Development 126, 3869-3879 (1999)
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DEV1377



# Pathogenesis of cleft palate in TGF-β3 knockout mice

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*Accepted 1 June; published on WWW 5 August 1999*

## SUMMARY

**We previously reported that mutation of the transforming growth factor-β3 (TGF-β3) gene caused cleft palate in homozygous null (–/–) mice. TGF-β3 is normally expressed in the medial edge epithelial (MEE) cells of the palatal shelf. In the present study, we investigated the mechanisms by which TGF-β3 deletions caused cleft palate in 129 × CF-1 mice. For organ culture, palatal shelves were dissected from embryonic day 13.5 (E13.5) mouse embryos. Palatal shelves were placed singly or in pairs on Millipore filters and cultured in DMEM/F12 medium. Shelves were placed in homologous (+/+ vs +/+, –/– vs –/–, +/– vs +/–) or heterologous (+/+ vs –/–, +/– vs –/–, +/+ vs +/–) paired combinations and examined by macroscopy and histology. Pairs of –/– and –/– shelves failed to fuse over 72 hours of culture whereas pairs of +/+ (wild-type) and +/+ or +/– (heterozygote) and +/–, as well as +/+ and –/– shelves, fused within the first 48 hour period. Histological examination of the fused +/+ and +/+ shelves showed complete disappearance of the midline epithelial seam whereas –/– and +/+ shelves still had some seam remnants.**

**In order to investigate the ability of TGF-β family members to rescue the fusion between –/– and –/– palatal shelves in vitro, either recombinant human (rh) TGF-β1, porcine (p) TGF-β2, rh TGF-β3, rh activin, or p inhibin was added to the medium in different concentrations at specific times and for various periods during the culture. In untreated organ culture –/– palate pairs completely failed to fuse, treatment with TGF-β3 induced complete palatal fusion, TGF-β1 or TGF-β2 near normal fusion, but activin and inhibin had no effect. We investigated ultrastructural features of the surface of the MEE cells using SEM to compare TGF-β3-null embryos (E 12.5-E 16.5) with +/+ and +/– embryos in vivo and in vitro. Up to E13.5 and after E15.5, structures resembling short rods were observed in both +/+ and –/– embryos. Just before fusion, at E14.5, a lot of filopodia-like structures appeared on the surface of the MEE cells in +/+ embryos, however, none were observed in –/– embryos, either in vivo or in vitro. With TEM these filopodia are coated with material resembling proteoglycan. Interestingly, addition of TGF-β3 to the culture medium which caused fusion between the –/– palatal shelves also induced the appearance of these filopodia on their MEE surfaces. TGF-β1 and TGF-β2 also induced filopodia on the –/– MEE but to a lesser extent than TGF-β3 and additionally induced lamellipodia on their cell surfaces. These results suggest that TGF-β3 may regulate palatal fusion by inducing filopodia on the outer cell membrane of the palatal medial edge epithelia prior to shelf contact. Exogenous recombinant TGF-β3 can rescue fusion in –/– palatal shelves by inducing such filopodia, illustrating that the effects of TGF-β3 are transduced by cell surface receptors which raises interesting potential therapeutic strategies to prevent and treat embryonic cleft palate.**

Key words: Palatal development, Cleft palate, Medial edge epithelial cells, Transforming growth factor-β3 (TGF-β3), Transgenic mouse, Filopodia, Lamellipodia, Cell adhesion molecules, Organ culture

## INTRODUCTION

The birth defect, cleft palate arises from a failure in the multi-step process of palate development. These steps include palatal shelf growth, elevation, fusion between paired shelves, and disappearance of the midline epithelial seam with migration and differentiation of mesenchymal cells (Ferguson, 1988). Molecular mechanisms underlying these events are being elucidated and cleft palate likely results from defects in multiple genes. Recent investigations utilizing transgenic mice have resulted in the production of cleft palate and hence the implication of a number of genes in palate development, including cytokines and their receptors (e.g., TGF-alpha/EGF receptor (Miettinen et al., 1999), TGF-β2 (Sanford et al., 1997), TGF-β3 (Proetzel et al., 1995; Kaartinen et al., 1995), activin-βA (Matzuk et al., 1995a), activin-receptor type II (Matzuk et al., 1995b), follistatin (Matzuk et al., 1995c), retinoic acid receptor gamma (Lohnes et al., 1993, 1994), endothelin (Kurihara et al., 1994) and sek4/nuk1 (Orioli et al., 1996), and homeobox genes and transcription factors (e.g.,

Barx1, Tissier-Seta et al. (1995); Msx1, Satokata and Maas (1994); Msx2, Winograd et al. (1997); Hoxa2, Gendron-Maguire et al. (1993), Rijli et al. (1993); Mhox, Martin et al. (1995); Pax9, Peters et al. (1997); Dli2, Dli3, Mo et al. (1997); rae28, Takihara et al. (1997) and deltaEF1, Takagi et al. (1998). The temporal and spatial expression of TGF-β1, TGF-β2 and TGF-β3 during development of the mouse palate have been extensively examined. These experimental studies suggest that TGF-βs act as regulators at the stages of palatal shelf elongation and fusion. TGF-β3 expression is localized in the medial edge epithelium just before secondary palatal fusion (E13.5 in mice) (Pelton et al., 1990; Fitzpatrick et al., 1990). On the following day (E14.5), the expression of TGF-β1 was detected in the MEE, whereas that of TGF-β2 was limited to the mesenchymal cells. Construction of TGF-β3 knockout mice demonstrated that TGF-β3-null mutant embryos developed cleft palate so that all TGF-β3-null pups died within 24 hours after birth (Proetzel et al., 1995). The unique feature of the TGF-β3 knockout mice is that no other morphological anomalies occur in the craniofacial region or in other organs, with the exception of the lung. In sharp contrast, TGF-β1 knockout mice do not develop cleft palate (Shull et al., 1992; Kulkarni et al., 1993). Cleft palate was observed but at lower incidence rates (23%) in TGF-β2 knockout mice (Sanford et al., 1997) which also exhibit multiple additional anomalies. Moreover, the proposition that TGF-β3 plays a crucial role in palatal shelf fusion was substantiated by the fact that inhibition of normal TGF-β3 activity either by antisense oligonucleotide or neutralizing antibody, resulted in a failure of palate fusion in vitro (Brunet et al., 1995). Here we investigate the molecular mechanisms by which TGF-β3 regulates normal and cleft palate formation using wild-type and TGF-β3-null mice.

## MATERIALS AND METHODS

### Genotyping of TGF-β3 knockout mice

We used the Manchester colony of TGF-β3 knockout mice (129 × CF1), which were previously constructed by Proetzel et al. (1995). Mice heterozygous with respect to the TGF-β3 gene (+/–) were mated to produce TGF-β3-null embryos (–/–), as well as heterozygous (+/–) and homozygous normal (+/+) embryos. The day of finding a vaginal plug was designated day 0.5. Pregnant mice were killed on embryonic days 12.5-16.5 (E13.5-16.5) and the embryos recovered. Embryos were individually labelled and decapitated for further study and the bodies used for genotyping by PCR. Genomic DNA was prepared as described by Laird et al. (1991). PCR analysis used two sets of primers for TGF-β3: one primer set was 5′ TGGGA GTCAT GGCTG TAACT 3′ (in intron 5) and 5′ GATGC GATGT TTCGC TTGGT 3′ (in pMC1neo). These primers amplified a 700 bp fragment for the mutated allele. PCR conditions were 31 cycles of 95°C for 20 seconds, 59°C for 20 seconds, 72°C for 1 minute, followed by one cycle of 72°C for 10 minutes. Another primer set was 5′ TGGGA GTCAT GGCTG TAACT 3′ (in intron 5) and 5′ CACTC ACACT GGCAA GTAGT 3′ (in intron 6). PCR products were a 400 bp fragment for the wild-type TGF-β3 allele and a 1,300 bp fragment for the mutated allele. PCR conditions were 31 cycles of 95°C for 20 seconds, 56ºC for 25 seconds, 72°C for 1 minute, followed by one cycle of 72°C for 10 minutes. PCR products were analyzed by Southern analysis using an ethidium bromide stained 2% agarose gel. Careful labelling of all samples enabled the genotype to be assigned to individual palatal shelves in various experimental conditions.

