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LIGANDS & SIGNALING COMPONENTS OF THE TRANSFORMING GROWTH FACTOR β FAMILY

-LOCAL REGULATORS OF INHIBIN PRODUCTION IN OVARIAN GRANULOSA CELLS

Jonas Bondestam

Program for Developmental and Reproductive Biology
Biomedicum Helsinki
and
Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki
Helsinki, Finland

Academic Dissertation

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Helsinki 2002
Supervisor

Docent Olli Ritvos
Program for Developmental and Reproductive Biology
Biomedicum Helsinki
and
Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki
Helsinki, Finland

Reviewers

Professor Outi Hovatta
Department of Clinical Science
Division of Obstetrics and Gynecology
Huddinge University Hospital
Karolinska Institute
Huddinge, Sweden

and

Professor Ulf-Håkan Stenman
Department of Clinical Chemistry
Helsinki University Central Hospital
Helsinki, Finland

Opponent

Professor Axel P. N. Themmen
Department of Internal Medicine
Erasmus University MC
Rotterdam, The Netherlands
To Pia
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This thesis is based on the following original publications referred to in the text by their Roman numerals. In addition, some unpublished data are presented.


ABBREVIATIONS

ab antibody
ACVRII activin receptor type II
ACVRIB activin receptor type IIB
Ad adenovirus
ALK activin receptor-like kinase
AMH anti-Müllerian hormone
AMHRRII anti-Müllerian hormone type II receptor
ATP adenosine triphosphate
BMP bone morphogenetic protein
BMPRII bone morphogenetic protein type II receptor
bp base pair
BSA bovine serum albumin
C- / COOH- carboxyterminal
cAMP cyclic adenosine 3’, 5’-monophosphate
CDMP cartilage derived morphogenetic protein
cDNA complementary deoxyribonucleic acid
C. elegans Caenorhabditis elegans
CRE cAMP responsive element
DES diethylstilbestrol
DMEM Dulbecco’s modified eagle’s medium
E estradiol
ELISA enzyme-linked immunosorbent assay
EST expressed sequence tag
FCS fetal calf serum
FISH fluorescence in situ hybridization
FSH follicle stimulating hormone
GDF growth differentiation factor
GDNF glial derived neurotrophic factor
GnRH gonadotropin-releasing hormone
gss genome survey sequence
hCG human chorionic gonadotropin
hGL human granulosa-luteal
IVF in vitro fertilization
kDa kilodalton
LH luteinizing hormone
MAPK mitogen-activated protein kinase
m.o.i multiplicity of infection
mRNA messenger ribonucleic acid
N- / NH3- aminoterminus
OP osteogenic protein
P progesterone
PAC P1 artificial chromosome
PBS phosphate-buffered saline
PCOS polycystic ovary syndrome
PCR polymerase chain reaction
PGC primordial germ cell
RT reverse transcription
ser/thr serine/threonine
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
SARA Smad anchor for activation
SBE Smad binding element
SDS sodium dodecyl sulfate
SSC saline sodium citrate
TGFβ transforming growth factor β
TGFβRII transforming growth factor β type II receptor
The mammalian transforming growth factor β (TGFβ) superfamily comprises over 40 ligands, which are multifunctional regulators of cellular growth, differentiation and death. These factors signal by binding to a limited number of highly conserved transmembrane type I (activin receptor-like kinase, ALK) and type II receptor serine/threonine (ser/thr) kinases. Intracellularly the signal is transmitted to the nucleus by phosphorylated Smad signaling proteins. During the course of this thesis project we first determined the chromosomal loci of the type II activin receptors, the type I receptor ALK7 and the activin binding protein follistatin. An attempt to clone new ser/thr kinases resulted in the identification and isolation of the human ALK7 cDNA, the mRNA of which was shown to be widely expressed in the adult human. Furthermore, adenovirus-mediated overexpression of the constitutively active ALK7 resulted in specific phosphorylation of Smad2.

Inhibin hormones, which belong to the TGFβ superfamily, are produced in the ovary and act as negative regulators of follicle stimulating hormone (FSH) release from the anterior pituitary. We set out to study the involvement of different TGFβ superfamily members and their signaling components in the complex regulation of intraovarian inhibin subunit expression. The bone morphogenetic proteins (BMPs) form the largest subgroup within the TGFβ superfamily and we showed that cultured human granulosa-luteal (hGL) cells express all components needed for BMP signaling and further, that recombinant BMPs selectively induce the expression of the inhibin βB-subunit in these cells leading to a production of dimeric inhibin B. Using recombinant adenoviruses we selectively overexpressed various components of the TGFβ superfamily signaling machinery in hGL cells and showed that overexpression of constitutively active ALK1-7 and Smad1 and Smad2 proteins stimulates the production of inhibin B. Next, using cultured rat granulosa cells, growth differentiation factor-9 (GDF-9), another member of the TGFβ superfamily, was shown to stimulate the expression of all three inhibin subunits resulting in a production of dimeric inhibin A and B. Finally, GDF-9 stimulation of the rat granulosa cells was shown to induce Smad2 phosphorylation, indicating that either ALK4, ALK5 or ALK7 might function as a putative type I receptor for GDF-9.
INTRODUCTION

In mammalian organisms the various members of the transforming growth factor β (TGFβ) superfamily are known to be involved in most aspects of cellular growth, differentiation and death. Although most of these polypeptides differ clearly in function they share a limited number of receptors. The receptor molecules are highly conserved transmembrane serine/threonine kinases, which can be divided in two distinct groups based on function and structure, the type I (or activin receptor-like kinases, ALKs) and type II receptors. Upon ligand binding these binding moieties form tetrameric complexes consisting of two type II and two type I receptors, after which Smad signaling proteins are activated by the type I receptors. Activin and TGFβ type I receptors specifically activate a different set of Smad signaling proteins than do the bone morphogenetic protein (BMP) type I receptors. In addition, there are inhibitory Smads which oppose the signaling cascade. Several of the knockout mouse models generated for the serine/threonine kinase receptors and Smads either die during embryonic development or show severe developmental defects, indicating that the loss of one signaling component can not necessarily be compensated for by another (reviewed in Massagué et al., 2000).

The mammalian ovary is responsible for producing fertilizable oocytes and female sex steroids. In addition to the pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), the TGFβ superfamily member growth differentiation factor-9 (GDF-9) has been shown to be indispensable for successful murine folliculogenesis (Dong et al., 1996), a process where ovarian primordial follicles are recruited to grow (reviewed in Findlay et al., 2002; Matzuk et al., 2002). However, even though GDF-9 has recently been reported to signal via the BMP type II receptor (BMPRII) (Vitt et al., 2002), its type I receptor(s) remains yet to be characterized. Consequently, prior to our studies it was not known whether stimulation of granulosa cells with recombinant GDF-9 would lead to Smad activation.

The inhibins are TGFβ family members produced by ovarian somatic cells, and in addition to auto/paracrine intraovarian effects these hormones are capable of suppressing FSH release from anterior pituitary cells. During the human menstrual cycle the two main forms of inhibin in the serum, inhibin A (a dimer of the inhibin α- and βA-subunits) and inhibin B (a dimer of the inhibin α- and βB-subunits) fluctuate in specific and distinct patterns. It has previously been shown using cultures of human granulosa-luteal (hGL) cells that the expression of different inhibin subunits can be specifically regulated by various growth factors, including activin and TGFβ (reviewed in Findlay et al., 2001). Even so, the possible involvement of various BMPs, type I receptors and downstream Smad signaling proteins in the complex regulation of inhibin subunit expression had not been addressed prior to this study.

The following review of the literature first focuses on giving a background on the TGFβ superfamily and the signaling machinery used by its members. Thereafter the roles of ovarian BMPs and the regulation of inhibin subunit expression in the ovary will be discussed.
1. GENERAL CHARACTERISTICS OF THE TRANSFORMING GROWTH FACTOR β SUPERFAMILY

1.1. Historical background

Of the approximately 30,000 human genes (Lander et al., 2001) some 40 form a group known as the transforming growth factor β (TGFβ) superfamily. The prototype for these polypeptides, TGFβ, was isolated in the beginning of the 1980s (Roberts et al., 1981; Derynck et al., 1985) and since then the family of known members has rapidly expanded to its present size. TGFβ was originally named after its ability to cause a phenotypic transformation of cultured epithelial cells. Soon later, it was shown to inhibit the growth of most epithelial and haematopoietic cells and to regulate the production of extracellular matrix by mesenchymal cells. The effects of a specific TGFβ family member appeared to vary depending on the particular type and state of a cell. At present the various members of the TGFβ superfamily are acknowledged to participate in almost all forms of biological events including cellular growth, differentiation, morphogenesis, sexual development, fertility and apoptosis. Based on structural similarities the different polypeptides of the TGFβ superfamily are divided into several subgroups. These include the TGFβs, the activins and inhibins, the bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs), and a heterogeneous group consisting of more distantly related factors such as anti-Müllerian hormone (AMH) and inhibin-α (reviewed in Kingsley, 1994; Massagué et al., 2000) (Table 1).

Table 1. Mammalian TGFβ superfamily subgroups and members.

<table>
<thead>
<tr>
<th>TGFβ</th>
<th>BMP/GDF</th>
<th>Activin</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ1</td>
<td>BMP-2</td>
<td>Activin βₐ</td>
<td>AMH</td>
</tr>
<tr>
<td></td>
<td>BMP-4</td>
<td>Activin β₁₀</td>
<td>Inhibin-α</td>
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<tr>
<td>TGFβ2</td>
<td>BMP-5</td>
<td>Activin βᵦ</td>
<td>Lefty A</td>
</tr>
<tr>
<td></td>
<td>BMP-6</td>
<td>Activin βₑ</td>
<td>Lefty B</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>BMP-7/OP-1</td>
<td>GDNF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMP-8α/OP-2</td>
<td>Neurturin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMP-8β/OP-3</td>
<td>Persephin</td>
<td></td>
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<td></td>
<td>BMP-14/GDF-5/CDMP-1</td>
<td>Artemin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMP-13/GDF-6/CDMP-2</td>
<td>MIC-1/GDF-15</td>
<td></td>
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<tr>
<td></td>
<td>BMP-12/GDF-7/CDMP-3</td>
<td>Nodal</td>
<td></td>
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<tr>
<td>GDF-1</td>
<td>GDF-1</td>
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<td>GDF-2</td>
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<td>GDF-9</td>
<td>BMP-9/GDF-2</td>
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<td>BMP-10</td>
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<tr>
<td>BMP-11/GDF-11</td>
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<td></td>
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<tr>
<td>GDF-8/Myostatin</td>
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<td></td>
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<tr>
<td>BMP-3/Osteogenin</td>
<td></td>
<td></td>
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<tr>
<td>BMP-3b/GDF-10</td>
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<tr>
<td>GDF-9</td>
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<td></td>
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<tr>
<td>GDF-9B/BMP-15</td>
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<tr>
<td>BMP-16, -17, -18 (unpublished patents)</td>
<td></td>
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</table>

AMH, anti-Müllerian hormone; BMP, bone morphogenetic protein; CDMP, cartilage derived morphogenetic protein; GDF, growth differentiation factor; GDNF, glial derived neurotrophic factor; MIC-1, macrophage inhibitory cytokine-1; OP, osteogenic protein
1.2. Biosynthesis and structural properties of TGFβ family members

All TGFβ superfamily members appear to be produced in the same manner. First, a larger precursor molecule of roughly 400-500 amino acids is synthesized. This molecule includes an N-terminal signal peptide, a pro-region and the mature C-terminal region (Fig. 1). The signal peptide directs the protein to the endoplasmatic reticulum/Golgi, where the pro-region is proteolytically cleaved at a conserved RXXR (R, arginine; X, any amino acid) site to form the mature peptide, which is significantly smaller than the pro-peptide (reviewed in Kingsley, 1994). The pro-region, which is proposed to be needed for proper folding of the mature peptide (Gray and Mason, 1990), is not very well conserved among different TGFβ members. However, orthologues of a specific ligand usually have highly homologous pro-regions. In the case of TGFβ1-3 and myostatin the pro-region also participates in the regulation of the biological activity of these peptides by forming non-covalently linked complexes with them, which are biologically inactive (Derynck et al., 1985; Gentry et al., 1988; Miyazono et al., 1988; Wakefield et al., 1988; Lee and McPherron, 2001). Another typical feature of most TGFβ family members is the seven conserved cysteines in their mature region. Six of these cysteines form a knot-like structure, whereas the remaining fourth cysteine is responsible for linking the two ligand monomers to each other, thus forming a dimer via a disulfide bond (Daopin et al., 1992; Schlunegger and Grutter, 1992). Interestingly, some TGFβ family proteins including the oocyte-derived GDF-9 (McPherron and Lee, 1993) and GDF9-B/BMP-15 (Dube et al., 1998; Laitinen et al., 1998) lack the conserved fourth cysteine that is involved in peptide dimerization. It is, however, presumed that hydrophobic interactions between the two dimers are sufficient for linking them together.

Fig. 1. Schematic drawing of a representative member of the TGFβ family. The 4th cysteine (in black) is missing in GDF-9 and GDF-9B/BMP-15. Modified from Kingsley, 1994.

1.3. The discovery of TGFβ receptors

Because of the multitude of cellular events initiated by TGFβ ligands, it was originally believed that their signaling system would be far more complex than it eventually has turned out to be. In the late 1980s three different proteins were found to bind TGFβ1 and these were subsequently named type I, type II and type III receptors based on their different molecular sizes (Cheifetz et al., 1987; Boyd and Massagué, 1989). By using a mutant cell line resistant to TGFβ-induced growth inhibition it was soon shown that de facto only the type I and II receptors were needed for TGFβ signaling (Laiho et al., 1990). At present seven mammalian type I and five
type II receptors have been identified. The type I and II receptors are structurally related yet functionally different transmembrane ser/thr kinases, with highly conserved properties within the two subfamilies. These receptors appear to be responsible for the binding of all TGFβ superfamily members, with the exception of the GDNF subfamily, which signals via a receptor system consisting of the GDNF-receptor-αs and a tyrosine kinase called RET (reviewed in Massagué, 1996; Piek et al., 1999b).

1.3.1. Mammalian type II serine/threonine kinases

In the early 1990s Mathews and Vale used expression cloning strategies to identify the murine type II receptor for activin, which turned out to be a receptor ser/thr kinase, now called ACVRII (Mathews and Vale, 1991). Soon after, a second activin receptor named ACVRIIB was cloned and the murine receptor was furthermore shown to have four alternative splicing variants with distinct affinities for activin (Attisano et al., 1992; Mathews et al., 1992; Hildén et al., 1994). Once more, using expression cloning strategies, the type II receptor for TGFβ was identified (Lin et al., 1992) and soon after in 1994 a mammalian type II receptor for AMH was isolated (Baarends et al., 1994; di Clemente et al., 1994). The following year the cloning of a human type II receptor for BMPs was reported (Kawabata et al., 1995a; Liu et al., 1995; Nohno et al., 1995; Rosenzweig et al., 1995). The five type II receptors consist of approximately 500 amino acids (1000 amino acids for a long form of BMPRII), the molecular weights of which vary between 70 and 85 kDa. These receptors all have N-glycosylated cysteine-rich extracellular domains which are involved in ligand recognition and binding. However, the overall sequence homology between the extracellular domains of the type II receptors is small being the region determining ligand specificity. A single transmembrane domain follows the extracellular domain and intracellularly are the highly homologous ser/thr rich kinase domains located, followed by carboxy-terminal tails (reviewed in Derynck and Feng, 1997) (Fig. 2). The three-dimensional structure of TGFβ has been described as analogous to that of a “curled hand”, including fingers and a palm formed by β-strands (Daopin et al., 1992) and the ligand has been found to undergo a mild confirmational change in response to interaction with its type II receptor through its finger-like structures (Hart et al., 2002). Recently, the crystal structures of the human TGFβRII (Hart et al., 2002) and ACVRII (Greenwald et al., 1999) receptor ectodomains were determined. The extracellular domain of ACVRII is overall quite similar to that of TGFβRII and it comprises seven β-strands arranged in three antiparallel sheets. Interestingly the topology includes a three-finger toxin fold unrelated to but reminiscent of several neuro- and cardiotoxins (Greenwald et al., 1999).

