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Short Communication

TGF-β_3 is required for the adhesion and intercalation of medial edge epithelial cells during palate fusion

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ABSTRACT Transforming growth factor-beta3 (TGF-β_3) plays a critical role during palate development, since mutations of the *TGF-β_3* gene give rise to cleft palate in both humans and mice. Striking alterations have been reported in the behaviour and differentiation of medial edge epithelial (MEE) cells in *TGF-β_3* knockout mouse palates. In the present paper, we provide evidence of alterations in MEE intercellular adhesion in *TGF-β_3 -/-* mouse palates using immunohistochemistry with monoclonal antibodies to a panel of cell adhesion and cytoskeletal molecules including E-cadherin, α and β catenin, β actin, vinculin and β_2 integrin. *In vitro* labeling of opposing MEE with two different lipophilic markers and subsequent analysis by confocal microscopy revealed that wild type MEE cells intercalate as soon as the midline epithelial seam forms. This finding indicates that the palate may elongate in a dorso-ventral direction by means of convergent extension, as occurs in other embryonic developmental processes. In contrast, this intercalation does not occur in the *TGF-β_3 -/-* MEE but it can be rescued by the exogenous addition of TGF-β_3. Thus, the substantial alteration of MEE intercellular adhesion observed in *TGF-β_3 -/-* palates may account for the defect in palatal shelf adhesion and the formation of cleft palate.

KEY WORDS: *MEE, palate development, TGF-β_3, cell adhesion, cell intercalation*

In mammals, the development of the secondary palate is a complex process in which contact between the epithelia covering the tips of the palatal shelves (the medial edge epithelium or MEE), together with their adhesion and "disappearance" from the midline seam are of major importance. Several growth factors have been reported to influence these events, but among them, Transforming Growth Factor beta 3 (TGF-β_3) is likely to play the most important role, since mutation of the *TGF-β_3* gene causes cleft palate in both mice (Proetzel *et al*., 1995; Kaartinen *et al*., 1995) and humans (Lidral *et al*., 1998).

The absence of TGF-β_3 in *TGF-β_3* knockout mice (*TGF-β_3 -/-*) leads to striking alterations in MEE development. In the wild type palate, immediately prior to palatal shelf contact, the MEE becomes multilayered, its most superficial cells bulge (bulging cells) (Martínez-Álvarez *et al*., 2000a), and microvilli, filopodia and lamellipodia develop on its apical surface (Taya *et al*., 1999). A several cell layer thick midline epithelial seam (MES) forms just after opposing MEE contact, which rapidly thins to become two to three cells thick. Subsequent to programmed cell death (Martínez-Álvarez *et al*., 2000b), epithelial-mesenchymal transformation (Fitchett and Hay, 1989) and migration to the oral and nasal palatal epithelia (Carette and Ferguson, 1992), MES cells disappear and the palatal mesenchyme becomes confluent in the midline. However, in the *TGF-β_3 -/-* palate, although palatal shelves grow, approach each other and contact, appropriate adhesion does not occur and a cleft palate develops (Proetzel *et al*., 1995; Kaartinen *et al*., 1995). Immediately prior to palatal shelf contact, the *TGF-β_3 -/-* MEE is only one cell layer thick and its surface has few of the characteristic bulging cells (Martínez-Álvarez *et al*., 2000a), microvilli, filopodia or lamellipodia (Taya *et al*., 1999). When these palatal shelves are placed in contact *in vitro,* their adhesion is poor or non-existent and MEE cells do not transform into mesenchyme (Kaartinen *et al*., 1997). The simultaneous alteration of all these processes in *TGF-β_3 -/-* palates strongly suggests a problem with cell adhesion.

