

# Smad4 (DPC4) – a potent tumour suppressor?

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**Summary** The recently described family of Smad molecules are essential mediators of transforming growth factor  $\beta$  (TGF- $\beta$ ) signalling. To date, seven members of this family have been identified, each of which plays a specific and separate role in mediating TGF- $\beta$  superfamily gene transcription. At least two different Smads, Smad2 and Smad4 (DPC4), have been implicated in human cancer and appear to have tumour-suppressor functions. Loss of function of Smad4 is most strongly associated with human pancreatic and colorectal malignancy. Furthermore, work from several different groups has suggested associations between Smad4 loss and malignancy in a number of other tissues. Here, we present a review of the current state of the literature implicating the central Smad mediator, Smad4, in the development of cancer.

**Keywords:** Smad4; DPC4; tumour suppressor; cancer

Some of the earliest theories of cancer predisposition have proposed a close relationship between the processes that control normal and malignant development. More recent work has established that the cellular pathways critical to embryonic development do indeed contain a number of genes which function as tumour-suppressor genes. Twenty-seven years ago, Knudson (1971) first proposed the existence of tumour-suppressor genes (TSGs) based on an analysis of the predisposition to retinoblastoma. Since that time, nearly 20 TSGs, including the human retinoblastoma gene (*RB*) itself, have been identified and cloned and, in many cases, mouse models of gene deficiency have been generated by gene targeting. For example, animals heterozygous for *Rb-1* (the mouse homologue of the human *RB* gene) develop tumours with almost 100% penetrance. This strain has also demonstrated the absolute requirement for *Rb-1* in development, as homozygous null animals die at day 13 of gestation, (e.g. Clarke et al. 1992). Other TSGs have also revealed essential developmental roles. Thus, *Apc*-deficient embryos die shortly after implantation (Moser et al. 1989) and p53-deficient mice show increased rates of neural tube abnormality (Armstrong et al. 1995; Sah et al. 1995).

The Smad4 gene (also termed DPC4) is located on chromosome 18q21 and is perhaps the most recent addition to this group of genes that show both developmental and tumour-suppressor functions. In 1996, Smad4 was discovered after genetic analysis of a panel of pancreatic carcinomas, earning its original name of DPC4 from the fact that it was homozygously deleted in a third of the cancers, hence deleted in pancreatic carcinoma-4 (Hahn et al. 1996a). Investigation of the gene showed that it had sequence homology to the *Drosophila melanogaster* mothers against dpp (*MAD*) protein, a transforming growth factor  $\beta$  (TGF- $\beta$ ) signalling homologue (Hahn et al. 1996a), and the *Caenorhabditis elegans* Mad homologues *sma-2*, *sma-3* and *sma-4* (Sekelsky et al. 1995). The presence of

strong homology to these genes implies that, as well as being a tumour-suppressor gene, Smad4 is important in TGF- $\beta$  signalling and mammalian development. Its association with human neoplasia may entirely be a consequence of this significance, as is becoming apparent for several other TSGs.

## TGF- $\beta$ family

The TGF- $\beta$  superfamily is one of the largest groups of polypeptide growth and differentiation factors and mediates a wide range of biological processes in both vertebrates and invertebrates (Kingsley, 1994). Various functional criteria have been used to group the superfamily (of currently around 25 different molecules) into three classes: TGF- $\beta$ s, activins and bone morphogenetic proteins (BMPs). Different members of these groups are variously implicated in the regulation of wound healing, immune responses and, more importantly, in control of growth pathways. The TGF- $\beta$  family itself consists of at least five genes encoding distinct proteins in vertebrates, referred to as TGF- $\beta$ 1–5. The biological effects of TGF- $\beta$  are mediated by specific TGF- $\beta$  receptors at the surface of the target cells, which fall into two classes dependent upon structure. The two types of receptor, both functional serine/threonine kinases, form heteromeric complexes which bind different ligands and initiate different intracellular responses. Essentially, the type I receptors appear to be less selective and can bind different ligands dependent on the more limited ligand specificity of the type II receptor with which they associate (Wrana et al. 1992a,b; Attisano et al. 1993; Massague et al. 1993). Association of the two receptors leads to downstream phosphorylation events which eventually lead to transcription of the appropriate TGF- $\beta$  superfamily gene (Maciassilva et al. 1996). Until recently, these downstream events have remained undetermined.