1.3.2. Mammalian type I serine/threonine kinases

After the discovery of the TGFβRII (Lin et al., 1992), there was a need to clone the TGFβ type I receptor, since the dual requirement of both type I and type II receptors for the mediation of TGFβ signals had already been postulated earlier using mutant cell lines resistant to TGFβ-induced growth inhibition (Laiho et al., 1990). In 1993, using reverse transcriptase-PCR (RT-PCR) with degenerate primers against highly conserved motifs within the kinase domain of the type II receptors, the existence of several new orphan ser/thr kinase receptors was reported (Matsuzaki et al., 1993; ten
Dijke et al., 1993). These receptors have been named “activin receptor-like kinases” (ALKs), due to their high sequence similarities with the activin type II receptors. It became, however, soon evident that the type I receptors clearly formed their own subgroup, since they shared a higher degree of sequence similarity among themselves than with the type II receptors. The functional activin and TGFβ receptors were thereafter shown to be heteromeric receptor complexes consisting of a combination of type I and II receptors (Wrana et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Franzén et al., 1993; Bassing et al., 1994; ten Dijke et al., 1994a). In 1994 two type I receptors found to bind BMP-4 and BMP-7 were reported (Koenig et al., 1994; ten Dijke et al., 1994b), and in 1996 a seventh mammalian ALK, denoted ALK7, was identified (Rydén et al., 1996; Tsuchida et al., 1996). An additional eighth ALK, zALK8, has been cloned from the zebrafish (Yelick et al., 1998), but it has also been proposed that it is actually the fish orthologue of the mammalian ALK2 receptor (Payne et al., 2001). All type I receptors are approximately 500 amino acid 55-65 kDa proteins, and they share several general structural motifs with the type II receptors. Like the type II receptors the ALKs have cysteine-rich extracellular domains with putative glycosylation sites, a short transmembrane region, and an intracellular ser/thr rich kinase domain. In contrast to the type II receptors, the ALKs have a functionally important conserved ~30 amino acids glycine/serine (G/S) rich region with a typical SGSGSG sequence, denoted the GS-box, immediately preceding the kinase domain. The C-terminal tails of the ALKs are also substantially shorter than those of the type II receptors (Massagué, 1998) (Fig. 2). So far the crystal structure has been determined only for the ALK3 receptor ectodomain. This comprises two β-sheets and one α-helix and the overall structure has been compared to an open left hand (Kirsch et al., 2000).

Both receptors have characteristic extracellular typically clustered cysteine (C) rich regions, followed by short single hydrophobic transmembrane domains. The type II receptors have a constitutively active kinase and a ser/thr rich C-terminal tail. The type I receptors have a conserved glycine/serine (GS)-box, to which the immunophilin FKB12 binds in the inactive state of the receptor. P, phosphorylation site. Adapted from Kingsley, 1994.

Fig. 2. Schematic drawing of a type II and a type I ser/thr kinase receptor.
1.3.3. Functional characteristics of the type I and II receptors

In order to mediate signaling by TGFβ family members a tetrameric complex of two type II and two type I receptors has to be formed (Wrana et al., 1992; Attisano et al., 1993; Ebner et al., 1993; Franzén et al., 1993; Yamashita et al., 1994). Even though the type II receptors for activin and TGFβ are able to bind their respective ligands alone, this is not sufficient for initiation of signal transduction. BMPs bind to both type I and type II receptors alone, but with rather low affinity. However, the affinity of BMPs for their respective receptors is greatly increased when both type I and II receptors are present simultaneously (ten Dijke et al., 1994b; Rosenzweig et al., 1995). Most type II receptors, with the exception of AMHRII, bind several ligands and can form complexes with different type I receptors. Furthermore, the existence of heterotetrameric receptor complexes with different type II and type I receptors has also been postulated (Yamashita et al., 1994; Weis-Garcia and Massagué, 1996; Gilboa et al., 2000). This possibility would greatly increase the number of unique receptor combinations available for the TGFβ family ligands.

The type II receptor, as exemplified by TGFβRII, is constitutively phosphorylated at several serine residues and ligand binding does not seem to affect its phosphorylation status (Luo and Lodish, 1997). However, upon ligand binding a receptor type I and II tetrameric complex is established and the constitutively active type II receptor then transphosphorylates the type I receptor at several serine and threonine residues in its GS-box (Wieser et al., 1995), and for example, ALK5 is additionally phosphorylated at Ser165 (Souchelnytskyi et al., 1996). The type I receptors have a Leucine-Proline sequence immediately preceding the GS-box, and the immunophilin FKBP12 has been shown to interact with this region (Wang et al., 1994; Charng et al., 1996; Okadome et al., 1996; Wang et al., 1996; Chen et al., 1997). The phosphorylation of the GS-box leads to conformational changes of the type I receptor leading to the release of FKBP12, which is thus prevented from further interaction with the receptor. The kinase of the type I receptor is then able to interact with its substrate, the different members of the Smad signaling protein family. Interestingly, FKBP12 null mice do not show impaired type I receptor signaling, indicating that other related factors may compensate for the loss of this protein (Shou et al., 1998). The kinase domain of the type I receptor contains a characteristic L45 loop, which is directly involved in Smad recognition (Feng and Derynck, 1997). Type I receptors have a conserved threonine, or glutamine, immediately before the N-terminal beginning of the kinase domain. When this particular amino acid is changed to the acidic aspartate (i.e., T/Q-D), the receptor will become constitutively active (ca) and signal independently of ligand binding and association to type II receptors. Similarly, by replacing a highly conserved lysine, which is involved in ATP binding, for arginine (i.e., K-R), the receptor will lose its ability to activate intracellular targets and becomes kinase defective (Wieser et al., 1995; Weis-Garcia and Massagué, 1996) and as a result act as a dominant negative mutant.

1.4. Additional TGFβ family member binding proteins

1.4.1. Type III receptors

Betaglycan is a cell surface proteoglycan originally detected in a screen for TGFβ receptors. It was named the TGFβ type III receptor since its molecular size was larger
than those of the type I and type II receptors (Cheifetz et al., 1988). The affinity of TGFβ2 is normally low for its ser/thr kinase receptors. Nonetheless, after it has bound to betaglycan the receptor affinity is greatly increased. Furthermore, betaglycan has been shown to bind inhibin (Lewis et al., 2000). Endoglin is a betaglycan related cell surface glycoprotein that has been shown to bind various TGFβ superfamily members when co-expressed with ser/thr kinases (Cheifetz et al., 1992; Barbara et al., 1999).

1.4.2. Other binding proteins

In contrast to the TGFβs, which form latent complexes with their precursor molecules, this has not been shown for BMPs. Instead, several antagonistic proteins that are able to bind to BMP ligands have been identified. “The Differential screening-selected gene aberrative in neuroblastoma” (DAN) family of secreted cysteine-knot proteins include Cerberus, Gremlin, Caronte and Noggin. These proteins are able to block BMP signaling by binding to the ligand and thus preventing it from interacting with its receptors (reviewed in Miyazono, 2000). Furthermore, BMPs have been shown to interact with a pseudoreceptor called “BMP and activin membrane-bound inhibitor” (Bambi). Bambi was first detected in Xenopus, but based on structural similarities it was later recognized as being the same protein as the nma gene product, that had been identified earlier in a human melanoma cell line (Degen et al., 1996; Onichtchouk et al., 1999). Bambi is structurally a type I receptor, but it lacks a functional kinase domain and hence cannot phosphorylate intracellular Smads.

The follistatin protein was purified in 1987 from follicular fluid and shown to be a secreted glycoprotein of 29-32 kDa (Robertson et al., 1987; Ueno et al., 1987). Soon it became evident that follistatin was able to block the effects of activins, but not inhibins, by binding to them through their common β-subunits (Nakamura et al., 1990; Shimonaka et al., 1991). In addition to binding to activin and inhibin, follistatin has been shown to block the biological activities of several BMPs, including GDF-9B/BMP-15 (Iemura et al., 1998; Otsuka et al., 2001a; Amthor et al., 2002). Mice with a targeted disruption of the follistatin gene die shortly after birth and have multiple developmental defects including decreased muscle mass and abnormal skeletal development (Matzuk et al., 1995c). The phenotype is more severe than that of the activin knockouts (Vassalli et al., 1994; Matzuk et al., 1995b), supporting the hypothesis that the bioactivity of additional growth factors might be regulated by follistatin in vivo.

1.5. Intracellular signaling molecules

1.5.1. The Smad family

The human homologues to Drosophila Mad (mother against dpp) and C. elegans sma proteins, called Smad1-8, were identified by screening human expressed sequence tag (EST) databases and cDNA libraries (Sekelsky et al., 1995; Chen et al., 1996; Eppert et al., 1996; Hoodless et al., 1996; Lechleider et al., 1996; Liu et al., 1996; Riggins et al., 1996; Savage et al., 1996; Yingling et al., 1996; Zhang et al., 1996; Imamura et al., 1997; Nakao et al., 1997a; Topper et al., 1997). Based on structural and functional similarities and differences the Smad proteins fall into three classes:

1) The receptor-regulated Smads (R-Smads) consist of two groups. Firstly, Smad1, Smad5 and Smad8, activated by ALK1, ALK2, ALK3 and ALK6. Secondly, Smad2
and Smad3, which are activated by ALK4, ALK5 and ALK7. 2) The common mediator Smad (co-Smad) Smad4, which forms complexes with the R-Smads. 3) The inhibitory Smads (I-Smads) Smad6 and Smad7, which oppose R-Smad signaling and function (reviewed in Massagué and Chen, 2000; Yue and Mulder, 2001).

1.5.2. Principles for Smad activation

The molecular weight of the Smad proteins ranges from 42 to 69 kDa. These factors consist of two highly conserved domains, of which the N-terminal domain is termed Mad Homology (MH)1 and the C-terminal domain MH2. The two MH domains are linked by a short proline-rich linker region, which appears to participate in the crosstalk between Smads and representatives of the mitogen-activated protein kinase (MAPK) family (Kretzschmar et al., 1997). In the basal state the MH1 domain of R-Smads functions as an inhibitor of the MH2 domain by binding to it. The R-Smad becomes activated and undergoes a conformational change after ALK receptor-mediated phosphorylation of its MH2 domain C-terminal SSXS sequence (Macias-Silva et al., 1996) (Fig. 3). Smad4 and the I-Smads lack this motif and thus cannot be phosphorylated by ALKs. The MH2 domain of the activated R-Smads can form complexes with other R-Smads of the same signaling class, and will then further associate with Smad4. Smad3 is believed to preferentially form trimers, whereas Smad2 supposedly also forms dimers. In addition the formation of Smad hexamers has been proposed (reviewed in Massagué and Wotton, 2000; Yue and Mulder, 2001). The definite stoichiometry of the Smad complexes has not been determined yet. The activated Smad complex is able to move into the nucleus where the MH1 domain can bind to DNA, either alone (except Smad2 which lack a DNA-binding region) or in complexes with several other transcription factors, e.g., Fast-1 in the case of Smad2 (reviewed in Massagué and Wotton, 2000; Yue and Mulder, 2001). The “Smad anchor for activation” (SARA) is a membrane bound protein that has been shown to recruit Smad2 and Smad3 to type I receptors by binding to their respective MH2 domains (Tsukazaki et al., 1998; Wu et al., 2000). The R-Smads contain a highly conserved region, the L3 loop, which determines type I receptor specificity. This region is invariant between Smad2 and Smad3, and between Smad1, Smad5 and Smad8, respectively (Lo et al., 1998). The L3 loop of Smad4 seems to be critical for its ability to form complex with R-Smads (Shi et al., 1997). Smad1 and Smad2 can translocate into the nucleus even without Smad4. However, Smad4 seems to be needed in order to stabilize the R-Smad-DNA complex and might additionally promote initiation of transcription (Liu et al., 1997).

(A) In the inactive state the MH2 domain binds to the MH1 domain. (B) Upon activation by phosphorylation of the MH2 domain SSXS sequence the R-Smad is able to form complexes with Smad4. Adapted from Massagué, 1998.

Fig. 3. Schematic picture of an R-Smad.
Smad6 and Smad7 are inhibitory (I)-Smads, which have been shown to function as antagonizers of R-Smad signaling. Smad6 seems to be mainly an antagonist of BMP signaling whereas Smad7 has been shown to antagonize signaling by both TGFβ, activin and BMPs (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997a; Tsuneizumi et al., 1997; Hata et al., 1998; Hanyu et al., 2001; Liu et al., 2002). Two main levels of I-Smad interference with the Smad signaling pathways seem to exist. On the one hand, when overexpressed, I-Smads can block Smad signaling by binding to the R-Smad-type I receptor interaction site through their MH2 domains (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997a). On the other, at lower expression levels, this is not necessarily the case and Smad6 has been shown to compete with Smad4 for binding to R-Smads, thus blocking the formation of R-Smad-Smad4 complexes (Hata et al., 1998). Furthermore, a novel antagonistic mechanism for I-Smads has been proposed. The I-Smads have been shown to be present in the cell nucleus and it is possible that they bind to and block R-Smad DNA binding sites without, however, initiating transcription (Bai and Cao, 2002). The expression of I-Smads is upregulated after TGFβ family member-induced activation of R-Smads. R-Smads and I-Smads seem to form a negative feedback loop to possibly prevent excessive stimulation of the cell (reviewed in Miyazono, 2002).

1) A ligand (e.g., activin) binds to the 2) constitutively active type II receptors, which then 3) transphosphorylate the recruited type I receptors in their GS-domains. 4) An R-Smad is released from its anchor (SARA), 5) becomes phosphorylated and forms complexes with Smad4. 6) Ultimately, the Smad-complex moves to the nucleus where it might bind to DNA and affect gene transcription. In contrast to activins and TGFβs, BMP proteins signal by binding simultaneously to type I and II receptors.

**Fig. 4.** Schematic drawing of the signaling chain from ligand binding to Smad activation.
1.5.3. Other signaling cascades activated by TGFβ family members

In addition to the Smad signaling pathways there is a rapidly growing body of evidence indicating that also members of the mitogen-activated protein kinase family (MAPK) cascade can be activated by different TGFβ family ligands. The MAPKs include three main groups: the extracellular signal-regulated kinases (ERKs), the c-Jun-N-terminal kinases (JNKs)/stress-activated protein kinases (SAPKs) and p38. These are all intracellular ser/thr kinases, which can be activated within minutes in response to extracellular stimuli, e.g., stimulation with TGFβ, and further transmit the response to the nucleus. Despite this, a possible direct activation site(s) for MAPKs on the type I and/or II receptors has not yet been identified. Interestingly, some MAPKs can phosphorylate R-Smads in their proline-rich linker-regions and prevent them from entering the nucleus (reviewed in Piek et al., 1999a; Mulder, 2000; Yue and Mulder, 2001). Thus, a cross-talk between Smads and the MAPKs clearly exists, but more studies are needed to determine the precise nature of this interaction.

1.6. Null mice models of ser/thr kinases and Smads

By creating null mice lacking a specific ser/thr kinase receptor or Smad protein, it has become possible to study the in vivo involvement of these factors. Knockout mice for all five type II and six out of seven type I receptors have been generated thus far. Several of the null mice lacking one of the ser/thr kinase receptor die during early embryonic development and, not surprisingly, a disruption of a specific Smad is also often embryonically lethal (Table 2).

