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Original Article

Developmental expression of Smad1-7 suggests critical function of TGF-β/BMP signaling in regulating epithelial-mesenchymal interaction during tooth morphogenesis

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ABSTRACT Members of the transforming growth factor-β family (e.g. TGF-β, BMP and activin) are critical regulators of tooth morphogenesis. The basic TGF-β signaling engine consists of a receptor complex that activates Smads and a Smad-containing complex that controls transcription of the downstream target genes. Little is known about the expression of endogenous Smads during tooth morphogenesis. Using a cRNA probe or antibody which specifically recognizes the expression of each Smad molecule, we provide a comprehensive endogenous Smad expression analysis during tooth morphogenesis. BMP signaling is transmitted through Smad1 and 5 which are first expressed within the dental lamina and later expand into condensed dental mesenchyme at the bud stage. As tooth development advances into the cap and bell stage, BMP signaling Smads are strongly localized within the inner enamel epithelium (IEE) and cranial neural crest derived dental mesenchyme (DM), indicating their critical role in regulating epithelial-mesenchyme interaction during tooth morphogenesis. Smad2 and 3 are responsible for transmitting TGF-β/activin signaling and show unique expression patterns during tooth morphogenesis. They are localized within the nuclei of both IEE and DM, suggesting that TGF-β-activated Smads are critical for regulating tooth development. Smad4, the common Smad, is expressed in both dental epithelium and mesenchyme throughout all stages of tooth morphogenesis. The expression of inhibitory Smads (Smad6 and 7) largely overlaps with receptor regulated Smads, indicating that negative feedback on BMP/TGF-β signaling is critical throughout all stages of tooth morphogenesis. Our results suggest that both receptor-regulated and inhibitory Smads are important regulators of tooth morphogenesis. The selective activation of Smad, as indicated by nuclear translocation, may suggest selective activation of different members of the TGF-β superfamily during tooth development.

KEY WORDS: *Tooth morphogenesis, Smad1-7, TGF-β, BMP*

Introduction

Members of the transforming growth factor-β superfamily mediate a wide range of biological activities, including cell proliferation, differentiation, extracellular matrix formation, and induction of homeobox genes, suggesting that TGF-β signaling is important in pattern formation and organogenesis during embryonic development. TGF-β superfamily includes the TGF-βs, activins and bone morphogenetic proteins (BMPs). Each ligand in the TGF-β family signals through the transmembrane serine-threonine kinase receptors. Specifically, binding of the ligand initiates the assembly of heteromeric complex of type II and type I receptors. Within the receptor complex, the type II receptor phosphorylates type I receptor. The activated type I receptor then phosphorylates the receptor-regulated Smad (R-Smad) and subsequently initiates the downstream Smad signaling pathway (Massague, 1998).

Smads function as signal transducers of TGF-β superfamily members in organisms ranging from worms to humans (Massague, 2000). Different members of the Smad family have distinct signaling functions. Smad1, 2, 3, 5 and 8 interact with and are phosphorylated by specific type I serine/threonine kinase receptors, and thereby act in a pathway-restricted manner. In particular, Smad2 and Smad3 are phosphorylated and translocated to the nucleus

Abbreviations used in this paper: BMP, bone morphogenetic protein; DM, dental mesenchyme; IEE, inner enamel epithelium; PS1, phosphorylated Smad1; PS2, phosphorylated Smad2; TGF, transforming growth factor.

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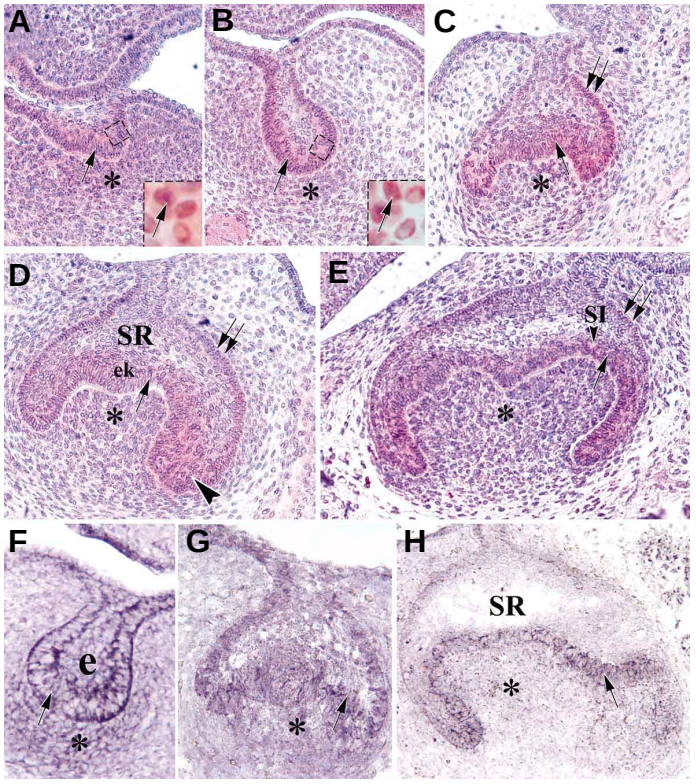


Fig. 1. Developmental expression of Smad1 during tooth morphogenesis *in vivo*. **(A)** *At the lamina stage (E12.5), Smad1 was present in dental lamina (arrow) and mesenchyme (*). Red is indicative of positive staining. Insert: positive staining of PS1 (phosphorylated Smad1) within dental epithelium.* **(B)** *At the bud stage (E13.5), Smad1 was localized within dental epithelium (arrow) and mesenchyme (*). Insert: positive PS1 staining.* **(C)** *At the cap stage (E14.5), Smad1 was localized to the inner (arrow) and outer (double arrow) enamel organ epithelium as well as dental mesenchyme (*).* **(D)** *At the early bell stage (E15.5), Smad1 was expressed mainly within the inner enamel organ epithelium, enamel knot (ek) and dental mesenchyme. SR, stellate reticulum.* **(E)** *At the late bell stage, Smad1 was specifically associated with the inner enamel organ epithelium (arrow), stratum intermedium (SI) and dental mesenchyme.* **(F-H)** In situ *hybridization revealed the expression of* Smad1 *mRNA from the bud to the late bell stage of tooth development. (F) E13.5, (G) E14.5, (H) E16.5. Positive signal is in dark purple. (*) dental mesenchyme. SR, stellate reticulum.*

after stimulation by TGF-β (Eppert *et al.,* 1996; Zhang *et al.,* 1996; Nakao *et al.,* 1997a) or activin (Chen *et al.,* 1996), whereas Smad1, 5 and 8 are activated following BMP stimulation (Hoodless *et al.,* 1996; Liu *et al.,* 1996; Kretzschmar *et al.,* 1997; Suzuki *et al.,* 1997; Nakayama *et al.,* 1998).

