Temporal and spatial expression of TGF-β 2 in tooth crown development in mouse first lower molar

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Transforming Growth Factor β2 (TGF-β2) is involved in the regulation of many important cellular processes during tooth development. In this study we systematically characterized the expression pattern of TGF-β2 *in vivo* and further analyzed its possible roles during different developmental stages of mouse first lower molar using immunofluorescence histochemical method with confocal microscopy. TGF-β2 signaling was detected in different developing stages in both dental epithelium and surrounding dental mesenchyme. For the first time, we found that the basement membrane and epithelial cells in the basal layer showed no immunostaining from embryonic day 11 to 13; the primary enamel knot and secondary enamel knot exhibited pronounced immunostaining with different expression patterns at embryonic day 14 and 16. In addition, the mature ameloblast lost immunoreactivity, but the secretory ameloblast still exhibited positive immunoreaction at day 2 of postnatal development. Collectively, the temporospatial distribution patterns of TGF-β2, especially in the basement membrane, epithelial cells in the basal layer, enamel knot, mature odontoblast and ameloblast, suggested a close association between TGF-β2 signaling and tooth crown development, and indicated that TGF-β2 might participate in tooth initiation, epithelial morphogenesis, formation of dentine matrix, and ameloblast differentiation.

Key words: transforming growth factor β2, tooth development, molar, immunofluorescence histochemistry.

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Transforming growth factor βs (TGF-βs) belong to a class of multifunctional growth factors that control several biological functions *in vivo*, such as cell growth, differentiation, migration, motility, adhesion, apoptosis, and extracellular matrix synthesis and degradation, and play a critical function in regulating tissue repair and regeneration process (Roberts *et al.*, 1987; Sporn *et al.*, 1987; Massague, 1990; Nilsen-Hamilton, 1990; Kingsley, 1994; Lawrence, 1996). TGF-βs are expressed during tooth development (Cam *et al.*, 1990; Vaahtokari *et al.*, 1991; Thesleff *et al.*, 1992; Heikinheimo *et al.*, 1993; Begue-Kirn *et al.*, 1994; Chai *et al.*, 1999; Sassa *et al.*, 2007) and involved in many cellular activities as a pivotal role (Thesleff *et al.*, 1992; Heikinheimo *et al.*, 1993; Chai *et al.*, 1994; Dobie *et al.*, 2002; Huojia *et al.*, 2005). The temporally distinct expression patterns of TGF-βs family members during tooth formation may be related to their specific cellular functions at distinct stages of development (Heikinheimo *et al.*, 1993; Nakashima *et al.*, 1994; Toyono *et al.*, 1997).

TGF-β2 is an isoform of TGF-βs polypeptides sharing structural and functional characteristics. It plays a critical role in regulating tooth morphogenesis, including inhibiting enamel organ epithelial and ectomesnechymal cell proliferation and differentiation (Chai *et al.*, 1994). Studies on TGF-β2-overexpressing transgenic mice showed that TGF-β2 stimulates odontoblast differentiation in maturing dentine to increase the dentine mineral apposition rate, showing that TGF-β2 is important to stimulate the formation of functional dentine (DenBesten *et al.*, 2001). In addition, TGF-β2 has also been shown to regulate epithelial differentiation of the developing teeth and odontogenic tumors (Heikinheimo *et al.*, 1993).

Previous investigations, on immunohistochemical localization of TGF-β2, were limited to several selected stages of tooth development (Heikinheimo *et al.*, 1993; Chai *et al.*, 1994; Chai *et al.*, 1999).

However, no studies thus far have systematically characterized the expression patterns of TGF-β2 *in vivo* during all stages of tooth crown development, from the initiation stage to the apposition stage. To obtain more information on the role of TGF-β2 during tooth crown development, we used mouse first lower molar as a model and comprehensively investigated the temporal and spatial patterns of TGF-β2 protein expression in various stages of the developing mouse first lower molar.