1.7. Ser/thr kinase receptor and Smad involvement in human disease

1.7.1. Tumorigenesis

The anti-mitogenic tumor suppressive effect of TGFβ is lost in several tumor-derived cell lines and it has been proposed that mutations in the TGFβ signaling cascade are involved in a large fraction of pancreatic and colorectal cancers (reviewed in de Caestecker et al., 2000; Massagué et al., 2000). Inactivating mutations of the TGFβ type II receptors have been detected in colorectal and gastric cancers (Markowitz et al., 1995; Lu et al., 1998). Furthermore, ser/thr kinase receptors are also mutated in some hereditary pre-malignant syndromes. Mutations of ALK3 have been shown to be responsible for different variants of juvenile polyptic (JP) syndromes, which are characterized by the high risk of affected individuals to develop malignant tumors from pre-existing benign polyps (Howe et al., 2001; Zhou et al., 2001). Smad deletions have been reported in several cancers, e.g., the chromosomal region 18q21 where Smad4 is located is deleted in more than 50% of human pancreatic carcinomas (Hahn et al., 1996) and in several colorectal cancers (Thiagalingam et al., 1996; Salovaara et al., 2002). Smad4 has also been shown to be mutated in a subset of families with juvenile polyposis (Howe et al., 1998) (Table 3).

1.7.2. Other disorders

Mutations of the ALK1 receptor have been shown to cause hereditary haemorrhagic telangiectasia (HHT) type 2. The presenting symptoms often include frequent epistaxis and patients have mucocutaneous telangiectasia; later in life gastrointestinal
bleeding may occur (Johnson et al., 1996). Additionally, arteriovenous malformations are sometimes seen. However, these are more common in hereditary haemorrhagic telangiectasia type 1, a related more severe disease, caused by mutations in endoglin, a TGFβ binding protein (McAllister et al., 1994). Mutations in BMPRII lie behind the rare autosomal dominant genetic disorder familial primary pulmonary hypertension (FPPH). This disease eventually leads to pulmonary hypertension and cor pulmonale by the fifth decade of life (Deng et al., 2000; Lane et al., 2000). The TGFβ family member AMH causes regression of the Müllerian ducts in males by binding to AMHRII. Patients with an inactivating mutation in the gene for AMHRII will show a persistent Müllerian duct syndrome (PMDS) phenotype. These patients are genetically males and normally virilized but will also have organs derived from the Müllerian ducts, including the uterus, Fallopian tubes and the upper vagina (Imbeaud et al., 1995) (Table 3).

Table 2. General characteristics of null mice lacking ser/thr kinase receptors and Smads.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Null mouse phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK1</td>
<td>die by E11.5* due to arteriovenous malformations</td>
<td>Oh et al., 2000; Urness et al., 2000</td>
</tr>
<tr>
<td>ALK2</td>
<td>die before E9.5, multiple gastrulation defects</td>
<td>Gu et al., 1999; Mishina et al., 1999</td>
</tr>
<tr>
<td>ALK3</td>
<td>die at E9.5</td>
<td>Mishina et al., 1995</td>
</tr>
<tr>
<td>ALK4</td>
<td>early embryonic death, impaired gastrulation</td>
<td>Gu et al., 1998</td>
</tr>
<tr>
<td>ALK5</td>
<td>embryonic death before E10.5, vascular defects</td>
<td>Larsson et al., 2001</td>
</tr>
<tr>
<td>ALK6</td>
<td>impaired female reproduction, mild chondrogenic defects</td>
<td>Yi et al., 2000; Yi et al., 2001</td>
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<tr>
<td>ACVRII</td>
<td>FSH↓, female infertility, skeletal defects</td>
<td>Matzuk et al., 1995a</td>
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<tr>
<td>ACVRIIIB</td>
<td>abnormal left-right axis formation, die postnatally</td>
<td>Oh and Li, 1997</td>
</tr>
<tr>
<td>BMPRII</td>
<td>die at E9.5</td>
<td>Beppu et al., 2000</td>
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<tr>
<td>TGFβRII</td>
<td>vascularization defects, embryonic death</td>
<td>Oshima et al., 1996</td>
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<tr>
<td>AMHRII</td>
<td>male pseudohermaphroditism</td>
<td>Mishina et al., 1996</td>
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<tr>
<td>Smad1</td>
<td>die at E9.5, defective allantois formation</td>
<td>Lechleider et al., 2001</td>
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<tr>
<td>Smad2</td>
<td>embryonic death before E12.5</td>
<td>Nomura and Li, 1998; Waldrip et al., 1998</td>
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<td>Smad3**</td>
<td>defective immune response, colorectal cancer, reduced fertility of female mice</td>
<td>Zhu et al., 1998; Datto et al., 1999; Yang et al., 1999b; Tomic et al., 2002</td>
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<tr>
<td>Smad4</td>
<td>die before E8.5</td>
<td>Sirard et al., 1998; Yang et al., 1998</td>
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<td>Smad5</td>
<td>Angiogenetic defects, die between E10.5 and E11.5</td>
<td>Chang et al., 1999; Yang et al., 1999a</td>
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<td>Smad6</td>
<td>cardiovascular defects</td>
<td>Galvin et al., 2000</td>
</tr>
</tbody>
</table>

* E, embryonic day.
** The Smad3 null mice reported by Zhu et al. had a disruption of exon 2 resulting in colorectal cancer, however, the female mice were fertile. In contrast, Smad3 null mice with a deletion of exon 8 reported by Yang et al. did not develop tumors but the females were later reported to be infertile.
Table 3. Chromosomal loci and human diseases associated with mutated / abnormally expressed ser/thr kinase receptors and Smads.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Disease/Disorder</th>
<th>Locus</th>
<th>References</th>
</tr>
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<tr>
<td>ALK1</td>
<td>human hereditary telangiectasia I</td>
<td>12q11-q14</td>
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<td>?</td>
<td>2q23-q24</td>
<td>Röijer et al., 1998a</td>
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<td>10q22.3</td>
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<td>12q13</td>
<td>Röijer et al., 1998a; Su et al., 2001</td>
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<tr>
<td>ALK5</td>
<td>various cancers</td>
<td>9q33-q34</td>
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<tr>
<td>ALK6</td>
<td>?</td>
<td>4q23-q24</td>
<td>Ide et al., 1998</td>
</tr>
<tr>
<td>ALK7</td>
<td>?</td>
<td>2q24.1-3</td>
<td>Study II</td>
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<td></td>
<td>2q22.2-q23.3</td>
<td>Study I</td>
</tr>
<tr>
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<td>3p22</td>
<td>Ishikawa et al., 1998; Kosaki et al., 1999, Study I</td>
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<td>BMPRII</td>
<td>familial primary pulmonary hypertension</td>
<td>2q33</td>
<td>Deng et al., 2000; Lane et al., 2000</td>
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<tr>
<td>TGFβRII</td>
<td>gastrointestinal cancer</td>
<td>3p22</td>
<td>Mathew et al., 1994; Markowitz et al., 1995; Parsons et al., 1995; Lu et al., 1998; Tanaka et al., 2000</td>
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<tr>
<td>AMHRII</td>
<td>persistent Müllerian duct syndrome</td>
<td>12q13</td>
<td>Imbeaud et al., 1995</td>
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<td>Smad2</td>
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<td>18q21.1</td>
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2. BONE MORPHOGENETIC PROTEINS, RECEPTORS AND SMADS IN THE MAMMALIAN OVARY

2.1. General characteristics of ovarian function

The female ovary plays dual yet closely related roles. On the one hand it is responsible for the generation of fertilizable oocytes; on the other it is the main production site for the female sex steroids, estradiol and progesterone. The pituitary gonadotropins FSH and LH are key players in both events. Even so, during recent years it has become evident that also oocyte secreted polypeptides of the TGFβ superfamily are needed for normal folliculogenesis in mammals (reviewed in Erickson and Shimasaki, 2001; Findlay et al., 2002). Especially the involvement of different BMPs and GDF-9 for ovarian function will be discussed below.

2.1.1. Early stages of follicular development

The female gametes are derived from primordial germ cells (PGCs) which is one of the first embryonic cell lineages to be established (reviewed in Buehr, 1997). In the mouse different members of the BMP family (BMP-2/-4/-8b) were recently shown to promote the formation of PGCs from pluripotent precursor cells, but the possible involvement of these growth factors for human PGC formation is not presently known (Ying et al., 2000; Ying and Zhao, 2001). In humans the germ cells are relocated in the gonadal ridges by the fifth week of embryonic development and are then termed oogonia. Through mitotic division the oogonia will form clusters surrounded by a flat layer of epithelial cells. Some oogonia will be arrested in the prophase of the first meiotic division, and are from that stage on referred to as primary oocytes, but the majority of the oogonia will continue to grow through mitosis. However, by the seventh month most of them will have undergone apoptosis. A single epithelial cell layer now surrounds the remaining primary oocytes and this unit is referred to as the primordial follicle. Some of these will eventually start to grow already during fetal life, but the majority remain in a resting state (reviewed in Buehr, 1997).

2.1.2. Later stages of follicular development

At birth the human ovary contains a bounded number of follicles; some 50 years later at the beginning of menopause the number of follicles have diminished to less than 1000, mainly through apoptotic cell death. Even though some follicles enter the growth phase already prior to puberty they undergo apoptosis at an early developmental stage because of lack of sufficient levels of gonadotropins. During puberty under the influence of FSH, over a growth period of 50 days or more, a primordial follicle might eventually become the (usually) single dominant follicle destined to ovulate (Fig. 5). As the primordial follicle (φ 30-60 μm) starts to grow its surrounding monolayer of flat pregranulosa cells will change to the cuboid granulosa cells characterizing the primary follicle. With continuing follicular growth secondary follicles develop. The stromal cells of the secondary follicles develop a blood supply through angiogenesis. The secondary follicles (φ 80-100 μm) are surrounded by several layers of granulosa cells and also the oocytes are larger than those of the earlier stage follicles. As the secondary follicles develop, the surrounding layer of
stromal cells will differentiate into theca interna and externa cells. Some cells within the theca interna differentiate into epitheloid cells, and simultaneously the oocytes start to secrete a matrix, which forms the zona pellucida layer. From now on the follicle is referred to as a preantral follicle (φ 150 µm) (reviewed in Gougeon, 1996; Gougeon, 1998). Follicles are able to grow to these stages without FSH stimulation, an event that is referred to as basal follicular growth. The ovaries of patients with an inactivating mutation of the FSH receptor gene have primary follicles, whereas secondary follicles are rarely found (Aittomäki et al., 1996). Altogether, relatively little is known about the regulation of basal follicular growth in humans. Nonetheless, some oocyte-derived ligands of the TGFβ family might be indispensable since inactivating mutations of the GDF-9 gene in mice (Dong et al., 1996) and the GDF-9B/BMP-15 gene in sheep (Galloway et al., 2000) lead to an arrest in folliculogenesis at the primary stage such that no secondary follicles evolve.

During a time period of approximately two months, the growing preantral follicle reaches a diameter of 200-400 µm, now known as the early antral follicle. The terminology refers to the fluid-filled cavities, antri, of these follicles, which separate the majority of the granulosa cells, referred to as the mural granulosa cells, from immediate contact with the oocyte. Two to three layers of granulosa cells, which form the cumulus oophorus, still surround the oocyte. Follicles with a diameter of 2-5 mm during the late luteal phase form the group of selectable follicles. The number of selectable follicles decreases with increasing age; women under the age of 40 have an estimated mean of approximately 12-22 selectable follicles per menstrual cycle. One of these follicles will then become the dominant follicle destined to ovulate during the next menstrual cycle and this selection is accomplished during the subsequent follicular phase. The diameter of the selected follicle rapidly increases from 5.5-8 mm to the 15-27 mm of the mature pre-ovulatory Graafian follicle (reviewed in Gougeon, 1996; Gougeon, 1998; McGee and Hsueh, 2000).

2.1.3. The end of the follicular lifespan

In humans the serum levels of LH peak some 36-40 hours prior to ovulation. The oocyte completes its meiotic division, and two daughter cells are produced, the first polar body and the secondary oocyte, respectively. Only one of them, the haploid secondary oocyte, is fertilizable. Soon following ovulation the remaining granulosa cells, theca interna cells and invading fibroblasts form a highly vascularized structure called the corpus luteum. Under the influence of LH the granulosa cells are luteinized, and start to produce progesterone. If pregnancy does not occur within the coming 14 days, the corpus luteum diminishes and ultimately becomes a corpus albicans. However, in the case of pregnancy human chorionic gonadotropin (hCG) secreted by the placenta will maintain the corpus luteum for more than four months, after which it starts slowly to degenerate (reviewed in McGee and Hsueh, 2000).
2.1.4. Ovarian steroid hormone production

The ovary is the main production site for the female sex steroids, estradiol and progesterone. According to the classical “two cell, two gonadotropin” theory the ovarian steroid hormone production requires an intimate co-operation between theca and granulosa cells. First, progestin and androgens are synthesized in the theca cells under the influence of LH by the combined actions of the cholesterol side chain cleavage (P450scc), 17α-hydroxylase (P45017α) and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymes. Second, the androgens are aromatized to estrogens in the granulosa cells by P450 aromatase (P450 arom), the expression of which is controlled by FSH (reviewed in Erickson and Shimasaki, 2001). It is, however, noteworthy that both cell types have the capability of producing both androgens and estrogens. Nevertheless, the majority of the androgens are synthesized in the theca cell whereas the granulosa cells synthesize most of the estrogens (reviewed in Gougeon, 1996).

2.2. Expression of BMPs in the mammalian ovary

The BMPs form the largest subgroup within the TGFβ superfamily. Even though originally identified as factors capable of inducing de novo bone growth, these multipotent polypeptides are now acknowledged to participate in almost all aspects of cellular differentiation (reviewed in Massagué et al., 2000). Recently, at least seven members of the BMP/GDF family have been identified in the mammalian ovary. In
Ligands & Signaling Components of the TGFβ Family

1989, transcripts of BMP-6 were reported to be expressed in murine oocytes (Lyons et al., 1989) and later using Northern blotting the mRNAs encoding BMP-3, BMP-3b and BMP-2 were detected in whole rat and/or human ovaries (Hino et al., 1996; Takao et al., 1996). Furthermore, BMP-3 has been shown to be expressed in cultured human granulosa-luteal (hGL) cells and its expression levels appear to be hormonally regulated (Jaatinen et al., 1996). Shimasaki and colleagues have localized the mRNAs of BMP-4 and BMP-7 to the theca cells of rat preovulatory follicles (Shimasaki et al., 1999). GDF-9, a distant relative of the TGFβ superfamily originally cloned in 1993 (McPherron and Lee, 1993), has been shown to be expressed in rodent (McGrath et al., 1995; Fitzpatrick et al., 1998; Joyce et al., 2000), ovine/bovine (Bodensteiner et al., 1999) and human oocytes (Sidis et al., 1998; Aaltonen et al., 1999; Teixeira Filho et al., 2002). GDF-9 is nowadays considered to be a member of the BMP subgroup of the TGFβ superfamily (Vitt et al., 2002). The closely related homologue of GDF-9, GDF-9B/BMP-15, is also expressed in the oocytes of various mammals (Dube et al., 1998; Laitinen et al., 1998; Aaltonen et al., 1999; Galloway et al., 2000; Teixeira Filho et al., 2002).