We examined the localization in the *TGF-β_3 +/+* and *-/-* MEE of a panel of proteins including the cell adhesion molecules E-cadherin and β_2 integrin, the intracellular attachment proteins α and β catenin and vinculin, and β actin (Fig. 1). The presence of E-cadherin, α and β catenin and β actin was investigated since these proteins are components of the E-cadherin based cell-cell

Abbreviations used in this paper: E, embryonic day; ECM, extracellular matrix; MEE, medial edge epithelium; MES, midline epithelial seam; TGF-β_3, transforming growth factor-beta3; *TGF-β_3 +/+, +/-* or *-/-, TGF-β_3* wild type, heterozygous or homozygous null.

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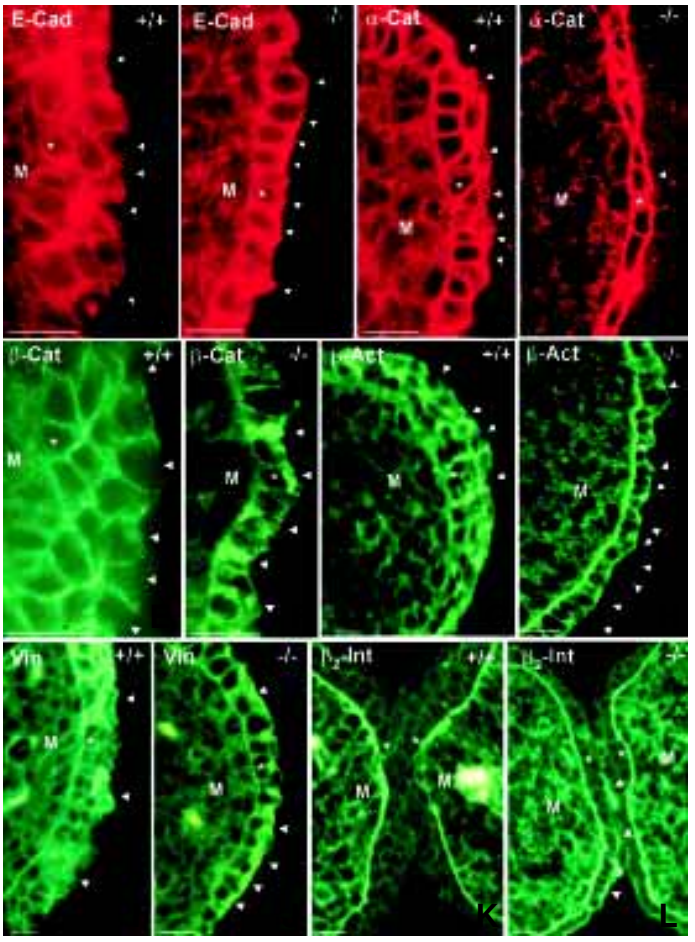

Fig. 1. Immunolocalization of cell adhesion and cytoskeletal molecules in E14.5 *TGF-*β_3 *+/+* (A,C,E,G,I,K) and *-/-* (B,D,F,H,J,L) mouse palatal shelves. (A) *E-cadherin (E-Cad) is localized circumferentially in all basal cells and basolaterally in the superficial cells (arrowheads) in the +/+ Medial Edge Epithelium (MEE).* **(B)** *This immunostaining is located in all MEE cell surfaces in -/- palates. Arrowheads indicate E-cadherin presence in the external MEE surface.* **(C)** *α catenin (α-Cat) immunostaining is distributed circumferentially in +/+ MEE basal cells and basolaterally in the superficial cells (arrowheads).* **(D)** *However, in the -/- palate, α catenin is observed in all MEE cell surfaces. The arrowhead indicates α catenin location in the MEE external surface.* **(E)** *In the +/+ MEE, β catenin (β-Cat) immunolabeling shows a honeycomb pattern, and it is located circumferentially in the basal cells and basolaterally in the superficial cells (arrowheads).* **(F)** *In the -/- MEE, β catenin immunostaining was observed as irregular accumulations in the cytoplasm (arrowheads).* **(G)** *β actin (β-Act) filaments are aligned circumferentially in all basal cells and basolaterally in the superficial +/+ MEE cells (arrowheads).* **(H)** *However, β actin is mostly located in the external and basal surfaces of the -/- MEE. Arrowheads indicate the superficial location of β actin in these cells.* **(I)** *The distribution pattern of vinculin (Vin) is intense and irregular in the +/+ MEE, showing a "patched" appearance (arrowheads).* **(J)** *This pattern is different in -/- palates, in which vinculin is mostly located at the external (arrowheads) and basal MEE surfaces.* **(K)** *When opposing +/+ MEE contact at the midline, β_2 integrin (β_2-Int) is restricted to the palatal mesenchyme and it is not present in the MEE.* **(L)** *In contrast, in the -/- palates, β_2 integrin is observed in the mesenchyme and between contacting MEE (arrowheads). Asterisks indicate the most basal MEE cells. M, mesenchyme. Scale bars, 10μm.*