### Palatal shelf organ culture

Palatal shelves were cultured according to the method of Brunet et al. (1995). Briefly, palatal shelves were removed at E13.5 or 14.5 from –/–, +/– or +/+ embryos (Fig. 1). The dissected palatal shelves were placed on 0.8 μm Millipore filters keeping the paired shelves with their medial edge epithelia (MEE) in close apposition without apparent distortion of the tissue shape. Care was taken to discern the anterior-posterior orientation of each shelf because it is known that fusion normally progresses along an anterior-posterior gradient in vivo. To facilitate alignment of palates into pairs the Millipore paper was cut in the shape of a trapezium so that the anterior portion of both paired shelves was always placed on the pin-pointed side. Various genotypic combinations of paired shelves were cultured as follows: (1) +/+ and +/+, +/– and +/–, –/– and –/–, in which the paired shelves were collected from the same embryos, and (2) +/+ and +/–, +/+ and –/–, +/– and –/–, in which the paired shelves were dissected from different embryos. The total number of palatal shelves used in the experiments are shown in Tables 1-4. Paired palatal shelves on Millipore filters were initially cultured using conventional Trowell organ culture techniques in Minimal Essential Medium (ICN, UK) supplemented with 300 μg/ml L-glutamate, 50 μg/ml glycine, 100 μg/mg ascorbate, 1% penicillin/streptomycin at 37°C in a 5% $CO_2$ air environment. After 6 hours culture the palate pairs were submerged in 20 μl Dulbecco's Minimal Essential Medium/Ham's F12 Growth Medium (DMEM/F12) 1:1 (ICN, UK), supplemented with 1% L-glutamine, 1% ascorbate and 1% penicillin/streptomycin for up to a further 96 hours. For the purposes of describing time-sequential changes of palatal fusion in vitro, the experimental time of transferring the tissue to the submerged system was designated as time zero. We investigated the effects on palatal fusion of various members of the TGF-β superfamily (Kingsley, 1994) at a range of concentrations added to the medium: agents tested were recombinant human (rh) TGF-β1 (100 ng/ml), porcine (p) TGF-β2 (100 ng/ml), rh TGF-β3 (10 ng/ml; 100 ng/ml) (TGF-β isoforms were purchased from R&D Systems, USA), rh activin (100 ng/ml, Autogen Bioclear, Belgium), and p inhibin (100 ng/ml, NIBSC, UK). By adding the TGF-β3 at various times we investigated the critical stage when it was required for shelf fusion in vitro using pairs of palatal shelves dissected from TGF-β3-null embryos at E14.5. Cultures were maintained for up to 48 hours using the same medium as used above, except for addition of rh TGF-β3 (10 ng/ml) at specified time schedules (see Tables). Times of fixing palatal shelves were 0, 12, 24, 36 and 48 hours for single palatal shelf culture and 0, 24, 48, 72 and 96 hours for palatal paired culture.

### Assessment of the normal and impaired fusion processes in vitro

At the end of the scheduled culture period, the paired specimens were examined macroscopically to determine the progression of shelf fusion. The categories and criteria adopted were: (1) total fusion having no apparent separation between the shelves, (2) partial fusion, and (3) non-fusion or complete discontinuity between the paired shelves. Histologic examination confirmed that fusion between the shelves was accompanied by gradual disappearance of the MEE and concomitant mesenchymal continuity. In some cases, the diagnosis was difficult because of degeneration of the oral epithelium, suggesting problems with the culture conditions. Those cases were excluded from further analyses and were not counted in the fusion ratios shown in Tables 1-4.

### Specimen preparation

For examination by light microscopy, all tissue specimens obtained in vivo and after culture were fixed in 4% paraformaldehyde, dehydrated in alcohol, immersed in chloroform, embedded in paraffin wax. Serial frontal sections, 4 μm thick, were prepared along the anterior-posterior axis of the separated shelves or fused palate and were stained with hematoxylin and eosin. For scanning electron microscopy

(SEM), palatal shelves obtained in vivo and after culture were processed as follows: all tissue specimens were fixed by 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) at 4ºC for 12 hours, rinsed in cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 2 hours. After dehydration using ethanol, the specimens were critical point dried with liquid $CO_2$. The dried specimens were coated with a thin layer of gold prior to observation in a Cambridge Stereoscan 360 microscope. Samples used for scanning electron microscopy (SEM) were 5 of each TGF-β3 genotype at E13.5, 15.5 and 16.5 and 8 of each genotype at E14.5 in vivo and were at least 5 of each genotype at 12, 24, 36 and 48 hours after the onset of culture at E13.5 in vitro. Quantification of surface structures on SEM images was carried out on images captured using a 3-CCD JVC colour camera with an 18-108/25 zoom lens. The software was PC Image (Foster Findlay Ass., USA) and 3-4 separate areas from each scan were analysed, each measuring 157.25 $\mu m^2$. Thresholding of the images allowed accurate counting of the surface structures.

For transmission electron microscopy (TEM), shelves were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4) at room temperature for 4 hours, rinsed in cacodylate buffer, postfixed in 1% osmium tetroxide containing 0.8% potassium ferrocyanide in 0.1 M sodium cacodylate for 2 hours. After embedding in Epon 812 resin (TAAB, England), ultrathin sections in silver or grey colour were cut and double-stained using uranyl acetate and lead citrate prior to observation in a JEOL 2000 EX II TEM. Ten samples of each genotype at E14.5 were used for TEM.

### Immunofluorescence

Cryosections (7μm) were cut through the developing palate of both wild-type and TGF-β3 knockout embryos at E14, using a Leitz 1720 Cryostat. The sections were fixed and incubated with a 1/100 dilution of primary antibody to either TGF-βRI or TGF-βRII (Santa Cruz, USA) for 1 hour at room temperature or overnight at 4°C. The antibodies recognise epitopes corresponding to amino acid sequences within the carboxy terminus of each receptor. A secondary FITC-linked antibody was used to detect binding of antibody to the receptors. Photographs were taken on a Leica DMRB microscope.

## RESULTS

### Palatogenesis in *TGF-β3*$^{+/+}$, *TGF-β3*$^{+/-}$ and *TGF-β3*$^{-/-}$ embryos in vivo

In all mouse embryos, the bilateral palatal shelves arose from the maxillary processes at E12.5, followed by their vertical growth down the sides of the tongue at E13.5 and their elevation to a horizontal position above the dorsum of the tongue around E14.5.

In +/+ and +/– embryos the horizontal growth of the bilateral shelves lead to the contact, adherence and fusion of their MEE to form the midline seam which disappeared between E14.5 and E15.5. The initial contact between the bilateral medial edges took place at an anteroposterior position corresponding to the second and third rugae, thereafter fusion spread anteriorly and posteriorly. During this intermediate stage of palatal closure between E14.5 and E15.5, two gaps usually remained visible anteriorly (smaller in size) and posteriorly (larger). No gaps were seen after E15.5. By E16.5, palatal fusion was complete histologically (Fig. 2A) with disappearance of the MEE seam throughout the entire palate (Fig. 2Ba,b,c,d). The first, second and third rugae were continuous on each side across the central fusion line (Fig. 2A).

