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Genomic analyses facilitate identification of receptors and signalling pathways for growth differentiation factor 9 and related orphan bone morphogenetic protein/growth differentiation factor ligands

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Recent advances in genomic sequencing allow a new paradigm in hormonal research, and a comparative genomic approach facilitates the identification of receptors and signalling mechanisms for orphan ligands of the transforming growth factor β (TGFβ) superfamily. Instead of purifying growth differentiation factor 9 (GDF9) receptor proteins for identification, we hypothesized that GDF9, like other ligands in the TGFβ family, activates type II and type I serine/threonine kinase receptors. Because searches of the human genome for genes with sequence homology to known serine/threonine kinase receptors failed to reveal uncharacterized receptor genes, GDF9 likely interacts with the known type II and type I activin receptor-like kinase (ALK) receptors in granulosa cells. We found that co-treatment with the bone morphogenetic protein (BMP) type II receptor (BMPRII) ectodomain blocks GDF9 activity. Likewise, in a GDF9-non-responsive cell line, overexpression of ALK5, but none of the other six type I receptors, conferred GDF9 responsiveness. The roles of BMPRII and ALK5 as receptors for GDF9 were validated in granulosa cells using gene 'knock-down' approaches. Furthermore, we demonstrated the roles of BMPRII, ALK3 and ALK6 as the receptors for the orphan ligands GDF6, GDF7 and BMP10. Thus, evolutionary tracing of polypeptide ligands, receptors and downstream signalling molecules in their respective 'subgenomes' facilitates a new approach for hormonal research.

Key words: BMP/GDF9/granulosa cell/ovary/TGFβ receptors

Introduction

Advances in the sequencing of diverse genomes allow an unprecedented opportunity to analyse the evolution of genes, including polypeptide ligands and receptors. In the human genome, many genes belong to distinct families because of their derivation from a common ancestor gene. In addition to sequence homology, these paralogous genes most often perform similar functions. Identification of paralogous genes in distinct families serves as the first step in building hypotheses for testing the structure and function of previously uncharacterized genes. Because ligand and receptor families have co-evolved, analyses of the subgenomes of extracellular protein ligands and their transmembrane receptors provide a new paradigm with which to match orphan ligands with their cognate receptors (Leo *et al*., 2002).

Transforming growth factor β (TGFβ) family ligands usually initiate signalling by binding to type I and type II serine/threonine kinase receptors, leading to the phosphorylation of Smad proteins (Massague, 1998). Analysis of the human genome indicated the presence of seven type I and five type II serine kinase receptors (Manning *et al*., 2002). Owing to their common evolutionary origin, more than 30 related members of the TGFβ superfamily likely interact with this limited set of receptors thereby activating Smad proteins (Mazerbourg *et al*., 2005). In the present review, we will summarize a genomic view of TGFβ superfamily ligands, their receptors and downstream signalling pathways and the ovarian functions of growth differentiation factor 9 (GDF9). Using a

genomic approach, we will then describe the identification of the type II and type I receptors for GDF9 and several related bone morphogenetic protein (BMP) and GDF proteins.

The TGFβ family ligands

The TGFβ superfamily of ligands is a group of more than 30 multifunctional polypeptide growth factors that include TGFβ proteins, activins/inhibins, BMPs, GDFs and others (Figure 1). These ligands regulate cell proliferation, differentiation and apoptosis, which are essential for embryonic development, organogenesis, bone formation, reproduction and other physiological processes (Chang *et al*., 2002). They are synthesized as large precursor molecules that are cleaved proteolytically by members of the subtilin-like proprotein convertase (SPC) family and the BMP1/Tolloid-like proteinase to release a C-terminal peptide of 110–140 amino acids (Kingsley, 1994; Massague, 1998; Constam and Robertson, 1999; Wolfman *et al*., 2003; Ge *et al*., 2005). The large N-terminal prodomain of the TGFβ ligand precursor is a key determinant in regulating the secretion and processing of these ligands (Thomsen and Melton, 1993; Constam and Robertson, 1999). In the case of TGFβ, BMP7, BMP9, myostatin and GDF11, the propeptide and the mature domain remain non-covalently associated after cleavage, resulting in the secretion of a latent complex (Gray and Mason, 1990; Bottinger *et al*., 1996; Hill *et al*., 2002; Brown *et al*., 2005; Ge *et al*., 2005; Gregory *et al*., 2005).

