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Original Article

The TGF-β type III receptor is localized to the medial edge epithelium during palatal fusion

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ABSTRACT During palatal fusion, the medial edge epithelial cells (MEE) but not the oral/nasal palatal epithelium, selectively undergo epithelial-mesenchymal transformation. It is known that this process is regulated, at least in part, by endogenous TGF-β3. One conceivable mechanism is that restricted expression of TGF-β receptors (TβRs) in a subpopulation of cells may localize TGF-β responsiveness (Brown *et al.*, 1999). However, TGF-β type II receptor (TβR-II) is expressed by all palatal epithelial cells during palatal fusion (Cui *et al.*, 1998) and therefore cannot localize TGF-β3 responsiveness. To investigate the role of TGF-β type III receptor (TβR-III) in MEE transformation, we examined the expression pattern of TβR-III in the developing palate from E12 to E15 mice *in vivo* and *in vitro* by immunohistochemistry and compared the expression pattern to that of type I receptor (TβR-I). The expression of TβR-III was temporo-spatially restricted to the MEE during palatal fusion, while the expression of TβR-I was primarily localized in all palatal epithelia, consistent with the expression patterns of TβR-II and TGF-β3 (Cui *et al.*, 1998). These results support our hypothesis that TβR-III localizes and mediates the developmental role of TGF-β3 on MEE transformation by specific expression in the MEE. TβR-III may modulate TGF-β3 binding to TβR-II in the MEE cells to locally enhance TGF-β3 autocrine signaling through the TβR-I/TβR-II receptor complex, which contributes to MEE selective epithelial-mesenchymal transformation.

KEY WORDS: *immunohistochemistry, medial edge epithelium, palatogenesis, TβR-I, TβR-III.*

Introduction

Mammalian palatal fusion requires that the medial edge epithelium (MEE) disappear from the midline. The MEE that remain viable may either migrate to join the oral and nasal surface epithelia or transform to a mesenchymal phenotype (for review, see Shuler, 1995). The TGF-β family, especially, TGF-β3 is important in the regulation of this process (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990; Gehris *et al.*, 1991; Brunet *et al.*, 1995; Kaartinen *et al.*, 1995, 1997; Proetzel *et al.*, 1995; Sun *et al.*, 1998). Absence of endogenous TGF-β3 inhibits palatal fusion and alters MEE fate with MEE retained in the midline (Kaartinen *et al.,* 1995, 1997; Proetzel *et al.*, 1995).

TGF-β family members initiate their cellular action by binding to specific cell surface proteins. Three major types of TGF-β receptors have been identified by receptor affinity labeling assays, and all of them have been proved important to relay the effects of TGF-β into the cell. Both type I (TβR-I) and type II (TβR-II) receptors are transmembrane serine/threonine kinases indispensable for TGF-β signaling. Type III receptor (TβR-III), also termed betaglycan, is a membrane-anchored protein lacking a cytoplasmic kinase domain, and serves as a direct modulator of TGF-β access to the signaling receptor. In the presence of activated TGF-β ligands, TβR-III forms a transient heteromeric complex with TβR-II and presents TGF-β directly to TβR-II. Upon binding to TGF-β ligand, TβR-II serine/threonine kinase triggers heterodimerization with TβR-I and further transphosphorylates TβR-I, which in turn displaces TβR-III from TGF-β ligand binding. The TβR-I/TβR-II complex subsequently transphosphorylates intracellular SMAD proteins, which results in the propagation of a phosphorylation signal into the nucleus (for reviews, see Heldin *et al.* 1997; Massagué, 1998).

Our previous study using immunohistochemistry co-localized TGF-β3 and TβR-II in serial sections during murine palatogenesis *in vivo* and *in vitro* (Cui *et al.*, 1998). We found that expression of TGF-β3 and TβR-II in the palate began at E13 when the palatal shelves were in a vertical orientation. TGF-β3 and TβR-II proteins

Abbreviations used in this paper: AV, atrioventricular; MEE, medial edge epithelium; TGF-β, transforming growth factor beta; TβR-I, TGF-β receptor type I; TβR-II, TGF-β receptor type II; TβR-III, TGF-β receptor type III.