All TGF-β3-null mice exhibited cleft palate. Clefts were of two principle types: partial cleft palate (frequent) and total cleft palate (rare). In the common partial cleft palates, a large gap posteriorly and a much smaller gap anteriorly remained even at E16.5 (Fig. 3A). When the shelves contacted in the partial clefts, fusion was abnormal as the first, second and third rugae did not become continuous at the central fusion line and this region also appeared thin in an oronasal direction. Partial palatal fusion between bilateral –/– shelves appeared to be arrested at the normal intermediatory stage characteristic of E14.5-15.5 +/+ embryos. In the anterior cleft, no fusion occurred between the bilateral palatal shelves and nasal septum (Fig. 3Ba). Histologically, in the partially fused region, a few elongated islands of the MEE seam persisted and the normal epithelial triangles at the junctions of the midline seam and the nasal/oral epithelia were markedly reduced or absent (Fig. 3Bb,c). The bilateral palatal shelves failed to fuse in the posterior cleft region but the mesenchymal cells condensed under the lining MEE and membranous ossification was initiated in adjacent regions (Fig. 3Bd). Histological examination showed that, in all genotypes, the epithelial cells lining the oral and nasal surfaces of the palate appeared similar morphologically: the oral squamous cells exhibited parakeratosis, while the nasal epithelial cells were ciliated columnar.

Immunocytochemistry using antibodies to TGF-βRI and RII revealed there were no differences in localisation or intensity

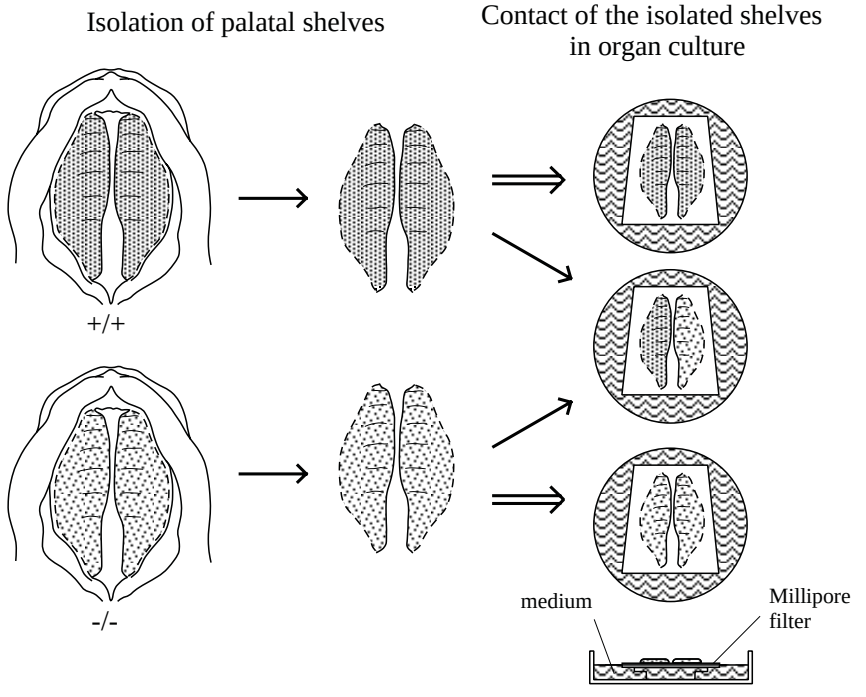


**Fig. 1.** Diagram of culture procedures. Palatal shelves were removed at E13.5 or 14.5 from –/– or +/+ embryos. Broken lines represent incision lines. A pair of the dissected shelves were placed on Millipore filters and then transferred to the culture system. Double arrows indicate the combination of the bilateral shelves, whereas the oblique single arrows indicate the recombination of shelves obtained from embryos with different genotypes.

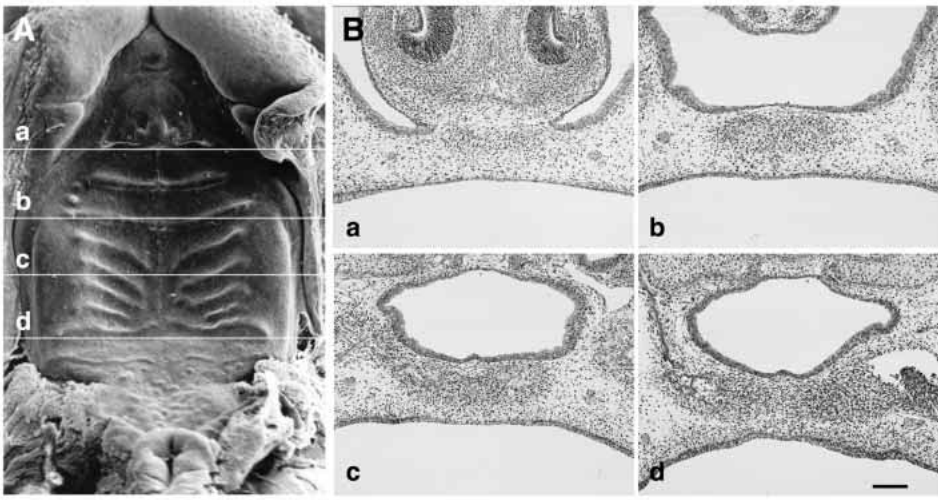


**Fig. 2.** (A) SEM images of the completely fused palate in the wild-type (+/+) embryo (E 16.5). (Ba-d) Micrographs of the serial frontal sections (HE stain). Note the disappearance of the MEE from anterior through posterior regions. Scale bar indicates 100 μm.

of TGF-β receptors between TGF-β3 knockout and wild-type palates in vivo (Fig. 4). Receptor I was observed in all the palate epithelia including the oral epithelium, nasal epithelium and medial edge seam (Fig. 4A,C) RII staining was intense in the MEE seam but weak in the oral and nasal epithelia (Fig. 4B,D). TGF-β3 staining was intense in wild type palatal MEE seam cells but absent in knockout palates. There was no difference in the intensity or distribution of TGF-β1 and TGF-β2 staining in the heads of wild-type and knockout mice.

## Palatogenesis in vitro

In a series of culture experiments using bilateral palatal shelves explanted at E13.5, there was absolutely no fusion at any anteroposterior level between the –/– pairs over 96 hours of culture (Table 1; Fig. 5C). By contrast, paired +/+ palatal shelves began to fuse after 24 hours (Table 1). Even at this time, nasal and oral epithelial triangles had formed and seam degeneration had commenced. By 72 hours in culture, fusion was complete and the midline seam had largely disappeared (Table 1; Fig. 5A). Fusion of pairs of +/– shelves progressed more slowly (Table 1) and was not complete until at least 96 hours in culture. The temporal sequence for each of the genotypes in culture was highly reproducible with only marginal fluctuation of a few hours.

When a –/– palatal shelf was paired with either a +/+ or +/– shelf, partial fusion occurred (Table 2; Fig. 5B). In such combinations the midline seam did not disappear completely and some epithelial islands were observed even up to 72 hours in culture. The oral and nasal MEE epithelial triangles on the –/– shelf side were very thin or missing (Fig. 5Ba). Complete fusion was never seen in these pairs and about 25% of pairs had not fused at all even after 72 hours (Table 2). Combinations of +/+ and +/– palatal shelves from different embryos fused normally and completely (Table 2) in a fashion similar to normal +/+ pairs (Table 1). –/– palate pairs from different animals failed to fuse (Table 2), a result identical to that obtained using –/– palatal pairs from the same embryo (Table 1).