The action of Smad4 (common Smad, Co-Smad) differs from other members of the Smad family. After ligand stimulation and phosphorylation of pathway-restricted Smads, Smad4 forms hetero-oligomers with R-Smads. In mammalian cells, Smad4 forms complexes with Smad2 and Smad3 after activation by TGF-β or activin, whereas, it forms complexes with Smad1 and Smad5 after activation by BMP (Massague, 1998). The activated Smad complex will then move into the nucleus where it binds with transcriptional co-activators or co-repressors to regulate the TGF-β signaling target gene expression (Massague, 2000).

Smad6 and Smad7 diverge structurally from other members of the Smad family (Hayashi *et al.,* 1997; Imamura *et al.,* 1997; Topper *et al.,* 1997), they function as inhibitors of TGF-β, activin and BMP signaling. One of the mechanisms proposed to explain the inhibitory effects of Smad6 and Smad7 is that each of these Smads can bind to diverse TGF-β family receptors and interfere with phosphorylation of R-Smads. For instance, Smad7 associates stably with the TGF-β receptor complex and inhibits TGF-β-mediated phosphorylation of Smad2 and Smad3. Alternatively, another mechanism which may help to explain the selective inhibition of BMP signaling by Smad6 suggests that, at low concentration, Smad6 can compete with Smad4 for binding to activated Smad1 and block the BMP signaling pathway (Hata *et al.,* 1998). Because transcription of the inhibitory Smad gene is induced by stimulation of TGF-β (Nakao *et al.,* 1997b; von Gersdorff *et al.,* 2000; Ito *et al.,* 2001), inhibitory Smads may produce autoregulatory negative feedback in the signal transduction of the TGF-β superfamily.

TGF-β family members are expressed in a time- and tissue-specific manner and serve as morphogens for organogenesis during embryonic development. In particular, the presence of TGF-β subtypes is obvious throughout the critical stages of epithelial-mesenchymal interactions related to the formation of tooth germ (Heine *et al.,* 1987; Massague, 1990; Pelton *et al.,* 1990; Hall, 1992; Chai *et al.,* 1994). Functionally, for instance, TGF-β2 exerts a negative regulation on proliferation of enamel organ epithelial cells during early stages of tooth development while attenuation of TGF-β type II receptor signaling has revealed its function in regulating the size and stage of tooth development (Chai *et al.,* 1994, 1999). Until recently, however, regulation of intracellular TGF-β signaling during tooth morphogenesis has not been clearly defined.

Smad proteins of the TGF-β superfamily are widely expressed in embryonic and extraembryonic tissues during embryogenesis (Dick *et al.,* 1998; Flanders *et al.,* 2001). Targeted disruptions of *Smad* genes have revealed important biological functions of these intracellular signaling molecules. But because of the early embryonic lethality associated with the *Smad* null mutation, such as *Smad1*$^{-/-}$, *Smad2*$^{-/-}$, *Smad4*$^{-/-}$, or *Smad5*$^{-/-}$, it has been very difficult to investigate the biological function of TGF-β/activin/BMP signaling Smad during organogenesis (Weinstein *et al.,* 2000; Tremblay *et al.,* 2001). Recently, using an *in vitro* organ culture model, we have demonstrated that Smad2 and Smad7 are critical factors in orchestrating TGF-β-mediated tooth development. Different Smads may have differential activities in regulating TGF-β-mediated cell proliferation and apoptosis. And the effectiveness of TGF-β signaling is highly sensitive to the level of *Smad* gene expression (Ito *et al.,* 2001). Clearly, in order to understand how different members of TGF-β/activin/BMP signaling Smads are orchestrated, we need to examine the spatial and temporal distribution of Smads during tooth morphogenesis.

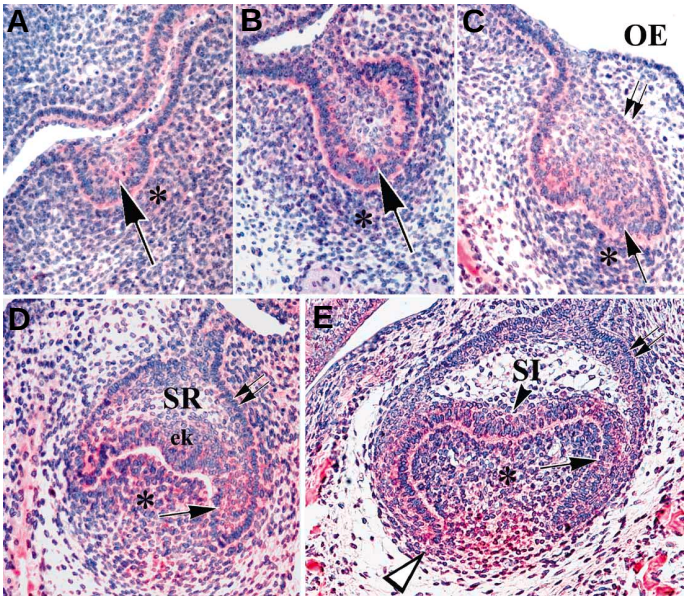
Here, we have investigated the expression pattern of Smad1, Smad2, Smad3, Smad4, Smad5, Smad6 and Smad7 to determine which members of the TGF-β superfamily are active during embryonic tooth morphogenesis. Additionally, anti-phosphorylated Smad1 or Smad2 antibody has identified the activated form of Smad, thus, suggesting its functional role in regulating tooth morphogenesis. Our study shows that there are unique patterns of expression of receptor-

regulated and inhibitory Smads while Smad4, the common Smad, is present in both dental epithelium and mesenchyme throughout different stages of tooth morphogenesis. This study is a critical step towards the understanding of how TGF-β superfamily signaling network is coordinated in regulating tooth morphogenesis.

