Transforming growth factor β signalling in vitro and in vivo: activin ligand–receptor interaction, Smad5 in vasculogenesis, and repression of target genes by the δ EF1/ZEB-related SIP1 in the vertebrate embryo

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Abstract

The identification and characterization of components of the transforming growth factor β (TGF β) signalling pathway are proceeding at a very fast pace. To illustrate a number of our activities in this field, we first summarize our work aiming at the selection from a large collection of single residue substitution mutants of two activin A polypeptides in which D27 and K102, respectively, have been modified. This work has highlighted the importance of K102 and its positive charge for binding to activin type II receptors. Activin K102E, which did not bind to high-affinity receptor complexes, may be a valuable β chain, when incorporated in recombinant inhibin to unambiguously detect novel inhibin binding sites at the cell surface. We then illustrate how Smad5 knockout mice and an overexpression approach with a truncated TGF β type II receptor in the mouse embryo can contribute to the identification of a novel TGF β $\rightarrow$ T β RII/ALK1 $\rightarrow$ Smad5 pathway in endothelial cells in the embryo proper and the yolk sac vasculature. We conclude with a summary of our results with a Smad-interacting transcriptional repressor but focus on its biological significance in the vertebrate embryo. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Activin; Angiogenesis; Bone morphogenetic protein; Brachyury; Smad; Smad-interacting protein

1. Introduction

Many recent observations maintain the ligands of the transforming growth factor type β (TGF β) family in the frontline of research in medicine and life sciences. These include the mapping of mutations affecting these genes and that lead to human congenital malformation (Storm and Kingsley, 1996; Polinkovsky et al., 1997; Thomas et al., 1997), the documentation of the activities of their encoded proteins in many biological processes ranging from cell differentiation to tissue repair,

angiogenesis and bone metabolism, and their link to pathology including cancer (Blobe et al., 2000; Gold, 1999). In the vertebrate embryo, the TGF β system is a key regulator of inductive interactions in early development (Mummery and van den Eijnden-van Raaij, 1999), including mesendoderm formation and neurogenesis (Dale and Jones, 1999; Agius et al., 2000; Bottner et al., 2000; Harland, 2000; Kimelman and Griffin, 2000), patterning (Dosch et al., 1997; Wilson et al., 1997), establishment of left-right asymmetry (Capdevila et al., 2000), as well as later in organogenesis (Grapin-Botton and Melton, 2000) and epithelial–mesenchymal communication (Jernvall and Thesleff, 2000). After birth, the system is a critical inhibitor of epithelial cell growth, a potent suppressor of many different cell types

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implicated in immune and hematopoietic function, a strong promoter of connective tissue growth and fibrosis (Massagué et al., 2000), and an important player in the hypothalamus–pituitary–gonadal axis and oocyte–follicle cell interaction (Lau et al., 1997; Elvin et al., 2000).

The past few years have witnessed a significant progress in the identification, the structure–function analysis and in vivo study of components of the TGF β signalling system, and their crosstalk with non-TGF β signalling pathways involving Wnt, Hedgehog, Delta/Notch, tumor necrosis factor and interferon cytokines (Massagué and Chen, 2000a; Massagué and Wotton, 2000b; Miyazono, 2000). The progress in the TGF β field ranges from the ligands themselves to their specific inhibitory binding proteins and proteases that cleave these binding proteins, the signalling and ancillary receptors (Massagué and Weis-Garcia, 1996; ten Dijke et al., 1996; Chong et al., 2000; Lewis et al., 2000), the receptor-activated Smad proteins and their non-Smad partners (Heldin et al., 1997; Massagué, 1998; Verschueren and Huylebroeck, 1999), the identification of direct target genes (Attisano and Wrana, 1998, 2000), and the study of non-Smad pathways some of which involve TGF β -activated kinase (TAK1) and p38 kinase (Zhang and Derynck, 1999).