Materials and Methods

Tissue preparation

ICR female mice were mated overnight and the detection of the vaginal plug was considered as embryonic day 0 (E0) (Vital River Laboratory Animal Technology Co., Ltd, Beijing, China). The embryos were harvested from E11 to E18 (n=102, 10-14 embryos per each time point). ICR mouse pups at day 0, 1 and 2 (D0, D1 and D2) of postnatal development were obtained (n=39, 10-14 pups per each time point). All experimental protocols were designed in compliance with the recommendation of the Beijing Experimental Animal regulation board (SYXK/JING/2005/0031). Heads were fixed in 4% paraformaldehyde (pH 7.2-7.4) for 24 hours at 4°C. Tissues from E18, D0, D1 and D2 were decalcified in 10% ethylenediamine tetraacetic acid (EDTA) for 24 hours. After dehydration through a graded series of ethanol solutions, tissues were embedded in paraffin. Coronal sections (5 μm) were cut and mounted on poly-L-lysine coated slides.

Histological staining and identification

Representative sections from each age group were stained with Mallory's phosphotungstic acid-hematoxilin (PTAH) stain to identify the various stages of odontogenesis.

Immunofluorescence histochemistry analysis

After paraffin removal in xylene and descending ethanol series, enzymatic antigen retrieval was performed by boiling the tissue sections in 10mM Sodium Citrate buffer (PH 6.0) for 10 min. After washing with phosphate buffered saline (PBS) (PH 7.4), sections were incubated with 1% (w/v) bovine serum albumin (BSA)/PBS for 20 min to block non-specific sites. All antibodies were diluted in PBS. Anti-TGF-β2 primary antibody (1:25, Abcam, Cambridge, UK) was applied overnight at 4°C, followed by Alexa Fluor 488-conjugated secondary antibody (1:200, Invitrogen, CA, USA) at room temperature (RT) for 2 hours. After repeated washing, sections were counterstained with Hochest 33342 (1:5000, Invitrogen, CA, USA) at RT for 5 min. Primary antibody was replaced by purified IgG (1:25, Invitrogen, CA, USA) in the negative control. The images were acquired by means of Olympus BX-51 microscope connected to an Olympus DP70 cooled digital color camera (Olympus, Japan) and Leica TCS IRE2, SP2/AOBS confocal microscope (Leica, Germany).

Determination of specificity of anti-TGF-β2 antibody

To test the specificity of anti-TGF-β2 antibody, western blot analysis was performed. Recombinant human TGF-β1, TGF-β2 and TGF-β3 proteins (Sigma, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) with a final concentration of 40 μg/mL. Protein samples were electrophoretically resolved using a 4-12% sodium dodecyl sulfate-polyacrylamide gradient gel (Invitrogen, CA, USA) and transferred to Immobilon-P membrane (Millipore, MA, USA). The membranes were incubated in blocking buffer overnight at 4°C, followed by anti-TGF-β2 primary antibody (1:1000, Abcam, Cambridge, UK) for 1 hour and a horseradish peroxidase-conjugated secondary antibody (1:5000, Cell Signaling, MA, USA) for 1 hour at RT. Proteins were visualized by enhanced chemiluminescence. By immunoblotting with anti-TGF-β2 antibody, an immunopositive band was detected in TGF-β2 protein sample at about 25.0 kDa; no band was detectable when TGF-β1 and TGF-β3 protein samples were examined (*not show*), indicating that the anti-TGF-β2 antibody does not identify other TGF-β isoforms.

Results

After Alexa Fluor 488-conjugated secondary antibody specifically combined with the primary antibody, all the locations for TGF-β2 protein expression showed green spots when excitation was performed at 490 nm under microscope. At the initiation stage (E11, E12) of the first lower molar, oral epithelium progressively increased in thickness