adhesion system, and are usually involved in epithelial adhesion during development (Nagafuchi *et al.*, 1994). The presence of β_2 integrin and vinculin was analyzed to examine MEE cell adhesion to the extracellular matrix. In the *TGF-*β_3 *+/+* MEE, the distribution of E-cadherin (Fig. 1A), α catenin (Fig. 1C), β catenin (Fig. 1E) and β actin (Fig. 1G) was mostly regular and circumferentially located in the basal cells and basolaterally placed in the superficial cells. Vinculin was present in all MEE cells, but different labeling intensities gave a "patched" appearance to the epithelium (Fig. 1I) which may indicate different states of adhesion among MEE cells. Thus, the areas of more intense vinculin localization may correspond to cells that are more actively involved in the movements leading to cellular redistribution which causes the multicellular organization observed in the preadhesion MEE (Brinkley, 1984).

The presence of all these molecules in the MEE was remarkably altered in *TGF-*β_3 *-/-* palates. *TGF-*β_3 *-/-* MEE cells showed abundant E-cadherin and α catenin even in apical regions of the cells (Fig. 1 B,D). In contrast to the honeycomb-like disposition observed in the *TGF-*β_3 *+/+* MEE (Fig. 1E), β catenin immunolabeling in *TGF-*β_3 *-/-* MEE cells was observed as irregularly distributed accumulations in the cytoplasm (Fig. 1F). The monolayered nature of the *-/-* MEE was clearly apparent. Important changes were also observed in the location of β actin and vinculin in these palates, as these proteins were mostly concentrated in the apical and basal cell surfaces and were regularly distributed (Fig. 1 H,J). Interestingly, comparative analysis of β_2 integrin localization in contacting *TGF-*β_3 *+/+* and *-/-* palates demonstrated that β_2 integrin is absent from the apical surface of the *TGF-*β_3 *+/+* MEE (Fig. 1K), whereas it is present on the opposing apical surfaces of the *TGF-*β_3 *-/-* MEE (Fig. 1L).

Just after palatal shelf adhesion, the midline epithelial seam progresses from being formed by several cell layers to becoming one cell layer thick (Martínez-Álvarez *et al.*, 2000b). At the same time, the palate elongates in a dorso-ventral direction. This process is strongly indicative of the existence of convergent extension by medio-lateral cell intercalation in the MEE, a phenomenon which has not yet been characterized in palate fusion. However, this mechanism is commonly employed during embryonic development when (as in the palate) a whole tissue needs to extend without increasing its number of cells (reviewed in Keller *et al.*, 2000). We examined the possibility that medio-lateral MEE cell intercalation takes place during the progression of the midline epithelial seam in the mouse palate and evaluated if this mechanism requires the presence of TGF-β_3. Left and right palatal shelves were labeled with the lipophilic markers DiO or DiI respectively. Paired palatal shelves were then apposed and cultured for 18 hours, as this is the culture period which allows better visualization of different aspects of the seam (unpublished observation). When fixed and subsequently visualized by confocal microscopy, DiO labeled cells show a green fluorescence while cells labeled with DiI appear red. Therefore, if cell intercalation occurred during epithelial seam development, green and red cells would appear mixed forming a row when observed in the same confocal section. As expected, wild type palate cultures showed red and green colored MEE cells intermingled in the same confocal plane (Fig. 2 A,B), forming several (Fig. 2A) or just one (Fig. 2B) layer, thus indicating that MEE cells intercalate during *in vitro* palate fusion. In contrast, all *TGF-*β_3 *-/-* cultures showed two differently colored separated bands when observed by confocal microscopy (Fig. 2C). These bands correspond to the differently labeled opposing MEE, whose superficial cells appeared either totally separated or just apposed, even after having been cultured for 4 days (Fig. 2D), and had not mixed. The addition of TGF-β_3 to the culture medium of both 18 hour and four day *TGF-*β_3 *-/-* palate cultures rescued MEE cell intercalation, as red and