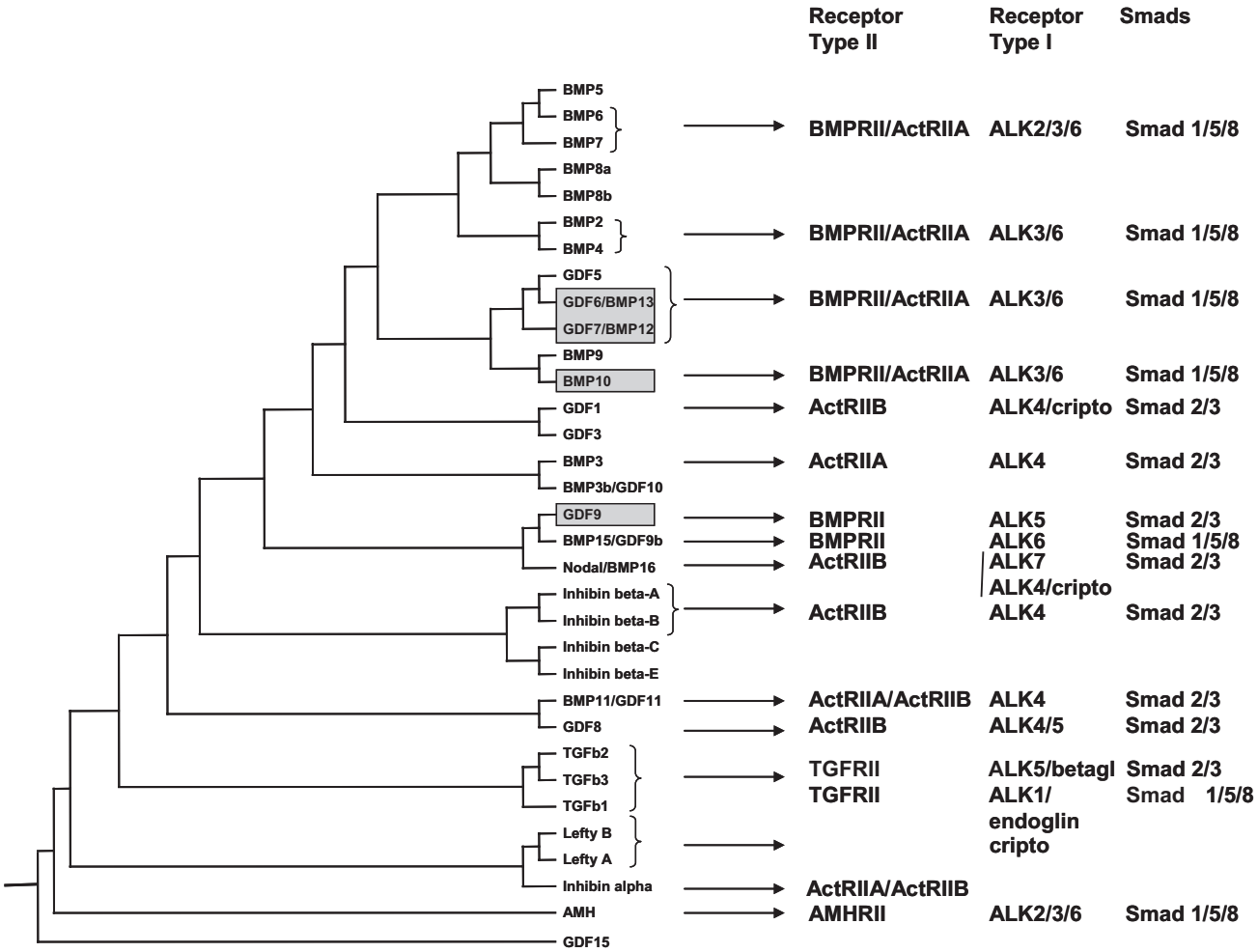

Figure 1. Phylogenetic relationship of paralogous TGFβ/GDF/BMP ligands, as well as characterized receptors and signalling pathways for individual ligands. The alignment of 35 TGFβ-related ligands was performed using the C-terminal region containing the cystine-knot structure, starting from the first invariant cysteine residue. Phylogenetic analyses were performed based on multiple sequence alignment using the ClusterW algorithm (http://www.ch.embnet.org/software/ClustalW.html) and the TreeView drawing software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Based on published literature [BMP6 (Ebisawa *et al*., 1999); BMP7 (Yamashita *et al*., 1994; Liu *et al*., 1995); BMP2 and BMP4 (Koenig *et al*., 1994; Yamaji *et al*., 1994; Liu *et al*., 1995; Nohno *et al*., 1995; Rosenzweig *et al*., 1995; Kawabata *et al*., 1998); GDF5 (Nishitoh *et al*., 1996); GDF6, GDF7 and BMP10 (Mazerbourg *et al*., 2005); GDF1 (Cheng *et al*., 2003); BMP3 (Daluiski *et al*., 2001); GDF9 (Vitt *et al*., 2002; Kaivo-Oja *et al*., 2003; Roh *et al*., 2003; Mazerbourg *et al*., 2004; Kaivo-Oja *et al*., 2005); BMP15/GDF9b (Moore *et al*., 2003); Nodal/BMP16 (Gritsman *et al*., 1999; Reissmann *et al*., 2001; Yan *et al*., 2001; Yeo and Whitman, 2001; Bianco *et al*., 2002); activin (inhibin beta) (Mathews and Vale, 1991; Mathews *et al*., 1992; Carcamo *et al*., 1994; Attisano *et al*., 1996); BMP11/GDF11 (Oh *et al*., 2000); GDF8 (Rebbapragada *et al*., 2003); TGFβ (Lin *et al*., 1992; Attisano *et al*., 1993; Franzen *et al*., 1993; ten Dijke *et al*., 1994a; Lux *et al*., 1999; Oh *et al*., 2000; Goumans *et al*., 2003); Lefty (Cheng *et al*., 2004); inhibin α (Lewis *et al*., 2000); and AMH (Baarends *et al*., 1994; di Clemente *et al*., 1994; Clarke *et al*., 2001; Josso *et al*., 2001; Visser *et al*., 2001; Jamin *et al*., 2002)], the type II and type I receptors as well as the intracellular signalling Smad proteins for individual ligands are listed. The ligands GDF9, GDF6, GDF7 and BMP10 under discussion in this review are highlighted. Betag, betaglycan. Reproduced with permission from Mazerbourg *et al.* (2005).

Despite a low degree of sequence similarity (~35%), the C-terminal peptides of diverse TGFβ ligands share a conserved structure with six cysteine residues known as the cystine knot (Vitt *et al*., 2001), thereby allowing the formation of a common structural scaffold (Scheufler *et al*., 1999; Thompson *et al*., 2003). In most ligands, an extra cysteine is present and engaged in an intermolecular disulphide bond that is necessary for the assembly of homo- or heterodimers. For ligands such as GDF9 and BMP15, both of which are missing the extra cysteine, homodimers are likely non-covalently associated. Although the structural scaffold of different monomers is conserved, analyses of the crystal structures of their dimerized forms showed unique conformational arrangements for TGFs, activins and BMPs (Scheufler *et al*., 1999; Thompson *et al*., 2003). TGFβ2, TGFβ3, BMP2 and BMP7 dimers have an extended symmetric arrangement described as an 'open form', whereas activin dimers have a compact folded-back conformation described as a 'closed form' (Thompson *et al*., 2003). Specific ligand dimers with distinct patterns of surface charge and hydrophobicity likely lead to differential interactions with the cell-surface receptors.