Treatment of pairs of palatal shelves with rh TGF-β3 (10 ng/ml) induced complete palatal fusion (Table 3; Fig. 5D). For –/– palatal shelves cultured in the presence of TGF-β3 palatal fusion began at 24 hours and these palates were completely fused histologically by 72 hours. However only about 75% of the –/– palatal pairs were induced to fuse by exogenous TGF-β3 (Table 3). Exogenous addition of TGF-β3 to pairs of +/+ or +/– palates accelerated their fusion macroscopically and histologically (Tables 1, 3). The same results were obtained with doses of TGF-β3 at 10-100 ng/ml. Experiments in which TGF-β3 was added for defined periods of time to the culture media and then removed by washing the cultures and replacing with control medium, followed by harvest at 72 hours showed that TGF-β3 need only be present for a very short time period at the start of the culture: 3-6 hours after the onset of

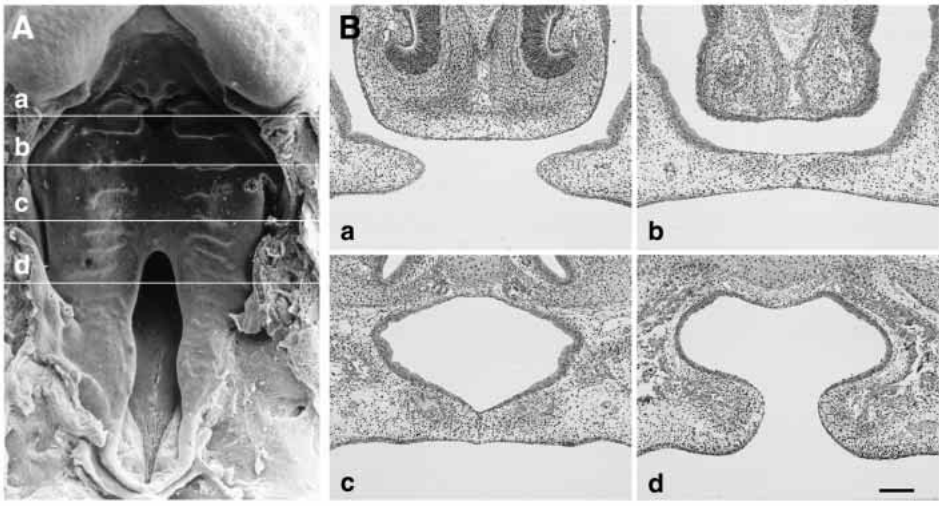


**Fig. 3.** (A) SEM images of the partially fused palate in the homozygous null TGF-β3 (–/–) embryo (E16.5). (B a-d) Micrographs of the serial frontal sections (HE stain). Note the small cleft in the anterior portion and a wide cleft in the posterior. Rugae were not present along the central fusion line of the anterior palate. Scale bar indicates 100 μm.

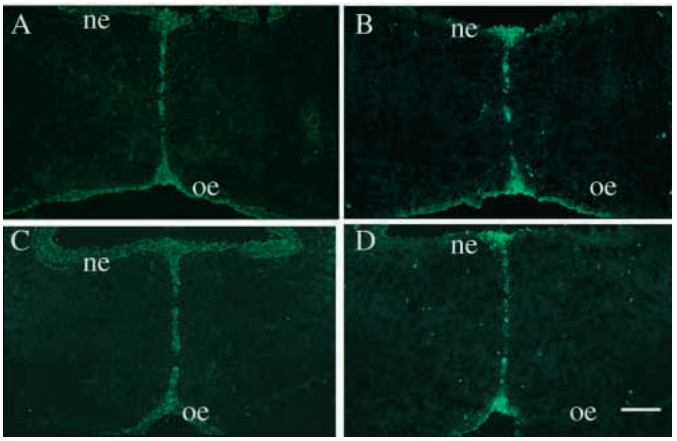


**Fig. 4.** Frontal cryosections through the developing palate at E14 from wild-type (A,B) and TGF-β3 knockout (C,D) embryos immunostained with antibodies to identify TGF-βRI (A,C) and TGF-βRII (B,D). ne, nasal epithelium; oe, oral epithelium. Scale bar, 150 μm.

submerged culture (Fig. 6). This fusion was histologically complete even with the brief 3-6 hours of TGF-β3 treatment. Exogenous TGF-β3 had no effect on –/– palatal fusion if given before 3 hours or after 12 hours in submerged culture (Fig. 6).

TGF-β1 and TGF-β2 also induced palatal fusion in the –/– shelves, but a much higher dose of each protein was necessary to induce fusion (Table 3). Moreover, this fusion was incomplete and patchy, often limited anteroposteriorly and over a short nasal-oral distance. Recombinant human TGF-β2 gave the same result as porcine TGF-β2 (data not shown). However, in the TGF-β1 or TGF-β2 treated –/– shelves the fusion was only partial when assayed histologically: the midline epithelial seam was largely intact with only partial disruption even after 72 hours culture (Fig. 5E,F; Table 3). The histological appearance was similar to that in the partially fusing zones of the –/– palates in vivo (Fig. 3C). Furthermore, exogenous addition of rh activin (5 pairs of –/– shelves) or p inhibin (3 pairs of –/– shelves) had no effect on palatal fusion. Occasionally the MEE cells between apposing shelves contacted each other but did not fuse or disappear. In these treatment cases, keratinization was observed. No bone formation occurred in any of the cultured palatal shelves.

## Ultrastructural appearance of the MEE of various genotypes in vivo and in vitro

Up to E13.5 in all genotypes, structures resembling short rods were observed on the surface of the MEE cells in vivo. These structures were scattered on the MEE cell surfaces and concentrated at intercellular junctions in both +/+ and –/– embryonic palates. Just prior to commencement of cell contact between the opposing MEE, at E14.5, a lot of filopodia like structures appeared on the surface of the MEE cells in +/+ embryos and the number of short rod structures decreased (Fig. 7A). By contrast, only short rod-like structures were visible on the MEE cells in –/– embryos at the same embryonic stage (Fig. 7B). Culture of single unpaired palatal shelves from wild-type embryos in normal medium also revealed the appearance of filopodia on the surface of the MEE cells (Fig. 7C). Filopodia began to appear after 12 hours in culture in +/+ embryos. Under identical in vitro conditions, single –/– palatal shelves did not develop filopodia (Fig. 7D) and resembled their in vivo appearance (Fig. 7B). Importantly, addition of TGF-β3 to the culture medium of single palatal shelves from –/– embryos induced the appearance of these filopodia on the surface of the MEE cells (Fig. 8A). Quantification of these surface structures using image analysis is summarised in Fig. 9. In untreated palates in vivo, 30.28% of the surface was covered with filopodia in wild-type mice, compared with only 8.58% in TGF-β3 knockout palates (Fig. 9A). In vitro the figures were similar, although the TGF-β3 null palates had

**Table 1. Incidence (%) of complete fusion in organ culture using bilateral palatal shelves dissected from individual embryos**

| Time of culture (hours) | Genotype of embryos (E 13.5) used | | |
|---|---|---|---|
| | –/– | +/+ | +/– |
| 0 | 0 (0/3) | 0 (0/3) | 0 (0/4) |
| 24 | 0 (0/7) | 60 (3/5) | 50 (4/8) |
| 48 | 0 (0/6) | 83 (5/6) | 71 (5/7) |
| 72 | 0 (0/5) | 100 (7/7) | 89 (8/9) |
| 96 | 0 (0/2) | 100 (1/1) | 100 (4/4) |

Figures in the parenthesis correspond to the fused cases versus the total number of cultured shelf pairs.