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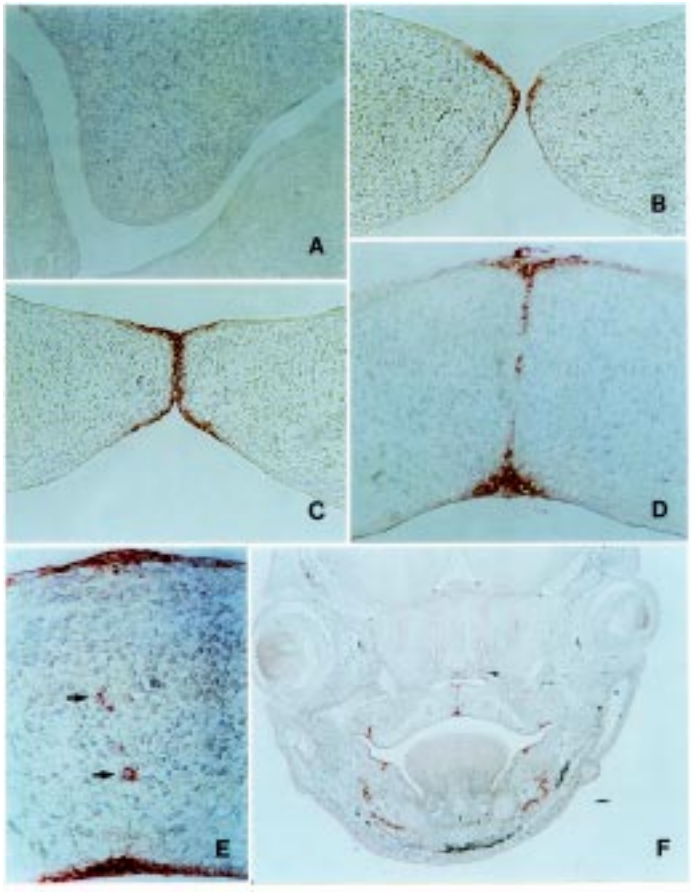

Fig. 1. Immunolocalization of TβR-III in palate tissue *in vivo*. (A) *E12; the palatal shelf has grown into the oral cavity. TβR-III is not detectable in palatal epithelium or mesenchyme.* **(B)** *E14; the palatal shelves are in a horizontal orientation above the tongue. Immunostaining of TβR-III is localized exclusively to the MEE.* **(C)** *Early E14.5; the initial contact of MEE from the opposing palatal shelves in the midline. Immunostaining of TβR-III is mainly localized in the MEE as well as in the oral/nasal epithelium adjacent to the midline.* **(D, F)** *Late E14.5; the midline seam is disrupted as palate fusion progresses. The distribution of TβR-III in the midline is discontinuous and accompanied by intense staining concentrated in the oral and nasal epithelial triangle areas.* **(E)** *E15; after palate fusion and mesenchyme confluence, immunostaining of TβR-III is not found in the palatal mesenchyme except in transformed MEE cells (arrows).*

were localized in the epithelial cells. This epithelial distribution remained during palatal shelf elevation, medial edge epithelial adherence, and midline epithelial seam disruption. After palatal fusion, weak expression of TGF-β3 and TβR-II were also present in the mesenchyme. Immunohistochemical localization of TGF-β3 and TβR-II in palatal shelves in organ culture had patterns that were consistent with the *in vivo* results. We concluded that TGF-β3 exerted its developmental role in an autocrine fashion through TβR-II binding. It was not clear, however, why only MEE selectively underwent epithelial-mesenchymal transformation while all palatal epithelia expressed TGF-β3 and TβR-II.

To investigate the role of TβR-III in MEE transformation, we examined the expression pattern of TβR-III in the developing palate from E12 to E15 *in vivo* and *in vitro* by immunohistochemistry and compared the expression pattern to that of TβR-I. We hypothesized that TβR-III localized and mediated the developmental role of TGF-β3 on MEE transformation by specific expression in the MEE. TβR-III may modulate TGF-β3 access to TβR-II in MEE to locally enhance TGF-β3 autocrine signaling through the TβR-I/TβR-II receptor complex, which contributes to MEE selective epithelial-mesenchymal transformation.