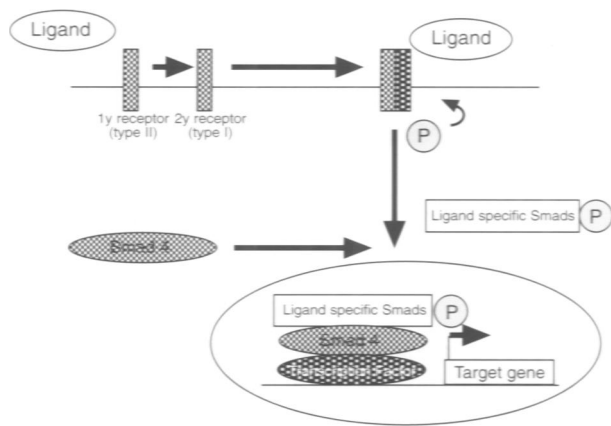
A role for Smad4 has been proposed within the effector arm of TGF- $\beta$  on the basis of homology to *Mad*, a gene characterized by its interaction with the *Drosophila* homologue of the TGF- $\beta$  superfamily member BMP. Smad4 is not the only gene to have been implicated in this way. Indeed, analysis of *Mad* revealed it to be homologous to the *sma* genes of *C. elegans*, and seven distinct

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**Figure 1** TGF- $\beta$  superfamily signalling and Smads. In the presence of TGF- $\beta$  or other TGF- $\beta$  superfamily member (here indicated by 'ligand'), the type I and type II receptors dimerize and become phosphorylated to initiate Smad-dependent signal transduction. This process is thought to be critically dependent on the activity of Smad4 (see text)

vertebrate *smad/Mad* genes have subsequently been cloned of which Smad4 (or DPC4) is one. These genes are now referred to as Smad1–7 according to the proposed nomenclature of Derynck et al (1996). Individual Smad proteins may mediate specific TGF- $\beta$  superfamily signals in development. For example, Smads 1 and 5 have been proposed to mediate BMP signal transduction as they can functionally substitute for BMP2/4 in *Xenopus* embryos. Smads 2 and 3 are implicated in TGF- $\beta$  and activin induction, and Smad 7 is currently thought to be an inducible antagonist of TGF- $\beta$  signalling. However, Smad4/DPC4 is apparently common to all the ligand-specific Smad pathways and would appear to have a role as a central mediator in TGF- $\beta$  superfamily signalling (Lagna et al. 1996; Heldin et al. 1997; Figure 1).

## MADs

The primary structures of MAD proteins do not contain any motif that clearly indicates their function. MADs are proteins of approximately 450 amino acids with highly conserved N- and C-terminal domains and a variable proline-rich intervening region. Smad4 has a structure consistent with a MAD-related protein: there are conserved N- and C-terminal regions (termed MH1 and MH2 respectively) connected by a poorly conserved linker domain rich in serine, threonine and proline residues (Wrana and Attisano, 1996). This structure seems to suggest that the MH domains share a tertiary structure critical to the regulation and function of the protein. In support of this, all the mutations identified in genetic screens map either to the MH1 or the MH2 domain, and often involve alterations in highly conserved residues.

Although MAD function within the nucleus is still largely unclear, it has been observed that the C-domains of various MAD proteins (and Smad4) display transcriptional activity when bound to DNA via a GAL4 DNA binding domain (Liu et al. 1996). The importance of the C-terminal region is further emphasized by studies of malignancy (see below), which reveal that the primary hotspot for Smad4 mutations is within the C-terminal domain (Savage et al. 1996). This is confirmed by the recently described crystal structure of the Smad4 C-terminal domain, which shows that the majority of tumour-derived mutations map to five amino acids that are involved in essential

intermolecular contacts (Shi et al. 1997). These observations raise the possibility that MAD proteins function by transactivation. In *Xenopus* embryos, Smad2 has been shown to interact with FAST-1, a transcription factor with a novel winged helix structure. Furthermore, Smad4 co-immunoprecipitates with this complex (Chen et al. 1997).

Although these data suggest a role for Smads in regulating transcription, the exact nuclear function of these heteromeric Smad complexes remains largely unknown. It has been shown that Smad3 and Smad4 can form a DNA-binding complex that activates transcription of a reporter gene. Furthermore, it has been demonstrated that MAD protein can bind DNA (Xin et al. 1996), and Smad4 itself has been shown to be a DNA-binding protein (Liu et al. 1997). Recently, it has emerged from functional assay studies in a Smad4 null cell line that the molecule does indeed appear to have distinct activation and ligand response domains within it (Caestecker et al. 1997). This suggests a model for Smad4 similar to other archetypal signalling molecules: in the absence of ligand, the N-terminal domain and possibly the middle linker region may obscure the activation domain at the C-terminal end of the molecule. After ligand activation, this results in exposure of the activation domain and may allow the binding of other molecules. The apparent role of Smad4 appears to be in mediating the actions of the other Smads.