2.2.1. Expression of BMP receptors in the mammalian ovary

BMPs bind to BMPRII in combination with ALK2/3/ or 6, additionally activin type II receptors may be used (reviewed in Massagué, 1998). By in situ hybridization ALK3, ALK6 and BMPRII have recently been shown to be expressed in rat, murine and ovine granulosa cells and oocytes (Shimasaki et al., 1999; Wilson et al., 2001; Yi et al., 2001). Furthermore, by immunohistochemical analyses the BMPRII, ALK3 and ALK6 proteins have been detected in ovine ovaries (Souza et al., 2002). The BMP type II receptor was recently shown to be expressed in hGL cells together with ALK2 and ALK3 (Erämaa et al., 1995, Study III). ALK3 is a well documented receptor for the BMP2/4 subfamily (Koenig et al., 1994; ten Dijke et al., 1994b; Yamaji et al., 1994; Nohno et al., 1995), whereas the members of the BMP5-8 subfamily also use ALK2 and ALK6 for their signaling (ten Dijke et al., 1994b; Ebisawa et al., 1999). Several BMPs have further been shown to interact with the activin type II receptors (Yamashita et al., 1995), which are abundantly expressed in, for example, hGL cells (Erämaa et al., 1995). GDF-9 was recently shown to interact with BMPRII (Vitt et al., 2002).

2.2.2. Smad expression in the mammalian ovary

Smad3, the first Smad to be shown to display ovarian expression, was originally detected in murine granulosa cells by in situ hybridization (Kano et al., 1999). Recently, the expression of Smad1-8 in postnatal rat ovaries has been reported (Drummond et al., 2002) and the expression levels of Smad2 and Smad3 have been shown to fluctuate depending on the developmental stage of the rat follicle (Xu et al., 2002). More specifically, the expression of both proteins was strong in small follicles but almost lost in large antral follicles while Smad2 expression increased again in luteal cells (Xu et al., 2002). We have shown by Northern blotting that the transcripts for Smads1-6 are detectable in hGL cells (Study III). Human Smad2 and Smad3 transcripts have been detected in human oocytes by RT-PCR (Österlund and Fried, 2000) and in human ovarian sections at the protein level (Pangas et al., 2002). Thus, it seems evident that all known Smad signaling proteins are expressed in the mammalian ovary.
2.3. Animal models showing involvement of BMPs for ovarian function

In 1996, the phenotype of mice deficient in GDF-9 was reported. Strikingly, female GDF-9 null mice were infertile due to a blockade of folliculogenesis at the primary follicular stage, whilst male mice were unaffected (Dong et al., 1996). Interestingly, sheep with mutations in the GDF-9B/BMP-15 gene, a close homologue of GDF-9, are infertile and their follicles do not develop beyond the primary stage. Moreover, heterozygotes for the mutations have an increased ovulation rate resulting in twins and triplets, indicating that the dosage of this protein is critical (Galloway et al., 2000). In contrast to this finding, the reproductive function of female mice with a targeted disruption of the GDF-9B/BMP-15 allele is only mildly impaired, resulting in slightly lowered fertilization rates (Yan et al., 2001). Recently, hyperprolific Booroola ewes were shown to have a mutation in the intracellular domain of their ALK6 receptors, resulting in a replacement of glutamine for arginine (Q249R) (Mulsant et al., 2001; Souza et al., 2001; Wilson et al., 2001). At present, no functional data on the bioactivity of the mutated receptor has been published. As mentioned earlier, mice deficient in ALK6 show impaired reproductive function even though they appear to ovulate histologically mature oocytes. The exact cause is unknown but ALK6 null mice show defective cumulus cell expansion combined with prolonged ovulatory cycles, which might (in part) explain the condition (Yi et al., 2001).

2.4. Biological effects of recombinant BMPs in the ovary

The in vitro effects of recombinant BMPs have mainly been studied in rodent granulosa cell cultures. On their own, BMP-4 and BMP-7 show no apparent effect on steroid hormone production in rat granulosa cells. Despite this, both factors have been shown to on the one hand stimulate FSH-induced estradiol production, and on the other suppress FSH-induced progesterone production (Shimasaki et al., 1999). Further, a direct in vivo injection of BMP-7 into rat ovaries resulted in a recruitment of primordial follicles into primary, preantral and antral stages; in vitro, BMP-7 was shown to act as a granulosa cell mitogen. The in vivo injection of BMP-7 further led to significantly fewer ovulated oocytes and lower serum progesterone levels as compared to untreated control animals (Lee et al., 2001). BMP-6 is highly expressed in oocytes (Lyons et al., 1989), but, knockout mice do not show impaired reproductive function (Solloway et al., 1998). Otsuka et al. have demonstrated that cultured rat granulosa cells respond to treatment with recombinant BMP-6 protein. Like most of the other BMPs tested, BMP-6 on its own did not affect basal steroid hormone production. Despite this, unlike BMP-7, BMP-6 was not found to be a granulosa cell mitogen. Co-treatment with FSH and BMP-6 inhibited FSH-induced progesterone production, without affecting estradiol production (Otsuka et al., 2001b). Recently BMP-2 was shown to increase estradiol and inhibin A production in cultured sheep granulosa cells, without affecting cell proliferation (Souza et al., 2002).

Several recent studies have described the in vitro effects of the recombinant GDF-9 and GDF-9B/BMP-15 proteins. Recombinant GDF-9 was found to increase the diameter of cultured rat preantral follicles and, moreover, to increase the production of the inhibin α-subunit in neonatal rat ovarian explant cultures (Hayashi et al., 1999). Using cultured mouse granulosa cells, recombinant mouse GDF-9 was shown to induce hyaluronan synthase 2 (HAS2), cyclooxygenase-2 (cox-2), steroidogenic acute
regulator protein (StAR) mRNAs as well as progesterone production. On the other hand, GDF-9 was found to suppress LH receptor and urokinase plasminogen activator (uPA) mRNA expression (Elvin et al., 1999). Recombinant GDF-9 protein caused oocytectomized cumulus cell complexes to expand, an effect that was mimicked by neither GDF-9B/BMP-15, nor BMP-6 (Elvin et al., 1999; Elvin et al., 2000). In cultures of preantral rat granulosa cells GDF-9 is mitogenic and dose-dependently downregulates the steroidogenic effects of FSH. On its own, GDF-9 stimulates progesterone production in granulosa cells from preovulatory follicles and estradiol production in granulosa cells from both small antral and preovulatory follicles, respectively (Vitt et al., 2000a). Recently, using cultures of proliferating human granulosa and theca cells GDF-9 was found to block the cAMP- and forskolin-induced synthesis of progesterone and androgens, respectively (Yamamoto et al., 2002). Intraperitoneal injections of GDF-9 twice daily over a span of 7-10 days in immature female rats led to a significant increase in ovarian weight. Histological analysis of the ovaries revealed that the number of growing follicles had increased (Vitt et al., 2000b). Recently, in line with results derived from rodent studies, GDF-9 was shown to promote human follicular growth in vitro (Hreinsson et al., 2002).

So far, only one group has reported the successful production of bioactive, C-terminal FLAG-tagged, human GDF-9B/BMP-15 recombinant protein (Otsuka et al., 2000). The protein was found to stimulate thymidine incorporation in cultured rat granulosa cells. When given alone, GDF-9B/BMP-15 did not alter basal steroid production by the granulosa cells. However, co-treatment with FSH caused a significant inhibition of FSH-induced progesterone production (Otsuka et al., 2000). Further, GDF-9B/BMP-15 has been shown to suppress FSH-induced steroidogenic enzyme mRNA levels, supposedly through downregulation of the FSH receptor mRNAs (Otsuka et al., 2001c). Recently, follistatin was shown to abolish the biological effects of GDF-9B/BMP-15, when granulosa cells were co-treated with both proteins (Otsuka et al., 2001a). It has also been shown that stimulation with recombinant GDF-9B/BMP-15 induces kit ligand (KL) mRNA expression in cultured rat granulosa cells and that the mitogenic effect of this protein is partially dependent on KL (Otsuka and Shimasaki, 2002). In cultured mouse granulosa cells recombinant GDF-9 has been shown to have the opposite effect on KL mRNA expression (Joyce et al., 2000), further indicating that the biological effects of GDF-9 and GDF-9B/BMP-15 clearly differ from each other.

Table 4. Summary of the mitogenic and steroidogenic effects of BMPs and GDF-9 in rodent granulosa cell cultures.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Mitosis</th>
<th>Progesterone</th>
<th>Estradiol</th>
<th>+FSH</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP-4</td>
<td></td>
<td></td>
<td></td>
<td>E↑, P↓</td>
<td>Shimasaki et al., 1999</td>
</tr>
<tr>
<td>BMP-6</td>
<td></td>
<td></td>
<td></td>
<td>P↓</td>
<td>Otsuka et al., 2001b</td>
</tr>
<tr>
<td>BMP-7</td>
<td>↑</td>
<td></td>
<td></td>
<td>E↑, P↓</td>
<td>Shimasaki et al., 1999, Lee et al., 2001</td>
</tr>
<tr>
<td>GDF-9</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>E↓, P↓</td>
<td>Elvin et al., 1999; Elvin et al., 2000; Vitt et al., 2000a</td>
</tr>
<tr>
<td>GDF-9B/ BMP-15</td>
<td>↑</td>
<td></td>
<td></td>
<td>P↓</td>
<td>Otsuka et al., 2000</td>
</tr>
</tbody>
</table>

↑ stimulation; ↓ suppression; E, estradiol; P, progesterone
3. OVARIAN INHIBINS AND THE REGULATION OF OVARIAN INHIBIN SUBUNIT EXPRESSION

3.1. Historical background of inhibins

The history of the distantly related TGFβ family member inhibin dates back to the 1920s when its existence was first postulated (Mottram and Cramer, 1923). The pituitary of castrated male rats had been found to contain hypertrophic “castrate” cells, and it was therefore hypothesized that the testis would normally secrete some yet unidentified inhibitory factor preventing the development of these cells. By injecting an aqueous testicular solution into castrated male rats, the appearance of the “castrate” cells was prevented and the factor thought to be responsible was termed inhibin (McCullagh, 1932). In the 1970s it was shown that the “inhibin effect” was due to suppression of FSH secretion (Setchell and Jacks, 1974). In 1985, four independent groups finally reported the molecular structure of inhibin (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985; Robertson et al., 1985). Inhibin was shown to consist of an α- and a β-subunit of 18 kDa (in its fully processed form) and 14 kDa, respectively, linked together by a disulfide bond (Fig. 5).

![Diagram of the composition of inhibin and activin subunits]

The human inhibin α-subunit can be proteolytically cleaved into a proregion, a larger αN-fragment and the mature αC-terminal region. The proforms of the inhibin β-subunits are proteolytically cleaved as well. Inhibins are heterodimers of an α- and a β-subunit, whereas activins are homodimers of two β-subunits. The two monomers are linked by disulfide bonds to form dimers (32 kDa inhibin A and 31 kDa inhibin B, with the fully processed α- and β-subunits).

Fig. 5. Schematic drawing of the composition of the proforms of the inhibin subunits and the mature forms of dimeric inhibins and activins.

Originally two different β-subunits, termed βA and βB were cloned, and both of them are expressed in the ovary (Mason et al., 1985). Additionally, three more β-subunits termed βC, βD (found in Xenopus) and βE have since been cloned (βC and βE from...
liver cDNA libraries), but they appear not to be involved in ovarian function (Hötten et al., 1995; Oda et al., 1995; Fang et al., 1996). Soon after the cloning of the inhibin subunits, it was observed that the β-subunits could dimerize even in the absence of the α-subunit, hence forming a protein called activin, opposing the biological effects of the inhibins. Activin was shown to stimulate FSH secretion, thus having the opposite effect of inhibin (Ling et al., 1986).

3.2. Intraovarian effects of inhibin

In addition to the sex steroids and gonadotropin-releasing hormone (GnRH), dimeric inhibins participate endocrinologically in the control of pituitary FSH release (reviewed in Bernard et al., 2002). Furthermore, inhibins are also believed to have intraglandular auto/paracrine effects, they have e.g., been shown to augment basic and LH-induced androgen production by theca cells *in vitro* (Hillier et al., 1991b). However, since a unique inhibin signal transduction pathway has not been elucidated the activity of inhibin in assays may in several cases be interpreted as an inhibition of activin activity (reviewed in Bernard et al., 2002). Knockout mice deficient in the inhibin α-subunit (and circulating inhibin dimers) develop ovarian tumors of gonadal sex cord-stromal origin with 100% penetrance as early as 4 weeks of age (Matzuk et al., 1992), indicating that inhibin might function as a potent tumor suppressor within the ovary.

3.3. Inhibin receptors

As mentioned previously most TGFβ family members signal via tetrameric complexes of cell surface ser/thr kinase receptors. Recent evidence suggest that inhibins can form high affinity complexes with betaglycan and ACVRII, thereby restricting the number of activin type II receptors available for activin signaling (Lewis et al., 2000). Furthermore, the presence of another inhibin binding protein (InhBP, originally denoted p120) has been shown (Chong et al., 2000). Even though these inhibin binding proteins have been reported, the existence of a specific inhibin receptor cannot be ruled out, since the proteins mentioned above are not known to activate downstream signaling components in response to inhibin binding (reviewed in Bernard et al., 2002).

3.4. Assays for determination of dimeric inhibins

Serum inhibin levels in women were measured already in the late 1980s. Nonetheless, the radioimmunoassay used failed to distinguish between different forms of inhibin, and it furthermore also detected free α-subunits (McLachlan et al., 1987). After the development of highly specific assays for inhibin A and inhibin B, it has been shown that these two forms of dimeric inhibins fluctuate in distinct manners during the menstrual cycle. Nonetheless, no biological differences are known to exist between the two inhibin dimers. Both inhibin assays are based on highly specific monoclonal antibodies against the βA or βB-subunits, respectively. These antibodies are adsorbed onto the plate and for detection a monoclonal Fab alkaline phosphatase conjugated anti-inhibin α-subunit antibody is used (Groome et al., 1994; Groome et al., 1996). The cross-reactivity for the inhibin B ELISA is 0.5% with inhibin A (Groome et al., 1996).
3.5. Inhibin subunit mRNA expression in the rat ovary

In the rat ovary the inhibin α-subunit is expressed in granulosa cells in follicles of all stages, whereas the two β-subunits are expressed in primary follicles onwards, but not in the corpora lutea (Meunier et al., 1988; Woodruff et al., 1988; Findlay et al., 2001). Recently, using a quantitative RT-PCR approach, the relative expression levels of the inhibin α-, βA-, and βB-subunits have been determined in postnatal rat granulosa cells (Drummond et al., 2000). In line with previous studies the α-subunit was found to be produced in excess over the two β-subunits, with its expression levels rising in parallel with follicular development. At postnatal day four (primordial and primary follicles present) and day eight (preantral follicles present), the mRNA levels of the two β-subunits did not differ significantly. In contrast, at day twelve (antral follicles present) the mRNA levels of the βA-subunit were twice as high as those of the βB-subunit. This supports the hypothesis that the βB-subunit would be predominantly produced by growing follicles. Furthermore, diethylstilbestrol (DES) treatment of immature rats for four days decreased the expression levels of both inhibin β-subunits, without affecting those of the α-subunit (Drummond et al., 2000).

3.5.1. Dimeric inhibin levels during the rat estrus cycle

The inhibin dimer-specific human ELISA assays have been shown to be applicable to the rat and consequently female rat serum and plasma inhibin levels have been determined throughout the four-day rat estrus cycle (Woodruff et al., 1996; Arai et al., 2002). The serum levels of inhibin A have been found to rise steadily from metestrus to peak in proestrus, whereas inhibin B serum levels are quite uniformly elevated in metestrus, diestrus and proestrus. Both serum inhibin levels were shown to reach their nadir during the secondary FSH surge, which is consistent with their regulatory role on FSH secretion. Since both inhibins are elevated at the time of the primary FSH surge, GnRH is able to overcome the inhibitory effects of both inhibins. There is a strong negative correlation between circulating inhibin B and FSH during the rat estrus circle, and it has been suggested that in rodents inhibin B is the primary regulator of serum FSH levels (Woodruff et al., 1996). In a more recent study plasma inhibin levels were measured and correlated to the ovarian inhibin RNA content (Arai et al., 2002). Changes in plasma inhibin concentrations were shown to coincide with the expression pattern of the inhibin β-subunit mRNAs with a 3-6 hour-delay (Arai et al., 2002) and, furthermore, the plasma inhibin levels measured correlated well with the results obtained previously (Woodruff et al., 1996).