We start here with a summary of the results from a concluded mutational analysis of the prototype ligand activin A (Wuytens et al., 1999) and compare these results briefly with those obtained by other groups studying TGF β (Daopin et al., 1992; Schlunegger and Grütter, 1992; Huang et al., 1999; Qian et al., 1999), nodal-related proteins (Ezal et al., 2000), and bone morphogenetic proteins (BMP2 and BMP7) and their receptor interaction (Griffith et al., 1996; Kirsch et al., 2000a,b). Our prime interest at present is, however, to study activin, BMP and TGF β signalling in vertebrate development, with increasingly visible emphasis on functional analysis in vivo. We focus here on the angiogenesis phenotype in the Smad5 deficient mouse embryo (Chang et al., 1999) and its striking reminiscence of the vascular phenotype observed in other mouse models, including one in which we overexpressed a truncated TGF β type II receptor (T β RII) (Goumans et al., 1999). We then summarize our recent work on a Smad-interacting protein (SIP1), which is a novel member of the δ EF1/ZEB family of transcriptional repressors. Irrespective of its likely complex mode of action, which is proposed to involve interaction with R-Smads (Verschueren et al., 1999) but also non-Smad co-repressors (such as CtBP-1 and -2; van Grunsven, unpublished results), the picture is emerging that this multi-zinc finger DNA-binding protein is an important regulator of the expression of many essential genes, including earlier characterized TGF β -responsive genes, in the vertebrate embryo.

2. Results

2.1. Type II receptor binding of activin A

It is somehow surprising that still few structure–function data are available for ligands of the TGF β family. In our study (for details, see Wuytens et al., 1999), the primary targets for mutagenesis of activin A were charged individual amino acids (excluding the cysteines in the mature region, see Mason, 1994), which by analogy with the prototype structures of TGF β 2 and BMP7, may be located in the presumed four solvent-accessible domains (N-terminal thumb, loop 1, loop 2 and the C-terminal segment of the conserved α helix, respectively; Fig. 1) of the 25 kDa mature activin A homodimer.

Based on the activities of the mutant polypeptides in two bio-assays (production of follicle stimulating hor-

Fig. 1. Schematic diagram of the activin A monomer (taken from Wuytens et al., 1999 and adapted from Mason, 1994), containing the N-terminal thumb region (N-term.), loop 1 (with fingers 1 and 2), the conserved α helix, and loop 2 (with fingers 3 and 4). The proposed structure is based on the known structures of TGF β 2 and BMP7 (Daopin et al., 1992; Schlunegger and Grütter, 1992; Griffith et al., 1996). Residues that, when mutated individually or in combination (for details, see Wuytens et al., 1999), did not significantly alter wild-type activity are highlighted in light gray. Mutant activins with higher (D27K), lower (K102A), and no activity (K102E) compared with wild-type activin (like K102R) are highlighted in dark grey. Cysteine residues are boxed and cysteine bonds are presented as solid, gray bars. The cysteine bond at residue 80 represents the intermolecular disulfide bond.

none in primary pituitary cells, and induction of mesoderm and the immediate-early activin-responsive *Brachyury* gene in *Xenopus* embryo animal caps, respectively), 4 out of 39 mutant proteins (D27K, K102A, K102E and K102R) produced in a vaccinia virus system were selected for further investigation, produced first in insect cells and then purified to homogeneity. D27K activin has a 2-fold higher specific bio-activity and binding affinity to an ActRIIA/ALK4 receptor complex than wild-type activin, whereas K102E activin had no detectable biological activity (confirmed by Fischer et al., 2000) and did not bind to any of the activin receptors (including ActRII homodimers) but still bound to follistatin-288. K102R and wild-type activin bound to all the activin receptor combinations tested and were equipotent in bioassays. The positive charge of amino acid 102 of activins is thus essential for biological activity and type II receptor binding. Both D27 and K102 are conserved in BMP7 and GDF5, which have been shown to bind to ActRII (Yamashita et al., 1995; Nishitoh et al., 1996). It has yet to be shown for the three ligands that the K102 residue directly contacts the pocket formed by F42/W60/F83 in ActRII (Gray et al., 2000). We are tempted to propose that the incorporation of a K102E β_A -chain in recombinant inhibin A ($\alpha\beta_A$), which normally binds to ActRII through its β chain (Xu et al., 1995), may provide an alternative tool to identify and characterize inhibin-binding proteins other than the type III TGF β receptor betaglycan (Lewis et al., 2000), p120 (Chong et al., 2000) and ActRII.