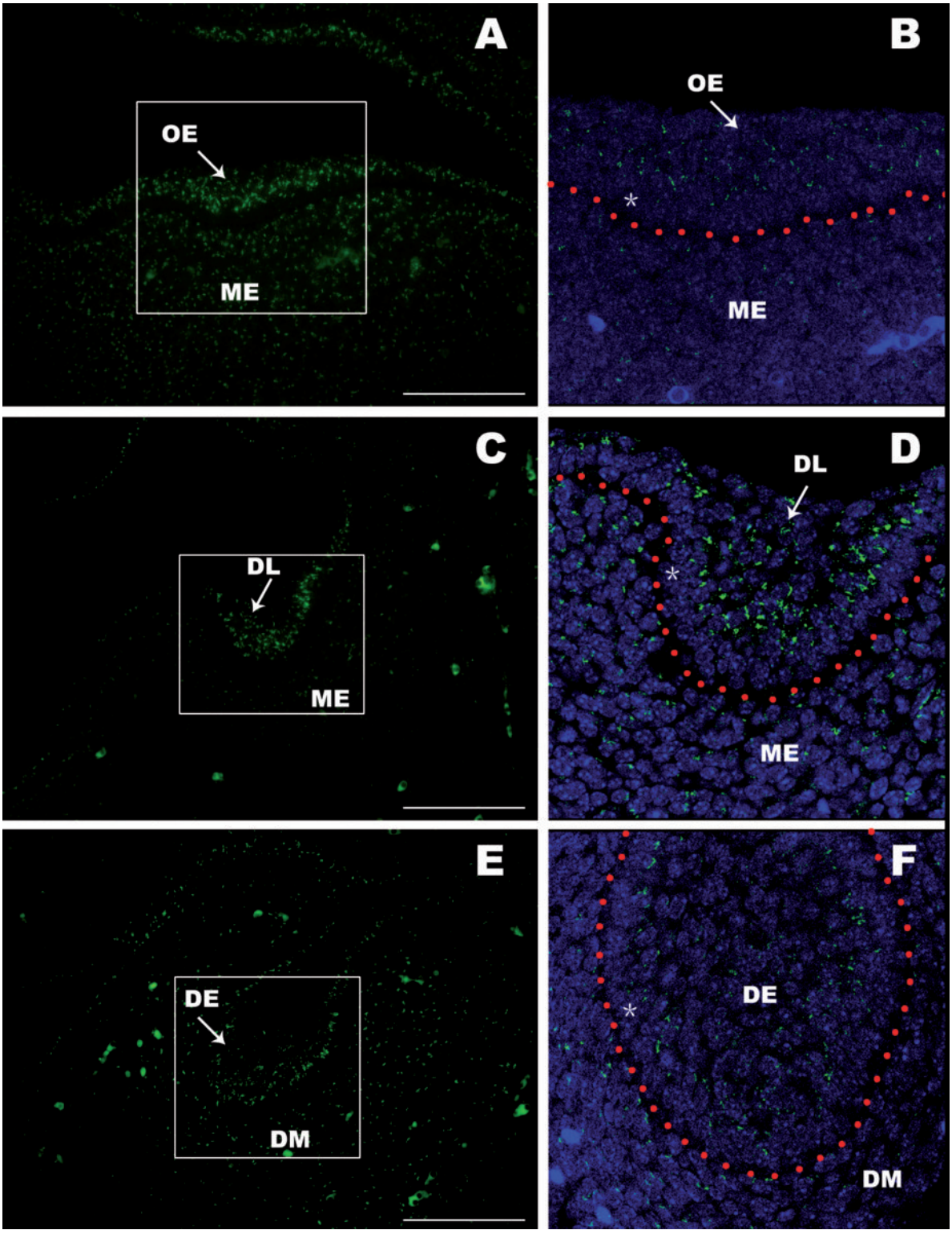

Figure 1. TGF-β2 expression patterns in the initiation stage and bud stage of ICR mouse developing first lower molar. (A, B) At E11, TGF-β2 was present within both the thickening epithelium and surrounding mesenchyme, but absent in the BM (red spot line) and epithelial cells in the basal layer (⁎). Boxed area in A is enlarged shown as B by confocal microscopy. (C, D) At E12, TGF-β2 was localized within the invaginating epithelium and surrounding mesenchyme, but the BM (red spot line) and epithelial cells in the basal layer (⁎) were negative for TGF-β2 staining. Boxed area in C is enlarged shown as D by confocal microscopy. (E, F) At E13, TGF-β2 was localized within the dental epithelium and surrounding mesenchyme, but absent in the BM (red spot line) and epithelial cells in the basal layer (⁎). Boxed area in E is enlarged shown as F by confocal microscopy. A, C, E were obtained under Olympus BX-51 microscope. B, D, F were acquired under Leica TCS IRE2, SP2/AOBS confocal microscope. DE: dental epithelium; DL: dental lamina; DM: dental mesenchyme; ME: mesenchyme; OE: oral epithelium. Bar= 50 μm.

and formed dental lamina. There was diffuse staining of TGF-β2 within both thickening epithelium and surrounding mesenchyme. Intense immunostaining was more evident in the oral epithelium (OE) (Figure 1 A, D). In contrast, the basement membrane (BM) and epithelial cells in the basal layer showed no immunostaining (Figure 1 B, D). Later in development, bud-shaped dental epithelium and adjacent dental mesenchyme maintained positive expression for TGF-β2 at the bud stage (E13). At the tip of dental epithelium, where enamel knot (EK) started to appear, we identified pronounced staining for TGF-β2 (Figure 1 E). However, both the BM and dental epithelial cells in the basal layer were negative (Figure 1 F).