**Table 2. Incidence (%) of complete fusion in organ culture using different palatal shelf combinations at E13.5**

| Time of culture (hours) | Genotype of embryos (E 13.5) used | | |
|---|---|---|---|
| | –/– vs –/– | –/– vs +/–* –/– vs +/+ | +/– vs +/+* +/– vs +/– +/+ vs +/+ |
| 0 | 0 (0/2) | 0 (0/3) | 0 (0/10) |
| 24 | 0 (0/1) | 86 (6/7) | 69 (9/13) |
| 48 | 0 (0/1) | 80 (4/5) | 100 (13/13) |
| 72 | 0 (0/1) | 75 (3/4) | 100 (2/2) |

*No significant differences were obtained in these combinations so the data were pooled.

**Table 3. Incidence (%) of fusion of pairs of palatal shelves in vitro with addition of various TGF-β isoforms to the culture medium**

| Agents used | Time of culture (hours) | Genotype of embryos (E 13.5) used | | |
|---|---|---|---|---|
| | | –/– | +/+ | +/– |
| rh TGF-β3 | 0 | 0 (0/3) | 0 (0/2) | 0 (0/4) |
| | 24 | 50 (1/2) | 100 (3/3) | 80 (8/10) |
| | 48 | 64 (9/14) | 100 (10/10) | 95 (18/19) |
| | 72 | 75 (3/4) | 100 (3/3) | 100 (4/4) |
| rh TGF-β1 | 0 | 0 (0/1) | 0 (0/2) | 0 (0/8) |
| | 24 | 60***** (3/5) | 86 (6/7) | 71 (5/7) |
| | 48 | 56***** (5/9) | 100 (8/8) | 100 (14/14) |
| | 72 | 50***** (1/2) | 100 (1/1) | 100 (1/1) |
| p TGF-β2 | 0 | 0 (0/1) | 0 (0/1) | 0 (0/2) |
| | 24 | 33***** (1/3) | 75 (3/4) | 75 (6/8) |
| | 48 | 50***** (5/10) | 100 (6/6) | 100 (7/7) |

Concentrations of TGF-β isoforms added to the culture media were rh TGF-β3: 10 ng/ml, rh TGF-β1: 100 ng/ml and p TGF-β2: 100 ng/ml.

*****Fusion of –/– palatal shelves with rhTGF-β1 or pTGF-β2 was partial and patchy both anteroposteriorly and oronasally as opposed to fusion with rhTGF-β3 which was complete.

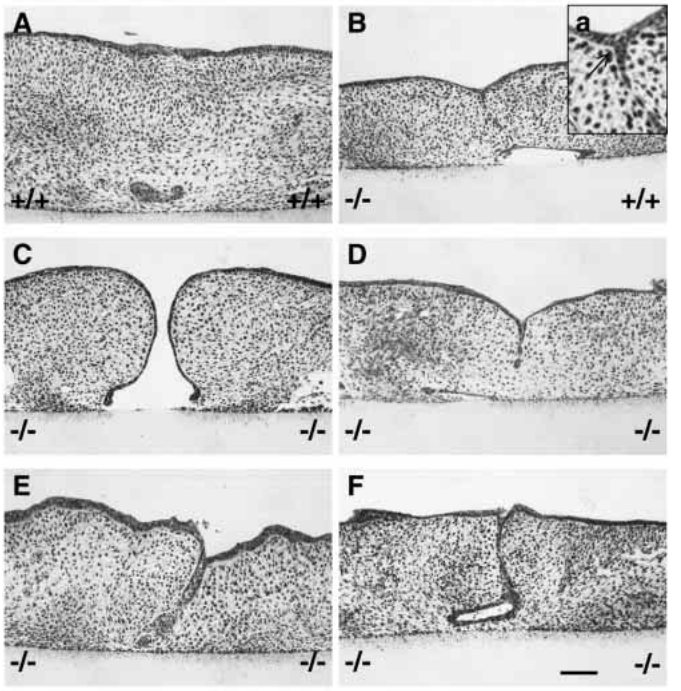


**Fig. 5.** Micrographs showing the outcome of recombination of embryonic palatal shelves in organ culture. (A) Complete fusion and disappearance of MEE using the paired +/+ shelves. (B) Progression of fusion but delay of midline seam disappearance in the recombined pair of –/– and +/+. (Ba) High-power micrograph of oral epithelial triangle from Fig. 4B. Arrow shows –/– shelf side of oral epithelial triangle. (C) No fusion between the paired –/– shelves after 96 hours in culture. (D-F) Rescue effected by TGF-β isoforms in culture media. The agents used and their concentrations were (D) rh TGF-β3 (10 ng/ml), (E) rh TGF-β1 (100 ng/ml), and (F) p TGF-β2 (100 ng/ml). All specimens were cultured for 72 hours. Note the continuity of mesenchymal cells with islands of MEE in the cultures with rh TGF-β3 (D). Addition of either TGF-β1 or TGF-β2 induced partial fusion (E,F). Their MEE seam remained prominent, as compared with cultures rescued by TGF-β3. Scale bar indicates 100 μm.

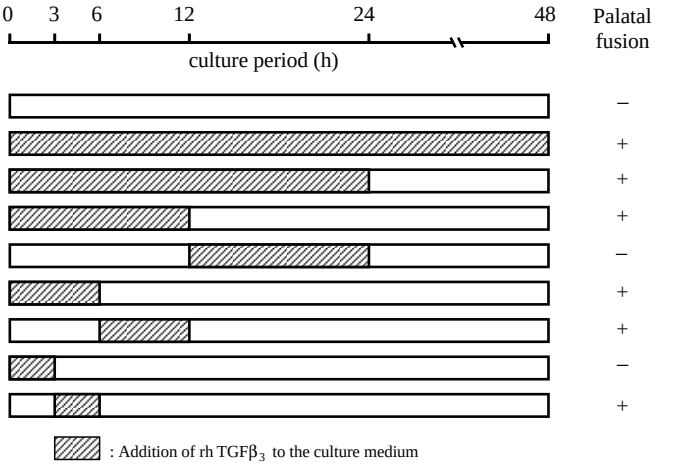


**Fig. 6.** Critical periods during which rh TGF-β3 rescued *TGF-β3*$^{-/-}$ palatal shelf fusion in culture. Exposure to rh TGF-β3 for only short periods, i.e., between 3 and 6 hours or between 6 and 12 hours, was sufficient to achieve fusion between –/– shelves. However, rh TGF-β3 could not rescue fusion if given before 3 hours or after 12 hours in culture.

even fewer surface structures (32.05% wild-type, 4.75% knockout; Fig. 9B). Upon treatment with recombinant TGF-β3, filopodia were induced significantly on the TGF-β3 knockout palates (49.1%) and also on the wild-type palates (53.1%; Fig. 9C).

Single palatal shelves from +/+ embryos cultured in medium supplemented with TGF-β3 developed more filopodia, which appeared earlier than in unsupplemented media. Furthermore, single palatal shelves from –/– embryos cultured with TGF-β1 or TGF-β2 also developed MEE surface structures: sparse filopodia but numerous lamellipodia (Fig. 8B,C). The number of filopodia induced by rh TGF-β1 or p TGF-β2 was less than that induced by rh TGF-β3, even though these proteins were at a much higher dose than TGF-β3. Further, no lamellipodia were found in cultures treated with TGF-β3, but they were numerous in cultures treated with TGF-β1 or TGF-β2.

By TEM the MEE cells of all genotypes consisted of two layers: an outer flat cell layer and an inner cuboidal cell layer along the basement membrane. In the wild-type embryos filopodia were observed on the surface of the MEE cells (Fig. 10A). Their cytoplasm contained actin filaments, whilst the surface of the filopodia was covered with filamentous material which stained positively with ruthenium red indicating the presence of proteoglycan (Fig. 10A,B). The proteoglycan rich filopodia structures were absent from the MEE of the –/– embryos which instead had a flat surface with short rod-like processes on their surfaces (Fig. 10C,D).