Results

Immunolocalization of endogenous TβR-III in palate in vivo and comparison with that of TβR-I

At E12 when the palatal shelves have first grown into the oral cavity as extensions of the maxillary processes, immunolocalilzation of TβR-III was not detectable in either palatal epithelium or mesenchyme (Fig. 1A). The expression of TβR-III appeared at E13 when palatal shelves were in a vertical position along the lateral sides of the tongue. Immunostaining was weak, but localized exclusively to the epithelial cells. The expression of TβR-III was increased dramatically upon palatal shelf elevation and midline seam formation (E14-14.5). Immunostaining was localized mainly to the MEE. No detectable signal was found in the palatal mesenchyme (Fig. 1B, C). As development continued, the midline seam was disrupted. The distribution of TβR-III in the midline became discontinuous and accompanied by intense staining concentrated in the oral and nasal triangle areas (Fig. 1D, F). By E15 MEE disappeared from the midline and mesenchyme confluence, no immunostaining was seen in the palatal mesenchyme except in a few MEE island and mesenchymal-like cells (Fig. 1E). Because these mesenchymal-like cells were very close to the location of the previous midline epithelial seam, based on observations from serial sections, we considered them transformed MEE cells.

In contrast to the restricted expression of TβR-III in MEE, the expression of TβR-I in the developing palate is temporo-spatially broader. The expression of TβR-I in palate appeared at E12 during the initial outgrowth from the maxillary prominence. Intense immunostaining was found in the entire palatal epithelium and in some mesenchymal cells (Fig. 2A). Distribution of TβR-I appeared as globules in cells, which might reflect local high expression and concentration of receptors or antibody aggregation in focal areas of cells. As palatal shelves grew along the lateral sides of the tongue (E13), immunostaining remained in palatal epithelium but is less in the mesenchyme (Fig. 2B). This epithelium-favored distribution pattern remained constant as the palatal shelves elevated to a horizontal orientation, contacted in the midline, and a single layer of MEE cells were present in the midline (E14-14.5). The distribution of TβR-I was in oral epithelium, nasal epithelium and midline epithelial seam, but not in the underlying mesenchyme (Fig. 2C). As development continued, the midline seam was disrupted and the distribution of TβR-I in the midline became discontinuous (Fig. 2D, E). At E15 after palatal fusion and mesenchymal confluence, weak expression of TβR-I was also present in the mesenchyme in addition to the continuous localization in the oral and nasal epithelium.

Immunolocalization of endogenous TβR-III/TβR-I in palate tissue in vitro and comparison with the in vivo expression pattern

Based on previous experience (Shuler *et al.*, 1991; Cui *et al.*, 1998), organ cultured palatal shelves were recovered after differ-

ent time periods that match *in vivo* stages of palatal development. After 7 hours in organ culture the medial edges of opposing palatal shelves remained apart, and TβR-III was not found in the palatal shelves (Fig. 3A). As organ cultures continued to the 24-36th hour, the palatal shelves became adherent and an epithelial seam formed in the midline. The TβR-III immunostaining became pronounced in the midline seam but not in the oral epithelium or in the mesenchyme (Fig. 3B). Intense staining of the (nasal) epithelium in contact with the Millipore filter represented an artifact resulting from the organ culture condition. As the midline seam disrupted around the 48th hour of organ culture, the distribution of TβR-III in the midline was only in epithelial islands (Fig. 3C). After 60 hours in culture, MEE disappeared from the midline resulting in palatal mesenchyme confluence. At that point immunostaining of TβR-III was observed in the oral epithelial triangle area (Fig. 3D).

Seven hours after initial organ culture the medial edges of opposing palatal shelves remained apart, and the expression of TβR-I was observed in the epithelium covering the shelves (Fig. 4A). As organ cultures continued to the 24-36th hour, the palatal shelves were adherent and a two-cell layer thick seam of epithelial cells was present in the midline of the palate. The TβR-I immunostaining was localized to the epithelial cells covering the palatal shelves and in the midline seam, but not in the underlying mesenchyme (Fig. 4B, C). TβR-I remained epithelial specific as the midline MEE seam was reduced to a single layer of cells. Once the MEE midline seam became fragmented around 48 hours of organ culture, the distribution of TβR-I lost its continuity in the midline. By the time of mesenchyme confluence after 60 hours in organ culture, weak staining was also seen in the palatal mesenchyme (Fig. 4D).

The results from immunolocalization of TβR-III/TβR-I in organ cultured palatal tissues replicated the pattern of expression observed *in vivo*.