3.6. Inhibin subunit expression in the human and primate ovaries

In the human ovary, no mRNA expression for the inhibin subunits has been detected in primordial follicles, even though weak immunohistochemical staining has been reported for the βA- and βB-subunits (Rabinovici et al., 1992; Yamoto et al., 1992). Growing human and primate follicles from preantral up to the preovulatory stage show a moderate to high expression of the mRNAs for the inhibin α-subunit and a low expression for the βA-subunit, whereas the inhibin βB-subunit mRNA is abundantly expressed in granulosa cells from small antral follicles (Schwall et al., 1990; Fraser et al., 1993; Roberts et al., 1993; Jaatinen et al., 1994). The mRNA levels of the βB-subunit fall sharply in preovulatory follicles, accompanied by a
marked increase in the levels of the $\beta_A$- and $\alpha$-subunits. Interestingly, in addition to the granulosa cells, theca cells of human antral follicles also appear to express all three inhibin subunit proteins, as recently shown by immunostaining (Pangas et al., 2002). Human luteinized granulosa cells express all three inhibin subunits, but the $\beta_B$-subunit is expressed at almost undetectable levels (Davis et al., 1987; Roberts et al., 1993; Erämaa et al., 1993). In a recent paper Fujiwara et al. used RT-PCR to determine the relative expression levels of the three inhibin subunits in different stages of human follicles. The expression levels of the $\alpha$- and $\beta_A$-subunit increased steadily with concomitant follicular growth, however, in this study no correlation between follicle size and $\beta_B$-subunit expression level was detected (Fujiwara et al., 2001). Further studies are needed to determine the exact relationship between follicle size and inhibin subunit expression levels, but the relative lack of human study material remains problematic.

3.6.1. Dimeric inhibin levels during the human menstrual cycle

During the human menstrual cycle the serum levels of inhibin A are low in the early follicular phase, rise in the late follicular phase to finally peak one day before the LH surge. Thereafter they fall briefly before peaking again in the mid-luteal phase. Dimeric inhibin A seems to originate mainly from the preovulatory follicle and the corpus luteum (Groome et al., 1994). In contrast to the inhibin A levels, the serum concentration of inhibin B is high during the midfollicular phase, decreases momentarily before ovulation, and rises shortly two days after ovulation, after which it drops to low levels for the rest of the cycle (Groome et al., 1996). Dimeric inhibin B therefore seems to be mainly derived from preantral and small antral follicles. In humans and primates a clear correlation between serum inhibin B levels and serum FSH levels exist especially during the follicular phase and the preovulatory gonadotropin surges (Groome et al., 1996).

3.7. In vitro regulation of inhibin subunit expression

The mechanisms regulating differential inhibin A and B synthesis are not thoroughly understood (reviewed in Bernard et al., 2001). Gonadotropins acting via cAMP-dependent pathways are well known stimulators of all three inhibin subunits inhibin production in both rodent and human granulosa cell cultures (Bicsak et al., 1986; Turner et al., 1989; Hillier et al., 1991a). Since the expression levels of the inhibin $\alpha$-, $\beta_A$- and $\beta_B$-subunits vary during different stages of follicular development, and since inhibin A and inhibin B have different serum profiles, it has been postulated and shown that the expression of each of the three subunits can be differentially regulated by local factors including TGF$\beta$ family members, leading to a production of either dimeric inhibin A or B. The regulation of the expression levels of the inhibin subunits has mainly been studied using granulosa cells derived from either immature, alternatively diethylstilbestrol (DES)-primed immature rats or, in the case of humans, using granulosa-luteal cells obtained from patients undergoing in vitro fertilization (IVF) programs (Drummond et al., 2000).

In cultures of rat granulosa cells the inhibin mRNA levels for the $\alpha$- and $\beta_A$-subunits are upregulated after stimulation with activin A and TGF$\beta$ (LaPolt et al., 1989; LaPolt et al., 1990). The inhibin $\alpha$-subunit has also been shown to be upregulated by
recombinant GDF-9 in cultures of neonatal rat ovarian explants (Hayashi et al., 1999). Furthermore, the production of both dimeric inhibin A and B is upregulated in cultures of rat granulosa cells after stimulation with TGFβ or addition of meiotically inactive bovine oocytes, a source of, for example, GDF-9 (Lanuza et al., 1999; Sendai et al., 2001). This effect is exaggerated in the presence of FSH. Taken together, in the rat, several of the tested TGFβ family members appear to upregulate and modulate the FSH-induced expression levels of all three inhibin subunits, leading to a production of inhibin A and B dimers.

Using cultured hGL cells, activin A and TGFβ have been shown to upregulate the mRNA expression levels of the inhibin βB-subunit, whereas gonadotropins preferentially stimulate the steady state mRNA levels of the inhibin α- and βA-subunits (Erämaa et al., 1995; Erämaa and Ritvos, 1996; Liu et al., 2001). Also, in accordance with the mRNA data the production of dimeric inhibin B, but not inhibin A, by these cells was increased in response to stimulation with activin A (Vänttinen et al., 2000). Based on these studies it appears that the three inhibin subunits are somewhat differentially regulated in human and rodent granulosa cells. Whether this discrepancy reflects the use of different stage granulosa cells or whether it is an interspecies difference is not presently known.
AIMS OF THE STUDY

At the beginning of this study the following specific aims were set out:

• to map all TGFβ family associated ser/thr kinase receptors with unknown chromosomal loci together with follistatin, in an attempt to study possible disease associations with their respective loci (I and II).

• to search for novel ser/thr kinase receptors (II).

• to determine whether cultured hGL cells express BMP receptors and Smads and, further, whether these cells respond to treatment with recombinant BMPs and their possible impact on inhibin production (III).

At a later stage of the study when new adenoviral tools and recombinant GDF-9 protein became available additional goals were set:

• to study the feasibility of recombinant adenovirus methodology for selective overexpression of different ALKs and Smads in hGL cells and their direct impact on inhibin production (IV).

• to determine the effects of recombinant GDF-9 protein on inhibin production and possible Smad activation in cultured rat granulosa cells (V).
MATERIALS AND METHODS

Please refer to the individual studies (I-V) and references for more detailed protocols.

1. Human granulosa-luteal cells

Human GL cells were obtained from women in conjunction with oocyte aspiration prior to IVF. The ovaries were stimulated by administering a human GnRH analog (Suprecur, Hoechst, Frankfurt am Main, Germany) and human recombinant gonadotropin (Puregon, Organon, Oss, The Netherlands or Gonal-F, Ares-Serono, Geneva, Switzerland). Thirty-six to 37 h prior to oocyte retrieval patients were administered a total dose of 10 000 IU hCG (Pregnyl, Organon or Profasi, Ares-Serono). The granulosa cells from 1-6 patients aspirated the same morning were pooled, enzymatically dispersed with 1% hyaluronidase (Sigma Chemicals Co, St Louis, MO, USA) in Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO, Grand Island, NY, USA) at 37°C for 30 min. Separation of the granulosa cells from erythrocytes was achieved by centrifugation through Ficoll-Paque (Pharmacia Corp., North Peapack, NY, USA). After this the cells were either recovered for RNA extraction or plated on plastic culture dishes (Greiner Bio-One, Frickenhausen, Germany) at densities specified in studies III-IV and cultured in DMEM supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 100 IU/ml penicillin 100 µg/ml streptomycin (GIBCO) at 37°C in a 95% air – 5% CO2 humidified environment. Cell culture media were changed every other day until the cells were stimulated with recombinant proteins or infected with adenoviruses, as detailed below.

2. Rat granulosa cells

Immature 23 days old female Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA, USA) and diethylstilbestrol-containing (DES; Sigma) capsules were implanted subcutaneously 72 h prior to killing the rats with CO2 (Hsueh et al., 1984). Ovaries were retrieved and the follicles were punctured in L-15 Leibovitz medium (Life Technologies, Gaithersburg, MD, USA) using 25 gauge needles. After removal of ovarian debris the granulosa cells were collected by centrifugation for 10 min at 500 x g after which the cells were dispersed by repeated suspension in McCoy’s 5a modified medium (Mediatech, Herndon, VA, USA) supplemented with 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (reagents obtained from BioWhittaker, Wakersville, MD, USA) and 1 ng/ml recombinant FSH (Puregon, Organon) unless otherwise stated. An aliquot of the cells were counted after staining with trypan blue, and finally the cells were cultured in Falcon polypropylene tubes (Becton Dickinson, Franklin Lakes, NJ, USA) or plated on 35 x 10 mm culture dishes (Beckton Dickinson) at densities specified in study V at 37°C in a 95% air – 5% CO2 humidified environment.

3. MIN6 cells

MIN6 cells were originally established from mouse insulinomas and this cell line shows several morphological characteristics of pancreatic β cells (Miyazaki et al., 1990). Furthermore, it has been shown to endogenously express ALK7 (Watanabe et al., 1999). MIN6 cells were cultured on 6-well plates (Greiner Bio-One) in DMEM (GIBCO) supplemented with 15% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 4.5 g glucose/l, 5 µl β-mercaptoethanol/l, 2% HEPES and 3.4 g NaHCO3/l (reagents obtained from GIBCO) at 37°C in a 95% air – 5% CO2 humidified environment.

4. 293T cells

Human fetal 293T cells (Graham et al., 1977; DuBridge et al., 1987) were grown in DMEM (GIBCO) supplemented with 10% FCS 2 mM L-glutamine, 100 IU/ml penicillin 100 µg/ml streptomycin (reagents obtained from GIBCO) at 37°C in a 95% air – 5% CO2 humidified environment in standard 175 ml plastic culture flasks (Greiner Bio-One). The cells were passaged twice weekly.
5. In vitro treatment of cells

To study the activation of Smads and stimulation of inhibin subunits the following recombinant proteins were used. Recombinant BMP-2, BMP-3, BMP-3b, BMP-4 and BMP-6 used in Study III were gifts from Dr. Rosen (Genetics Institute, Cambridge, MA, USA) and recombinant activin A a gift from Dr. Eto (Ajinomoto Inc., Kawasaki, Japan), respectively. In the other studies, recombinant activin A and BMP-2 were purchased from R&D Systems (Minneapolis, MN, USA). Recombinant human follistatin (288-amino acid follistatin) and hCG were supplied by the National Hormone and Pituitary Program (Baltimore, MD, USA) and recombinant FSH from Organon. The production of recombinant rat GDF-9 protein has been described (Hayashi et al., 1999). Briefly, 293T cells were transfected with an expression vector containing the rat GDF-9 cDNA. Selection of clones stably expressing GDF-9 was done using Zeocin (Invitrogen, San Diego, CA, USA). Conditioned media were harvested after 4 days of serum-free culture and finally concentrated. Descriptions of the stimulation media and treatment times can be found in studies II-V.

6. Recombinant adenoviruses

High titer stocks of recombinant Smad1/2/6/7, constitutively active (ca) and kinase defective (kd) ALK1-7 and LacZ adenoviruses were grown in 293T cells. The original cloning and generation of these viruses has been reported earlier (Fujii et al., 1999). Briefly, a kit based on a previously described method (Miyake et al., 1996) for making recombinant human serotype 5 adenoviruses was purchased from Takara Biomedicals (Takara Shuzo Co., Shiga, Japan). The respective Smad and ALK cDNAs were cloned into the vector pAxCAwt, which contains a potent β-actin promoter and cytomegalovirus (CMV) enhancers. After preparation of cosmids in competent cells (Eschericia coli), co-transfection of the cosmids with adenoviral DNA into 293 cells was performed, after which recombinant adenoviruses were generated through homologous recombination. The recombinant adenovirus vector used lack the E1 and E3 genes needed for propagation of the virus. However, these genes are provided by 293 cells, hence the adenoviruses can propagate in them. For the generation of high titer stocks 293T cells were seeded on gelatin-coated culture flasks (Greiner Bio-One). After achieving 50-70% confluency (within 1-2 days), the cells were infected with the recombinant adenovirus. Within 2-3 days the cells started to detach and show cytopathic effects, after which they were harvested and lysed using repeated freezing and thawing. These steps were repeated three times and for the final round of amplification twelve 175 ml culture flasks were used. Purification of the adenoviruses from the final pool of lysed cells was achieved by ultracentrifugation for 90 min through CsCl gradients of densities 1.3 and 1.6. The virus band was retrieved and purified using PD-10 columns (Pharmacia) and the fraction containing the virus was determined using spectrophotometry. Virus aliquots were stored at -80°C in PBS containing 10% glycerol. Tittrations of the recombinant adenoviruses were performed in 293T cells according to the TCID50 method described in the adenovirus manual supplied by Takara Biomedicals (http://bio.takara.co.jp/BIO_EN/default.asp). All adenoviral work was carried out in a P2 level classified laboratory. Infections of granulosa and MIN6 cells were done in serum-free media for 1-2 h, after which the media were changed and the cells were cultured for 8-72 h depending on the experiment as detailed in studies II-V.

7. Transfection of rat granulosa cells

Cultured rat granulosa cells were transfected with inhibin-α promoter luciferase and control RSV-β-galactosidase plasmids under serum-free conditions for 4 h at 37°C using lipofectamine (GIBCO) (Feligner et al., 1987). After culturing for 24 h in media supplemented with 1% FCS the cells were lysed and the supernatants analyzed using a Monolight 2010 luminometer (Analytical Luminescence Lab., San Diego, CA, USA).

8. RNA extraction and preparation of filters

Total RNA from cultured hGL cells or cytoplasmic RNA from freshly isolated hGL cells was extracted with the guanidine isothiocyanate-cesium chloride method (Chirgwin et al., 1979) or using the modified Nonidet P-40 lysis procedure (Favaloro et al., 1980), respectively. Rat granulosa cell total RNA was extracted using an RNeasy extraction kit provided byQiagen (Valencia, CA, USA). RNA
samples were quantitated by absorbance measurement at 260 nm. For Northern blots, indicated amounts of RNA were size-fractionated in 1.5% agarose gels, after which they were transferred to Hybond-N nylon filters (Amersham Pharmacia Biotech, Little Chalfont, UK). For dot blot studies, RNA samples were denatured and spotted onto nylon membranes using a 96-well Minifold device (Schleicher and Schuell, Keene, NH, USA). Finally, the filters were baked at 80°C for 1 h prior to UV-cross-linking for 6 min using a Reprostar II ultraviolet illuminator.

9. Preparation of cDNA probes and hybridizations

Double-stranded cDNA probes were labeled by random priming (Feinberg and Vogelstein, 1983) with [α-32P]-α-deoxy-CTP (3000 Ci/mmole; Amersham) using a Prime-a-gene kit (Promega, Madison, WI, USA). In the case of the human inhibin βB-subunit a single-stranded cDNA probe was generated using PCR as previously described (Erämaa et al., 1995). Purification of the probes was achieved using Nick columns (Pharmacia). Hybridizations were performed overnight at temperatures indicated in studies II-V, in hybridization solution containing 50% formamide, 6 x SSC, 0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% BSA, 100 µg/ml salmon sperm DNA, 100 µg/ml yeast RNA and 0.5% SDS, or in the case of commercial filters, in hybridization solution provided by the manufacturer (Clontech Laboratories, Palo Alto, CA, USA). The probes were used at a concentration of 1-3 x 10^6 cpm/ml. The filters were washed two to four times for 20 min with 0.1-1 x SSC, 0.1-1% SDS at 50-65°C with continuous agitation. For detection the filters were exposed to Fujifilm phosphoimaging plates, after which the plates were analyzed using a Fujifilm IP-Reader Bio-Imaging Analyzer BAS 1500 (Fuji Photo co. Ltd., Tokyo, Japan) and MacBAS software provided by the manufacturer on an Apple Macintosh PC (Apple Corp., Cupertino, CA).