## DISCUSSION

All *TGF-β3*$^{-/-}$ mouse embryos had a cleft palate, whereas all *TGF-β3*$^{+/-}$ or *TGF-β3*$^{+/+}$ embryos had a normal intact palate in vivo. In this study we show that organ culture of palatal shelves in defined medium produces a similar result, namely total failure of *TGF-β3*$^{-/-}$ shelves to fuse but complete fusion of pairs of either *TGF-β3*$^{+/-}$ or *TGF-β3*$^{+/+}$ palatal shelves. This result confirms that the pathogenetic mechanism underlying cleft palate in the *TGF-β3*$^{-/-}$ mouse is a failure of palatal shelf fusion.

Interestingly, this failure of fusion of *TGF-β3*$^{-/-}$ palatal shelves can be partially rescued in vitro. Co-culture of a –/– palatal shelf with either a +/+ or +/– palatal shelf resulted in fusion in approximately 75% of cases although the morphologies of midline seam disappearance and epithelial triangles were altered. This suggests that TGF-β3 protein synthesized in the +/+ or +/– shelf diffuses across and induces phenotypic changes in the *TGF-β3*$^{-/-}$ MEE so allowing them to fuse, and that TGF-β3 normally signals by an autocrine/paracrine (as opposed to an intracrine) mechanism in the palatal MEE. The normal distribution of TGFβ receptors on the MEE of wild-type and knockout palates is in keeping with these findings. These co-culture experiments also suggest that the dose of TGF-β3 required to induce MEE fusion is low: a suggestion strengthened by the normal fusion of +/– palatal shelves in vivo and in vitro despite an estimated 50% reduction in TGF-β3 levels.

These interpretations are given further credence by the

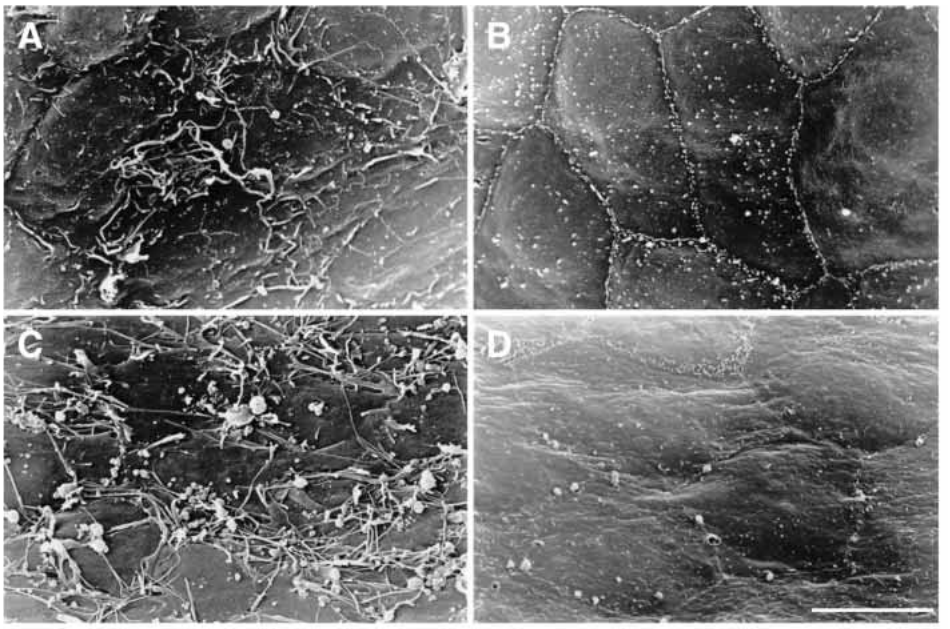


**Fig. 7.** SEM micrographs showing the membrane surfaces of MEE cells in TGF-β3-null (–/–) and wild-type (+/+) embryos in vivo and in vitro. (A) At E14.5, just before fusion, a number of filopodia appear on the surface of the MEE in +/+ embryos. (B) However, none were observed in –/– embryos at the same time, although short rods were seen. When the wild-type palatal shelves were dissected at E13.5 and cultured for 24 hours (C), similar projections appeared on the cell surface but not on the –/– MEE (D). Scale bar indicates 5 μm.

experiments demonstrating that exogenous TGF-β3 added to the culture medium at doses of either 10 or 100 ng/ml induces fusion of about 75% of *TGF-β3*$^{-/-}$ palatal shelf pairs. Furthermore experiments involving the application of TGF-β3 to such cultures for defined time periods demonstrate that TGF-β3 is only required for a short time period of a few hours to induce MEE fusion in pairs of *TGF-β3*$^{-/-}$ palatal shelves. Previously we have inhibited palatal fusion in cultures of wild-type palatal shelves by addition of either neutralizing antibody or antisense oligonucleotides against TGF-β3 (Brunet et al., 1995). These inhibitors were also only required for a short time period: between 24-36 hours of culture of E13 palatal pairs (equal to 0-12 hours of culture of E14 palatal shelves). Collectively these data indicate that TGF-β3 is normally only active for a very short time to induce changes in the MEE and so allow palatal fusion. This makes sense in the context of normal palatal morphogenesis, otherwise the MEE of the vertical palatal shelves might fuse with the epithelium of the floor of the oral cavity. It may also explain a lot of earlier experimental data (Ferguson et al., 1984; Ferguson, 1988) indicating both that the timing of palatal fusion was a critical developmental event (which if disrupted would result in cleft palate) and that the ability of mouse palatal shelves to fuse with other embryonic structures in vitro depended critically on the age and differentiation status of the palatal shelves and the other embryonic epithelia (Ferguson et al., 1984). Finally a recent study (Sun et al., 1998) indicates that exogenous addition of TGF-β3 to cultured chick palatal shelves which normally do not fuse can induce abnormal fusion.

In situ hybridisation studies indicate that TGF-β3 is expressed in the palatal MEE cells between E13.5 and 15.5 (Pelton et al., 1990; Fitzpatrick et al., 1990). This suggests that

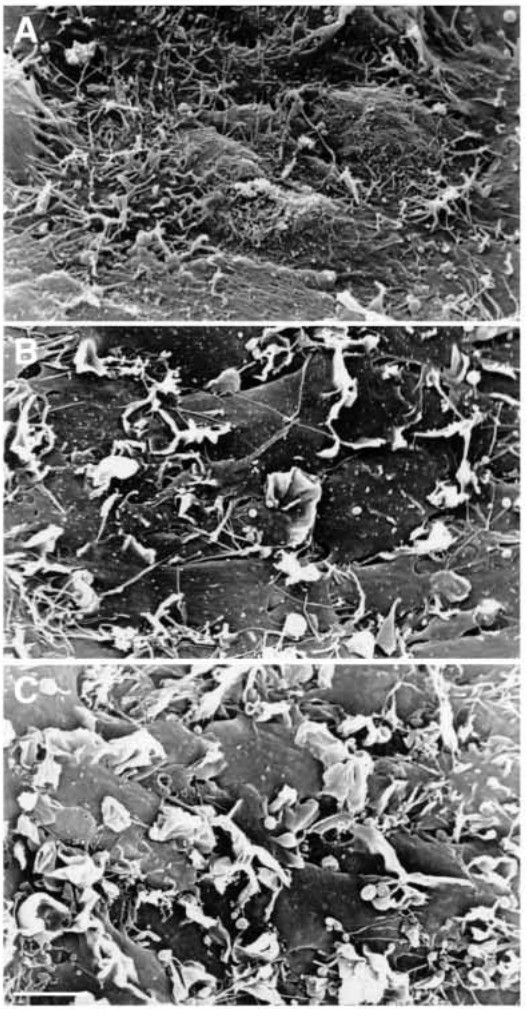


**Fig. 8.** SEM micrographs of the –/– MEE cells after culture for 12 hours with addition of TGF-β isoforms to the media. (A) TGF-β3 rescued palatal shelf fusion and also induced the formation of filopodia-like projections on the MEE surfaces. (B) TGF-β1 and (C TGF-β2 also modified the MEE cell surface structures but induced predominantly lamellipodia and some filopodia. Lamellipodia were not seen on the MEE of cultures treated with TGF-β3. Scale bar indicates 5 μm.