Discussion

During palatal fusion MEE cells but not oral/nasal palatal epithelium selectively undergo epithelial-mesenchymal transformation/migration (Fitchett and Hay, 1989; Shuler *et al*., 1991, 1992; Carette and Ferguson, 1992; Griffith and Hay, 1992). It is known that this process is regulated, at least in part, by endogenous TGF-β3 (Kaartinen *et al.,* 1995, 1997; Proetzel *et al*., 1995). One conceivable mechanism is that restricted expression of TβRs in a subpopulation of cells may localize TGF-β responsiveness (Brown *et al*., 1999). TβR-II, however, is expressed by all palatal epithelial cells during palatal fusion (Cui *et al*., 1998) and therefore cannot localize TGF-β3 responsiveness. To investigate the role of TβR-III in MEE transformation, we examined the expression pattern of TβR-III in the developing palate *in vivo* and *in vitro* by immunohistochemistry and compared the expression pattern to that of TβR-I. As was TβR-II, TβR-I was expressed by all palatal epithelium during palate development. However, the expression of TβR-III was temporo-spatially restricted to a subgroup of palatal epithelial cells and correlated with the process of MEE epithelial-mesenchymal transformation. The expression TβR-III was not identified in the palate during initial morphogenesis, but dramatically increased at the time of midline epithelial seam formation. The expression of TβR-III was exclusively localized to the MEE cells. As MEE disappeared, the distribution of TβR-III was lost in the midline but

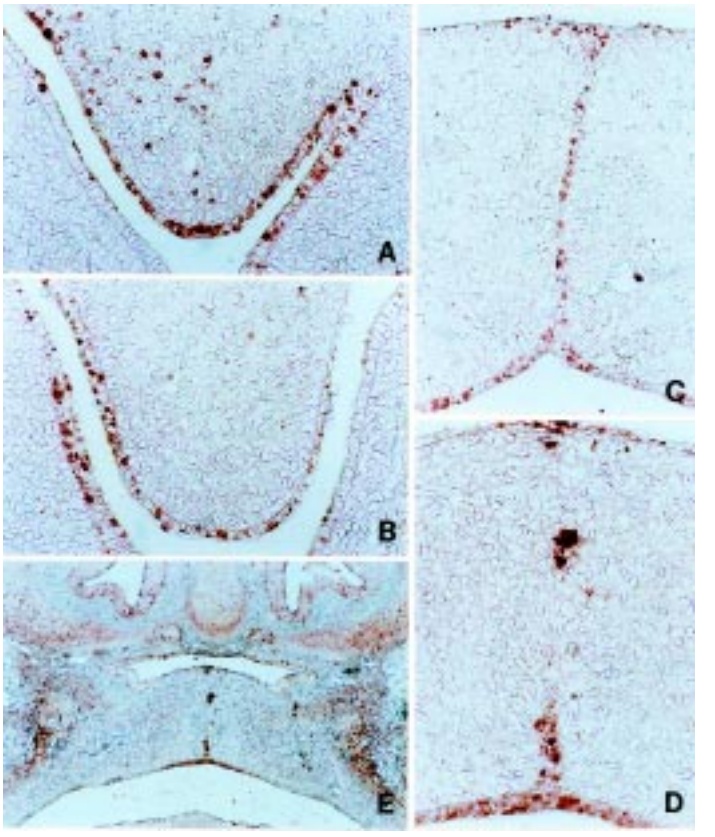

Fig. 2. Immunolocalization of TβR-I in palate tissue *in vivo*. (A) *E12; the palatal shelf initially outgrows from the maxillary prominence. The distribution of TβR-I is seen in the entire palatal epithelium and in some mesenchymal cells.* **(B)** *E13; the palatal shelf is in a vertical orientation along the lateral side of the tongue. The distribution of TβR-I remains in the palatal epithelium, but is much less in the mesenchyme.* **(C)** *Early E14.5; a single layer of MEE cells is present in the midline. TβR-I is distributed in the midline epithelial seam as well as in the oral and nasal epithelium, but not in the underlying mesenchyme.* **(D, E)** *Late E14.5; the midline seam is disrupted by the progress of palate fusion. The distribution of TβR-I is present in discontinuous islands in the midline, but persists in the oral and nasal epithelium.*

persisted in some transformed MEE cells. This expression pattern is consistent with a role for TβR-III in localizing and mediating the effects of TGF-β3 on MEE epithelial-mesenchymal transformation.