Results

Developmental Expression of BMP Signaling Smads during Tooth Morphogenesis

BMP signaling is critical for the initiation and subsequent morphogenesis of tooth organ (Thesleff and Sharpe, 1997). BMP signals through its cognate receptors and downstream Smad1 and Smad5. To understand the biological significance of BMP signaling Smads during tooth formation we investigated spatial and temporal distribution of Smad1 and Smad5. At the initiation of tooth formation, Smad1 was detected mainly in the dental lamina (Fig. 1A, arrow) while there was faint Smad1 expression in the cranial neural crest (CNC)-derived dental mesenchyme (Fig. 1A). At the bud stage, Smad1 was localized to both dental epithelium and mesenchyme (Fig. 1 B,F). Significantly, phosphorylated Smad1 was localized within the nuclei of dental epithelial cells, indicating that activated Smad1 had translocated into the nucleus to regulate downstream target gene and it plays an important role in regulating early tooth development (Fig. 1 A,B, inserts). As tooth morphogenesis advanced into the cap stage, Smad1 was associated with inner and outer enamel organ epithelium and dental mesenchyme (Fig. 1 C,G). At the early bell stage, Smad1 positive staining began to shift towards both the inner enamel organ epithelium and dental mesenchyme, suggesting that it plays an important role in regulating the critical epithelial-mesenchymal interaction for the differentiation of ameloblast and odontoblasts, respectively. Noticeably, the enamel organ epithelium at the junction between inner and outer layers showed strong Smad1 staining (Fig. 1D, arrowhead),



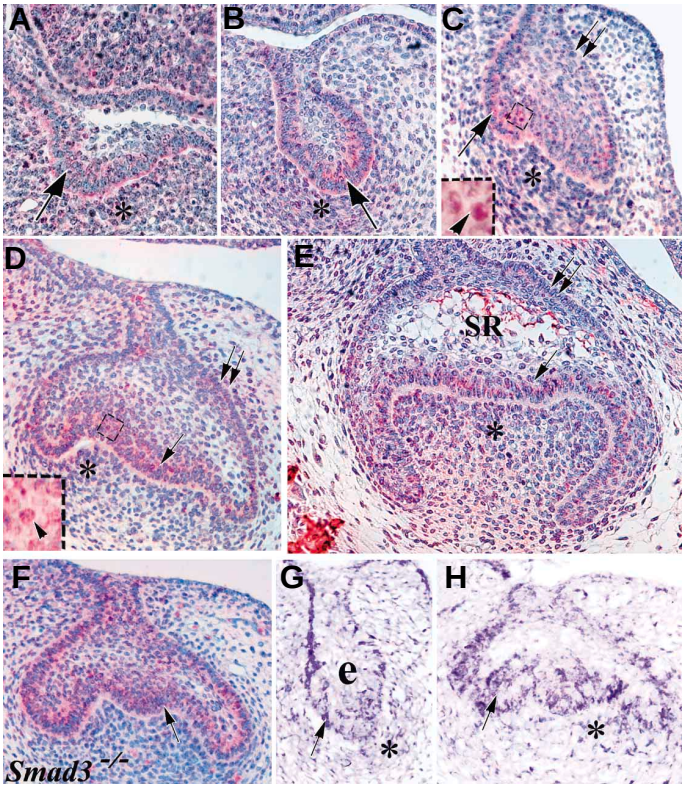


Fig. 2. (Left) Developmental expression of Smad5 during tooth morphogenesis *in vivo*. (A) *At the lamina stage, Smad5 was present in the dental lamina (arrow) and CNC-derived mesenchyme (*). Red is indicative of positive staining.* **(B)** *At the bud stage (E13.5), Smad5 was detected within dental epithelium. Odontogenic mesenchyme showed faint Smad5 expression.* **(C)** *At the cap stage (E14.5), Smad5 was strongly expressed in inner enamel organ epithelium (arrow) and was less prominent in dental mesenchyme (*). OE, oral epithelium.* **(D)** *At the early bell stage, Smad5 was mainly localized to inner enamel organ epithelium, including the enamel knot and CNC-derived dental mesenchyme.* **(E)** *At the late bell stage, Smad5 expression was restricted to the inner enamel organ epithelium, dental mesenchyme and follicle (open arrow). SR, stellate reticulum. SI, stratum intermedium.*

Fig. 3. (Right) Developmental expression of Smad2 during tooth morphogenesis *in vivo*. (A) *At the dental lamina stage, Smad2 was expressed within the dental epithelium (arrow) and mesenchyme (*).* **(B)** *At the bud stage, Smad2 was present within the dental epithelium and CNC-derived mesenchyme. Notice that majority of the staining was in the cytoplasm.* **(C)** *Using antibody to localize the expression of phosphorylated Smad2 (PS2) at the late bud stage, we showed that activated Smad2 was present within dental epithelium and mesenchyme. Insert = positive PS2 staining within dental epithelium (arrow points to positive nucleus staining with anti-PS2).* **(D)** *At the cap stage, Smad2 was associated with inner enamel organ epithelium and CNC-derived dental mesenchyme while outer enamel organ epithelium (double arrow) also showed some positive staining. Insert shows positive PS2 staining within dental epithelium.* **(E)** *At the bell stage, Smad2 was present within inner enamel organ epithelium, stellate reticulum (SR) and dental mesenchyme (*).* **(F)** *In* Smad3 *null mutant sample, Smad2 was detected at the identical location where endogenous Smad3 was expressed in this cap stage tooth organ (see Fig. 3D).* **(G,H)** In situ *hybridization demonstrated the spatial and temporal distribution of* Smad2 *mRNA during tooth morphogenesis. Positive staining is in deep purple. Specifically,* Smad2 *was localized to dental epithelium and mesenchyme at E13.5 (G). Later on,* Smad2 *was mainly localized to the inner enamel epithelium and dental mesenchyme at the cap stage (H).*