The TGFβ family of ligands interacts with a limited number of serine/threonine kinase receptors

The molecular signalling pathways for several ligands of the TGFβ superfamily (TGFβ, activins, BMP2 and BMP7) have been investigated intensively. Members of the TGFβ superfamily were shown to initiate signalling by assembling serine/threonine kinase receptor complexes that activate downstream Smad transcription factors (Kawabata *et al*., 1998; Massague, 1998; Figure 2). The receptor serine/threonine kinase family in the human genome comprises 12 members (Figure 3; Manning *et al*., 2002). There are five type II serine–threonine kinase receptors: the BMP receptor type II (BMPRII), the anti-mullerian hormone receptor type II (AMHRII), the TGFβ receptor type II (TGFRII) and the activin receptors type II (ActRIIA and ActRIIB) (Figure 3). In addition, there are seven type I receptors designated as activin receptor-like kinases (ALKs) (ten Dijke *et al*., 1993; Figure 3). Both types of receptors consist of ~500 amino acids and are organized into an amino terminal extracellular ligand-binding domain with 10 or more cysteines, a transmembrane region and a carboxyl terminal serine/threonine kinase domain. Type II receptors have autophosphorylation activity (Lin *et al*., 1992; Mathews and Vale, 1993; Wrana *et al*., 1994; Attisano *et al*., 1996). After forming a complex with the ligand, the type II receptor phosphorylates the type I receptor at a glycine- and serine-rich motif (the GS domain) just upstream of the C-terminal kinase domain. The phosphorylation of the GS domain activates the type I receptor kinase, leading to the phosphorylation of downstream R (receptor) Smad proteins (Wrana *et al*., 1994; Wieser *et al*., 1995; Souchelnytskyi *et al*., 1996; Figure 2). Structural data on the TGFβ ligands and their

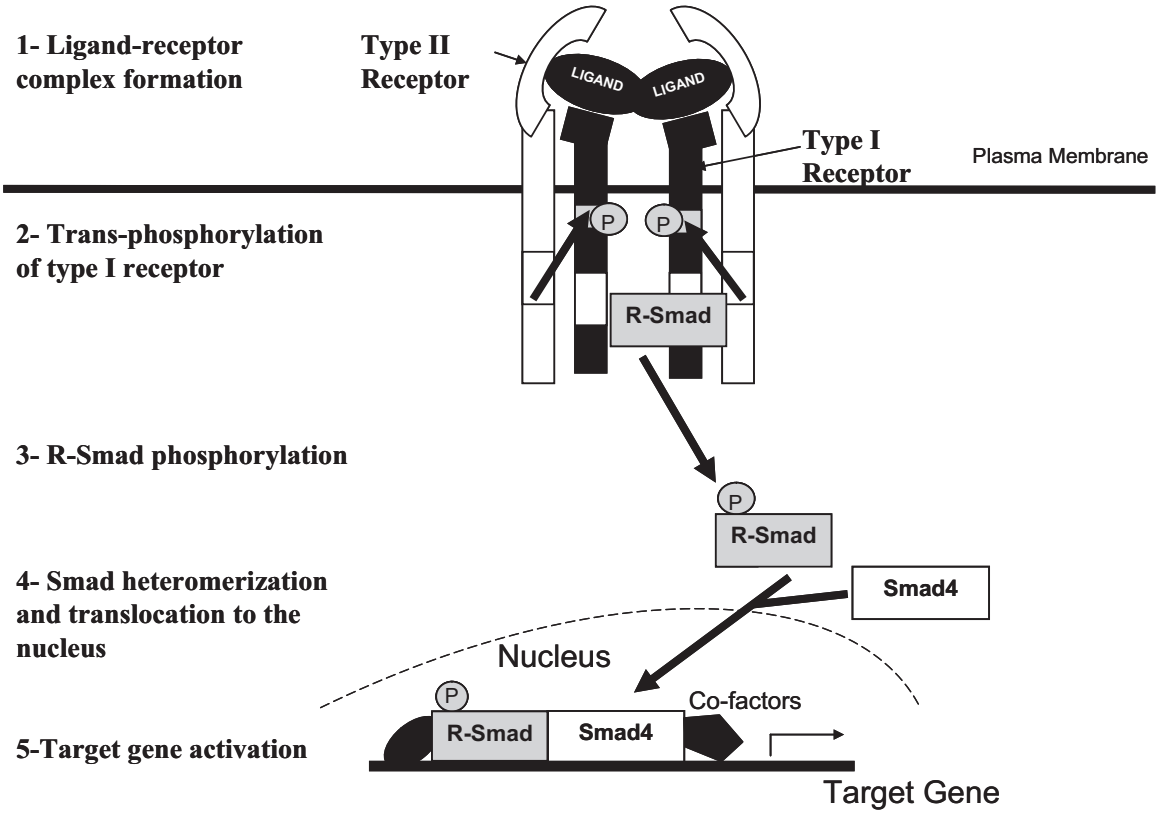

Figure 2. Schematic representation of the signalling pathways for the TGFβ family ligands. The dimeric ligands bind to two types of serine/threonine receptors namely type I and type II receptors. Formation of the tetramer receptor (two type I and two type II) allows phosphorylation of the type I receptor by the type II receptor on the GS domain, resulting in the activation of the type I receptor kinase. Type I receptors specifically recognize and phosphorylate Receptor-Smads (R-Smad). Phosphorylated R-Smads, in turn, associate with a common (Co)-Smad, Smad4. The complexes of R-Smads/Smad4 translocate to the nucleus and interact with specific DNA motifs. However, effective binding to particular gene regulatory sites is enabled and modulated by diverse DNA-binding factors and transcriptional co-activators or co-repressors.