2.2. *Smad5* and *T β RII* mouse models indicate involvement of *Smad5* in non-BMP signalling

2.2.1. *In vivo Smad5* gain-of-function studies

Smad5 is highly similar to *Smad1* and, together with *Smad8*, is convincingly shown to be directly involved as a receptor-regulated Smad (R-Smad) in BMP signalling downstream of ALK3 and ALK6-containing receptor complexes (Heldin et al., 1997; Kawai et al., 2000). Other ligands like GDF5 and anti-Müllerian hormone may involve ALK6 $\rightarrow$ *Smad1* signalling (Nishitoh et al., 1996; Gouedard et al., 2000), and BMP7 signalling can also occur through ALK2/ActRII receptor complexes (Yamashita et al., 1995). Ectopic expression of full-length *Smad5* in the amphibian embryo induces ventral mesoderm similar to BMP2, BMP4 or *Smad1*, as seen from the altered morphology of the embryo that reflects a ventralized phenotype (see also Suzuki et al., 1997; our unpublished results, Fig. 2B). Loss of the dorsal marker *gooseoid* from, and expansion of the ventral markers *Xvent1* and *XWnt8* to dorsal marginal zone explants of the early gastrula, as shown by RT-PCR, further confirms this conclusion (Fig. 2A).

Fig. 2. *Smad1* and *Smad5* induce ventral cell fates in *Xenopus*. (A) Expansion of the expression domain of endogenous *Xvent1* mRNA, normally a ventrolateral mesodermal marker, to isolated dorsal marginal zones (DMZ), indicating the ventralizing activity of *Smad1* and *Smad5*. Embryos were micro-injected with different amounts (indicated in ng RNA per blastomere) of RNA encoding full-length *XSmad1* and *Smad5*. DM zones were explanted at the early gastrula stage and incubated until sibling embryos reached stage 19. Total RNA was isolated and analyzed by RT-PCR assays for expression of *Xvent1*, *gooseoid* (*gsc*), muscle (*m.*) actin, *Xwnt8*, and histone H4 (*H4*) mRNA as indicated. Embryo, ventral marginal zone (VMZ) and DMZ on the left were uninjected controls (Co; shown for DMZ); RT-PCR of uninjected control DMZ sample without prior reverse transcription. (B) Phenotype of embryos micro-injected at the 4-cell stage into all four blastomeres with different amounts (indicated in ng RNA per blastomere) of RNA encoding full-length *Xsmad1* and *Smad5*.

2.2.2. *In vivo Smad5* loss-of-function studies

Smad5 mRNA is present in the mouse embryo throughout early post-implantation development (Chang et al., 1999) and is strongly expressed in the urogenital system at mid-gestation (E12.5; Fig. 3). To determine whether *Smad5* is essential for mouse embryogenesis or, alternatively, *Smad5* deficiency is not embryonic lethal but the mice would develop pathology after birth (for example, and based on the high expression level during embryogenesis, postnatal gonadal tu-

mors may develop in mutant mice), we have generated in collaboration with M. Matzuk (Houston) a loss-of-function mutation of Smad5 using gene targeting in ES cells (Chang et al., 1999). Smad5 null mice die late in gestation (i.e. between E9.5 and E11.5) in contrast to other Smad deficient mouse embryos (for a recent review, see Goumans and Mummery, 2000), and display variable morphological defects, including at E8.0 the developing amnion, gut and heart (the latter being reminiscent of BMP2 knockout mice; Zhang and Bradley, 1996). At later stages, the mutant embryos fail to undergo proper heart looping and body turning, are edematous, and have (likely secondary) cranio-facial and neural tube abnormalities.

Three major and general conclusions could be made based on careful analysis of the phenotypes. Firstly, despite the expression of Smad5 mRNA (Meersseman et al., 1997; Chang et al., 1999) and protein (Bosman and Zwijsen, unpublished results) from gastrulation onwards, Smad5 only becomes essential later in embryogenesis. All mutant embryos indeed proceed beyond the egg cylinder stage, undergo gastrulation, and initiate organogenesis. Secondly, at least four developmental programs depend on Smad5, i.e. the ventro-lateral morphogenesis for correct development of gut and heart, the formation of allantois and amnion, the maintenance of the vascular integrity of the yolk sac and (at later stages) the embryo proper, and the determination of left-right asymmetry (for details on the latter phenotype, see Chang et al., 2000). Thirdly, the lesions in the yolk sac are reminiscent of BMP4 (Winnier et al., 1995), TGF β 1 (Dickson et al., 1995) and T β RII (Oshima et al., 1996) knockout mice.

Fig. 3. Smad5 is strongly expressed in the urogenital system at mid-gestation in the mouse embryo. Bright-field photograph of in situ hybridization with anti-sense RNA probe for Smad5 at mid-gestation (E12.5; sagittal section) in the mouse embryo. The Smad5 gene is in fact expressed ubiquitously at low to moderate levels, but note the very high expression levels of the gene in the urogenital system.