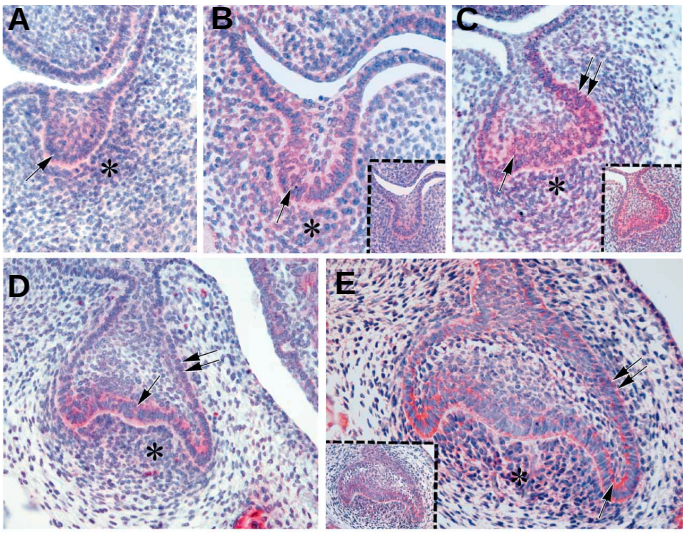


Fig. 4. (Left) Developmental expression of Smad3 during tooth morphogenesis *in vivo*. **(A)** *At the lamina stage, Smad3 was present within dental epithelium (arrow) and condensed mesenchyme (*). Pink-red denotes positive staining.* **(B)** *At the bud stage, Smad3 was localized to dental epithelium. Insert illustrates expression of Smad1 on the adjacent section.* **(C)** *At the late bud stage, both dental epithelium and CNC-derived mesenchyme showed positive Smad3 staining. Insert shows expression of Smad1 on the adjacent section.* **(D)** *At the cap stage, Smad3 was mainly localized within inner enamel organ epithelium (arrow) and dental mesenchyme (*). Outer enamel organ epithelium showed only faint Smad3 staining (double arrow).* **(E)** *At the bell stage, Smad3 was localized to the inner enamel organ epithelium and adjacent dental mesenchyme. Insert: expression of Smad1 on the adjacent section.*

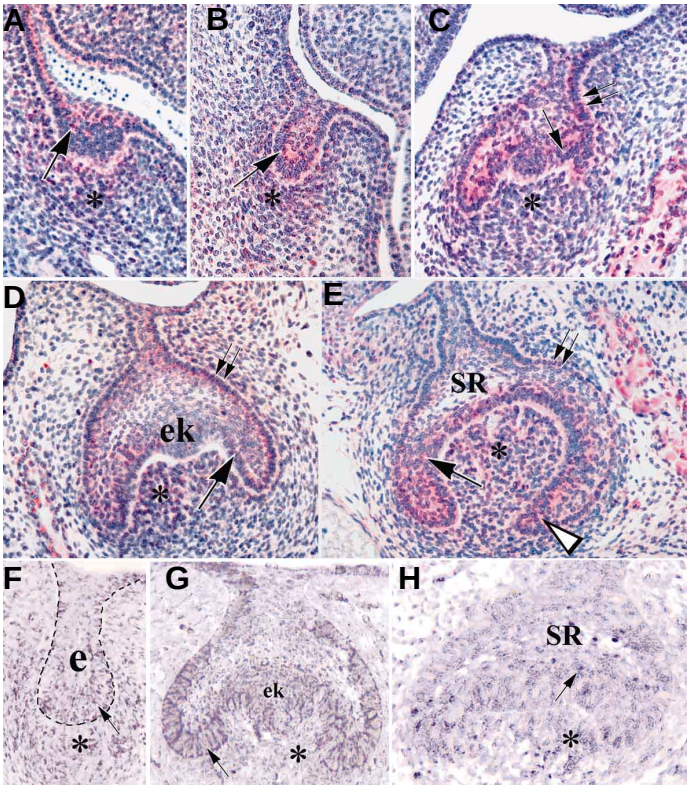


Fig. 5. (Right) Developmental expression of Smad4 during tooth morphogenesis *in vivo*. **(A)** *At the lamina stage, Smad4 was present in dental epithelium (arrow) and condensed mesenchyme (*). Red is indivative of positive staining.* **(B)** *At the bud stage, Smad4 was localized to the dental epithelium and mesenchyme.* **(C)** *At the cap stage, positive Smad4 staining was located within inner (arrow) and outer (double arrow) enamel organ epithelium and dental mesenchyme.* **(D)** *At the early bell stage, Smad4 was present in inner and outer enamel organ epithelium, enamel knot (ek) and CNC-derived dental papilla (*).* **(E)** *At the late bell stage, Smad4 expression was prominent at the inner enamel organ epithelium and its adjacent dental mesenchyme. Notice the strong staining at the folding between inner and outer enamel organ epithelium (open arrow). SR: stellate reticulum which showed faint Smad4 staining.* **(F-H)** In situ *hybridization revealed identical Smad4 expression pattern as the one shown by immunolocalization. (F) E13.5, (G) E14.5, (H) E15.5. Positive signal is dark blue.*

indicating the significant functional role of BMP signaling during the continued development of this portion of the epithelium which ultimately will lead to the formation of epithelial diaphragm to guide root development. The stellate reticulum showed faint staining of Smad1 (Fig. 1D). The enamel knot serves as a signaling center and guides future cusp development. Smad1 was localized in the enamel knot at the early bell stage (Fig. 1D), indicating an active involvement of BMP signaling. At the late bell stage, Smad1 was mainly localized to the inner enamel organ epithelium and stratum intermedium (Fig. 1 E,H), indicating the active role of BMP signaling during the differentiation of ameloblasts.

The spatial and temporal distribution of Smad5 was very similar to that of Smad1, indicating the concerted action of these two receptor-regulated Smads in transducing BMP signaling during tooth morphogenesis. Specifically, Smad5 was present in the dental lamina and CNC-derived mesenchyme at the initiation of tooth formation (Fig. 2A). At the bud stage, Smad5 was detected in dental epithelium but not in the CNC-derived dental mesenchyme (Fig. 2B). As tooth morphogenesis progresses into the cap stage, Smad5 was abundant in dental epithelium and less prominent in the CNC-derived dental mesenchyme while oral epithelium was free of Smad5 expression (Fig. 2C). In the early bell stage, Smad5 was localized mainly to the inner enamel organ epithelium, enamel knot and CNC-derived dental mesenchyme (Fig. 2D). In the late bell stage, Smad5 expression was further restricted to inner enamel organ epithelium, the junction of inner and outer enamel epithelium, CNC-derived dental mesenchyme and follicle (Fig. 2E), while outer enamel organ epithelium, oral epithelium and stellate reticulum remained to be free of Smad5 expression.