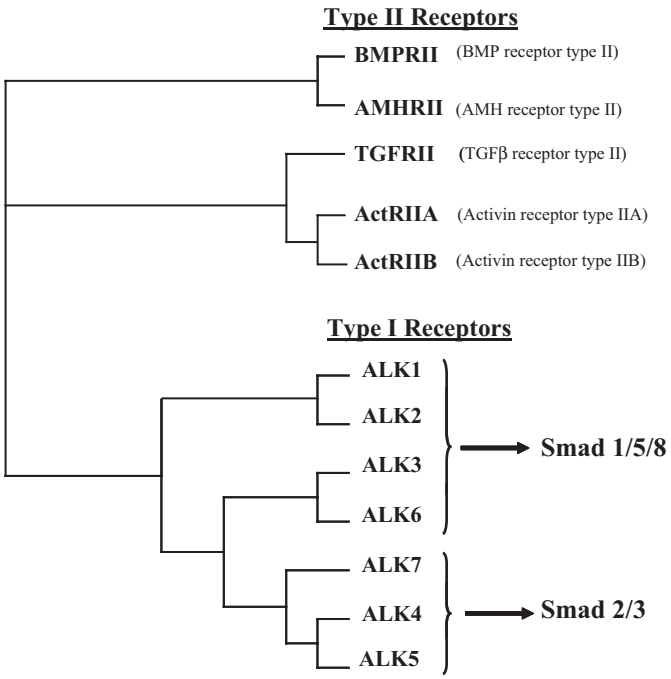

Figure 3. Phylogenetic relationship of the type II and type I serine/threonine kinase receptors. Phylogenetic analyses of all known human serine/threonine kinase receptors were performed based on multiple full-length sequence alignment using the ClusterW algorithm (http://www.ch.embnet.org/software/ClustalW.html) and the TreeView drawing software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Smad proteins involved in the signalling pathway associated with individual type I receptors are indicated. The GenBank accession numbers for individual human receptor protein sequence are BMPRII, NP_001195; AMHRII, Q16671; TGFRII, NP_001020018; ActRIIB, NP_001097; ActRIIA, NP_001607; ALK1, NP_000011; ALK2, NP_001096; ALK3, NP_004320; ALK6, NP_001194; ALK4, NP_004293; ALK5, NP_004603; ALK7, AAM93495.

receptor complexes support a model of oligomeric receptor assembly that does not involve a direct receptor–receptor interaction (Kirsch *et al*., 2000; Greenwald *et al*., 2003). Furthermore, two general modes of ligand binding have been described. One mode involves direct ligand binding to the type II receptor that then guides the ligands into an orientation that is competent to interact with type I receptors. For example, TGFβ and activin form a complex with the type II receptor and recruit the type I receptor (Mathews and Vale, 1991; Wrana *et al*., 1992; Attisano *et al*., 1996). In contrast, both type I and type II receptors are needed for the binding of several BMP ligands. In these cases, high-affinity ligand binding was detected only when both type I and type II receptors are co-expressed (ten Dijke *et al*., 1994b; Rosenzweig *et al*., 1995).

For some ligands, access to the receptor is controlled by membrane-anchored proteins that act as accessory receptors or co-receptors. The membrane-anchored proteoglycan betaglycan, also known as the TGFβ type III receptor, mediates TGFβ binding to the type II receptor (Brown *et al*., 1999). Recently, betaglycan was identified further as a co-receptor that increases the affinity of inhibin for the activin and BMP type II receptors (Lewis *et al*., 2000; Wiater and Vale, 2003). Endoglin is another accessory receptor for the binding of TGFβ to ALK1 (Cheifetz *et al*., 1992; Lebrin *et al*., 2004). The secretory protein, cripto, mediates the binding of nodal and GDF1 to activin receptors (Yeo and Whitman, 2001; Cheng *et al*., 2003). Recently, the glycophosphatidylinositol (GPI)-anchored proteins of the repulsive guidance molecule (RGM) family, Dragon and RGMa, have been shown to be co-receptors of BMP2 and BMP4 (Babitt *et al*., 2005; Samad *et al*., 2005).

Two downstream Smad signalling pathways

The intracellular TGFβ signalling mediators are a group of phylogenetically related proteins, the Smads (Attisano and Tuen Lee-Hoeflich, 2001). The first member of this family is Mad (mothers against decapentaplegic), which was identified from genetic screens in *Drosophila melanogaster* (Sekelsky *et al*., 1995). Initial evidence that Smads function downstream of TGFβ receptors was provided by the ability of Mad mutations to inhibit signalling by the *Drosophila* BMP homologue (decapentaplegic) (Hoodless *et al*., 1996; Wiersdorff *et al*., 1996). Three MAD homologues were identified in *Caenorhabditis elegans* and called sma-2, sma-3 and sma-4 (Savage *et al*., 1996). Vertebrate homologues of sma and MAD were called Smad, as a contraction of the invertebrate gene names (Derynck *et al*., 1996). Smad proteins are divided into three structural domains (Attisano and Tuen Lee-Hoeflich, 2001). The N-terminal MH1 domain exhibits sequence-specific DNA-binding activity, except in the major splice form of Smad2, which contains an insert that prevents DNA binding. The C-terminal MH2 domain is involved in the interaction with the type I receptor and in the formation of the Smad complexes. The intermediate domain is divergent among Smads. It contains multiple sites of phosphorylation and allows specific crosstalk with other signalling pathways.