We propose that Smad5 deficiency is compensated during post-implantation development by other BMP R-Smads like Smad1, which coincides with the observation that deficiencies of the upstream components BMP2 and ALK3 cause a more severe, earlier phenotype (Zhang and Bradley, 1996; Mishina et al., 1995). Smad4 deficient mice have, like BMP4 and ALK3 mutant mice, no mesoderm (Sirard et al., 1998; Yang et al., 1998) but have an additional visceral endoderm defect. This more severe phenotype would then be in line with the more general role of Smad4 as common mediator or co-Smad. Smad2 deficient mice do not develop mesoderm either, and display an overgrowth of extraembryonic ectoderm into the epiblast (Nomura and Li, 1998; Waldrip et al., 1998; Weinstein et al., 1998), thereby mimicking deficiency of *nodal*, which likely exerts effects via ALK4, which activates Smad2 (see Agius et al., 2000 for a recent analysis of *Xnodal-related* proteins and interference of their mesoderm-inducing activity with truncated ALK4 receptor in *Xenopus*). In general, this gene targeting approach demonstrates that careful comparative analysis of the phenotype(s) of mutant mouse embryos can reveal ligand-receptor-intracellular effector relationships, some of which may yet not have been documented biochemically. This concept is also nicely illustrated in the Smad5 knockout mouse, as a number of observations support the existence of a novel TGF β $\rightarrow$ T β RII/ALK1 $\rightarrow$ Smad5 pathway in the vessel wall.

2.2.3. Smad5 in vasculogenesis/angiogenesis

The onset of vasculogenesis/angiogenesis and hematopoiesis in Smad5 mutant mouse embryos is normal in both extraembryonic tissue and the embryo proper, but soon afterwards the vessel homeostasis becomes abrogated in the yolk sac and the embryo. The anemic appearance of the Smad5 deficient yolk sac (Fig. 4D and E) is likely the result of loosely attached extraembryonic endoderm to extraembryonic mesoderm (Fig. 5H and I). This defect is phenocopied (Fig. 4A and C, Fig. 5D, F and G) in highly somatic chimeras obtained after aggregation of wild-type morulas with ES cells that overexpress a truncated kinase-deficient T β RII (Δ T β RII; Goumans et al., 1999). In such mice, the entire embryo proper (with the exception of few cells in the gut endoderm) and yolk sac mesoderm, which gives rise to the endothelial, hematopoietic and smooth muscle cells of the yolk sac vasculature, is derived from the mutant ES cells, whereas its endodermal compartment is derived from the aggregation partner (Fig. 4G). Defective vasculogenesis in the yolk sac (with a blistered appearance) is observed in the Δ T β RII mouse, but like in the Smad5 knockout mice, a primitive plexus is still formed and accumulation of red blood cells can be observed (Fig. 4A–F, Fig. 5). In addition, and similar to the Smad5 null embryos, another conclusion

Fig. 4. Morphology, anemia and vasculature of yolk sacs of highly chimaeric embryos resulting from morula aggregation with ES cells stably transfected with $\Delta T\beta RII$ expression vector (Goumans et al., 1999), and comparison with yolk sacs from $Smad5$ knockout mice (Chang et al., 1999). (A) E9.5 $\Delta T\beta RII$ chimera with yolk sac anemia and defective vasculogenesis. The white arrowhead indicates the enlarged pericardial sac. (B) E9.5 control embryo showing a well developed, blood filled vasculature. The black arrowhead indicates the position of the pericardial sac. (C) E9.5 $\Delta T\beta RII$ chimera with defective vasculogenesis. Note the accumulation of red blood cells (white arrowhead) and the formation of a primitive plexus (open arrowhead). (D) E9.5 $Smad5$ null ($-/-$) conceptuses within the yolk sac, which is devoid of a blood-filled network of vessels, but still develops a primitive plexus. (E) E9.5 $Smad5$ null ($-/-$) and heterozygous ($-/+$) conceptuses within their yolk sac. Note delayed development of $-/-$ embryo and open gut, together with confirmation of anemic phenotype of the yolk sac as compared with the $-/+$ embryo. (F) E9.5 control ($+/+$) conceptus within the yolk sac, which has a well developed vascular network containing red blood cells. (G) Schematic representation of the protocol used for the generation of highly chimaeric mouse embryos by morula aggregation. In these experiments, $\Delta T\beta RII$ expressing ES cells were aggregated with morulae from a mouse line expressing $LacZ$ ubiquitously. To bias towards the generation of high percentage chimaeric embryos, the ES cells were preaggregated in suspension (Goumans et al., 1999). From the insert (bottom right), which shows the staining of the E8.5 chimaeric embryo with the chromogenic substrate X-gal for $LacZ$, it follows that such high chimeras are nearly completely derived from the $\Delta T\beta RII$ ES cells.