green cells were observed mixed in a monolayered epithelial seam (Fig. 2E). Complete palate fusion occurred when *TGF*-β_3 -/- palates were cultured for 4 days in the presence of TGF-β_3 (Fig. 2F).

The abnormal presence of the molecules analyzed in this study (except β-catenin) in the apical surface of the *TGF*-β_3 -/- MEE is indicative of a change in the polarity of these cells. This is particularly significant in the case of the distribution of the β_2 integrin, which is present on the external surface of contacting *TGF*-β_3 -/- MEE but is absent from this location in the +/+ MEE palate. Since, after their contact, C57BL/6J *TGF*-β_3 -/- palatal shelves always separate resulting in a complete cleft palate, this superficially placed β_2 integrin does not facilitate the adhesion of opposing MEE, and it could even impair the normal MEE cell recognition and adhesion required for cell intercalation and palatal shelf adhesion.

Cell polarity shift constitutes a major modification in an epithelium (Eaton and Simons, 1995) and could be responsible for the totally different adhesion and behaviour observed in the *TGF*-β_3 -/- MEE cells. The mechanism(s) involved in this apparent shift of MEE cell polarity due to the absence of TGF-β_3 have yet to be determined. Variations in the composition of the extracellular matrix (ECM) surrounding these cells could cause this shift, since, for instance, the experimental addition of collagen to the external surface of epithelial cysts reorients the cell apicobasal axis, and the adherence of these cells to the new external collagen matrix is mediated by integrins (Wang *et al.*, 1990). TGF-β_s are required for epithelial cell polarization in other systems (Unda *et al.,* 2000), and this effect has been related to changes of the ECM composition (Wirl *et al.,*1995). Furthermore, the presence of some ECM molecules in both mouse (Gato *et al.*, 2001) and human (Bodo *et al.*, 1999) cleft palates is dependent on the effect of TGF-β_3, which raises the possibility that ECM molecules on the MEE surface could be altered in the absence of TGF-β_3.

An alteration of the E-cadherin cell-cell adhesion system could be also responsible for this apparent shift in MEE cell polarity. In epithelial tissues, the impaired function of cadherin receptors leads to the absence of the normal basolateral clustering of E-cadherin (Yap *et al.*, 1997), and in these situations E-cadherin is non-functional (Eaton and Simons, 1995). The irregular accumulation of β catenin in the cytoplasm of the *TGF*-β_3 -/- MEE cells (while it is regularly placed in the cell membrane in +/+ cells), together with the presence of both E-cadherin and α catenin all around these cells, suggest that the E-cadherin mediated MEE intercellular adhesion system may be altered. Brinkley (1984) observed a multilayered organisation of wild type MEE cells immediately prior to palatal shelf contact, that is likely required to strengthen the adhesion between opposing MEE. This is not due to cell proliferation, and it might be caused by cell redistribution (Brinkley, 1984). Since cadherins are required for the assembly of cells into multiple layers (reviewed in Braga *et al.*, 1997), an impaired cadherin mediated cell adhesion among *TGF*-β_3 -/- MEE cells is probably responsible for their inability to redistribute, thus remaining monolayered.