At the early cap stage (E14), tooth germ included enamel organ and dental mesenchyme. TGF-β2 was abundant in the outer dental epithelium (ODE), inner dental epithelium (IDE) and primary enamel knot (PEK), while less prominent signals

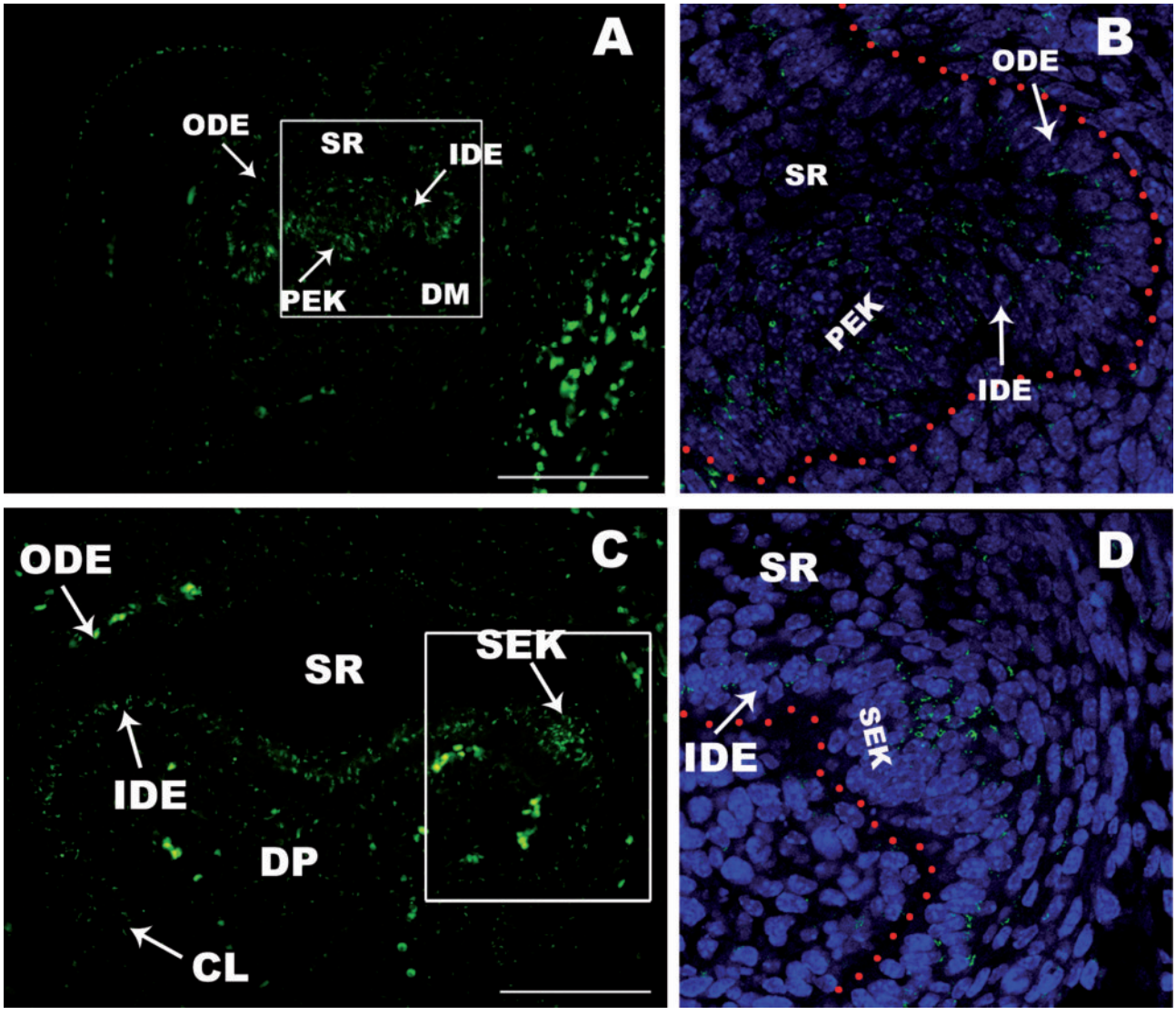

Figure 2. Different expression patterns of TGF-β2 between PEK and SEK in ICR mouse developing first lower molar. (A, B) At E14, TGF-β2 was mainly localized in the ODE, IDE and PEK. In PEK, cells adjacent to and away from the BM (red spot line) all showed intense immunostaining for TGF-β2. Boxed area in A is enlarged shown as B by confocal microscopy. (C, D) At E16, TGF-β2 was mainly localized to the cervical loop, IDE, SEK and adjacent dental mesenchymal cells. However, only cells away from the BM (red spot line) showed positive TGF-β2 staining in SEK. Boxed area in C is enlarged shown as D by confocal microscopy. A, C were acquired under Olympus BX-51 microscope. B, D were acquired under Leica TCS IRE2, SP2/AOBS confocal microscope. CL: cervical loop; DM: dental mesenchyme; DP: dental papilla; IDE: inner dental epithelium; ODE: outer dental epithelium; PEK: primary enamel knot; SEK: secondary enamel knot; SR: stellate reticulum. Bar = 50 μm.

were observed in the stellate reticulum (SR) and dental mesenchyme (Figure 2 A). In PEK, cells adjacent to and away from the BM were all positive for TGF-β2 expression, while the BM remained to be free of TGF-β2 expression (Figure 2 B). At E15, dental mesenchyme was divided into dental papilla and dental follicle by cervical loop. TGF-β2 was localized mainly in the ODE, cervical loop, IDE and dental mesenchymal cells adjacent to IDE (*data not shown*).

