid cellen en peritubulaire myoid cel-achtige cellijnen activine-A maken. Expressie van de inhibine β_A-subunit mRNA wordt meteen na isolatie van de cellen gevonden en neemt vervolgens toe gedurende de kweek van deze cellen. Dit is een aanwijzing dat peritubulaire myoid cellen *in vivo* ook activine-A zouden kunnen maken. Ondanks het feit dat activine-A enkele eigenschappen gemeen heeft met de peritubulaire myoid cel factor PmodS, zijn deze factoren waarschijnlijk niet hetzelfde. Eén van de verschillen tussen activine-A en PModS is de regulatie door androgenen. De uitscheiding van PModS wordt gestimuleerd door androgenen, terwijl de expressie van de inhibine β_A-subunit hierdoor niet gereguleerd wordt.

De resultaten, die in dit proefschrift gepresenteerd worden, alsmede enkele vragen rond de signaaltransductie van inhibine en activine worden bediscusieerd in **hoofdstuk 6**. Uit deze discussie blijkt dat activines een rol kunnen spelen in de regulatie van testis functie. Toekomstig onderzoek zal echter de gepresenteerde resultaten moeten verduidelijken. Mogelijkheden hiervoor worden eveneens geopperd.

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CURRICULUM VITAE

Johan de Winter werd geboren op 18 januari 1966 te Sassenheim. Na het behalen van het diploma VWO-B aan het Maaslandcollege te Oss, begon hij in 1984 met de studie biologie aan de Katholieke Universiteit Nijmegen (KUN). Gedurende deze studie verrichtte hij op de afdeling Dierfysiologie van de KUN (Prof. Dr. S.E. Wendelaar Bonga) onderzoek naar de regulatie van de MSH-afgifte door de melanotrope cel van *Xenopus laevis*. Verder werd op de afdeling Biochemie van de KUN (Prof. Dr. H.P.J. Bloemers) het chromatine domein van het c-sis proto-oncogen bestudeerd. Op 31 oktober 1989 werd het doctoraal examen in de fysiologisch/biochemische richting afgelegd. Van 1 december 1989 tot 1 september 1993 was hij werkzaam als assistent in opleiding op de afdeling Biochemie II (later Endocrinologie & Voortplanting) van de Erasmus Universiteit Rotterdam, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd. Sinds 1 september 1993 is hij werkzaam als wetenschappelijk onderzoeker bij het Hubrecht Laboratorium te Utrecht op het EG-project "Interactions of activins with type II activin receptors in differentiation systems: structure-function analysis and synthesis of dominant negative ligands and receptors".