Cadherin function is dependent on its interaction with the actin cytoskeleton. Our demonstration of rapid medial edge epithelial cell intercalation in the wild type MEE, that is abolished in the absence of TGF-β_3, associated with changes in the organisation and distribution of cell adhesion and cytoskeletal molecules is supported by previously reported TGF-β_3 induction of filopodia on the MEE surface (Taya *et al.*, 1999). This indicates that rapid cell movements mediated by Ras GTPases, e.g. Cdc42, take place in the wild type MEE (Taya *et al.*, 1999), and suggests that TGF-β_3 dependent rapid adhesion and intercalation of opposing MEE cells is a critical event in normal palatal fusion, whose disruption, e.g. by mutation of *TGF*-β_3, results in cleft palate.

Experimental Procedures

Animals

C57BL/6J *TGF*-β_3 +/- mice (Jackson Laboratories) were timed mated and the day of finding a plug was designated as day zero. Embryonic day (E) 13.5 and E14.5 *TGF*-β_3 +/+ and -/- mouse embryos were used. Embryo extraction and genotyping were performed as described in Martínez-Álvarez *et al.* (2000b).

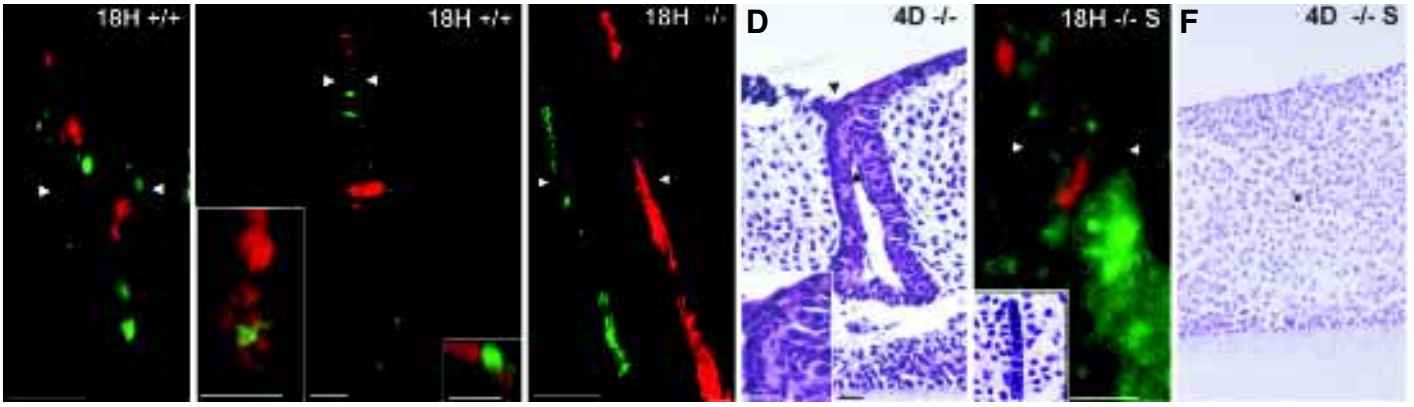

Fig. 2. Cell intercalation in E13.5 *TGF*-β_3 +/+ (A,B) and -/- (C,D,E,F) cultured palates. A,B,C and E are confocal sections of 18 hour cultured paired palatal shelves whose MEE were previously labeled either with DiI (right MEE) or DiO (left MEE). D, insert in E and F are haematoxylin and eosin stained sections of 18 hour (insert in E) and 4 day (D,F) cultured palates. (A) *After being cultured for 18 hours, TGF-β_3 +/+ palates show a row of mixed DiI (red) and DiO (green) labeled MEE cells in the midline.* **(B)** *A monolayer of intercalated red and green MEE cells is observed in some specimens. The inserts are high magnifications of longitudinal (left) and transverse (right) confocal sections from the specimen in (B) which presented intercalated green and red cells.* **(C)** *DiI and DiO labeled MEE cells have not intermingled in a TGF-β_3 -/- palate culture and form two rows of differently colored cells.* **(D)** *After having been cultured for 4 days, opposing TGF-β_3 -/- MEE adhere very poorly and the partial seam formed (area between arrowheads) has failed to narrow. The insert shows a high magnification of the adhered zone which clearly illustrates the aspect of opposing MEE cells at the contact zone.* **(E)** *The addition of TGF-β_3 to TGF-β_3 -/- labeled palate cultures, results in the mixing of red and green cells and the narrowing of the seam formed (insert).* **(F)** *Four day cultured TGF-β_3 -/- palates treated with TGF-β_3 show complete fusion (asterisk indicates plane of fusion). Arrows in (A) to (C) and (E) indicate the lateral borders of the epithelial seam. S, TGF-β_3 supplemented culture. Scale bars, 10 μm.*