10. Western blotting

Prior to Western blotting, the cells were washed once on ice with PBS, after which they were harvested in 1× Laemmli-buffer containing 10% β-mercaptoethanol. Cells were gently sonicated on ice for 10 s with a Soniprep 150 MSE sonicator (Sanyo Corp., Japan) and boiled for 3 min. Proteins were separated on 10-15% SDS-PAGE gels and electroblotted onto Hybond-C membranes (Amersham). For the detection of hemagglutinin (HA)-tagged ALK receptors a monoclonal rat anti-HA antibody (Boehringer Mannheim, Indianapolis, IN, USA; clone 3F10) was utilized, and for the detection of FLAG-tagged Smads a monoclonal anti-FLAG antibody (Sigma; clone M2) was used. The commercial antibodies were used according to protocols provided by the manufacturers. For the detection of C-terminally phosphorylated Smad1 and Smad2, membranes were blocked for 1 h at room temperature in TBS-Nonidet P40 containing 2.5% fat free dry milk, after which membranes were incubated with either a previously described anti-phosphoSmad1 (αPS1) antibody or anti-phosphoSmad2 (αPS2) antibody diluted 1:12000 in TBS-Nonidet P40 containing 2.5% fat free milk at 4°C overnight (Persson et al., 1998; Piek et al., 1999b). Membranes were washed 3 times for 10 minutes with TBS containing 0.05% Tween-20 or Nonidet P-40 prior to incubation for 1 h at room temperature with a secondary anti-rabbit antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) diluted 1:14000 in TBS-Nonidet P40 containing 2.5% fat free milk. After washing four times for 10 min with washing solution the immunoreactive proteins were detected using enhanced chemiluminescence (ECL PLUS kit; Amersham).

11. Protein content

The protein concentrations of cellular samples lysed with 0.1 M NaOH for 1 h at room temperature and neutralized with 0.1 M HCl were measured by the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA).

12. Enzyme-linked immunosorbent assay (ELISA)

Commercial ELISAs for the detection of immunoreactive dimeric inhibin A and B in the spent granulosa cell media were used according to the manufacturer’s (Serotec Ltd., Oxford, UK) instructions. The assays use monoclonal antibodies directed toward the human inhibin βA- and βB-subunits for capture and a Fab alkaline phosphatase conjugated monoclonal antibody against the human inhibin α-subunit for detection. The crossreactivity between the two assays is minimal, approximately 0.5% for inhibin A in the inhibin B ELISA and < 0.1% for inhibin B in the inhibin A ELISA (Groome
et al., 1994; Groome et al., 1996). A signal amplification kit (Life Technologies) was used in connection with the ELISAs. To achieve concentrations within the measuring ranges of the assays the spent culture medium was diluted with fresh medium.

13. Fluorescence in situ hybridization (FISH)

To obtain clones for FISH we screened a human total genomic P1 artificial chromosome (PAC) library (kind gift of Dr. Pieter de Jong, Roswell Memorial Institute, Buffalo, NY, USA) using specific primers for human activin type II receptors, follistatin and ALK7. FISH was performed on metaphase chromosomes derived from human peripheral blood leukocytes. The cells were cultured according to standard protocols and treated with 5-bromo-deoxyuridine (BrdU, 200 µg/ml) at an early replicating phase to induce banding pattern (Lemieux et al., 1992). Hybridization with biotin 11-dUTP (Sigma) labeled specific clones were carried out in 50% formamide, 10% dextran sulfate in 2 x SSC as described earlier (Pinkel et al., 1986; Lichter et al., 1988). Slides were stained with Hoechst 33258 (1 µg/ml) and exposed to UV light for 30 min, and counterstained with DAPI including antifading reagent (Vectashield, Vector Laboratories, Burlingame, CA). A multicolor image analysis was used for acquisition, display and quantification of hybridization signals of metaphase chromosomes with an system described earlier (Heiskanen et al., 1996).

14. ALK7 cDNA cloning

In a screen for new ser/thr kinases in databases on the Internet (http://www.ncbi.nlm.nih.gov/blast), a human genome survey sequence (gss) was found (GenBank AQ129133) showing 83% identity to the corresponding sequence of the rat ALK7 gene. PCR primers were subsequently designed to amplify the found sequence from human genomic DNA and the amplified PCR fragment was sequenced using an ABI PRISM 377 DNA sequencer (Perkin-Elmer, Applied Biosystems, Foster City, CA). A human brain ZAP II cDNA library with 9 × 10⁶ independent clones was screened after labeling the PCR fragment with the Megaprime DNA labeling system (Amersham). Hybridization was performed in a solution containing 50% formamide, 5 x SSC, 5 x Denharts, 0.1% SDS and 50 µg/ml salmon sperm DNA overnight at 37°C. The filters were washed in 2 x SSC, 0.1% SDS, twice at room temperature, and in 0.2 x SSC, 0.1% SDS twice at 55°C. A phagemid vector pBluescript SK(-) with a 3.5-kb insert containing the human ALK7 cDNA was obtained by excision from the lambda vector with the helper phage R408 as earlier described (Short et al., 1988).

15. Statistical methods

All statistical analyses were performed with Extatics software (Select Micro Sytems, Yorktown Heights, NY, USA) on a Macintosh PC. For single comparisons Student’s t test or Mann-Whitney’s U test were used and for multiple comparisons one-way analysis of variance (ANOVA) followed by Scheffé’s test or Duncan’s multiple range test were applied. Statistical significance was defined as a P value ≤ 0.05.
RESULTS AND DISCUSSION

1. CHROMOSOMAL MAPPING OF HUMAN ACTIVIN TYPE II RECEPOTRS, FOLLISTATIN AND ALK7 (I AND II)

Due to the multitude of developmental defects associated with the loss of activin type II receptors and follistatin previously reported in knockout mice (Matzuk et al., 1995a; Matzuk et al., 1995c; Oh and Li, 1997), we set out to determine the chromosomal localizations of the human orthologues. Ultimately we wanted to look for possible associations between the chromosomal loci of the orthologues and known chromosomal localizations of developmental disorders reminiscent of the respective knockout mice phenotypes. Also, since ser/thr kinase receptors mediate growth inhibition in a number of cell types, knowledge of their loci is likely to be useful for cancer geneticists.

We screened a human total genomic P1 artificial chromosome (PAC) library using specific primers for the human activin type II receptors, follistatin and ALK7. After labeling of identified positive clones with biotin, we performed FISH on metaphase chromosomes and determined the chromosomal localizations of the respective genes (as detailed in the Materials and Methods section).

1.1. Activin receptor type II (ACVRII)

We mapped ACVRII to 2q22.2-q23.3 (I, Fig. 1A). However, no known syndromes reminiscent of the knockout mice phenotype were found to be associated with this chromosomal region. Previous studies on mice deficient in activin type II receptors have shown that the lack of this receptor leads to multiple developmental defects. Briefly, ~20% of mice with a targeted disruption in the ACVRII gene showed alterations in mandible development frequently associated with cleft-palate malformations and died soon after birth. Nonetheless, the majority of the ACVRII null mice seemed morphologically normal and developed into adults. Among the viable mice, the reproductive performance of the female mice was affected and they did not undergo normal estrus cycles, probably due to low serum FSH levels. Male mice were fertile, even though they had smaller testes than wild-type mice (Matzuk et al., 1995a).

1.2. Activin receptor type IIB (ACVRIIB)

The human ACVRIIB gene was mapped to chromosome band 3p22 (I, Fig 1B), and the result is in agreement with a report by Ishikawa et al. (1998). ACVRIIB null mice have been shown to die postnatally within days. These mice showed severe cardiovascular defects including randomized heart position, malposition of the great arteries and ventricular and septal defects. Further developmental defects included renal abnormalities and an anomalous patterning of the vertebrae (Oh and Li, 1997). Double-knockout embryos for the two activin type II receptors died before gastrulation, indicating that the two receptors have only partially overlapping functions during mammalian development (Oh and Li, 1997). Interestingly, two
unrelated patients with the situs inversus syndrome have recently been reported to have missense mutations in the ACVRIIB gene (Kosaki et al., 1999).

1.3. Follistatin (FST)

We found that the follistatin gene, which codes for a protein known to bind for example activin, inhibin (Nakamura et al., 1990) and GDF9B/BMP15 (Otsuka et al., 2001a), maps to chromosome band 5q11.2 (I, Fig. 1C). Mice deficient in follistatin die within hours postnatally. The phenotype of these mice show growth retardation associated with decreased muscle mass, a shiny taut skin, skeletal defects, abnormal whiskers and tooth development and ultimately they fail to breathe (Matzuk et al., 1995c). On the contrary, follistatin-overexpressing transgenic mice show a dramatically increased skeletal muscle mass, supposedly through inhibition of myostatin activity (Lee and McPherron, 2001). Interestingly, a study reporting a link between the chromosomal region 5p14 and the polycystic ovary syndrome (PCOS) has been published (Urbanek et al., 1999). The authors considered follistatin as a candidate gene for PCOS and subsequently mapped it to this region (5p14), which is in disagreement with our results. Therefore, to confirm our own results we obtained a human genomic clone of follistatin (kind gift of Prof. Shunichi Shimasaki, University of California, USA) and repeated the FISH with this novel probe (unpublished data). The results were in accordance with our original, published findings. Furthermore, the International Radiation Hybrid Mapping Consortium recently mapped the follistatin gene to chromosome 5q (http://www.ncbi.nlm.nih.gov), in agreement with our results. Later, Urbanek et al. reported that no mutations were found in the follistatin gene of PCOS patients (Urbanek et al., 2000).

1.4. Activin receptor-like kinase 7 (ALK7)

The gene for ALK7 was mapped to 2q24.2-q3 (II, Fig. 3). Some patients lacking this chromosomal region have been described. They show severe developmental defects but whether this is due to loss of ALK7 is not currently known (Chinen et al., 1996; Nixon et al., 1997). A knockout mouse model for ALK7 has not been reported thus far. ALK7 is discussed in more detail below.

2. SEARCH FOR NOVEL SERINE/THREONINE KINASE RECEPTORS (II)

2.1. Cloning and characterization of ALK7

In 1998-2000, when this project was undertaken, it was not known whether the human genome would contain novel type I and/or type II ser/thr kinase receptors in addition to the previously reported ones. Furthermore, the receptors for the ovary-specific ligands GDF-9 and GDF-9B/BMP-15 were unknown, but likely to be ser/thr kinases. Therefore, in an attempt to find new receptors we performed Basic Local Alignment Search Tool (BLAST®) searches against genomic databases on the Internet (http://www.ncbi.nlm.nih.gov/blast) using known ser/thr kinases as templates. We identified a human genomic survey sequence (gss), part of which shared 83% homology with the transmembrane region of the rat ALK7 receptor. This is a lower degree of homology than that shown by the corresponding transmembrane regions of
most other human and rodent ALKs. Primers specific for the ALK7 resembling part of the gss were designed and PCR was performed using human genomic DNA as the template. The derived PCR-product was used for screening multiple-tissue Northern blot filters. The expression profile was highly similar to that previously reported for the rat ALK7 receptor (Rydén et al., 1996; Tsuchida et al., 1996), being most prominent in the brain. Therefore, a brain cDNA library was screened, after which the total open reading frame (ORF) for this receptor, the human orthologue of the rat ALK7, was obtained. Rat and human ALK7 receptors were found to exhibit a 93.5% level of homology in their amino acid sequence (II, Fig. 2).

It has previously been shown that ALK7 is expressed in mouse insulinoma derived MIN6 cells, which is in line with the fact that based on a RT-PCR study ALK7 appears to be the most abundantly expressed type I ser/thr kinase receptor in fetal rat pancreatic islets (Watanabe et al., 1999). Therefore, MIN6 cells were chosen for our ALK7 signaling studies. An adenovirus with a constitutively active mutant form of ALK7 (Ad-caALK7) was obtained and used to infect cutured MIN6 cells. By Western blotting the phosphorylation of both endogenous Smad2 and adenovirally derived Smad2 by Ad-caALK7 was shown (II, Fig. 4), which is in line with previous reports (Watanabe et al., 1999; Jörnvall et al., 2001). The phosphorylation status of Smad1/5/8 was not altered by ALK7. Thus, it seems evident that ALK7 signals through the same Smads as ALK4 and ALK5, whereas ALK1, ALK2, ALK3 and ALK6 form a functionally distinct subfamily. This is in line with the fact that the kinase domain of ALK7 is very similar to those of ALK4 and ALK5 (Tsuchida et al., 1996). However, the extracellular domains of these ALKs are clearly divergent, which is in agreement with their different ligand specificities/affinities.

ALK7 was originally cloned as an orphan receptor, without known ligands (Rydén et al., 1996; Tsuchida et al., 1996). A recent study by Reissmann et al. showed that ALK7 in association with ACVRIIB function as Nodal receptors in the early embryo (2001). Nodal has been described as an embryonic protein. Nevertheless, its mRNA was recently identified using RT-PCR in the postnatal mouse mammary gland (Bianco et al., 2002). To examine whether Nodal would be expressed in the adult human we obtained and labeled a human Nodal expressed sequence tag (EST; IMAGE clone #5239967) with [α-32P]-α-deoxy-CTP and performed Northern blot analyses of multiple human tissue mRNA filters. Even so, no clear expression of Nodal mRNA was detected (unpublished data). These results, combined with the fact that ALK7 is widely expressed in several tissues in the adult, might indicate that, in addition to mediating signaling by Nodal in the embryo, ALK7 serves as the receptor for other ligands in the adult. This hypothesis remains to be verified.

### 2.2. Ovarian ALKs

In parallel to the ALK7 cloning project we set out to clone all ALKs being expressed in the ovary. Using cDNA derived from ovaries of two-week-old mice we performed PCR, using degenerate primers corresponding to the highly conserved kinase domains of the ALKs, in a receptor cloning strategy that has previously been successfully used by others (ten Dijke et al., 1993). We sequenced approximately 200 independent clones and identified six out of seven known ALKs (Table 5). Sixty-five percent of the identified type I receptors were ALK3. However, much caution is needed when interpreting these results, since PCR primer stoichiometry might at least partially
explain the quantitative differences of different ALKs obtained (unpublished data). Taken together these results combined with our database screens and those of others (Vitt et al., 2002) suggest that no new type I nor type II ser/thr kinases remain to be discovered in the mammalian genome. Therefore, it seems likely that also GDF-9 and GDF-9B/BMP15 mediate their effects via a combination of known type I and II receptors.

Table 5. Expression pattern of ALKs in two-week-old mouse ovaries.