At the early bell stage (E16), PEK disappeared and secondary enamel knot (SEK) appeared. Strong staining of TGF-β2 was mainly localized to the cervical loop, IDE, SEK and adjacent dental mesenchymal cells (Figure 2 C). Interestingly, only cells away from the BM showed immunostaining for TGF-β2 in SEK (Figure 2 D). Weak immunostaining of TGF-β2 was detected in the ODE and SR (Figure 2 C). The BM remained negative (Figure 2 D). At the late bell stage (E18), dental papilla cells adjacent to the dental cusp differentiated into odontoblasts, which began to secrete dentine matrix at the tip of cusp. IDE cells began to polarize to form preameloblasts. High levels of TGF-β2 were found in the odontoblasts as well as dental papilla adjacent to the dental cusp. TGF-β2 signal was observed at low levels in the preameloblasts and preodontoblasts (Figures 3 A, B). At D0, preameloblast differentiated into ameloblast. The odontoblasts and adjacent ectomensenchymal cells in the coronal part of dental papilla continued to be strongly positive for TGF-β2, and the immunoreaction of TGF-β2 in the ameloblasts gradually increased (Figures 3 C, D). As the apposition stage proceeded, dentine matrix at the tip of cusp calcified into dentine and enamel matrix was secreted (D1).

More pronounced immunostaining of TGF-β2 was found in the ameloblasts. The odontoblasts and dental papilla adjacent to the dental cusp remained strongly positive (Figures 3E, F). While enamel matrix at the tip of dental cusp calcified into enamel (D2), the mature ameloblasts here had lost immunoreactivity. The secretory ameloblasts still exhibited positive immunoreaction at this stage (Figures 4 A-C). During the apposition stage, we found that the immunoreaction of TGF-β2 was present in the enamel matrix and dentin-enamel junction (DEJ), and in the same locations in the negative control as well (*not shown*), suggesting that it is a non-specific reaction.