Immunohistochemistry

Ten *TGF*-β_3 *+/+* and eleven *-/-* E14.5 mouse heads were used for immunochemistry. No less than four different specimens per antibody used were analyzed. Immunohistochemistry was performed as described in Martínez-Álvarez *et al.* (2000a). Primary antiboides used were rat IgG anti mouse E-cadherin (10 μg/ml) (Takara Biomedicals), mouse IgG anti recombinant α catenin (5 μg/ml) (Alexis), mouse IgG anti human β catenin (5 μg/ml) (Alexis), FITC-conjugated mouse IgG anti human β actin (1:250) (Sigma), mouse IgG anti chicken vinculin (1:100) (Sigma) or mouse IgG anti-human CD11a antigen (β_2 integrin) (1:50) (Novocastra). Incubation with these antibodies was followed by the addition of Cy™-3-conjugated rabbit anti rat or mouse IgG secondary antibodies (1:125) (Jackson ImmunoResearch) or Fluorescein conjugated horse anti mouse IgG secondary antibody (5 μg/ml) (Vector) following the manufacturers instructions. Fluorescence in sections was visualized using a Leica DMRB microscope and photographed using Kodak Ektachrome film.

Detection of MEE Cell Intercalation

Palatal shelves from each side of ten E13.5 *TGF*-β_3 *+/+* and twenty E13.5 *TGF*-β_3 *-/-* mouse embryos were dissected under sterile conditions in 1/1 Dulbecco's modified Eagle's medium/F12 (DMEM/F12) and separated. Several microdrops of either 2 μM DiO (SP-DiOC18(3)) (left palatal shelves) or DiI (DiIC18(5)-DS) (right palatal shelves) (Molecular Probes) in DMEM/F12 were delivered by microejection onto the corresponding MEE using a 30 μm diameter glass micropipette. Labeling was repeated twice with an interval of an hour. Palatal shelves were then washed in DMEM/F12, placed in pairs and apposed to allow fusion in Trowell´s tissue culture with DMEM/F12 + 1% ascorbic acid. Ten ng/ml TGF-β_3 was added to the culture medium in the relevant experiments. Cultures were then incubated for 18 hours at 37°C in a 5% CO_2 incubator. Cultured palates were collected, fixed for 1 hour in 4% formaldehyde in PBS at 4°C, and 10 of them per experimental condition were studied. The distribution of the labeled cells was analyzed *in toto* with an MRC-1024 confocal microscope (Bio-Rad, Hempstead, UK) using an helium-neon laser tuned to 543 nm with a BP 650/20 nm emission filter for DiI labeling and an argon laser tuned to 488 nm with a BP 515/10 nm emission filter for DiO labeling. Both longitudinal and transverse sections of the palates were obtained, and images containing red and green fluorescence in relevant sections were recorded and overlapped. Lasersharp (BioRad) software acquisition was employed and 3D reconstructions were performed using LaserVOX software (BioRad).

Histological Preparation

Twenty 18 hour and ten 4 day cultured palates from a total of thirty *TGF*-β_3 *+/+*, *-/-* and TGF-β_3 treated, *TGF*-β_3 *-/-* mice were processed for histology. Haematoxylin and eosin staining was performed following standard procedures. Sections were studied using a Nikon Optiphot light microscope and photographed with a Nikon FX 35A camera.

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