Functional studies have demonstrated that Smads can be grouped into three subfamilies: the receptor-regulated Smads (R-Smads), the common Smad (Co-Smad) and the inhibitory Smads (I-Smads). The R-Smads are phosphorylated by the type I receptor kinases on a conserved carboxyl terminal SSXS motif. ALK1, ALK2, ALK3 and ALK6 phosphorylate Smad1, Smad5 and Smad8, whereas ALK4, ALK5 and ALK7 phosphorylate Smad2 and Smad3 (Figure 3; Kretzschmar and Massague, 1998; Chen and Massague, 1999; Watanabe *et al*., 1999; Jornvall *et al*., 2001). R-Smads form heteromeric complexes with the Co-Smad, Smad4. Smad4 is a shared partner of the R-Smads and is not phosphorylated in response to ligands. The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear co-factors, regulate the transcription of target genes (Figure 2). Smad transcription factors bind DNA on promoter sequences defined as Smad-binding element (SBE). The minimal SBE sequence for Smad1, Smad3 and Smad4 contains only four base pairs 5′-AGAC-3′ (Yingling *et al*., 1997; Dennler *et al*., 1998; Shi *et al*., 1998; Zawel *et al*., 1998; Johnson *et al*., 1999). Smads also have been reported to bind to G/C-rich sequences (Kim *et al*., 1997; Labbe *et al*., 1998; Ishida *et al*., 2000). Specific Smad3 or Smad1/5/8-response elements have been identified in the promoter of target genes and used for constructing luciferase reporter genes activated selectively by TGFβ family members (Dennler *et al*., 1998; Kusanagi *et al*., 2000; Jornvall *et al*., 2001; Reissmann *et al*., 2001; Korchynskyi and ten Dijke, 2002). The consensus sequence of the promoter of the CAGA, BRE and GCCG reporters was derived from the TGFβ-induced plasminogen activator inhibitor type I gene (Dennler *et al*., 1998), the BMP-induced mouse gene, inhibitors of differentiation (Id) (Korchynskyi and ten Dijke, 2002) and the Mad-binding site in *Drosophila* BMP-like ligand

decapentaplegic responsive genes (Kusanagi *et al*., 2000), respectively. Because Smads bind DNA with low affinity and low specificity, they require co-operation with other sequence-specific binding factors to interact efficiently with promoters of target genes. Indeed, both the MH1 and MH2 domains interact with many proteins in the nucleus (Massague, 2000; Massague and Wotton, 2000; ten Dijke *et al*., 2000; Wrana, 2000). The Smad-interacting transcription factors dictate the precise response to ligands in different cell types and in co-operation with other signalling pathways (Derynck and Zhang, 2003; Shi and Massague, 2003).

In addition to R-Smads and Co-Smads, I-Smads (Smad7 and Smad6) form a distinct subclass of Smads that antagonize TGFβ signalling transduction. Although I-Smads contain a C-terminal MH2 domain, their N-terminal region has low similarity with the canonical MH1 domain. Smad7 stably interacts with all activated type I receptors to prevent R-Smad activation and downstream transcriptional modulation (Hayashi *et al*., 1997; Nakao *et al*., 1997a; Itoh *et al*., 1998; Souchelnytskyi *et al*., 1998). In contrast, Smad6 specifically competes with R-Smad1 for complex formation with Smad4, thus preferentially inhibiting the BMP pathway (Hata *et al*., 1998; Souchelnytskyi *et al*., 1998; Ishisaki *et al*., 1999).

Based on a genomic analysis of the entire repertoire of TGFβ/BMP/GDF ligands (33 ligands) (Chang *et al*., 2002; Manning *et al*., 2002; Figures 1 and 3), the TGFβ family members activate only two major intracellular signalling pathways characterized by the activation of the two different groups of intracellular Smad proteins, Smad1/5/8 and Smad2/3. As shown in Figure 1, TGFβ and activin interact with their respective type II receptors, followed by the activation of the type I receptors, ALK1 or ALK5 and ALK4, respectively. This, in turn, leads to the phosphorylation of the downstream Smad3 and Smad2 proteins (Macias-Silva *et al*., 1996; Nakao *et al*., 1997a,b). The stimulation of the CAGA promoter by these ligands is mediated by the Smad3 and Smad4 proteins (Dennler *et al*., 1998). In contrast, BMP2 binds to the type II receptors, BMPRII and ActRIIA, and the type I receptors, ALK3 and ALK6, leading to the activation of Smad1, Smad5 and Smad8 (Koenig *et al*., 1994; Yamaji *et al*., 1994; Liu *et al*., 1995; Nohno *et al*., 1995; Rosenzweig *et al*., 1995; Kawabata *et al*., 1998; Massague, 1998). In addition to interacting with these BMP receptors, BMP6 and BMP7 also can signal through ALK2 (Yamashita *et al*., 1994; ten Dijke *et al*., 1994b; Liu *et al*., 1995; Macias-Silva *et al*., 1998; Ebisawa *et al*., 1999). This subfamily of BMP ligands activates the intracellular factors Smad1, Smad5 and Smad8 followed by the stimulation of the BRE and GCCG promoters (Kusanagi *et al*., 2000; Korchynskyi and ten Dijke, 2002; Monteiro *et al*., 2004). It is apparent that combinatorial uses of a limited number of type I and type II receptors lead to differential Smad activation by the large number of ligands. Because many of the BMP and GDF proteins remain orphan ligands, we hypothesized that these ligands are likely to interact with the limited number of receptors to activate the two major downstream signalling pathways. We focused on GDF9 and several related orphan ligands (GDF6, GDF7 and BMP10) and identified their receptors and downstream signalling pathways using a genomic approach.