from these studies is that neither $TGF\beta$ nor $Smad5$ signalling is required for differentiation of extraembryonic mesoderm in endothelial cells, but is necessary for their subsequent organization into robust vessels. One difference between the $Smad5$ knockout and the over-expression $\Delta T\beta RII$ models is the deposition of fibronectin in the yolk sac, which is normal for $Smad5$ null mice (Fig. 5I, compare left ($+/+$) with right ($-/-$) panel) but discontinuous in the $\Delta T\beta RII$ mice (Goumans et al., 1999).

We also noted the increased and ectopic expression of the angiogenic and hematopoietic marker genes *flk-1* and ζ -globin in the impaired tissues in the $Smad5$ knockout mouse (Fig. 5J,K; Chang et al., 1999). The unique observation that aggregates of cells consisting of different cell types including endothelial cells, likely of allantois origin, are found on the amnion of $Smad5$ deficient embryos shows that $Smad5$ may normally be involved in restriction of allantois and may prevent hematopoietic cell differentiation in the amnion. This is

in line with the earlier proposal that the ectodermal component of the amnion, where Smad5 protein is present (Bosman and Zwijsen, unpublished results) can be involved in this negative regulation. The phenotypic analysis also raised the possibility that Smad5 is normally involved in the negative regulation of hematopoietic cell proliferation. This is supported by experiments in cell culture involving an anti-sense Smad5 RNA

2.2.4. *TGF β signalling cascades in vasculogenesis/angiogenesis*

the existence of the novel $\text{TGF}\beta \rightarrow \text{ALK1/T}\beta\text{RII} \rightarrow \text{Smad5}$ pathway in the yolk sac vasculature: ALK1 is expressed there, where Smad5 is present (Roelen et al., 1997; Bosman and Zwijsen, unpublished results), ALK1 can directly activate Smad1 and Smad5 (Macias-Silva et al., 1998), and the ALK1 knockout mice (Oh et al., 2000; Urness et al., 2000) fail to specify the differences between arteries and veins, which normally occurs at the earliest onset of angiogenesis, before the onset of circulation. ALK1 deficient mouse embryos develop shunts between arteries and veins, which is a phenotype not seen in endoglin deficient mice (Arthur et al., 2000), display excessive growth of endothelial cells and delayed differentiation of vascular smooth muscle cells and accompanying deficient localization to the perivascular region, and fail to confine hematopoiesis to the arteries (Oh et al., 2000; Urness et al., 2000). In addition, mutations in ALK1 cause the rare disease hereditary hemorrhagic telangiectasia-2 (HHT2, Abdalla et al., 2000), characterized by vascular dysplasia and continuous bleeding problems.

Taken together, the *in vivo* functional analysis of genes of the $\text{TGF}\beta$ signalling cascade clearly demonstrate that $\text{TGF}\beta$ is indeed important for proper vessel formation. Why endothelial cells developed two different $\text{TGF}\beta$ pathways, i.e. one driven by ALK5 and one by ALK1, to inhibit endothelial cell proliferation and to stimulate differentiation of mesenchymal precursor cells to vascular smooth muscle cells in response to the same ligand, is still unclear. With regard to its consequences, it is very interesting to compare in detail the phenotypes of two groups of knockout mice, the ALK1 and Smad5 knockout mice (here referred to as the ALK1 group) and the $\text{TGF}\beta 1$, $\text{T}\beta\text{RII}$, and endoglin knockout mice (the ENG group), respectively. The vascular phenotypes are identical within each group of targeted mice but different, although overlapping, between these two groups. All these mice exhibit normal differentiation of endothelial cells and vasculogenesis at embryonic day E8.5, have distended vessels in the yolk sac at E9.5, and are embryonic lethal around E10.5–11.5. Both groups show similar defects in the differentiation and/or recruitment of vascular smooth muscle cells. A major difference between the two groups is the vascular morphogenesis of the embryo proper at E9.5. The ENG group mutant embryos has relatively normal branching capillary network and lumen size at E9.5, whereas the capillaries of the ALK1 group mutant embryos fail to form a branching capillary network, and the blood vessels are greatly dilated at E9.5. The differences can probably be attributed to the fact that mutations in the ENG group disrupt both ALK1 and ALK5 pathways, whereas mutations of the ALK1 group disrupt only ALK1 signalling, leading to dominance of ALK5 in such mice. Activation of the latter