Developmental Expression of TGF-β/Activin Signaling Smads during Tooth Morphogenesis

Both TGF-β and activin are critical regulators during tooth morphogenesis. Although TGF-β or activin signals through its own type II and I receptors, it has been demonstrated that both of these signaling molecules rely on Smad2 and Smad3 as the intracellular signaling mediators (Massague, 1998). To understand the biological significance of TGF-β/activin signaling Smads during tooth formation we investigated spatial and temporal distribution of Smad2 and Smad3. At the initiation of tooth formation, dental

lamina was positive with Smad2 expression (Fig. 3A, arrow) while cranial neural crest (CNC)-derived dental mesenchyme also revealed positive Smad2 expression (Fig. 3A). As tooth development advanced into the bud stage, Smad2 was expressed within dental epithelium and CNC-derived dental mesenchyme (Fig. 3 B,G). Although Smad2 was present during the early stages of tooth development it was mainly localized with the cytoplasm of the dental epithelium. Using an antibody which recognizes the phosphorylated form of Smad2 we provide *in vivo* evidence that the activated form of Smad2 was first detected within inner enamel epithelium and CNC-derived dental mesenchyme at the late bud stage (Fig. 3C and insert), indicating an active role of Smad2 in mediating TGF-β/activin regulated tooth development. At the cap stage, Smad2 was mainly associated with inner enamel organ epithelium and dental mesenchyme (Fig. 3 D,H). At the bell stage, Smad2 was localized to inner enamel organ epithelium, stellate reticulum and dental mesenchyme, underlining the critical function of Smad2 in regulating TGF-β/activin-mediated terminal differentiation of ameloblasts and odontoblasts, respectively (Fig. 3E).

The expression of Smad3 was associated with enamel organ epithelium and CNC-derived dental mesenchyme throughout the different stages of tooth development. At the lamina stage, Smad3 was present within the dental lamina and condensed dental mesenchyme (Fig. 4A). As tooth development continues, Smad3 was mainly detected within the dental epithelium at the early bud stage (Fig. 4B) while it was present within both dental epithelium and CNC-derived dental mesenchyme at the late bud stage (Fig. 4C). At the cap stage, the presence of Smad3 was highly evident within the inner enamel organ epithelium and dental mesenchyme, suggesting a possible role in regulating epithelial-mesenchymal interaction during the advancement of tooth development (Fig. 4D). At the bell stage, Smad3 expression pattern remained similar to the one at the cap stage, demonstrating the continued functional involvement of Smad3 in mediating TGF-β/activin regulated tooth development (Fig. 4E).

The expression patterns of Smad2 and Smad3 largely overlap throughout embryogenesis (Flanders *et al.,* 2001). It has been shown that alternatively spliced Smad2 may function as Smad3 in transducing TGF-β signaling in *Smad3* null mutant mice, thus, compensating for the loss of Smad3 function (Yagi *et al.,* 1999). Here we examined the expression of Smad2 in *Smad3* null mutant sample and found that Smad2 was expressed in the location where endogenous Smad3 was supposed to be expressed, suggesting that it is indeed possible for alternatively spliced Smad2 to function as Smad3 *in vivo* (Fig. 3F). In addition, previous studies have also indicated that TGF-β and BMP signaling may exert antagonistic effects on the same cell during development. Here we have investigated the expression patterns of TGF-β and BMP signaling Smads (Smad1 and Smad3) on adjacent sections to test whether their expression patterns would overlap. Smad1 was localized to the identical location of Smad3 throughout the bud, the cap and the bell stages of tooth morphogenesis, suggesting a possible interaction between TGF-β and BMP signaling Smads (Fig. 4 B,C,E inserts).

Developmental Expression of Common Smad during Tooth Morphogenesis

Both TGF-β and BMP are critical regulators for tooth development. Although TGF-β and BMP signal through its own receptors and the specific receptor-regulated Smads, Smad4 functions as a common Smad which binds to receptor-regulated Smads and mediates both TGF-β and BMP signaling pathway during embryogenesis. Here, we show that Smad4 was widely expressed throughout all stages of tooth development. At the lamina stage, Smad4 was present in the dental epithelium and the adjacent dental mesenchyme (Fig. 5A). The expression of Smad4 was continuously detected throughout the dental epithelium and adjacent CNC-derived mesenchyme at the bud and cap stages (Fig. 5 B,C,F,G). At the early bell stage, inner and outer enamel organ epithelium, enamel knot and CNC-derived dental mesenchyme all showed Smad4 expression (Fig. 5D). And the expression pattern of Smad4 was similar at the late bell stage as compared to the one at the early bell stage, indicating the continued involvement of TGF-β/BMP signaling in regulating tooth development (Fig. 5 E,H).