Discussion

The present study revealed the temporospatial distribution patterns of TGF-β2 during tooth crown development. Localization is most intense at the stages of tooth development associated with critical events in histogenesis, morphogenesis, and cytodifferentiation. At the initiation stages (E11, E12) and bud stage (E13) of the first lower molar, we found that TGF-β2 was expressed in both thickening dental lamina and surrounding mesenchyme (Figures 1 A, C, E), but was negative in the BM and epithelial cells in the basal layer (Figures 1 B, D, F). However, the *in vitro* results from other groups (Chai *et al.*, 1994; Chai *et al.*, 1999) showed that TGF-β2 was present in the dental lamina epithelium, BM and adjacent dental mesenchyme. We speculate that maybe the slower development *in vitro* than *in vivo* results in the different expression patterns of TGF-β2 between *in vivo* and *in vitro*. Because TGF-β2 can inhibit the proliferation of ectodermally derived epithelium cells (Sporn *et al.*, 1987; Massague, 1990; Nilsen-Hamilton, 1990; Chai *et al.*, 1994) and dental epithelial cells in the basal layer exhibit the high proliferation rate during early tooth development (Vaahtokari *et al.*, 1991), it is reasonable to explain the negative expression of TGF-β2 in the BM and epithelial cells in the basal layer. Taken together, these data demonstrate that TGF-β2 is probably involved in tooth initiation. Tooth morphogenesis is regulated by reciprocal epithelial-mesenchymal interactions (Kollar *et al.*, 1970; Slavkin, 1974; Thesleff *et al.*, 1981; Ruch *et al.*, 1983). Enamel knot, a transient epithelial structure, acts as a signaling center, which provides positional information for tooth morphogenesis and regulates the growth of tooth cusp (Vaahtokari *et al.*, 1996; Thesleff *et al.*, 2001). Our immunolocalization studies by confocal microscopy clearly showed that cells in PEK and SEK were all positive for TGF-β2 polypeptide (Figures 2 B, D). However, we observed some differences between PEK and SEK in the immunoreactive locations of TGF-β2. In PEK, TGF-β2 was expressed in cells adjacent to and away from the BM (Figure 2 B). In contrast, in SEK, only cells away from the BM exhibited the immunostaining for TGF-β2 (Figure 2 D), suggesting that TGF-β2 may play a different role in PEK and SEK cells. During the transition from the cap stage to the bell stage, a localized remodeling of BM and folding of IDE initiate in the region where