The role of GDF9 in the ovary

The expression of GDF9 mRNA and protein is confined to the oocyte of primary and larger follicles in rats (Hayashi *et al*., 1999; Jaatinen *et al*., 1999), mice (McGrath *et al*., 1995; Dong *et al*., 1996) and humans (Aaltonen *et al*., 1999). In sheep, goats and cows, GDF9 mRNA is found in primordial follicles as well (Bodensteiner *et al*., 1999). Mutant mice with a deletion of the *GDF9* gene as well as sheep homozygous for *GDF9* gene mutations have demonstrated the important role of this oocyte factor in the stimulation of early follicular growth (Dong *et al*., 1996; McNatty *et al*., 2004). Furthermore, Vitt *et al*. (2000b) have shown that recombinant GDF9 is able to stimulate initial follicle recruitment *in vivo*. In contrast to FSH, which mainly stimulates pre-antral follicular growth (McGee *et al*., 1997), GDF9 treatment increases the number of primary and small pre-antral follicles (Vitt *et al*., 2000b). Moreover, *in vitro* treatment with GDF9 promotes the survival as well as the progression of human follicles to the secondary stage in organ culture (Hreinsson *et al*., 2002). GDF9 appears to be essential for folliculogenesis at the primary pre-antral follicle transition. However, follicular development progresses up to the pre-antral stage in *inhibin α/GDF9* double-null mice, suggesting that inhibin α mediates the optimal actions of GDF9 *in vivo* (Wu *et al*., 2004).

Experiments with recombinant GDF9 have shown that GDF9 regulates granulosa cell function in small antral and pre-ovulatory follicles. In studies using cultured granulosa cells, GDF9 promotes granulosa cell proliferation as reflected by increases in thymidine incorporation (Vitt *et al*., 2000a). GDF9 stimulates basal estradiol synthesis in differentiated and undifferentiated granulosa cells and stimulates basal progesterone synthesis in differentiated granulosa cells (Elvin *et al*., 1999a; Vitt *et al*., 2000a). In contrast, treatment with GDF9 inhibits FSH-induced estradiol and progesterone synthesis as well as LH receptor expression (Vitt *et al*., 2000a). Another important function of GDF9 is the suppression of Kit ligand expression in granulosa cells (Joyce *et al*., 2000), consistent with findings showing Kit ligand overexpression in *GDF9*-null mice (Elvin *et al*., 1999b). Similarly, the primary follicles of *GDF9*-null mice demonstrated an up-regulation of inhibin α subunit (Elvin *et al*., 1999b). Data on the effect of GDF9 on granulosa cell expression of inhibin subunits *in vitro* are more controversial. Indeed, in mouse granulosa cells of pre-ovulatory follicles, Varani *et al*. (2002) showed that GDF9 increased inhibin βB, but not inhibin α, subunit expression. These results contrast with data obtained with granulosa cells from rat pre-antral/antral follicles or human luteinized follicles (Hayashi *et al*., 1999; Kaivo-Oja *et al*., 2003; Roh *et al*., 2003). Treatment with GDF9 stimulated inhibin α- and β-subunit expression in rat granulosa cells, and both inhibin A and inhibin B production in rat and human granulosa cells (Hayashi *et al*., 1999; Kaivo-Oja *et al*., 2003; Roh *et al*., 2003). This discrepancy could be explained by species differences and by changes in the sensitivity of granulosa cells to GDF9 at different follicular stages.

The importance of GDF9 on theca cell function is still unclear. In primary cultures of theca cells, treatment with GDF9 augments androstenedione production (Solovyeva *et al*., 2000). *In vivo* injection of GDF9 led to an increase in the ovarian content of the theca cell marker, cytochrome *P*-450 17, 20 lyase (CYP17) (Vitt *et al*., 2000b). Furthermore, in *GDF9*-null mice, the follicular theca layer is absent (Dong *et al*., 1996). This was confirmed by the absence of expression of selective theca cell markers such as CYP17, LH receptor and c-kit mRNA (Elvin *et al*., 1999b). However, Wu *et al*. (2004) suggest that GDF9 could indirectly induce

theca cell recruitment *in vivo*, through changes in ovarian inhibin α expression.

GDF9 is a modulator of the peri-ovulatory responses in the ovary, and treatment with GDF9 induces cumulus cell expansion (Elvin *et al*., 1999a). Although some data suggest that GDF9 is not the only cumulus expansion-enabling factor (Dragovic *et al*., 2005), the importance of GDF9 in cumulus expansion has been confirmed by using RNA interference (Gui and Joyce, 2005). This controversy has been recently discussed by Pangas and Matzuk (2005). GDF9 regulates cumulus cell gene expression and suppresses the expression of genes normally found in mural granulosa cells (Elvin *et al*., 1999a). Treatment with GDF9 induces the expression of hyaluronan synthase 2, steroidogenic acute regulator protein (StAR), prostaglandin endoperoxide synthase 2 (Ptgs2), EP2 (PGE2 receptor; Elvin *et al*., 2000), pentraxin 3 (Varani *et al*., 2002), tumour necrosis factor-induced protein 6 (Varani *et al*., 2002), peroxiredoxin 6 (Leyens *et al*., 2004) and gremlin (Pangas *et al*., 2004). In contrast, GDF9 treatment inhibits LH receptor and urokinase plasminogen activator expression (Elvin *et al*., 1999a). The regulation of the expression of different ovarian genes by GDF9 is summarized in Table I.