Differential expression of TβR-II and TβR-III is also seen in the endothelial cells during the atrioventricular (AV) cushion formation of chick (Brown *et al*., 1996, 1999). In the developing heart, endothelial cells that line the lumen in the regions of the AV cushion and outflow tract undergo epithelial-mesenchymal transformation and participate in the formation of the valves and membranous septa (for reviews, see Eisenberg and Markwald, 1995; Fishman and Chien, 1997). Immunohistochemical localization revealed that TβR-II was expressed by all endothelial cells in the heart, whereas TβR-III was detected on the endocardial cells overlying the AV cushion and on a subset of migrating mesenchymal cells (Brown *et al*., 1996, 1999). Moreover, TβR-III-specific antisera successfully inhibited mesenchyme formation and migration in AV cushion explants, and mis-expression of TβR-III in non-transforming ventricular endothelial cells conferred the transformation response to

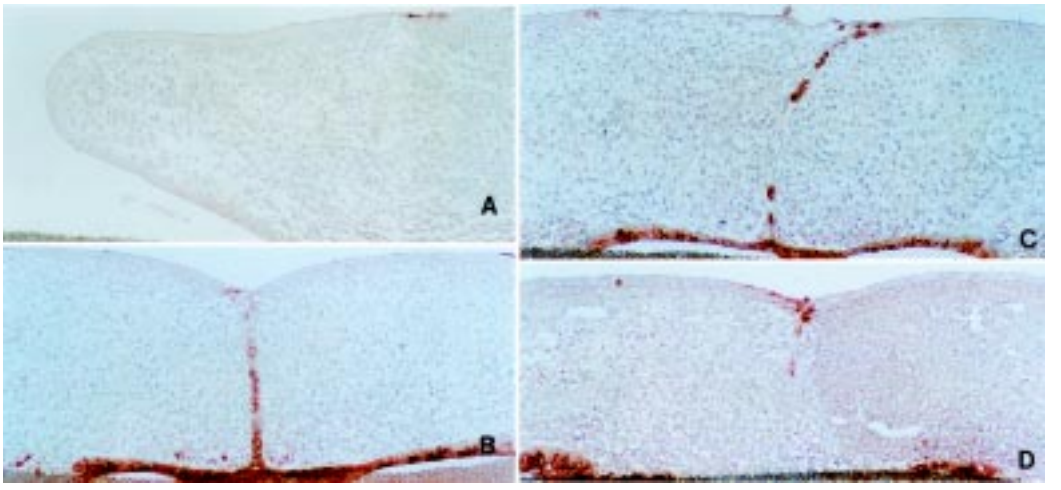

Fig. 3. Immunolocalization of TβR-III in palate tissue in culture. (A) *E13 +7h; the medial edges from opposing palatal shelves remain apart. The distribution of TβR-III is not detectable in the palate.* **(B)** *E13 +36h; a single-layer-MEE seam is present in the palate midline. The distribution of TβR-III is localized to the MEE, but not to the oral epithelium and palatal mesenchyme. Intense staining in the nasal epithelium in contact with a filter represents an artifact of the culture condition.* **(C)** *E13 +48h; the midline seam becomes fragmented. The distribution of TβR-III persists in the midline but looses its continuity.* **(D)** *E13 +60h; the palatal mesenchyme is confluent and the distribution of TβR-III is only seen in the oral epithelial triangle area.*

TGF-β2. These results support a model where TβR-III localizes transformation in the heart and plays an essential, non-redundant role in TGF-β signaling (Brown *et al.,* 1999).

TβR-III binds all three TGF-β isoforms with high affinity (Segarini *et al.*, 1989; Cheifetz and Massagué, 1991). In the presence of activated TGF-β, TβR-III forms a heteromeric complex with TβR-II. Thus, its main role is probably to make the TβR-II more accessible to the ligand (López-Casillas *et al.*, 1993; Moustakas *et al.*, 1993). In addition, by capturing and retaining ligand, TβR-III may protect the ligand from cellular degradation and/or inactivation, rendering more ligand available for binding to the signaling receptors (Chen *et al.*, 1997). Supportive evidence showed that expression of TβR-III in cells that lacked TβR-III significantly increased TGF-β1 binding to TβR-II by 2.5-fold or more (Wang *et al.*, 1991; López-Casillas *et al.*, 1993). In myoblasts, in the absence of TβR-III only a small population of TβR-II could bind TGF-β1 with high affinity, while a larger population of TβR-II had a much lower affinity for TGF-β1. Expression of TβR-III, however, converted all TβR-II receptors into one population with high affinity for TGF-β1 (López-Casillas *et al.*, 1993). Expression of TβR-III in human breast cancer cells MCF-7 not only increased TGF-β1 binding to the signaling receptors but also restored autocrine TGF-β1 activity in the cell. Thus, TβR-III may also be essential for an optimal level of autocrine TGF-β activity in cells (Chen *et al.*, 1997).