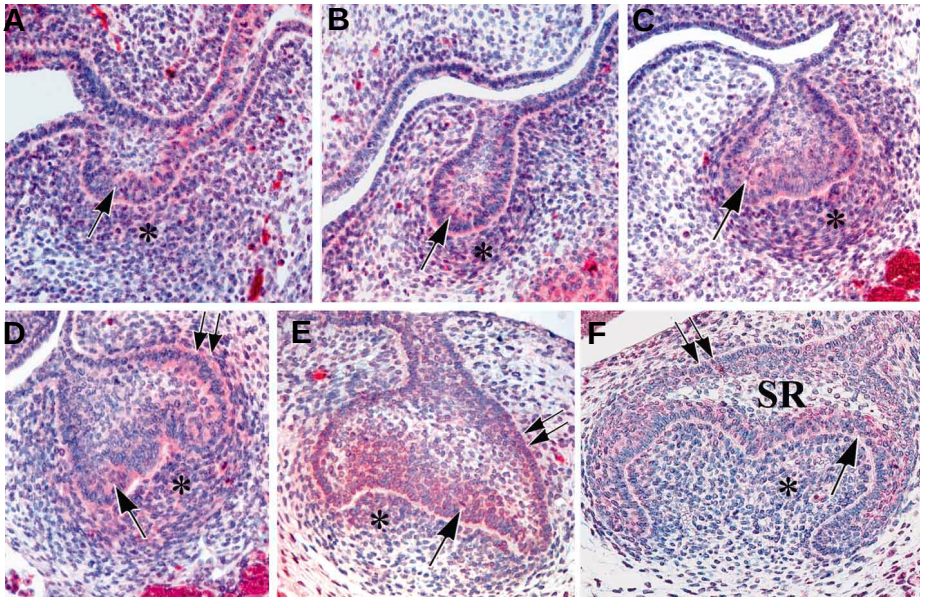


Fig. 6. Developmental expression of Smad6 during tooth morphogenesis *in vivo*. **(A)** *At the laminar stage, diffused Smad6 expression was evident in the dental lamina (arrow) and mesenchyme (*).* **(B)** *At the bud stage, Smad6 was present in dental epithelium and mesenchyme.* **(C)** *At the early cap stage, Smad6 was specifically expressed within enamel organ epithelium and CNC-derived dental mesenchyme.* **(D)** *At the late cap stage, Smad6 was present in both inner (arrow) and outer (double arrow) enamel organ epithelium as well as dental mesenchyme (*).* **(E,F)** *Smad6 expression had shifted to be predominately at the inner enamel organ epithelium and adjacent dental mesenchyme at the early and late bell stage, respectively. SR, stellate reticulum.*

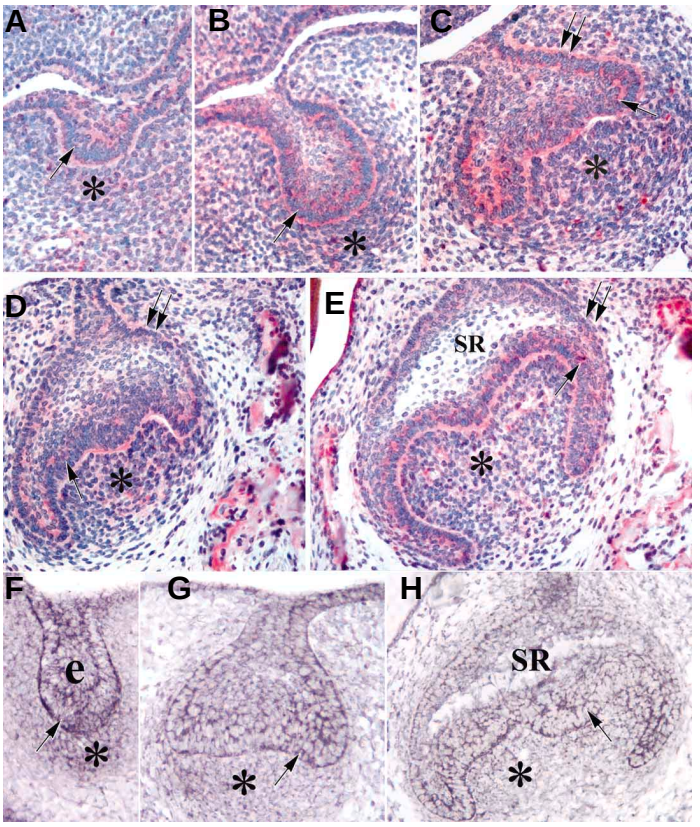


Fig. 7. Developmental expression of Smad7 during tooth morphogenesis *in vivo*. **(A)** *At the laminar stage, Smad7 was in both dental epithelium (arrow) and mesenchyme (*).* **(B)** *At the bud stage, strong Smad7 expression was evident in the dental epithelium and mesenchyme.* **(C)** *At the cap stage, Smad7 was localized to both enamel organ epithelium and dental mesenchyme.* **(D)** *At the late cap/early bell stage, Smad7 was present at the inner enamel organ epithelium (arrow) and adjacent dental mesenchyme (*). The outer enamel organ epithelium also showed positive Smad7 staining (double arrow).* **(E)** *At the late bell stage, Smad7 expression pattern remained similar to the early bell stage (D).* **(F-H)** In situ *hybridization revealed identical* Smad7 *expression patterns as shown by immunolocalization analysis. (F) E13.5, (G) E14.5, (H) E16.5.*

Developmental Expression of Inhibitory Smads during Tooth Morphogenesis

Inhibitory Smads disrupts TGF-β/BMP signal transduction by preventing phosphorylation of pathway-specific Smads. Smad6 is mainly responsible to inhibit BMP signaling while Smad7 is more involved in inhibiting TGF-β dependent signaling (Imamura *et al.,* 1997; Hata *et al.,* 1998; Massague 1998). Both Smad6 and Smad7 were expressed throughout all stages of tooth morphogenesis. Specifically, Smad6 and Smad7 were mainly present within the dental lamina at the initiation stage of tooth development (Figs. 6A, 7A). At the bud stage, Smad6 was present in the dental epithelium and mesenchyme (Fig. 6B), while Smad7 had identical expression pattern (Fig. 7 B,F). As tooth development reached the early cap stage, Smad6 was specifically expressed in enamel organ epithelium and dental mesenchyme, but it was not detected in the oral epithelium (Fig. 6C). Smad7 was widely expressed within the tooth germ at this stage, both in the dental epithelium and CNC-derived mesenchyme (Fig. 7 C,G). At the late cap and the bell stage, Smad6 expression had shifted its presence to be predominately at the inner enamel organ epithelium and CNC-derived dental mesenchyme (Fig. 6 D-F). The expression of Smad7 remained to be widely spread throughout both dental epithelium and mesenchyme at the late cap and the bell stage during tooth development (Fig. 7 D,E,H). Collectively, our data suggests that the negative feedback on TGF-β/BMP signaling is critical throughout all stages of tooth development.

Discussion

Members of the TGF-β family have been implicated in the control of cell proliferation, differentiation, apoptosis, extracellular matrix production, and organ morphogenesis (Massague, 1998). TGF-β signaling transduction molecules Smads are components of a critical intracellular pathway that transmits TGF-β signals from the cell surface to the nucleus. Significantly, Smad2, 3 and 7 have been demonstrated as important regulators for tooth morphogenesis (Ito *et al.,* 2001; Ferguson *et al.,* 2001). The present study seeks to elucidate the potential role of different Smads during tooth development. This comprehensive Smad expression analysis is to provide insight into the complexity of TGF-β superfamily signaling in regulating tooth morphogenesis.