Identification of GDF9 receptors and downstream signalling pathway

GDF9 belongs to the TGFβ superfamily (Figure 1). Phylogenetically, it is placed between the BMP2/4/6/7/8 and the activin/TGFβ subgroups of ligands but with a closer relationship to the BMPs (Newfeld *et al*., 1999; Vitt *et al*., 2002). Instead of performing GDF9 binding assays and purifying the GDF9 receptor proteins from granulosa cells for their identification, we hypothesized that GDF9, like other ligands in the same family, likely activates the limited number of known serine/threonine kinase receptors in the genome. To identify the type II receptor for GDF9, (Vitt *et al*., 2002) used the soluble form (the ectodomain of the receptor fused to the Fc-binding region of human IgG) of the type II receptors, BMPRII and ActRIIA, to study potential interactions with GDF9. Of interest, the stimulatory effects of GDF9 on granulosa cell proliferation were completely blocked, following co-incubation with the extracellular domain of BMPRII. Similarly, the BMPRII ectodomain was capable of blocking the inhibitory effect of GDF9 on FSH-induced progesterone production. In addition, direct interactions between GDF9 and BMPRII were demonstrated by co-immunoprecipitation of GDF9 with the ectodomain of BMPRII (Vitt *et al*., 2002), whereas ActRIIA was only minimally efficient in binding GDF9. Furthermore, the suppression of endogenous BMPRII biosynthesis using an antisense RNA approach completely blocked the stimulatory effects of GDF9 on the proliferation of rat granulosa cells *in vitro* (Vitt *et al*., 2002). These results showed that BMPRII is a receptor essential for GDF9 signalling in granulosa cells.

BMPRII has been demonstrated to mediate the actions of BMP2, BMP4, BMP6 and BMP7 through interactions with the type I receptors ALK2, ALK3 and ALK6 (Koenig *et al*., 1994; Yamaji *et al*., 1994; Liu *et al*., 1995; Nohno *et al*., 1995; Rosenzweig *et al*., 1995; Kawabata *et al*., 1998; Massague, 1998). To identify the type I receptor for GDF9 in granulosa cells, we took advantage of the availability of different promoter-luciferase constructs for analyses of downstream pathways of the TGFβ family ligands. The CAGA promoter is known to be activated by the TGFβ/activin pathway mediated by Smad3 (Dennler *et al*., 1998), whereas the activation of BRE and GCCG promoters is mediated by Smad1 and 5 (Kusanagi *et al*., 2000; Korchynskyi and ten Dijke, 2002; Monteiro *et al*., 2004). We transfected individual promoter-reporter constructs into cultured granulosa cells and found that GDF9 treatment induced the activation of the CAGA promoter, but not the BRE or GCCG promoters, in rat granulosa cells (Mazerbourg *et al*., 2004). Similar results also were found for human granulosa cells (Kaivo-Oja *et al*., 2005). Co-transfection with Smad7, but not Smad6, led to the suppression of the GDF9 stimulation of the CAGA promoter, confirming that GDF9 signalling does not involve the BMP-responsive pathway mediated by Smad1, Smad5 and Smad8. We further demonstrated that treatment with GDF9, like activin, increased the level of phospho-Smad3 and phospho-Smad2 in rat and human granulosa cells (Kaivo-Oja *et al*., 2003; Roh *et al*., 2003; Mazerbourg and Hsueh, 2003).

Following the identification of the downstream pathway for GDF9 in granulosa cells, we selected a cell line with minimal responsiveness to GDF9 but containing BMPRII to search for the

Table I. List of the genes up- or down-regulated by GDF9 in theca, granulosa and cumulus cells

	Up-regulated	Down-regulated
Theca cells	*CYP17* (Vitt *et al*., 2000b)	*Kit ligand* (Joyce *et al*., 2000)
Granulosa cells	*Urokinase plasminogen activator* (Elvin *et al*., 1999a)	*LH receptor* (Elvin *et al*., 1999a; Vitt *et al*., 2000a)
	Inhibins α, βA, βB (Kaivo-Oja *et al*., 2003; Roh *et al*., 2003)	*Inhibin α* (Elvin *et al*., 1999b)
	StAR (Elvin *et al*., 1999a)	
	TNF-induced protein 6 (Varani *et al*., 2002)	
Cumulus cells	*Hyaluronan synthase* (Elvin *et al*., 1999a; Gui and Joyce, 2005)	*LH receptor* (Elvin *et al*., 1999a)
	EP2 receptor (Elvin *et al*., 2000)	*Urokinase plasminogen activator* (Elvin *et al*., 1999a)
	Ptgs2 (Elvin *et al*., 1999a; Gui and Joyce, 2005)	
	Peroxiredoxin 6 (Leyens *et al*., 2004)	
	Pentraxin 3 (Varani *et al*., 2002)	
	TNF-induced protein 6 (Varani *et al*., 2002)	
	Gremlin (Pangas *et al*., 2004)	

CYP17, cytochrome *P*-450c17α; EP2, prostaglandin E2; Ptgs2, prostaglandin endoperoxide synthase 2; StAR, steroidogenic acute regulator protein; TNF, tumour necrosis factor.

type I receptor for GDF9. We overexpressed each of the seven type I receptors in the minimally responsive COS7 cells and found that the expression of ALK5, but not any other type I receptor, conferred GDF9 activation of the CAGA promoter (Figure 4). We further performed RNA interference experiments to conclusively demonstrate the important role of ALK5 as the type I GDF9 receptor in granulosa cells (Mazerbourg *et al*., 2004). Our data suggested crosstalk between the known BMPRII and ALK5 together with downstream Smad3 and Smad2 proteins (Figure 1). Our findings are consistent with earlier reports showing the expression of BMPRII, ALK5 and different Smad proteins by granulosa cells in developing follicles (Sidis *et al*., 1998; Shimasaki *et al*., 1999; Qu *et al*., 2000; Drummond *et al*., 2002; Xu *et al*., 2002). On the basis of structural data on the TGFβ ligands and receptors complexes supporting a model of co-operative oligomeric receptor assembly with no direct interaction between the receptors (Greenwald *et al*., 2003, 2004), we can hypothesize that GDF9 is first binding the high-affinity receptor, BMPRII, thus enhancing the affinity to the type I receptor, ALK5.