Perhaps one of the most powerful indicators of the importance of Smad4's role comes from some recently published papers by Sirard et al (1998) and Takaku et al (1998), who have produced Smad4 knockout mice. Both groups have found that complete inactivation of Smad4 results in embryonic death at around 7.5 d.p.c. due to failed gastrulation and poor anterior development, a phenotype reported to arise from reduced cell proliferation rather than increased apoptosis. Somewhat surprisingly, mice heterozygous for Smad4 do not display any increase in spontaneous tumorigenesis compared with wild-type mice. If the mechanism of loss of Smad4 is indeed through a two-step hit, then heterozygous mice would be predicted to be more cancer prone, especially within the pancreas and intestine. Following this reasoning, Takaku's group introduced the Smad4 mutation into the *Apc*<sup>>716</sup> background, a murine model for familial adenomatous polyposis. The resulting compound heterozygotes developed intestinal polyps which evolved into more aggressive tumours than those observed in the simple *Apc*<sup>>716</sup> heterozygotes, suggesting that mutations in Smad4 play a significant role in the progression of colorectal tumours.

## Smad4 and human neoplasia

The primary report ascribing TSG status to Smad4 revealed loss of function of this gene in 27% of human pancreatic malignancies (Hahn et al. 1996b). Two additional reports have now been published showing somewhat higher rates of Smad4 loss in this tumour type (48% and 53%; Table 1). The predominant mutation observed in these studies has been homozygous deletion of Smad4. In these cases, it remains possible that additional linked genes have been deleted, and that loss of Smad4 is irrelevant, particularly in view of the close proximity of Smad4 to other TSG loci. It is, therefore, of significance that a number of mutations have been identified within the Smad4 gene itself, strongly supporting a causal link between loss of function and malignancy.

These findings raise the possibility that Smad4 acts as a global TSG. However, Smad4 loss has been found to be relatively rare in a range of other tumour types (Table 1). One notable exception to

Table 1 DPC4 loss in various cancers

Cancer type	DPC4 loss (%)	Method of characterization	Type of mutation	Comments	Reference
Colorectal cancer	16	PCR, DNA seq	Four missense mutations and one 12-bp deletion	Thirty-one cancers were examined	Takagi et al (1996)
	22	IVSP	One dinucleotide substitution and three missense mutations		Thiagalingham et al (1996)
Colitis-associated neoplasia	33	PCR, DNA seq	Biallelic inactivation in one of three neoplasms shown to have allelic loss of 18q	Only six cancers examined	Hoque et al (1996)
Neuroblastoma	0	RT-PCR, SSCP	No mutations found in the DPC4 gene, although DPC4 mRNA expression was reduced	Limited role for DPC4 in this cancer	Kong et al (1997)
Prostate cancer	10	SB, PCR, SSCP	Allelic loss of chromosome 18 markers but no point mutations or deletions		MacGrogan et al (1997)
Oesophageal cancer	0	SB, PCR, SSCP			Verbeek et al (1996)
	0	IVSP	None	Only ten samples tested	Lei et al (1996)
	0	LOH, SSCP	None	No DPC4 loss despite high 18q21.1 loss	Barrett et al (1996)
Gastric cancer	0	PCR, DNA seq	None	Only ten samples tested	Lei et al (1996)
	2	LOH, PCR, DNA seq	One case of biallelic inactivation		Powell et al (1997)
Lung cancer	9	SB, PCR, SSCP	Two missense mutations and two frameshift mutations	DPC4 mutations were not present in all lung cancers carrying 18q21 deletions	Nagatake et al (1996)
	0	SB, PCR, SSCP			Verbeek et al (1996)
HNSCC		LOH, RT-PCR, DNA seq	One nonsense mutation in a cell line, 47% LOH in primary tumours	High frequency of LOH at the 18q region cannot be explained by DPC4	Kim et al (1996)
Bladder cancer	0	MA, PCR, DNA seq	None		Schutte et al (1996)
Breast cancer	12	MA, PCR, DNA seq	Homozygous deletion of the complete coding sequence of DPC4 (MDA-MB468 breast carcinoma line)		Schutte et al (1996)
	2	PCR, SSCP, SB	MD-MB-468 breast cancer cell line		Verbeek et al (1996)
	0	MA, PCR, DNA seq	None		Schutte et al (1996)
Hepatocellular cancer	7	PCR, LOH analysis	LOH only, no mutations assessed	Comparative study of LOH among various tumour-suppressor genes	Piao et al (1997)
	12	MA, PCR, DNA seq	Non-conservative amino acid replacement (cell line SW626)		Schutte et al (1996)
Renal cancer	0	MA, PCR, DNA seq	None		Schutte et al (1996)
Nerve cell cancers	0	MA, PCR, DNA seq	None		Schutte et al (1996)
Melanoma	0	MA, PCR, DNA seq	None		Schutte et al (1996)
Osteosarcoma	0	MA, PCR, DNA seq	None		Schutte et al (1996)
	0	PCR, SSCP, SB			Verbeek et al (1996)
Pancreatic cancer	48	MA, PCR, DNA seq	Fifteen homozygous deletions, nine intragenic alterations		Schutte et al (1996)
	53	PCR, DNA seq	G→A transitions and, more frequently, transversions	Thirty-eight tumours analysed in a three tumour-suppressor gene study	Rozenblum et al (1997)
	27	LOH, IVSP	Twenty-five homozygous deletions (out of 84), one truncated protein, six point mutations	The original DPC4 study	Hahn et al (1996)
Leukaemia	0	PCR, SSCP, SB			Verbeek et al (1996)
Oral cancer	6	PCR, SSCP, LOH	Two point mutations, one missense and one substitution	DPC4/DCC study	Watanabe et al (1997)