BMP is expressed in a gradient in the first branchial arch oral ectoderm and is critical for proper initiation of tooth development. Subsequently, BMP functions to regulate the expression of transcription factors, such as members of the *Pitx* or *Msx* family, to mediate epithelial-mesenchymal interactions during tooth morphogenesis

TABLE 1

DEVELOPMENTAL EXPRESSION OF SMADS 1-7 DURING TOOTH MORPHOGENESIS

	Smad1			Smad2			Smad3			Smad4			Smad5			Smad6			Smad7		
	E	M	SR/I	E	M	SR/I	E	M	SR/I	E	M	SR/I	E	M	SR/I	E	M	SR/I	E	M	SR/I
E12.5 lamina	+++ N	+		++	+		+++	+		++	+		++	+		++	+		+++	+	
E13.5 bud	+++ N	+		++	+		+++	+		++	+		++	0		++	++		+++	+	
E14.5 cap	+++	+	SR	+++ N	+	SR	+++	+		+++ N	+	SR	+++	+	SR	++	++		+++	+	SR
E15.5 early bell	+++ ek	+	SR	+++ N	++		++	+	SR	+++ N	++	SR	++	+		++	++		++	+	
E17.5 late bell	+++ IEE	+	SI	++ IEE	+		++ IEE	+	SI	++ IEE	++	SI	++ IEE	+		++	+	SI	++ IEE	+	SI

E, dental epithelium; M, dental mesenchyme; SR, stellate reticulum/SI, stratum intermedium; IEE, inner enamel epithelium. 0, no detectable staining; +, 0-33% of cells stained; ++, 33-66% cells stained; +++, >66% cells stained; N, positive staining in the nuclei; ek, enamel knot.

(Tucker and Sharpe, 1999; Jernvall and Thesleff, 2000). Smad1, Smad5 and Smad8 are activated by BMP type I receptors and are critical for carrying out the regulatory function of BMP signaling (Hoodless *et al.,* 1996; Nishimura *et al.,* 1998; Kawai *et al.,* 2000; for review see Massague, 1998, 2000). Targeted null mutation of *Smad1* results in pronounced defects in the formation of extra-embryonic tissues while *Smad5* null mutation prevents the formation and patterning of the embryonic proper, demonstrating that both of these BMP signaling pathway-specific Smads are involved in early embryogenesis (Chang *et al.,* 1999; Tremblay *et al.,* 2001). To date, there has not been any functional analysis of BMP signaling Smads in regulating tooth morphogenesis. Our study shows that Smad1 is actively involved in regulating the dental lamina formation, as indicated by positive staining of phosphorylated Smad1 (activated) within dental epithelial cells. Smad1 is likely responsible for regulating the proliferation of dental epithelial cells during early tooth development. Later on, Smad1 is expressed mainly in the inner enamel organ epithelium and the adjacent dental mesenchyme, suggesting that Smad1 mediated BMP signaling is critical for epithelial-mesenchymal interaction during tooth morphogenesis. Smad5 shares similar expression pattern as the one for Smad1, suggesting that concerted action of Smad1 and Smad5 is important for transducing BMP signaling during tooth development.

Previous studies have indicated that TGF-β and its cognate receptors are important regulators during early tooth development (Pelton *et al.,* 1990; Heikinheimo *et al.,* 1993; Chai *et al.,* 1994, 1999). Specifically, endogenous TGF-β signals through its cognate receptors to exert negative control on proliferation of dental epithelium to regulate the overall growth during early tooth development. Until recently, however, the mechanism of negative regulation on proliferation of dental epithelium by TGF-β is not well understood. Studies of our group and others have shown that the expression of Smad2 and Smad3 are present in dental epithelium during early stages (the lamina and the bud stage) of tooth development (Dick *et al.,* 1998; Flanders *et al.,* 2001; Ito *et al.,* 2001). Significantly, using the PS2 antibody, we have demonstrated that phosphorylated Smad2 is present in the nuclei of inner enamel epithelial cells starting at the late bud stage, indicating the active role of Smad2 in regulating tooth development. Functional analysis demonstrates that the effectiveness of TGF-β signaling is highly sensitive to the level of Smad gene expression (Ito *et al.,* 2001). The spatial and temporal distribution of Smad2 and Smad3 match precisely with the distribution of TGF-β ligand and its cognate receptors during early tooth development, thus demonstrating the important regulatory function of these intracellular signaling molecules. The expression of Smad2 and Smad3 is also detected in the dental mesenchyme at the late bud and early cap stage, suggesting that both TGF-β and activin have active roles in regulating cranial neural crest derived cells as well as epithelial-mesenchymal interaction during tooth development. Interestingly, analysis of tooth development in *activin receptor II* and *Smad2* mutants show that incisors and mandibular molars fail to develop but maxillary molars develop normally, suggesting regional specific requirement for Smad2-mediated activin signaling in regulating tooth development (Ferguson *et al.,* 1998, 2001).

There is no tooth development defect in *Smad3* null mutant mice. Adult *Smad3* null mutant mice have impaired mucosal immunity and diminished T cell responsiveness to TGF-β as well as form metastatic colorectal cancer (Zhu *et al.,* 1998; Yang *et al.,* 1999). Because of the high homology between Smad3 and Smad2 (> 95%) it has been speculated that the shorter form of Smad2 (alternatively spliced Smad2 without exon 3) may function as Smad3 in transducing TGF-β signaling, thus, can compensate for the *Smad3* null mutation (Yagi *et al.,* 1999). Smad2 localization analysis showed expression of Smad2 at the exact location where endogenous Samd3 supposed to be expressed in *Smad3* null mutant embryo (Fig. 3F), further supporting the possible functional compensation of Smad3 by alternatively spliced Smad2 in regulating tooth morphogenesis. Recently, Smad3 has been shown to play a more important role in TGF-β mediated pathogenetic events and be less involved during embryonic development (Ashcroft *et al.,* 1999). Further studies are needed to investigate the possible functional uniqueness of Smad3 in regulating TGF-β signaling during tooth development.