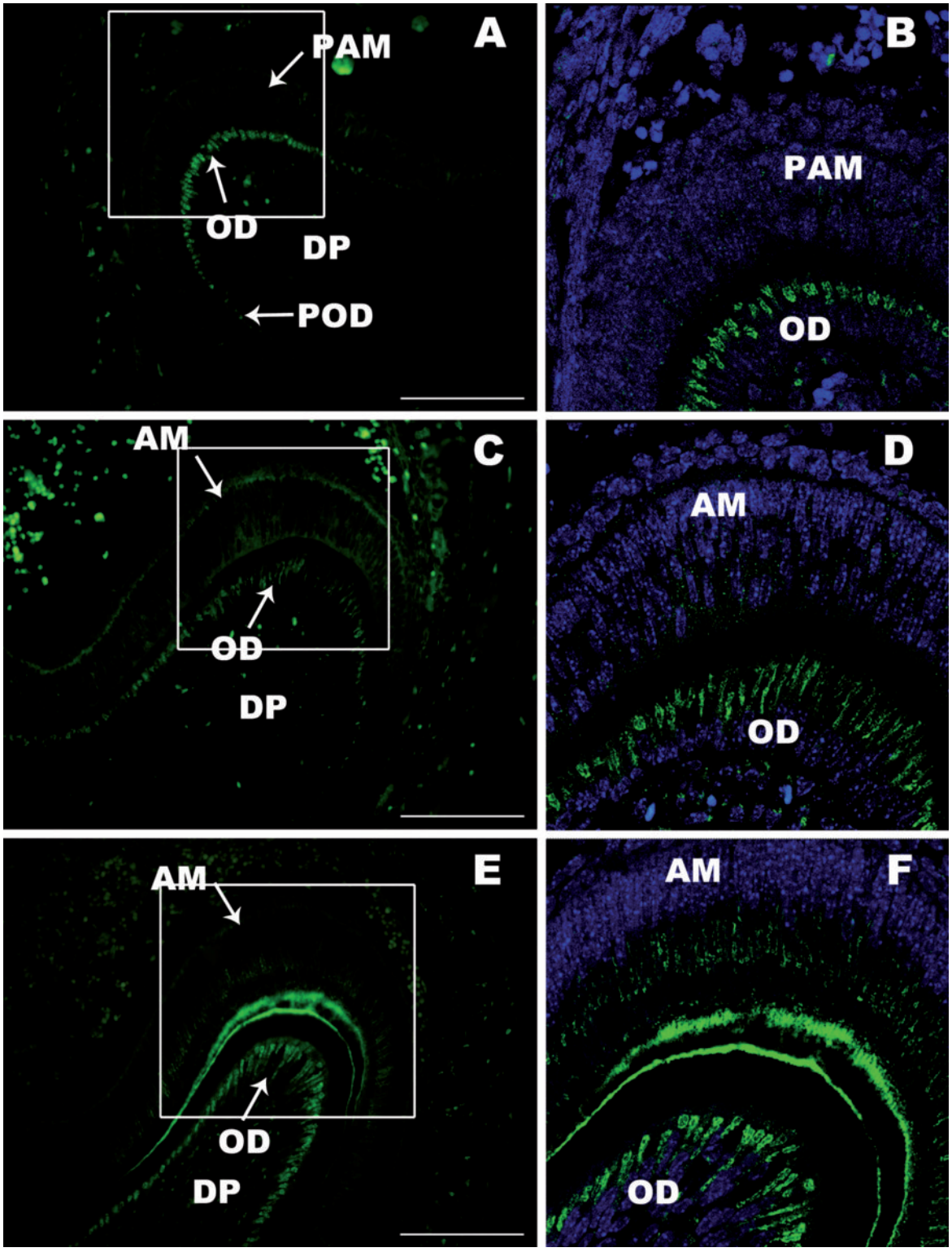

Figure 3. TGF-β2 expression patterns in the late bell stage and apposition stage of ICR mouse developing first lower molar. (A, B) At E18, TGF-β2 was present in the odontoblasts, dental mesenchyme adjacent to dental cusp, preodontoblasts and preameloblasts, but the odontoblasts and dental mesenchyme adjacent to dental cusp exhibited intense immunostaining for TGF-β2, and faint staining was seen in the preameloblasts. Boxed area in A is enlarged shown as B by confocal microscopy. (C, D) At D0, TGF-β2 expression patterns remained similar to E18. However, TGF-β2 staining in the ameloblasts gradually increased. Boxed area in C is enlarged shown as D by confocal microscopy. (E, F) At D1, TGF-β2 expression patterns remained similar to D0, but the ameloblasts and odontoblasts showed more pronounced immunostaining for TGF-β2. Boxed area in E is enlarged shown as F by confocal microscopy. A, C, E were acquired under Olympus BX-51 microscope. B, D, F were acquired under Leica TCS IRE2, SP2/AOBS confocal microscope. AM: ameloblast; DP: dental papilla; OD: odontoblast; PAM: preameloblast; POD: preodontoblast. Bar = 50 μm.

BM is in contact with EK cells, which appears as a major change associated with the initial cusp formation that precedes the formation of SEK (Lesot *et al.*, 1999). At the region of IDE folding, the motility of epithelial cells increases (Lesot *et al.*, 1999). Based on the different expression patterns of TGF-β2 between PEK and SEK, TGF-β2 may be involved in epithelial morphogenesis during the early tooth development. In addition, along with the observation that TGF-β2 can inhibit the proliferation of ectodermally derived epithelium cells (Sporn *et al.*, 1987; Massague, 1990; Nilsen-Hamilton, 1990; Chai *et al.*, 1994), the positive immunoreaction of TGF-β2 in EK supports the findings that the most EK cells neither divide nor incorporate BrdU (Vaahtokari *et al.*, 1991; Vaahtokari *et al.*, 1996; Coin *et al.*, 1999).