SSCP, single strand conformation polymorphism; MA, microsatellite analysis; SB, Southern blot; PCR, polymerase chain reaction; DNA seq, DNA sequencing; LOH, loss of heterozygosity; IVSP, in vitro synthesized protein assay; HNSCC, head and neck squamous cell carcinoma; RT-PCR, reverse transcriptase polymerase chain reaction.

this is in colorectal cancer, in which there is good evidence for low-frequency loss at this locus. Once again, mutations have been identified within the Smad4 gene, strongly arguing against the possibility that Smad4 loss is occurring as a consequence of other genetic events. Using similar evidence, a role for Smad4 can be argued in both lung and oral cancer, although the level of loss in these tumour types is very low. Of the other tumour types listed in Table 1, there is little evidence to directly implicate Smad4, although these studies do provide some support for an involvement of deletions of chromosome 18. The 12% losses of Smad4 in ovarian and breast cancer are perhaps misleading as this estimate of loss is derived from analysis of two cell lines, SW626 and MDA-MB468 respectively.

The relatively restricted tissue specificity of Smad4 inactivation in malignancy had suggested that the other MAD homologues may be targets of tumour suppression in specific tumour subsets. However, analysis of Smads 1, 3, 5 and 6 has revealed no mutations in a total of 167 tumours, including colon, lung, breast and pancreas (Riggins et al. 1997). Recently, 30 human-expressed sequence tags with homology to *Mad*, the *sma* genes of *C. elegans* and/or *Smad4* have been identified and five new genes termed *JV18-1*, *JV15-1*, *JV15-2*, *J5-1* and *JV-4* have been subsequently characterized (Riggins et al. 1996). One gene, *JV18-1*, was localized to 18q21, adjacent to *Smad4* and *DCC*, an area of frequent genetic loss in colorectal carcinoma. *JV18-1* is somatically mutated in 2 out of 18 colorectal carcinomas that had been selected on the basis of loss of heterozygosity of polymorphic markers on 18q (Riggins et al. 1996). Based on homology to other Smad genes, *JV18-1* has now been assigned the new nomenclature of Smad2 and is the second *Mad* homologue to be implicated in tumour suppression. The prevalence of *JV18-1/Smad2* mutations in other neoplasms is not well characterized to date, although there is evidence to suggest that Smad2 may be mutated in a subset of leukaemias and lymphomas (Ikezo et al. 1998) and also in lung carcinomas (approximately 4%) (Uchida et al. 1996). Disruption of the Smad2 gene during development results in a complete loss of embryonic germ-layer tissues (Waldrip et al. 1998), confirming that Smad2, like Smad4, has an essential role to play in development.

TGF- $\beta$  is well known for its antiproliferative activity in the majority of mammalian epithelial cells, and loss of TGF- $\beta$  responsiveness is documented to be associated with aggressive neoplasms (Pommier, 1992; Arteaga et al. 1993; Polyak, 1996). It has, therefore, been suggested that loss of components of the TGF- $\beta$  pathway or its related genes, such as Smad4, would be selected for in the clonal evolution of neoplasms. Significantly, two recent reports (Grau et al. 1997; Atfi et al. 1997) have linked Smad4 to other pathways including the SAPK/JNK cascade, implicating Smad4 in both the control of cell cycle arrest and apoptosis. This suggests that Smad4-dependent malignancy may arise after disruption of these key regulatory mechanisms.

In summary, Smad4 has been shown to be a critical effector of the TGF- $\beta$  response, a role apparently mediated through its control of other Smad genes. Smad4 appears to be the key regulatory protein of this signalling pathway, ultimately controlling transcription driven by the TGF- $\beta$  superfamily. Loss of Smad4 has been shown to be associated primarily with pancreatic malignancy, and to a lesser extent with colorectal cancer. Its involvement in other cancer types is currently either very limited or unproven. Characterization of the role played by Smad4 will throw light on the basic biology of pancreatic neoplasia and should also suggest new therapeutic approaches to this disease. Finally, a

determination of the role played by Smad4 in malignancy should provide an excellent paradigm for other components of this signalling pathway, perhaps leading to the identification of a family of genes with related TSG activity.

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