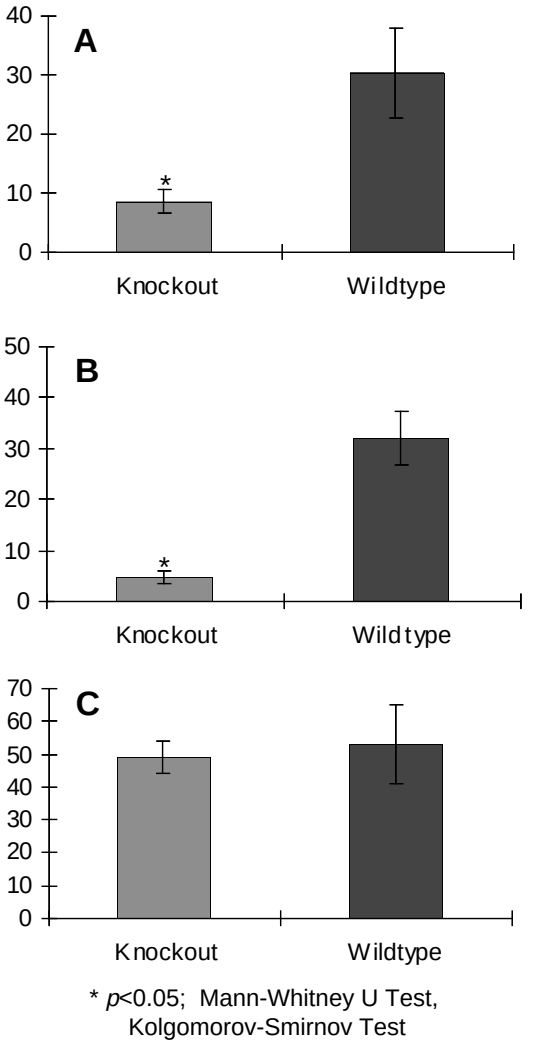


**Fig. 9.** Graphs showing the area of SEM micrographs covered by filopodia, expressed as a percentage of the total area, in wild-type and TGF-β3 knockout palates (A) in vivo ($P<0.05$, Mann-Whitney U test; Kolgomorov-Smirnov Test), (B) in vitro ($P<0.05$, Mann-Whitney U test; Kolgomorov-Smirnov Test), and (C) when treated in vitro with recombinant TGFβ3.

some other mechanisms such as translational regulation or activation of the latent TGF-β3 may be important in controlling the timing of the activity of TGF-β3 in vivo.

Importantly no other members of the TGF-β superfamily could induce the complete sequence of morphological changes seen in normal palatal fusion in *TGF-β3*$^{-/-}$ palatal shelves in culture. This suggests that TGF-β3 is recognized by a specific receptor complex in the palatal MEE capable of distinguishing TGF-β3 from the other TGF-β isoforms. Exogenous addition of TGF-β1 or TGF-β2 to *TGF-β3*$^{-/-}$ palatal shelves in vitro induced incomplete partial fusion. Often only a small anteroposterior section of the palatal shelf pairs fused and usually over a short oral-nasal distance. In all such cases the midline epithelial seam either did not form properly or persisted and failed to disappear. These effects are similar to the morphology of palatal fusion seen in vivo in the anterior part of the palate that fuses in partial cases of *TGF-β3*$^{-/-}$ cleft palate. This suggests that TGF-β1 or TGF-β2 may diffuse from the surrounding mesenchyme or from blood in the palatal circulation (TGF-β1 but not TGF-β2 or TGF-β3 are present in serum) in vivo and partially induce palatal fusion in the *TGF-β3*$^{-/-}$ palatal shelves. Such partial rescue does not occur in cultured *TGF-β3*$^{-/-}$ palatal shelves presumably because the TGF-β1 or TGF-β2 either diffuses out into the medium or is not present due to the absence of circulating blood in the palatal cultures. This view is supported by the decreased numbers of surface filopodia on the –/– palatal shelves in vitro compared to in vivo. Partial rescue may only occur over a defined anteroposterior region in vivo, as TGF-β1 is present at high concentrations in the osteogenic blastema. The fact that the fusion process is neither normal nor complete in the TGF-β1/TGF-β2 partially rescued *TGF-β3*$^{-/-}$ palatal shelves indicates once again the specificity of the TGF-β3 receptor and signalling pathways. Further evidence for such specificity comes from the fact that, despite the interaction of many intracellular signalling pathways even via SMADs (Imamura et al., 1997; Nakao et al., 1997), none of the numerous other growth factors present in the palatal MEE (e.g. FGFs, IGFs, PDGF etc.) appear capable of inducing fusion in *TGF-β3*$^{-/-}$ palatal shelves in vivo or in vitro. Neither TGF-β1 nor TGF-β2 knockout mice have isolated cleft palate (Shull et al., 1992; Kulkarni et al., 1993; Sanford et al., 1997). Further evidence for the specificity of TGF-β3 in inducing palatal fusion comes from the results of the present experiments in which the addition of either activin or inhibin did not induce palatal fusion in cultured *TGF-β3*$^{-/-}$ palatal shelves. Interestingly knockout mice where the genes for activin or activin-related molecules such as activin BA (Matzuk et al., 1995a), activin receptor type II (Matzuk et al., 1995b) and the activin binding protein follistatin (Matzuk et al. 1995c) have been deleted showed cleft palate as part of a complex phenotype in a small percentage of cases. These cleft palates may be the result of altered craniofacial anatomy rather than a failure of palatal fusion as cleft palate is a common occurrence in knockout mice usually as a result of physical effects on palatal shelf elevation (Ferguson, 1994). This hypothesis can be tested by culturing pairs of palatal shelves from the various activin knockout mice.

Importantly this study suggests the mechanisms by which TGF-β3 induces normal palatal MEE fusion and where its absence leads to cleft palate. Normally in vivo numerous filopodia appear on the surface of the MEE cells in +/+ and +/– palates just before contact of the opposing shelves, but they are absent in the *TGF-β3*$^{-/-}$ MEE. Likewise in vitro, *TGF-β3*$^{-/-}$ palatal shelves have no filopodia and fail to fuse whereas +/+ or +/– palatal MEE develop filopodia and these palatal shelves fuse. Exogenous addition of TGF-β3 to the culture medium both induced filopodia on the MEE of the *TGF-β3*$^{-/-}$ palatal shelves and caused them to fuse. Further, exogenous addition of TGF-β3 to cultures of +/+ or +/– palatal shelves induced filopodia earlier and in greater numbers than untreated cultures: such treated cultures also fused earlier. Finally the appearance of filopodia on the palatal MEE correlates with the timing of the presence of active TGF-β3 in the wild-type MEE in vivo and in vitro. Collectively, these data suggest that TGF-β3 causes filopodia to form on the surface of palatal MEE cells and that these filopodia are essential for normal palatal fusion. This view is strengthened by the observation that exogenous addition of TGF-β1 or TGF-β2 to cultures of *TGF-β3*$^{-/-}$ palatal shelves induces only a few filopodia but many lamellipodia on