During the late bell stage and apposition stage, a complex series of epithelial-mesenchymal interactions result in the differentiation of odontoblast and ameloblast, and the secretion of dentine and enamel matrix (Kollar *et al.*, 1979; Ruch, 1985). Our findings showed that odontoblasts exhibited a marked positive reaction for TGF-β2 antibody, with positive staining in the cytoplasm of odontoblasts (Figures 3 B, D, F). Pelton *et al.* and Heikinheimo *et al.* observed a strong TGF-β2 mRNA hybridization signal in odontoblast during the late bell stage (Pelton *et al.*, 1990; Heikinheimo *et al.*, 1993). Therefore, this suggests that TGF-β2 is synthesized and secreted by odontoblast during the late bell stage. Since TGF-β2 can induce the expression of Collagen type I (Fuchshofer *et al.*, 2005), which is the main component of dentine, our data further support the involvement of TGF-β2 in the formation of dentine matrix. Additionally, studies in TGF-β2-overexpressing transgenic mice showed that TGF-β2 stimulates odontoblast differentiation in maturing dentine to increase the dentine mineral apposition rate, indicating that TGF-β2 may be critical to stimulate the formation of functional dentine (DenBesten *et al.*, 2001). From the late bell stage to the apposition stage, we found that, following the gradually differentiated odontoblast and intensified expression of TGF-β2 in the odontoblast, preameloblast differentiated into ameloblast and the immunoreaction of TGF-β2 in the preameloblast and ameloblast gradually increased (Figures 3 B, D, F). Interestingly, the mature ameloblast had lost immunoreactivity as enamel matrix at the tip of dental cusp calcified into enamel (Figure 4 B), but the secretory ameloblast still remained positive (Figure 4 C). Saeki and DenBesten analyzed TGF-β2 overexpressing transgenic mouse and found incomplete enamel formation, suggesting that the defects in enamel result from ameloblast changes in response of underlying dentin to TGF-β2 overexpression (Saeki *et al.*, 2007). An inhibition of preameloblast proliferation or enhanced differentiation relative to exposure to higher levels of TGF-β2 is consistent with the known effects of TGF-β2 on epithelial cells (Derynck, 1994). On the basis of the above analysis, we speculate that TGF-β2 derived from odontoblast may be involved in ameloblast differentiation, demonstrating that TGF-β2 plays a potential role in epithelial-mesenchymal interactions during tooth development.

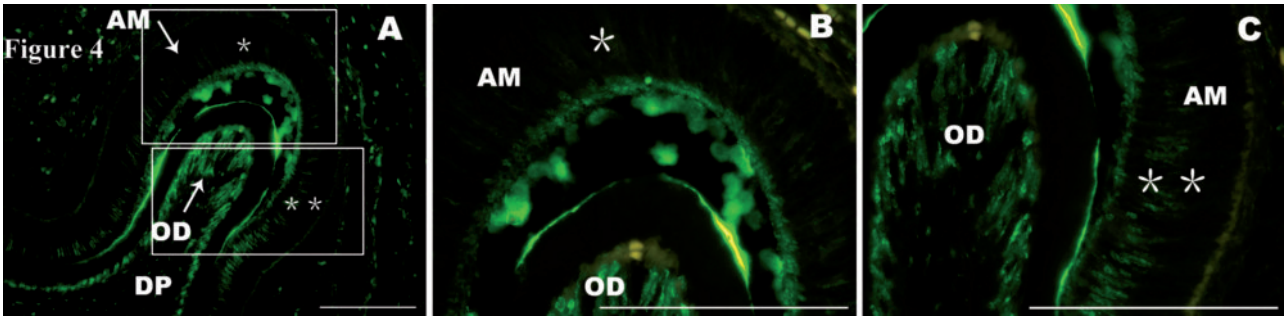

Figure 4. Difference of TGF-β2 expression patterns between the mature ameloblasts and secretory ameloblasts of ICR mouse developing first lower molar. (A, B, C) At D2, the ameloblasts, odontoblasts and dental mesenchyme still remained positive, while the mature ameloblasts (*) at the tip of dental cusp lost immunoreactivity for TGF-β2 and the secretory ameloblast (**) adjacent to dental cusp still exhibited positive staining. Boxed areas in A are respectively enlarged shown as B and C. A, B, C were acquired under Olympus BX-51 microscope. AM: ameloblast; DP: dental papilla; OD: odontoblast. Bar=50 μm.

In summary, our study indicates that TGF-β2 does exhibit characteristic temporospatial distribution patterns in the developing mouse first lower molar. TGF-β2 was detected in initiating dental lamina and surrounding mesenchyme, but was negative in BM and epithelial cells in the basal layer, suggesting TGF-β2 likely participates in tooth initiation. Different expression patterns of TGF-β2 between PEK and SEK showed the potential function of TGF-β2 in epithelial morphogenesis. Strong immunoreaction of TGF-β2 in differentiated odontoblast indicated the involvement of TGF-β2 in the formation of dentine matrix. Additionally, dynamic expression patterns of TGF-β2 in odontoblast, preameloblast and ameloblast suggested that TGF-β2 probably regulates ameloblasts differentiation through the epithelial-mesenchymal interactions.

Acknowledgements

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