We assume that TβR-III also enhances TGF-β3 binding to its signaling receptors in the cells. We believe that restricted expression of TβR-III is the key for TGF-β3 selectively regulating MEE in all palatal epithelium during palate fusion. It is likely that TβR-III facilitates TGF-β3 binding to TβR-II in MEE to enhance TGF-β3 autocrine through the receptor complex of TβR-I/TβR-II that contributes to MEE epithelial-mesenchymal transformation.

As stated earlier, TβR-III is a membrane-anchored proteoglycan. Due to its a relatively short cytoplasmic domain that lacks commonly recognized protein docking or kinase-like motifs, TβR-III is believed not to directly transduce TGF-β signal but regulate TGF-β access to the signaling receptors (López-Casillas *et al.*, 1991; Wang *et al.*, 1991). However, this opinion has been challenged (Brown *et al.*, 1999). TβR-III may also signal in response to the TGF-β ligand. The binding of TGF-β to TβR-III may alter the composition or activity of the TβR-I/TβR-II signaling complex to activate a unique set of downstream mediators that resulted in, for instance, AV cushion transformation (Brown *et al.*, 1999).

TβR-I and TβR-II are TGF-β signaling receptors. They both are necessary in the formation of a heteromeric receptor complex to transduce TGF-β signals into the cell. (Wrana *et al.*, 1992; Vivien *et al.*, 1995). In the developing palate, TβR-I and TGF-β3/TβR-II have a remarkable similarity in the distribution site from E13 to E15. They all localize predominantly to the palatal epithelium that includes oral epithelium, nasal epithelium and the midline epithelial seam, but are absent from the underlying mesenchyme. They are all lost from the midline when the MEE disappears. The pattern of TβR-I expression supports our previous conclusion that

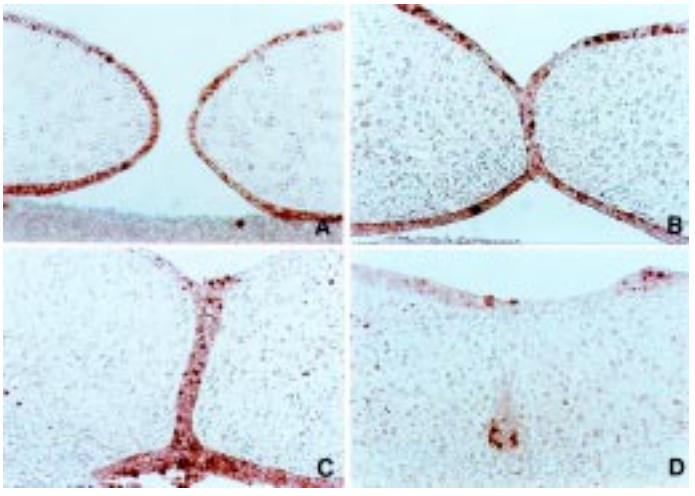

Fig. 4. Immunolocalization of TβR-I in palate tissue in culture. (A) *E13 +7h; the medial edges from opposing palatal shelves remain apart. The distribution of TβR-I predominates in the entire epithelium.* **(B, C)** *E13 +24~36h; a two-layer MEE seam is present in the midline of the palate (B, as anterior portion; C, as middle portion). The distribution of TβR-I is restricted to the MEE and the epithelium covering the palate tissue, but is not present in the underlying mesenchyme.* **(D)** *E13 +60h; palatal mesenchyme is confluent with some MEE islands remaining. TβR-I expression persists in the MEE, while weak immunostaining is seen in the palatal mesenchyme.*

the epithelium is the major target tissue of TGF-β signaling in the developing palate (Cui *et al*., 1998). Constant expression of TβR-I in palate prior to and during MEE epithelial-mesenchymal transformation provides evidence that TβR-I as well as TβR-II is required by TGF-β3 signaling in this event. The temporo-spatial coincidence of the receptor complex TβR-I/TβR-II and its ligand in palate further support our previous conclusion that TGF-β3 exerts its effect in an autocrine fashion (Cui *et al*., 1998).