receptor then induces production and secretion of proteinases, thereby leading to distended blood vessels.

Again, such studies illustrate the importance of developmental biology and the functional dissection of $\text{TGF}\beta$ signalling *in vivo* for better understanding of disease. Vasculogenesis and angiogenesis are complex processes in which endothelial cells, in an integrated and controlled manner, proliferate, differentiate, migrate, produce proteolytic enzymes for invasion, follow a cell–cell and cell–extracellular matrix program, deposit new basement membrane, and change their transport properties. Many of these endothelial cell functions will be monitored in the future using both *in vitro* and *in vivo* models, and will involve the study of the normal developmental mechanisms, including those regulated by $\text{TGF}\beta$ signalling. Doing so, important information will be obtained regarding the role of the various alternative $\text{TGF}\beta$ family signalling cascades. Thus, for instance, information about the relative role of $\text{TGF}\beta 1 \rightarrow \text{ALK1} \rightarrow \text{Smad5}$ versus the $\text{TGF}\beta \rightarrow \text{ALK5} \rightarrow \text{Smad2/3}$ or the $\text{activinA} \rightarrow \text{ALK4} \rightarrow \text{Smad2/3}$ pathways should be derived.

Activin A is a well-studied member of the $\text{TGF}\beta$ family in the fields of signal transduction, and vertebrate embryogenesis and fertility, but recently an important role for this ligand was demonstrated in initiation of tumor angiogenesis (Breit et al., 2000). It has been shown that activin A is an inhibitor of endothelial cell proliferation *in vitro*. However, unlike $\text{TGF}\beta$, activin A exhibits inhibitory activity in the vascular formation of the chorio-allantoic membrane assay *in vivo*. Interestingly, in addition to reduced number of capillaries, a considerable hemorrhage was observed in the site of application. This indicates an effect on vessel maturation reminiscent of the effect demonstrated in the knockout mice of the $\text{TGF}\beta$ system. Moreover, activin A expression is down-regulated by *N-myc* oncogene amplification in neuroblastoma cells, whereas the $\text{TGF}\beta$ levels were not affected. These data suggest a role of activin A in initiation of tumor angiogenesis and its involvement in the mechanisms by which genetic changes in cancer lead to enhancement of the tumor phenotype in the process of malignant transformation. The role of activin in angiogenesis is further supported by the fact that the activin-binding glycoprotein follistatin can regulate autocrine endothelial cell activity and induce angiogenesis (Kozian et al., 1997). The differential effect of activin A on angiogenesis compared with that of $\text{TGF}\beta$ is perhaps not so surprising as different ligands of the $\text{TGF}\beta$ family can result in different responses. However, it is difficult to explain this at the molecular level, as many effects of both $\text{TGF}\beta$ and activin A have been shown to be mediated by Smad2/3/4 complexes. Perhaps cell-type specific expression of other components of the signalling pathway, including hitherto unknown non-Smad interacting

Fig. 6. Schematic comparison (not drawn to scale) of δ EF1 and its subdomains with mouse SIP1 and *Xenopus* SIP1 (XSIP1) (A); and proposed model for high-affinity binding of full-length δ EF1 and SIP1 to bipartite sites in candidate target promoters (B). (A) The N- and C-terminal clusters of zinc fingers (NZF and CZF) are shown (C2H2 and C3H-type), together with the homeodomain-like sequence and the CtBP binding sites. The Smad-binding domain (SBD) in SIP1 is also indicated. The percentages of identical amino acid residues in these well-conserved regions are shown between δ EF1 and mouse SIP1, and between mouse SIP1 and XSIP1, respectively. (B) δ EF1 and SIP1 can bind DNA as a monomer to spaced bipartite CACCT elements in promoters of target genes (for details, see Remacle et al., 1999). We have indicated this spacing (N with number base pairs) for different promoters for which, at least in vitro, DNA binding could be demonstrated and only if, both half sites were intact. These promoters include *Xbra*, *E-cadherin* (*Ecad*), $\alpha 4$ integrin ($\alpha 41$) and mouse *folliculin* (*FS*).