<table>
<thead>
<tr>
<th>Ser/thr kinases identified</th>
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<tbody>
<tr>
<td>ALK1</td>
<td>1</td>
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<tr>
<td>ALK2</td>
<td>16</td>
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<td>ALK3</td>
<td>72</td>
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<td>ALK4</td>
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<td>ALK5</td>
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<td>ALK6</td>
<td>0</td>
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<tr>
<td>ALK7</td>
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3. EXPRESSION OF SERINE/THREONINE KINASE RECEPTORS AND SMADS IN HUMAN GRANULOSA-LUTEAL CELLS (III AND IV)

3.1. Ser/thr kinases

It has previously been shown that hGL cells express the receptors needed for TGFβ- and activin-mediated signaling (Erämaa et al., 1994; Erämaa and Ritvos, 1996). The expression of a BMP ligand in these cells, BMP-3, has also been observed (Jaatinen et al., 1996), but whether these cells would also express BMP receptors was not known when our studies were undertaken. To answer this question we performed Northern blot analysis and subsequently demonstrated the presence of the BMPRII and ALK3 receptors, but we failed to detect ALK6 in these cells (III, Fig. 1). The failure in detecting ALK6 should perhaps be re-evaluated (e.g., by using RT-PCR) since this receptor has recently been shown to be abundantly expressed in both rodent and ovine ovaries, and it appears to be needed for female fertility in mice and sheep (Shimasaki et al., 1999; Mulsant et al., 2001; Wilson et al., 2001; Yi et al., 2001; Souza et al., 2002). Using Northern blot hybridization we detected ALK7 mRNAs in the human ovary (Study II). However, its localization has not been specifically localized to, for example, the hGL cell compartment. Hence, with the possible exception of ALK1, ALK6, ALK7 and AMHRII all other type I and II ser/thr kinases appear to be expressed in human hGL cells. AMHRII has been reported to be expressed in rat granulosa cells of growing follicles, but its expression appears to be lost in the corpus luteum (Baarends et al., 1995). In our unpublished experiments we have failed to detect AMHRII expression in RNA derived from hGL cells, indicating that its expression might be lost in these human luteinized cells. Furthermore, we did not detect any Smad activation after stimulation of these cells with recombinant AMH (unpublished data). It seems plausible that in the human ovary the expression profile of different ser/thr kinase receptors changes with follicular growth, as has recently been shown to be the case for the activin receptors (Pangas et al., 2002).
3.2. Smads

By Northern blot hybridization analysis we detected mRNA transcripts for the R-Smads Smad1, Smad2, Smad3 and Smad5 and the common partner Smad, Smad4, in unstimulated hGL cells (III, Fig. 1) and by immunohistochemistry we showed the presence of the (phosphorylated) Smad1 and Smad2 proteins (IV, Fig. 2). We further evaluated how a prolonged culture time would affect the expression levels of the Smads. The Smad transcripts were found to steadily decline when compared to the expression levels found in freshly isolated cells, an effect most prominently observed for Smad3, but not evident for Smad1 nor Smad2 (III, Fig. 1). The inhibitory Smad Smad6 was barely detectable in unstimulated hGL cells, but its transcripts were induced after stimulation with recombinant BMP-2. Whether Smad7 and Smad8 are expressed in these cells has not been shown, however, within the rat ovary Smad8 has only been detected in oocytes (Drummond et al., 2002). Taken together our results confirm the presence of both TGFβ/activin and BMP signaling machinery components in hGL cells.

4. STIMULATION OF INHIBIN PRODUCTION IN HUMAN GRANULOSA-LUTEAL CELLS BY BONE MORPHOGENETIC PROTEINS (III)

Previous studies have revealed that activin and TGFβ are selective inducers of the inhibin βB-subunit (and dimeric inhibin B) in cultured hGL cells, whereas gonadotropins stimulate the expression of the α- and βA-subunits (and dimeric inhibin A) in a time- and concentration-dependent manner (Erämaa et al., 1995; Erämaa and Ritvos, 1996; Vänttinen et al., 2000; Liu et al., 2001). To test the possible involvement of BMPs in inhibin subunit regulation in hGL cells we used recombinant BMP-2, -3, -3b and –6. Initial experiments using 50-100 ng/ml of the respective ligands revealed that both BMP-2 and BMP-6 (representing two different subgroups within the BMP family), but not BMP-3 nor BMP-3b, were able to selectively induce the expression of the inhibin βB-subunit in cultured hGL cells. Neither the transcripts for the α- nor the βA-subunits were affected by any of the recombinant BMPs tested. As shown previously (Erämaa et al., 1995), hCG did not induce the transcripts of the inhibin βB-subunit, and it was further shown to suppress the stimulatory effects of BMP-2. By treating the cells with 20 µg/ml of cycloheximide for 30 min prior to stimulation with BMP-2, we showed that the stimulatory effect of BMP-2 on the inhibin βB-subunit was dependent on de novo protein synthesis (III, Fig. 4). Furthermore, since follistatin has been reported to block several BMPs, e.g., BMP-4 and GDF-9B/BMP-15 (Fainsod et al., 1997; Otsuka et al., 2001a), we wanted to determine whether it would also block the biological effects of BMP-2. Even though follistatin was able to block activin in our hGL assay, not even a 7.3-fold molar excess of follistatin was sufficient to block BMP-2-induced stimulation of the expression of the inhibin βB-subunit mRNA (III, Fig. 6). Finally we used a specific ELISA assay for dimeric inhibin B and showed that BMP-2 stimulated the synthesis of this protein in a dose- and time-dependent fashion, in concordance with the RNA data obtained previously (III, Fig. 7).
In this study (III) we showed for the first time that different BMP members are able to evoke a biological response in human granulosa cells. Furthermore we demonstrated that BMP receptors and downstream signaling protein Smads are expressed in these cells. Several BMPs have recently been identified in mammalian ovaries of different species (Lyons et al., 1989; Hino et al., 1996; Jaatinen et al., 1996; Takao et al., 1996; Shimasaki et al., 1999), which might indicate that these factors play important roles as local auto- or paracrine regulators of inhibin production. Like other TGFβ superfamily members studied, including TGFβ, activin A (Erämaa et al., 1995; Erämaa et al., 1996; Liu et al., 2001) and GDF-9 (unpublished data), we conclude that BMP-2 and BMP-6 also are able to selectively induce the expression of the inhibin βB-subunit in cultured hGL cells. Interestingly, recombinant BMP-3 proteins did not show any apparent effect, which is well in line with a recent report demonstrating that BMP-3 actually might function as an antagonist of BMP-mediated signaling (Daluiski et al., 2001). Further experiments should be undertaken to determine whether BMP-3 functions as an inhibitor of BMP-2-mediated synthesis of dimeric inhibin B also in the hGL system. It is also possible that other antagonists of BMP-mediated signaling in addition to BMP-3 are expressed in the human ovary, and recent data indicate that mRNAs for Bambi/nma are expressed in cultured hGL cells (unpublished data). Furthermore, genetic evidence from a strain of sheep where heterozygotes for a mutation of the GDF-9B/BMP-15 gene are hyperprolific and homozygotes infertile (Galloway et al., 2000), adds further evidence to the belief that the specific “dosage” of a certain BMP ligand might be crucial for its biological activity/activities, suggesting that specific inhibitors might also be present in the ovary.

We decided to use hGL cells as a model for inhibin subunit regulation since they are relatively easily obtainable and all three inhibin subunits are expressed in them, even though the expression levels of the βB-subunit are very low (Erämaa et al., 1993). Based on our results it is possible that during early human folliculogenesis the heavy stimulation of the βB-subunit seen in vivo is achieved by a concerted action of various members of the BMP family together with other growth factors such as activin and TGFβ. However, when interpreting the results of this study it is important to note that the hGL cells used in this study were not derived from small antral follicles, which are presumed to be the main expression site of the inhibin βB-subunit during folliculogenesis (Schwall et al., 1990; Fraser et al., 1993; Roberts et al., 1993), a hypothesis further supported by the rise in circulating inhibin B serum levels seen during the beginning of the follicular phase of the human menstrual cycle (Groome et al., 1996). Therefore, much caution is needed when drawing conclusions about the physiological relevance of the in vitro regulation of the different inhibin subunits observed in hGL cells.

A recent study described inhibin subunit regulation in human granulosa cell cultures derived from small antral follicles (Welt and Schneyer, 2001). FSH and cAMP were found to selectively induce secretion of inhibin A, but not inhibin B, which is in line with results obtained from the hGL model. However, in vivo the situation might be otherwise, because the rise in serum inhibin B levels during the follicular phase is accompanied by a rise in FSH. One possible explanation could be that FSH increases the number of granulosa cells, which indirectly results in an increase in the amount of dimeric inhibin B being produced. Interestingly, none of the different growth factors tested by Welt and Schneyer (activin A, insulin like growth factor (IGF)-I and insulin) were able to induce inhibin B production on their own, which is in disagreement with
our data. It would be useful to use human granulosa cells derived from small antral follicles to determine the role of different BMPs for inhibin B production. These cells are, however, not easily obtainable. Nevertheless, it seems evident that in the human granulosa cells the production of, on the one hand, inhibin A and, on the other, inhibin B are individually regulated.

5. OVEREXPRESSION OF ALKS AND SMADS IN HUMAN GRANULOSALUTEAL CELLS STIMULATES INHIBIN B PRODUCTION (IV)

Different members of the TGFβ superfamily, such as activin, TGFβ (Erämaa et al., 1995; Erämaa et al., 1996) and BMP-2 (III), have recently been shown to selectively induce expression of the inhibin βB-subunit in cultured hGL cells. The downstream signaling of these ligands is mediated through type I ser/thr kinase receptors that activate Smad signaling proteins and various MAPK family members in response to ligand binding. ALK1/2/3 and 6 are known to activate Smad1, Smad5 and Smad8, whereas ALK4/5 and 7 have been shown to activate Smad2 and Smad3. Recently, Smads1-8 were shown to be expressed in the rat ovary (Drummond et al., 2002), while we have shown that several representatives of the Smad family are expressed in the hGL cells (Study III). To investigate the direct effect of type I receptors and Smad signaling proteins on dimeric inhibin production we had to find a means of overexpressing these factors in cultured hGL cells. These nondividing primary cells are exceedingly difficult to transfect by standard liposome-mediated methods (<1% cells transfected, unpublished data), wherefore other methods had to be applied. Recombinant adenoviruses have been used as gene transfer vectors in numerous settings for the in vitro overexpression of a specific gene as well as in clinical gene therapy trials. Therefore, we evaluated the feasibility of these viral vectors for gene delivery in granulosa cell cultures. First, some general background on the use of recombinant adenoviruses will be presented below.

5.1. Recombinant adenoviruses

The discovery and isolation of adenoviruses dates back to 1953 when investigators tried to establish cell lines from adenoidal tissues removed from children and military recruits (Rowe et al., 1953). Thereafter adenoviruses have been shown to cause a multitude of human diseases including upper respiratory tract infection, conjunctivitis and gastroenteritis. The human adenoviruses form almost 50 different serotypes, which are further divided into six subgroups (denoted A-F). Serotypes 2 and 5 of subgroup C are the most commonly used backbones for the generation of recombinant adenoviral vectors. The genomes of serotype 2 and 5 adenoviruses consist of approximately 36 kilobases (kb) linear double-stranded DNA, which encodes 20-30 different proteins. The packaging capacity of the virus restricts the insertion of foreign cDNAs to approximately 8 kb. The adenoviruses are nonenveloped, icosahedral in shape and have a diameter of 60-90 nm (Fig. 6). Adenoviruses infect cells by first binding to specific extracellular "coxsackievirus-adenovirus receptors" (CAR) expressed on the surface of most cells (Bergelson et al., 1997). After receptor binding the complex associates with heterodimeric integrins which allows internalization of the virus through endocytosis. Inside the target cell the virus is partially uncoated, after which the DNA further migrates to the nucleus where its genes are transcribed to
mRNA (reviewed in Yeh and Perricaudet, 1997; Wang and Huang, 2000; Mizuguchi et al., 2001).

**Fig. 6.** Schematic drawing of an adenovirus.

After cellular transduction the adenoviral early transcriptional genes (E1-E4) are first transcribed. These are responsible for viral gene transcription, DNA replication, host immune suppression and inhibition of apoptosis of the infected cell (reviewed in Wang and Huang, 2000). The recombinant adenoviruses used in this study have deletions in E1 and E3, which prevent them from propagating in ordinary cells. E1 is absolutely needed for propagation whereas E3 encodes proteins associated with host defense mechanisms and is not needed for virus propagation (reviewed in Mizuguchi et al., 2001). For the generation of high titer virus stocks effective propagation of the virus is needed. This is achieved by infecting, for example, 293 or 911 cells that stably express the early transcriptional genes needed (E1) and, after concentration, virus stocks containing up to $10^{12}$ particles/ml can be achieved (reviewed in Wang and Huang, 2000).

5.2. Overexpression of adenovirally derived proteins in hGL cells

We used 293T cells to grow high titer stocks of different recombinant adenoviruses, including constitutively active and kinase defective ALKs and wild type Smads (Fujii et al., 1999). We found that human granulosa cells were very susceptible to adenoviral infection, indicating that they express high levels of adenoviral receptor molecules. Already a multiplicity of infection (m.o.i) value of one or even less produced in most cases a detectable amount of adenovirally derived protein (IV, Fig. 1). When m.o.i. values $>300$ were used the granulosa cells started to show cytopathic effects and detached from the plastic within 24 h, hence lower concentrations of viruses were routinely used. Primary luteinized granulosa cells form an optimal target for adenovirally-mediated gene transfer since they do not propagate in vitro and consequently the expression level of the transduced gene is not “diluted” by cell division. The expression of the virally derived gene product was detectable starting at 6 h post-infection and peaked 1-2 days post-infection. To our knowledge this was the first time recombinant adenoviruses were used to infect human granulosa cells in culture. Nonetheless, this technique has recently been applied to rat granulosa cells in

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**The protein shell is composed of 252 subunits, called capsomeres (240 hexons and 12 pentons). The viral genome consists of double stranded DNA, which is associated with a protein complex (terminal protein).**
We first infected cultured hGL cells with adenoviruses encoding constitutively active mutants of ALK1-7. The expression of the adenovirally-derived proteins was routinely confirmed by immunoblotting with antibodies against specific C-terminal tags (HA and FLAG for ALKs and Smads, respectively). We next showed these receptors to be biologically active by immunoblotting against phosphorylated Smad signaling proteins 6-12 h post-infection with a caALK virus (IV, Fig. 2). It seems evident that ALKs phosphorylate Smads in granulosa cells in the same way as has previously been observed in numerous other types of cells. We further wanted to examine the effect of caALK overexpression on the production of dimeric inhibin B. All seven ALKs were found to stimulate the production of inhibin B, the effect being most profound for ALK4 and ALK5 (IV, Fig. 4). This might be due to the fact that granulosa cells produce small amounts of activin and TGFβ, which in an auto/paracrine manner further stimulate the overexpressed type I receptor, leading to an “additive” effect on inhibin B production.

Interestingly, when Smad1 and Smad2 were overexpressed alone they were shown to become phosphorylated independently of addition of exogenous ligands (IV, Fig. 5). Others have recently addressed the reason for this intriguing finding known from numerous in vitro studies. It was shown that in the case of Smad3, SARA might serve as a guardian preventing monomeric Smad3 from forming activated trimers in the absence of proper ligand stimulation. High levels of overexpressed Smad3 possibly overcome the limits of SARA of preventing them from becoming aberrantly activated (Qin et al., 2002). But, whether this or similar mechanisms also apply for overexpressed Smad1 and Smad2 remains to be shown in the future.

The production of inhibin B was clearly stimulated after overexpression of Smad1 and Smad2 (IV, Fig. 5). This is in line with our previous results indicating that both TGFβ/activin- and BMP-activated signaling pathways converge on the same final stage, the stimulation of inhibin B production (Erämaa et al., 1995; Erämaa and Ritvos, 1996, Study III). In our unpublished results the stimulatory effect on dimeric inhibin A production by caALK1-7 was minimal, which is in line with RNA and protein data previously obtained by us and others (Erämaa et al., 1995; Erämaa and Ritvos, 1996; Tanimoto et al., 1996; Vänttinen et al., 2000; Liu et al., 2001, Study III). Finally, Smad7 overexpression effectively shut off the stimulatory effects of activin and BMP-2 on inhibin B production (V, Fig. 7), further indicating that Smads might participate in the regulation of inhibin production in hGL cells.