In summary, in this study we examined the expression pattern of TβR-III in murine palatal tissues from E12 to E15 *in vivo* and *in vitro* using immunohistochemistry and compared the expression pattern to that of TβR-I. The expression of TβR-III was temporo-spatially restricted to the MEE during palatal fusion, while the expression of TβR-I was primarily localized in the palatal epithelia, consistent with the expression patterns of TβR-II and TGF-β3. These results support our hypothesis that TβR-III localizes and mediates the developmental role of TGF-β3 on MEE transformation by specific expression in the MEE. TβR-III may modulate TGF-β3 binding to TβR-II in the MEE cells to locally enhance TGF-β3 autocrine activity through the TβR-I/TβR-II receptor complex, which contributes to MEE selective epithelial-mesenchymal transformation. The consistent expression pattern of *in vivo* and *in vitro* studies will allow us to further investigate the functional roles of TβRs *in vitro*.

Materials and Methods

Animals

Timed pregnant Swiss-Webster mice were used for these studies. The females were mated overnight and the presence of a vaginal plug used to determined day 0 and hour 0 (E0). The animals were maintained under standard conditions in the USC Vivarium.

Tissue preparation and experimental strategy

Based on known sequence of developmental events during palatogenesis, pregnant females were sacrificed by cervical dislocation at the following selected embryonic stages. E12, the palatal shelves emerged from the maxillary prominence. E13, the palatal shelves remained in a vertical position along the lateral sides of the tongue. E14, the palatal shelves became horizontal position above the tongue and the medial edges of the two palatal shelves contacted in the midline. E14.5, the midline epithelium was a single layer of cells or the seam was disrupted with some MEE remaining. E15, the mesenchymal portion of the two palatal shelves became continuous. The fetuses were dissected and the heads were fixed immediately in 4% paraformaldehyde-PBS at 4ºC, followed by routine procedures for embedding in paraffin. The coronal sections (5 μm) were mounted in serial order on poly-L-lysine coated slides. The tissues were examined by immunohistochemistry to analyze the expression pattern of endogenous TβR-III and TβR-I. To more readily compare the expression levels between different developmental stages, each slide contained sections from all developmental stages, from E12 to E15.

Palate culture

To compare the expression pattern of TβR-III/TβR-I during organ culture conditions with that of *in vivo*, palatal shelves were dissected from E13 fetal murine heads and placed in pairs on Millipore filters with their medial edges in contact. The palatal shelves were cultured at the air-medium interface in Grobstein organ culture dishes in BGJb medium (Gibco) at 37ºC and a 5% CO_2/air atmosphere. The organ cultures were maintained for up to 60 hours. The first specimens were collected at the 7th hour and the rest were continuously harvested every 12 hours. The procedures of tissue preparation and experimental strategy were the same as *in vivo*.

Immunohistochemistry

Sections were deparaffinized and rehydrated. The activity of endogenous peroxidase was quenched by treating tissue sections with 3% hydrogen peroxide in absolute methanol. Non-specific background was eliminated by incubating the tissue sections with non-immune serum. The primary antibody against TβR-III (goat polyclonal IgG, R&D Systems) or TβR-I (rabbit polyclonal IgG, Santa Cruz Biotechnology, Inc.) was incubated on the tissues at a concentration of 2 μg/ml overnight at room temperature. The immunizing epitope of TβR-III corresponds to the extracellular domain of the human homologue. The immunizing epitope of TβR-I is mapped within an internal domain of the human homologue origin (also designated ALK-5) and the antibody is mouse reactive. Incubation was followed by addition of a biotinylated secondary antibody. Streptavidin-peroxidase was then added to bind the biotin residues on the secondary antibody. The presence of peroxidase was revealed by addition of substrate chromogen solution. Peroxidase catalyzed the substrate (hydrogen peroxide) and converted the chromogen (AEC) to a red deposit, which demonstrates the location of the antigen TβR-III or TβR-I (HISTOSTAIN^{TM} BULK kit, Zymed Laboratories Inc.). As a negative control, goat/rabbit IgG was substituted for the primary antibody, respectively. Each of the experiments was repeated at least three times to show the consistence of the results.

Acknowledgments

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