partners of these Smads, may be revealed to solve this problem. In addition, this will only become clear by unambiguous demonstration that different downstream effector proteins and target genes are used in vivo, but we propose that crossing of appropriate and preferably vascular-restricted knockout mice for genes in these pathways, and the use of cells isolated from the different targeted mice that will provide a unique source of material, will be crucial to address the role of differential TGF β signalling. This is extremely important, because it will define the pathways to be targeted specifically for the treatment of cancer, and cardiovascular and other angiogenic diseases.

2.3. SIP1, a novel member of the δ EF1/ZEB family of transcriptional repressors, and its action on the activin-responsive *Xenopus Brachyury* promoter

Two subclasses of the Smad proteins, the R-Smads and co-Smads, transduce signals from the cell surface to the nucleus, where they participate in the regulation of gene expression. It has become evident that Smads should be envisaged as very versatile proteins, which integrate multiple signalling pathways and can directly affect target gene expression in many ways (Attisano and Wrana, 1998; Verschueren and Huylebroeck, 1999; Attisano and Wrana, 2000; Massagué and Chen, 2000a; Massagué and Wotton, 2000b; Wrana, 2000). Indeed, their direct binding to DNA and the interaction in the nucleus of Smad with non-Smad proteins, many of which are DNA-binding activators or repressors of

transcription, uncover a unique but complex mode of action in the nucleus of TGF β activated cells.

We are studying SIP1, which was identified using the yeast two-hybrid system. Although SIP1 interacts with the MH2 domain of R-Smads in yeast and in vitro, its interaction with full-length R-Smads in mammalian cells requires receptor-mediated Smad activation (Verschueren et al., 1999). SIP1 is a new member of the δ EF1 family of two-handed zinc finger/homeodomain proteins (Fig. 6A) and was recently recloned as ZEB2 (Postigo and Dean, 2000). Like δ EF1 (Sekido et al., 1997), SIP1 binds to 5'-CACCT sequences in different promoters, including the *Xenopus Brachyury* (*Xbra*) promoter, where two of such sites are present within the earlier defined 150 bp-long activin-responsive element (Latinkic et al., 1997). Overexpression of either full-length SIP1 or its C-terminal zinc finger cluster, which bind to the *Xbra*2 promoter in vitro with high and low affinity (Remacle et al., 1999), respectively, prevented expression of the endogenous *Xbra* gene in early *Xenopus* embryos. Therefore, SIP1, like δ EF1, is likely to be a transcriptional repressor, which may be involved in the regulation of at least one immediate activin-responsive gene in the frog embryo.

SIP1 was recently found to display unique DNA-binding properties (Remacle et al., 1999). SIP1 and δ EF1 contain two separated clusters of zinc fingers, one N-terminal and one C-terminal. These two clusters show high sequence homology and are also highly conserved between SIP1 and δ EF1. Each zinc finger cluster binds independently to a 5'-CACCT sequence.

However, high-affinity binding sites for full-length SIP1 and δ EF1 in the promoter regions of candidate target genes like *Xenopus Xbra2*, human $\alpha 4$ -integrin ($\alpha 4I$) and *E-cadherin (Ecad)* and mouse follistatin (Fig. 6B) are bipartite elements composed of one CACCT and one CACCTG sequence, the orientation and spacing of which can vary. Using transgenic *Xenopus* embryos, we demonstrate that the integrity of these two sequences is necessary for correct spatial expression of a *Xbra2* promoter-driven reporter gene. Deletion analysis and point mutations in putative transcription factor binding sites in this promoter define two repressor modules, which exert their effect at different stages during gastrulation. One repressor module is indeed defined by a bipartite binding site for SIP1 (Lerchner et al., 2000).

Both zinc finger clusters in full-length SIP1 must be intact for the high-affinity binding of SIP1 to DNA and for its optimal repressor activity (Remacle et al., 1999). Our results show that SIP1 can bind as monomer and contacts with the first zinc finger cluster the one target sequence, and with the second cluster the other (Fig. 6B). Although it cannot be excluded that δ EF1 proteins bind also to single sites in promoters in vivo, our work redefined the binding site and, consequently, candidate target genes for vertebrate members of the δ EF1 family. This novel mode of binding to bipartite sites via separated clusters of zinc fingers may apply also to other transcription factors that contain such clusters, like schnurri. This intracellular protein operates in dpp (BMP) pathways in *Drosophila* (Arora et al., 1995; Grieder et al., 1995) and was recently suggested to interact with Smad proteins, as well (Dai et al., 2000; Udagawa et al., 2000).