6. STIMULATION OF RAT GRANULOSA CELLS WITH GROWTH DIFFERENTIATION FACTOR-9 (V)

The aim of this part of the study was to determine whether treatment with recombinant GDF-9 would stimulate inhibin production in rat granulosa cells. BMPRII was identified by our collaborators to be a receptor for GDF-9 during the course of this study (Vitt et al., 2002) and it was further anticipated that GDF-9 stimulation of the granulosa cells would lead to R-Smad phosphorylation. Even so, whether the Smads would be of the activin/TGFβ or the BMP subgroup was not known prior to this study.
The mRNA levels of the inhibin α- and β-subunits have previously been shown to be upregulated in rodent granulosa cells by gonadotropins (Zhang et al., 1987; Turner et al., 1989) and several growth factors, including TGFβ and activin (LaPolt et al., 1989, LaPolt, 1990). It is important to realize that inhibin β-subunits can also dimerize to form activins, since at equimolar ratios of α- and β-subunits mainly activin is formed. Therefore one cannot necessarily interpret whether dimeric inhibin is being produced on the basis of inhibin subunit expression (Meunier et al., 1988). Mice deficient in GDF-9 have up to a three-fold increase in circulating FSH-levels (Dong et al., 1996), but whether this is caused by a decrease in circulating inhibin levels is not known. Furthermore, GDF-9 has been shown to stimulate the inhibin α-subunit content in explants of neonatal rat ovaries (Hayashi et al., 1999), but whether GDF-9 would also affect the transcription levels of the two β-subunits and, further, the production of inhibin dimers was not known prior to this study.

6.1. Production of dimeric inhibins in response to GDF-9 stimulation of rat granulosa cells

We used recombinant rat GDF-9 protein as well as recombinant human FSH and showed both of them to be inducers of the production of dimeric inhibin A and B protein, as well as the expression levels of all of the three inhibin subunit mRNAs (V, Fig. 1 and 3, respectively) in cultured rat granulosa cells. Interestingly, when administered together GDF-9 and FSH showed a supra-additive effect on dimeric inhibin production. The synergistic effect of GDF-9 and FSH was not observed at the mRNA level, suggesting that posttranscriptional actions of GDF-9 on inhibin synthesis might exist. Because recombinant rat inhibin standards are presently unavailable, the inhibin levels were measured against human standards. It is therefore noteworthy that the absolute inhibin concentrations measured possibly underestimate the real levels. Nonetheless, the changes in the ratios are likely to be maintained even if the inhibins were measured against rat inhibin standards (Lanuza et al., 1999).

To further decipher the stimulatory effects of GDF-9 on inhibin α-subunit expression, we transiently transfected rat granulosa cells with an inhibin α-subunit promoter-luciferase construct. Treatments with GDF-9, as well as with FSH, were shown to stimulate the inhibin-α gene promoter, but the combined effect was less than additive (V, Fig. 5). Interestingly a recent report indicated that women with PCOS might have aberrant expression of GDF-9 mRNAs (Teixeira Filho et al., 2002). It has furthermore been reported that PCOS patients may have up to 16-fold lower inhibin α-subunit mRNA expression within a single follicle as compared to similar-sized follicles of control patients, but whether this is due to the decreased expression of GDF-9 is not currently known (Fujiwara et al., 2001). In contrast, GDF-9 knockout mice have normal or slightly elevated inhibin α-subunit mRNA expression, whereas the expression of the βB-subunit mRNAs are dramatically decreased. Whether the slightly elevated FSH-levels seen in this animal model are due to low circulating levels of inhibin B has not been shown (Elvin et al., 1999).

Lanuza et al. have previously demonstrated the production of the two inhibin dimers to be differentially regulated in cultured granulosa cells derived from immature DES treated rats (1999). Under basal conditions a preference for the production of inhibin A over inhibin B was seen; moreover, the basal production of both inhibins was
clearly enhanced with addition of FSH to the media. TGFβ and activin A were found to preferentially stimulate the production of inhibin B, even though inhibin A levels were increased as well. Co-treatment with FSH and TGFβ led to an augmented stimulatory effect on both inhibin dimers. Furthermore, the addition of 15 freshly isolated bovine oocytes per culture well stimulated the production of inhibin B 100-fold and inhibin A 10-fold over control levels during 72 h culture. Since bovine oocytes, among other growth factors, have been found to express GDF-9 (Bodensteiner et al., 1999; Sendai et al., 2001) it is indeed possible that the effect seen on inhibin production was induced by this growth factor.

To study the impact of TGFβ on dimeric inhibin production in the native unstimulated ovary, cultures of post-natal ovarian cells derived from 4-, 8- and 12-day-old rats have been used (Drummond et al., 2000). In oocytes of various age it was shown that inhibin A was produced in excess over dimeric inhibin B, as is the case in granulosa cells derived from DES-treated rats (Lanuza et al., 1999). Ovarian cells from 4-day-old ovaries, which are rich in small primordial and primary follicles, mainly produce dimeric inhibin A when stimulated with TGFβ. Cell cultures containing granulosa cells derived from larger preantral and antral ovaries (8- and 12-day-old ovaries, respectively) were able to produce both dimeric inhibin B and A after stimulation with TGFβ (Drummond et al., 2000). Thus the differentiation state of the follicles seems to determine what form of dimeric inhibin is produced.

In our own unpublished experiments we stimulated cultured hGL cells with recombinant rat GDF-9. As has previously been shown when stimulating hGL cells with TGFβ, activin and different BMPs (Erämaa et al., 1995; Erämaa and Ritvos, 1996; Vänttinen et al., 2000; Liu et al., 2001, Study III), GDF-9 also selectively upregulated the expression levels of the inhibin βB-subunit and further stimulated the production of dimeric inhibin B in a concentration- and time-dependent manner. This is clearly in contrast to the rat model, where we showed GDF-9 to upregulate the mRNA levels of all three inhibin subunits as well as to stimulate the production of both dimeric inhibin A and B (Study V). It is therefore important to acknowledge that there appear to be fundamental differences in the regulation of the three inhibin subunits in humans as compared to rodents. Whether this is caused by interspecies differences or by differences in the granulosa cell models used remains to be elucidated. Presently no biological differences between the two inhibin dimers are known and hence the possible physiological need for different dimeric inhibins remains to be shown.

6.2. GDF-9 receptor signaling is mediated via Smad2

Previous studies have revealed that both the rat and human inhibin α-subunit promoters contain cAMP-response elements (CRE) (Pei et al., 1991; Schmitt et al., 2002). This is in the line with the fact that both FSH and pharmacological agents capable of increasing the intracellular content of cAMP (e.g., forskolin) are potent inducers of the inhibin α-subunit (Zhang et al., 1987; Turner et al., 1989). Since treatment of granulosa cells with recombinant GDF-9 stimulated the inhibin α-subunit promoter (V, Fig. 5), yet does not induce the production of intracellular cAMP (Vitt et al., 2000a), we set out to determine whether other downstream factors would be activated after stimulation with GDF-9. More specifically, we wanted to determine
whether intracellular Smad signaling proteins would be phosphorylated after treatment with GDF-9.

Recombinant GDF-9 protein was recently demonstrated to bind to BMPRII ectodomains (Vitt et al., 2002). Furthermore, by blocking the expression of endogenous BMPRII mRNAs using antisense oligos in cultured rat granulosa cells, the stimulatory effect of GDF-9 on granulosa cell proliferation as measured by thymidine uptake was abolished (Vitt et al., 2002). It is not, however, presently known what type I receptors GDF-9 might use for its signal transduction. BMPRII has previously been shown to form complexes with ALK2, ALK3 and ALK6 in mammalian cells (Koenig et al., 1994; Kawabata et al., 1995a).

Based on the known receptor binding data it was anticipated that GDF-9 stimulation would also lead to stimulation of the BMP-Smads, Smads1/5/8, through recruitment of one of the above mentioned BMP type I receptors. To address this question we cultured rat granulosa cells and subsequently stimulated them with different concentrations of recombinant GDF-9. Quite surprisingly, like activin A, GDF-9 was shown to time- and dose-dependently induce the expression of a 52 kDa protein, which was detected using an antibody against C-terminally phosphorylated Smad2. In contrast to BMP-2, stimulation with GDF-9 did not induce phosphorylation of Smad1 (V, Fig. 4). Hence, it seems likely that GDF-9 binds to a receptor complex consisting of BMPRIIs and either ALK4, ALK5, or, perhaps based on its expression pattern less likely, ALK7 (Table 6). BMPRII has been shown to interact with ALK5 in yeast, but to date this interaction has not been shown in mammalian cells (Kawabata et al., 1995b). Interestingly Smad2 and Smad3 but no activin type II receptor proteins were recently shown to be expressed in rat primary follicles (Drummond et al., 2002). This indicates that some other factor than activin (e.g., GDF-9) might utilize these signaling intracellular proteins during early stages of folliculogenesis.

Table 6. Type I and type II receptors and some examples of ligand binding.

<table>
<thead>
<tr>
<th>TGFβRII</th>
<th>BMPRII</th>
<th>ACVRII</th>
<th>ACVRIIB</th>
<th>AMHRII</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK1</td>
<td>TGFβ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALK2</td>
<td>BMP-7</td>
<td>BMP-7</td>
<td>BMP-7</td>
<td>AMH</td>
</tr>
<tr>
<td>ALK3</td>
<td>BMP-2</td>
<td>BMP-2</td>
<td>BMP-2</td>
<td>AMH</td>
</tr>
<tr>
<td>ALK4</td>
<td>GDF-9?</td>
<td>Activin</td>
<td>Activin</td>
<td></td>
</tr>
<tr>
<td>ALK5</td>
<td>TGFβ</td>
<td>GDF-9?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALK6</td>
<td>BMP-2</td>
<td>BMP-2</td>
<td>BMP-2</td>
<td>AMH</td>
</tr>
<tr>
<td>ALK7</td>
<td>GDF-9??</td>
<td>Nodal</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Since stimulation with GDF-9 does not increase intracellular cAMP content (Vitt et al., 2000a), it is likely that intracellular Smad signaling proteins activated by GDF-9 type I receptors might participate in the stimulation of the inhibin α-subunit promoter
We did indeed locate a CAGA consensus sequence in the rat inhibin α-subunit promoter, and Smad3 has been shown to bind to this Smad binding element (SBE) (Dennler et al., 1998). It is highly likely that stimulation with GDF-9 also leads to Smad3 phosphorylation, which could bind directly to the SBE of the inhibin α-promoter. However, this issue remains to be shown in future studies.

Our unpublished experiments have further shown that in cultured hGL cells GDF-9 activates Smad2, consistent with the rat model. Interestingly, stimulation with recombinant GDF-9 also appears to lead to Smad2 phosphorylation in various non-ovarian cell lines tested (e.g., 293 cells). This indicates that the receptor combination used is not fully ovary-specific in contrast to the biological effects of GDF-9, which appear to be tightly restricted to the ovary (Dong et al., 1996). What then keeps GDF-9 ovary-specific if its receptors are abundantly expressed in various tissues? Several possibilities exist. One is the existence of some intraovarian yet unidentified component which increases the affinity of GDF-9 for its receptors (e.g., analogous to Cripto in ALK4 mediated Nodal signaling, Reissmann et al., 2001), thus preventing it from “leaking” to the circulation and binding to non-ovarian BMP type II receptors. It might also be that the half-life of GDF-9 is short in vivo, wherefore it can only act in an auto/paracrine fashion because of rapid inactivation. The lack of the fourth cysteine that is utilized in dimerization suggests that GDF-9 dimers might not be very stable (McPherron and Lee, 1993). A fourth possibility is the existence of GDF-9 binding

Fig. 7. Possible mechanism for inhibin α-subunit gene activation by GDF-9.
proteins, however, at least follistatin does not seem to block the biological activities of GDF-9 (unpublished data).

A detailed understanding of the regulation of inhibin biosynthesis across folliculogenesis is important as it may shed light on pathophysiological conditions where follicular development is disrupted. Finally, as it is becoming clear that GDF-9 and GDF-9B/BMP-15 signaling pathways play essential roles in early mammalian folliculogenesis (Dong et al., 1996; Galloway et al., 2000), future studies will be needed to elucidate the complete signaling machinery utilized by these factors. Also the possible activation of other intracellular signaling proteins working in parallel to the Smads needs to be investigated.
SUMMARY AND CONCLUDING REMARKS

The members of the TGFβ superfamily are involved in a plethora of cellular events and their signaling machinery involves a set of markedly conserved transmembrane type I and II ser/thr kinase receptors and intracellular Smad proteins. Recent data indicate that several TGFβ superfamily members are vitally important for normal ovarian function and, furthermore, that they might participate in the regulation of inhibin production. We set out to study some of these growth factors and their receptor molecules with special emphasis on their role for ovarian inhibin production. On the basis of the results obtained during the course of this thesis study the following conclusions can be drawn:

• The chromosomal loci for the ACVRII, ACVRIIB, ALK7 and follistatin genes were determined using FISH. Even though we did not identify clear disease associations to their chromosomal loci this knowledge will undoubtedly support further studies.

• We identified and cloned the human ALK7 cDNA and found its mRNA to be widely distributed in most adult tissues. In human tissues, the expression of Nodal, the receptor’s sole known ligand, appears to be restricted to the embryo. Future studies will be needed to determine whether other ligands use ALK7 for their signaling. Despite extensive searches no novel type I nor type II ser/thr kinases were discovered.

• We first identified the transcripts of several BMP Smads and ser/thr kinases in human ovarian granulosa cells. Secondly we showed for the first time that various BMPs are able to selectively induce expression of the inhibin βB-subunit, and furthermore, production of dimeric inhibin B in cultured hGL cells. The stimulatory effect of the BMPs was suppressed by gonadotropins. It would be useful to study the effect of BMPs in cultures of granulosa cells derived from small antral follicles, since these are thought to be responsible for the production of the majority of inhibin B in vivo. Furthermore, our results point out the possible involvement of the BMP signaling pathway in human ovarian physiology.

• We showed that hGL cells could be effectively transduced with recombinant adenoviruses. Applying this novel methodology we selectively overexpressed all seven ALKs and various Smads in cultured hGL cells and showed that overexpression of the constitutively active ALKs lead to Smad phosphorylation and stimulation of dimeric inhibin B production. A similar effect was obtained after overexpression of Smad1 and Smad2, whereas Smad7 was able to block the stimulatory effect of both activin A and BMP-2 on inhibin production. Taken together our results indicate that R-Smads directly stimulate inhibin B production in these cells.
• Using cultured rat granulosa cells we demonstrated that GDF-9 stimulates the production of all three inhibin subunits as well as of dimeric inhibin A and B. This indicates that also in the human GDF-9 participates in the complex regulation of inhibin production. GDF-9 produced by the oocyte may act by stimulating the granulosa cells to produce inhibin, which might downregulate pituitary FSH secretion, thus affecting the progression of folliculogenesis. Finally, GDF-9 treatment of rat granulosa cells also leads to specific phosphorylation of Smad2, hence an emerging line of evidence indicates that ALK4, ALK5 or ALK7 participate in the functional receptor complex together with the GDF-9 type II receptor, BMPRII. Studies have been initiated to address which of these receptors are needed for GDF-9 mediated signaling.

• It is clear that further studies are needed to define the whole GDF-9 receptor complex at the molecular level. Since recombinant GDF-9 in the future might have the potential for being used in infertility treatments, in particular for stimulation of follicular growth \textit{in vitro}, a thorough understanding of its means of action is of fundamental importance. Equally interesting would be to block the effects of GDF-9 and hence prevent the progression of folliculogenesis at the primary stage, which could lead to the development of a novel non-steroidal contraceptive.
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Jonas Bondestam

Helsinki, November, 2002
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Local Regulators of Inhibin Production in Ovarian Granulosa Cells


Local Regulators of Inhibin Production in Ovarian Granulosa Cells


Ligands & Signaling Components of the TGFβ Family


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Local Regulators of Inhibin Production in Ovarian Granulosa Cells