Although GDF9 and BMP15 are the closest paralogues, BMP15 was found to activate the Smad1/5/8 pathway through interaction with BMPRII and ALK6 in rat granulosa cells (Moore *et al*., 2003). The fact that paralogues could activate different pathways is supported by the ability of TGFβ to stimulate both pathways through binding to ALK1 or ALK5 depending on the cellular context (Goumans *et al*., 2002). The use of distinct signalling pathways by GDF9 and BMP15 homodimers could explain their unique roles in follicular development. However, GDF9 and BMP15 could form homo- and/or heterodimers when produced in the same cell in culture, likely through non-covalent interactions (McPherron and Lee, 1993; Vitt *et al*., 2002; Liao *et al*., 2003). The formation of the GDF9/BMP15 heterodimers could modify their affinity for a given receptor complex and induce distinct physiological responses (Aono *et al*., 1995; Suzuki *et al*., 1997; Nishimatsu and Thomsen, 1998; Butler and Dodd, 2003). Similarly, GDF7 has been shown to enhance the axon-orienting activity of BMP7 (Butler and Dodd, 2003) likely through the formation of GDF7/BMP7 heterodimers. The ability of the heterodimer GDF9/BMP15 to bind a receptor complex and activate the Smad pathway remains to be demonstrated.

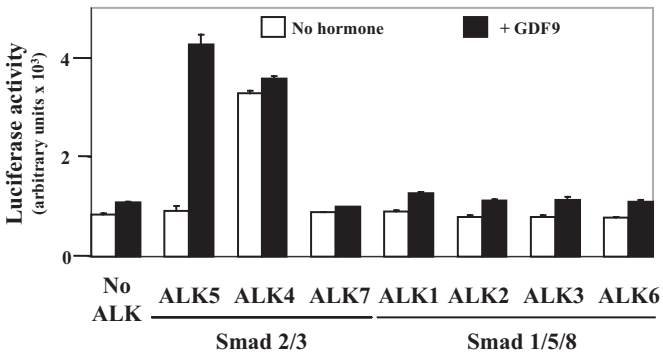

Figure 4. Overexpression of ALK5 confers GDF9 responsiveness in COS7 cells. Cells were transfected with 500 ng of the CAGA reporter and 30 ng of the plasmids encoding individual ALK proteins. Cells were incubated for 24 h with or without GDF9. The relative luciferase activity was normalized based on β-galactosidase activity. Modified with permission from Mazerbourg *et al*. (2004). Copyright 2004, The Endocrine Society.

The signalling pathway of other orphan ligands: GDF6, GDF7 and BMP10

The present genomic approach to identify cognate receptors and downstream Smad pathways for GDF9 can be applied to other GDF/BMP orphan ligands. Based on sequence comparison of their C-terminal cystine-knot domains, a subfamily of closely related ligands including GDF5, GDF6, GDF7, BMP9 and BMP10 can be identified (Figure 1). Among them, GDF6, GDF7 and BMP10 are orphan ligands likely signalling through the same limited number of type I and type II receptors. GDF6, also known as BMP13, is important for joint and cartilage formation (Storm *et al*., 1994). GDF7 (BMP12) is essential for the development of interneurons, sensory neurons and the seminal vesicle (Storm *et al*., 1994; Lee *et al*., 1998; Settle *et al*., 2001; Lo *et al*., 2005). In addition, BMP10 plays a role in heart development (Chen *et al*., 2004). We found that all three ligands formed homodimers. They activate a BMP-responsive promoter reporter (BRE) through the phosphorylation of Smad1/5/8 in a pre-osteoblast MC3T3 cell line (Mazerbourg *et al*., 2005). To identify the type I and type II receptors for GDF6, GDF7 and BMP10, we used overexpression and RNA interference approaches. In the minimally responsive COS7 cells, we individually overexpressed the seven type I receptors and identified ALK3 and ALK6 as candidate receptors for GDF6, GDF7 and BMP10 based on the stimulation of the BRE promoter. For the endogenous ALK proteins, our RT–PCR analyses indicated that ALK3, but not ALK6, was expressed in MC3T3 cells, suggesting that ALK3 is the type I receptor mediating GDF6, GDF7 and BMP10 signalling in this cell line. Indeed, transfection with the ALK3 small hairpin (sh) RNA suppressed GDF6, GDF7 and BMP10 stimulation of the BRE promoter. Using the same approach, we induced gene silencing of two type II receptors, BMPRII and ActRIIA, expressed by MC3T3 cells and involved in BMP2, BMP7 and GDF5 signalling (Koenig *et al*., 1994; Nishitoh *et al*., 1996; Macias-Silva *et al*., 1998). Because of the pronounced inhibitory effects of BMPRII shRNA on GDF6, GDF7 and BMP10 signalling, BMPRII is likely the preferential type II receptor for these three ligands (Mazerbourg *et al*., 2005). These data demonstrated that GDF6, GDF7 and BMP10 signal through the BMPRII/ALK3 receptor complexes in MC3T3 cells. However, one cannot rule out the possibility that other cells may use a different combination of the type I ALK3/ALK6 receptors and the type II BMPRII/ActRIIA receptors for signal transduction.

Conclusion

The present approach provides a genomic paradigm for matching paralogous polypeptide ligands with a limited number of evolutionarily related receptors capable of activating unique downstream signalling proteins. Instead of the traditional single-gene approach in hormonal research, the evolutionary tracing of polypeptide ligands, receptors and downstream signalling molecules in their respective subgenomes could allow the future prediction of receptors and downstream signalling pathways for other orphan ligands.

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