Smad4 serves as the common mediator for Smad-dependent signaling for TGF-βs, BMPs and activins during embryogenesis. The expression of Smad4 is widely detected within dental epithelium and cranial neural crest-derived mesenchyme during tooth morphogenesis. This expression pattern suggests that Smad4 acts as a central mediator for transducing signaling initiated by members of TGF-β superfamily. Previous studies have suggested that TGF-β may antagonize the function of BMP signaling in regulating tooth development (Chai *et al.,* 1999; Ito *et al.,* 2001). Our TGF-β and BMP signaling Smads co-localization analysis provides supportive physical evidence for a possible interaction between these intracellular signaling mediators (Fig. 4 B,C,E and inserts). And Smad4 is strategically positioned to mediate the outcome of simultaneous TGF-β and BMP signaling during tooth development.

The expression of inhibitory Smad is evident during tooth development. For example, Smad7 expression overlaps with the expression patterns of Smad2 and Smad3, suggesting a negative feedback on TGF-β signaling. Functional analysis has shown that Smad7 may mediate TGF-β induced apoptosis within dental epithelium, while TGF-β receptor activated Smads are involved in mediating epithelial cell proliferation (Ito *et al.,* 2001). Smad7 can bind onto the GS domain on TGF-β type I receptor and prevent the phosphorylation of Smad2 upon activation by TGF-β ligand. Negative regulation by Smad7 also plays an important role in the restriction and termination of signaling. This negative feedback could be a critical limiting factor on the range of TGF-β signaling and form a gradient to precisely regulate TGF-β ligand activity during organogenesis.

In summary, endogenous members of TGF-β superfamily peptide signal through their cognate receptors and Smads to regulate downstream target gene expression and exert control on the fate of dental epithelium and cranial neural crest derived mesenchyme that gives rise to ameloblasts and odontoblasts, respectively. This study, along with our previous study on the regulatory function of TGF-β type II receptors, clearly demonstrates the multiple levels of TGF-β signaling regulation and begins to provide insights into the complexity of TGF-β superfamily signaling that is operative simultaneously throughout different stages of tooth development (see Table 1 for summary of Smads 1-7 expression throughout all stages of tooth morphogenesis). Meanwhile, increasing evidence suggests that signaling by members of the TGF-β superfamily is not exclusively Smad dependent (Hartsough and Mulder, 1995; Hartsough *et al.,* 1996; Frey and Mulder, 1997; Yu *et al.,* 2002). Future studies addressing functional significance of interaction between Smad-dependent and Smad-independent pathways will significantly advance our understanding of the TGF-β signaling mechanism in regulating tooth morphogenesis.

Materials and Methods

Preparation of Animal Tissues

Timed-pregnant Swiss-Webster mice were sacrificed between postcoital day 12.5 (E12.5) to E18.5 (E18.5). Mouse embryos were collected and staged according to the external developmental characteristics (somite pairs) as described by Theiler (1989). The tissue was then fixed with either 4% paraformaldehyde for *in situ* hybridization analysis or Carnoy's fixative solution (10% Glacial Acetic Acid; 30% Chloroform; 60% Absolute Ethyl Alcohol, w/v) for immunohistochemistry. Paraffin blocks containing processed mouse tissue were sectioned coronally for Smad localization analysis.

In-Situ *Hybridization*

A 256 bp fragment of murine Smad2 cDNA subcloned into pBluescript II SK was digested with *Not* I and transcribed with *T7* RNA polymerase (Boehringer Mannheim) for an antisense probe. For a Smad2 sense probe, pBluescript II SK was cut with *Eco*RI and transcribed with *T3* polymerase. A 602 bp fragment of murine Smad7 cDNA subcloned into pCR2.1 was digested with *Hind* III and transcribed with *T7* polymerase for an antisense probe. Smad1, Smad4 or Smad7 cDNA was subcloned into pBluescript II SK. Antisense and sense probes were generated according to standard procedures. The RNA probes were labeled with digoxigenin labeling kit (Boehringer Mannheim).

Immunohistochemistry

Paraffin blocks containing E12.5 to E18.5 mouse tissue were sectioned (5 μm in thickness) and mounted onto slides coated with histostick (Accurate Chemical & Scientific Co., Westbury, NY). The slides were heated in a 60°C oven for 45 min and subsequently hydrated to water through a series of decreasing concentrations of ethanol. The immunohistochemical staining was performed by using the Zymed HistoStain SP kit (zymed.com). We have provided the detailed information (in parenthesis) on the concentration of primary antibody used in the study; commercial company or institution where the antibody was obtained; and published literature verifying the specificity of the antibody. The following is a list of primary antibodies used for this study: anti-Smad1 (0.2 μg/μl; Zymed laboratories, cat. # 51-1200, San Francisco, CA; Flanders *et al.,* 2001), anti-Smad2 (0.2 μg/μl; Santa Cruz Biotechnology, cat # sc-6200, Santa Cruz, CA; Flanders *et al.,* 2001), anti-Smad3 (1 μg/μl; Upstate Biotechnology, cat # 06-920, Lake Placid, NY; Ito *et al.,* 2001), anti-Smad4 (0.25 μg/μl; Transduction laboratories, cat # S71120, Lexington, KY; Ito *et al.,* 2001), anti-Smad5 (0.5 μg/μl; Zymed laboratories, cat # 51-3700), anti-Smad6 (0.2 μg/μl; Zymed laboratories, cat # 51-0900; Flanders *et al.,* 2001), anti-Smad7 (0.2 μg/μl; Dr. S. Souchelnytskyi, Ludwig Institute for Cancer Research; Ito *et al.,* 2001), anti-phosphorylated Smad1 (anti-PS1) (0.2 μg/μl; Dr. S. Souchelnytskyi; Nakao *et al.,* 1999), anti-PS2 (0.2 μg/μl; Dr. C-H. Heldin, Ludwig Institute for Cancer Research; Nakao *et al.,* 1999; Ito *et al.,* 2001). Adult mouse lung was used as positive control (Zhao *et al.,* 2000). Positive staining was indicated by orange-red coloration. The slides were counter-stained with hematoxylin to show histological details.

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