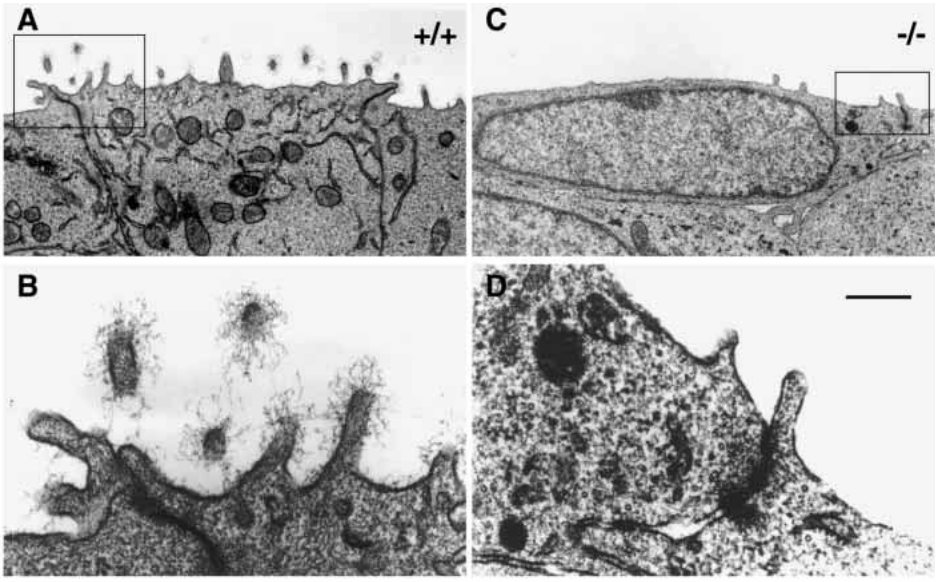


**Fig. 10.** TEM micrographs of MEE cells in the wild-type (+/+) and null mutant (–/–) palates (E14.5). (A) The wild-type MEE cells possessed a number of long projections (filopodia) on their surfaces, some of which were seen as isolated vesicles due to the plane of section. (B) Enlargement of boxed area in A. Note the dense proteoglycan-like filaments extending from the filopodia surfaces. (B) The –/– MEE possessed only a few short projections and no filamentous proteoglycan-like structures were visible (D) Enlargement of boxed area in C. Scale bar indicates 1 μm in A and C, 200 nm in B and D.

the surfaces of their MEE and that such treatments result in a partial abnormal palatal fusion discussed earlier. This observation also suggests how TGF-β3 might exert its isoform-specific effect. Recently Nobes and Hall (1995) reported that the small GTPases cdc42, rac and rho differentially induced filopodia and lamellipodia when microinjected into cultures of 3T3 fibroblasts. Injected cdc42 induced filopodia whereas ras and rho generated lamellipodia. These small GTPases control focal adhesion complexes and actin polymerization leading to the formation of filopodia or lamellipodia. This suggests that specific binding and signalling of TGF-β3 may induce active cdc42 and hence filopodia whereas specific binding and signalling from TGF-β1 and TGF-β2 may induce rac and/or rho and hence lamellipodia. This hypothesis is supported by the observation that TGF-β1 and TGF-β2 induce stress fiber bundles of alpha smooth muscle actin in fibroblasts (myofibroblasts): a rac/rho mediated event whereas TGF-β3 does not (Serini and Gabbianni, 1996).

These filopodia on the surface of normal prefusion MEE cells have been observed in previous descriptive accounts of palatal morphogenesis (Waterman et al., 1973, Schupbach and Schroeder, 1983) but we are the first to demonstrate their regulation and the crucial role they play in palatal fusion. How might they do this? First the filopodia will greatly increase the surface area of the MEE available for fusion. Second the filopodia may have concentrations of cell adhesion molecules or desmosomes (as have been described in other systems: Gumbiner, 1996) which would greatly facilitate fusion. In this regard it is of interest to note our observations of copious proteoglycan molecules on the surface of filopodia of the wild-type MEE but their absence from *TGF-β3*$^{-/-}$ palates in vivo when viewed by TEM. This suggests that these proteoglycan structures may be cell adhesion molecules which facilitate palatal fusion. Such an hypothesis is supported by the fact that palatal fusion is blocked by diazo-oxo-norleucine which is an inhibitor of glycosaminoglycan and glycoprotein synthesis (Green and Pratt, 1977). Interestingly in other systems TGF-β3 has been shown to stimulate the synthesis of perlecan (a heparin sulphate proteoglycan which also binds to TGF-β3; Iozzo et al., 1997) and hyaluronic acid (Ellis and Schor, 1998). Third, the filopodia will reduce the free surface charge energy of the MEE: something which is important in the contact and fusion of opposing palatal shelves (Curtis and Buultjens, 1973). Finally the filopodia may be diagnostic of increased motogenic and migratory activity. Such motogenic and migratory activity would be important in the rapid interdigitation of opposing palatal MEE cells following contact to secure a firm fusion: such rapid interdigitation is observed in vivo. Increased motogenic and migratory activity of the MEE would also be important for disruption of the midline epithelial seam either by epithelial migration into the triangles and onto the oral and nasal surfaces (Carrette and Ferguson, 1992) or by epithelial-mesenchymal transformation (Fitchett and Hay, 1989, Griffith and Hay, 1992,, Schuler et al., 1991, 1992). Interestingly *TGF-β3*$^{-/-}$ shelves show poor seam degeneration and epithelial triangle formation when they partially fuse in vivo or in vitro (partial rescue by TGF-β1 or TGF-β2, +/+ versus +/– shelf co-culture) indicating defective motogenic and migratory activity. Thus TGF-β3 may stimulate palatal medial edge epithelial cell adhesion, midline seam formation and disruption by a similar mechanism of inducing filopodia, motogenic and migratory activity. Such unified action through a single mechanism may explain why cultures of single isolated palatal shelves become transiently denuded in vitro as their MEE migrate (Ferguson et al., 1984). Interestingly TGF-β3 stimulates epithelial cell migration and transformation in other tissues e.g. cardiac cushions (Ramsdell and Markvald, 1997) and its differential effects on cell migration may explain its differential anti-scarring activity in wound healing (Shah et al., 1995, O'Kane and Ferguson, 1997). The thin midline fusion area in the partially fused *TGF-β3*$^{-/-}$ palates in vivo may be caused by defective cell migration and thus analogous to submucous cleft palate.

Importantly since extrinsic TGF-β3 can rescue palatal fusion and seam disruption in TGF-β3-null embryos in vitro, this suggests a possible novel therapeutic strategy to treat cleft palate in utero. Letterio et al. (1994) demonstrated that both exogenously administered and endogenous maternally produced TGF-β1 could pass from the maternal bloodstream across the placenta and into the embryos where it could rescue

the *TGF-β1*$^{-/-}$ phenotype. TGF-β1 occurs normally in the blood, TGF-β3 does not. Therefore if some cases of human cleft palate are caused by mutations in the TGF-β3 gene and if such cases could be detected by monitoring high risk (e.g., from family history) pregnancies using very high resolution ultrasound to detect failure of palatal shelf fusion, then in theory cleft palate could be treated in these embryos by intravenous administration of recombinant TGF-β3 to the mother. This TGF-β3 would cross the placenta and rescue palatal fusion in the defective embryos. The fact that this study shows that exogenous TGF-β3 is only required for a very short time in very low doses to rescue palatal fusion argues for the feasibility of such an approach. Furthermore exogenous TGF-β3 appears to have no adverse effects on palatal fusion in +/+ or +/– palates indicating that it may be safe in cases of a false negative diagnosis. However, much future in vivo experimentation will be required to demonstrate the feasibility and safety of such an approach.

We thank Tom Doetschmann and Gabi Proetzel from the University of Cincinnati for the initial collaboration generating the TGF-β3–/– mice. Cindy Qiu and Luci Bretznel kindly carried out immunostaining for the TGF-β receptors.

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