We have cloned the *Xenopus* orthologue of SIP1 and documented its expression in the marginal zone, the neuro-ectoderm, neural crest, neural tube and lateral plate mesoderm during amphibian development (van Grunsven et al., 2000). Our results indicate that SIP1 and *Xbra* mRNA expression briefly overlap early in development but then become rapidly mutually exclusive. We are now studying the role of SIP1 in mesoderm and neural induction further in this embryo and are investigating, whether also transcription activation by *Xbra* has direct effects on *XSIP1* transcription in addition to the documented repressor effect of SIP1 on *Xbra* transcription. *XSIP1* is highly similar to mouse SIP1 (Fig. 6A), including the sequences of the two zinc finger clusters and the Smad binding domain, and the location of active binding sites for the co-repressors CtBP-1 and -2 (van Grunsven, unpublished results). Preliminary results, obtained in collaboration with J. Smith's group and E. Bellefroid's group, show that SIP1 transcription can be induced in animal caps of the amphibian embryo by attenuation of BMP signals with either noggin or truncated BMP receptor. Conversely, overexpression of BMP leads to downregulation of SIP1 mRNA synthesis.

Further work in *Xenopus* will also have to address the direct link of SIP1 with activin/nodal effects in mesoderm induction and/or the BMP effects on dorsal-ventral patterning of mesoderm, neural cell specification, and neural crest cell generation and delamination from the overlying ectoderm, and the possible crosstalk of SIP1 with non-TGF β pathways in these processes. Our current work with SIP1 in the mouse is anticipated to reveal its function in neurogenesis including neural crest generation/migration, in somitogenesis, and in the lateral plate and posterior mesoderm, as these are processes/locations, where the SIP1 gene is expressed early in development and the SIP1 protein can have unique activities compared with δ EF1. SIP1 function may also overlap with δ EF1 later in development including in skeletogenesis (Takagi et al., 1998), as its expression pattern at midgestation (Fig. 7) overlaps but is not identical with this of δ EF1 (Takagi et al., 1998).

Acknowledgements

The TGF β group within the Huylebroeck lab would like to thank first D. de Kretser and D. Phillips for inviting us to the exciting meeting on activins, inhibins and follistatins in Melbourne. We also thank the many past and present external collaborators in the activin A, Smad5, T β RII and SIP1 projects, results of which were presented at the meeting. In particular, we thank F. Bosman, K. Devos and A. Van De Voorde at Innogenetics S.A., C. Niehrs for early work with Smads in

Fig. 7. In situ hybridization (dark-field image, E12.5 sagittal section) for SIP1 in the mouse mid-gestation embryo. Note the presence of SIP1 mRNA in the cranio-facial region (it remains to be determined whether this expression includes muscle cells), the sclerotome-derived condensations around the vertebral axis, the spinal cord and dorsal root ganglia, and the epithelium of multiple soft tissues.

Xenopus embryos, H. Chang in M. Matzuk's lab, M.-J. Goumans earlier in C. Mummery's lab and now in P. ten Dijke's lab, W. Lerchner and C. Papin in J. Smith's group, K. Opdecamp in E. Bellefroid's lab, J. Comijn and G. Berx in F. Van Roy's group, and P. Tylzanowski in F. Luyten's lab. We also thank T. Fotsis for stimulating discussions on and access to results on the differential effects of TGF β and activin A on endothelial cells, and H. Kondoh and Y. Higashi for providing precious δ EF1 plasmids and sharing preliminary expression data of SIP1 in the mouse. Part of the described work was supported by VIB and Innogenetics S.A. within the framework of a collaboration agreement, while additional support was obtained from the Fund of Scientific Research, Flanders (FWO-V; G.0296.98 and G.0243.01), EU-Training and Mobility of Researchers (TMR; CT98-0216) and the University of Leuven (OT/00/41). C.C. and E.B. are pre-doctoral fellows supported by IWT, and L.v.G. and A.Z. are supported by EU-TMR and FWO-V, respectively.

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