Developmental Biology **250,** 393–405 (2002)
doi:10.1006/dbio.2002.0792

TGF-β_3-Induced Chondroitin Sulphate Proteoglycan Mediates Palatal Shelf Adhesion

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In mammals, the adhesion and fusion of the palatal shelves are essential mechanisms in the development of the secondary palate. Failure of any of these processes leads to the formation of cleft palate. The mechanisms underlying palatal shelf adhesion are poorly understood, although the presence of filopodia on the apical surfaces of the superficial medial edge epithelial (MEE) cells seems to play an important role in the adhesion of the opposing MEE. We demonstrate here the appearance of chondroitin sulphate proteoglycan (CSPG) on the apical surface of MEE cells only immediately prior to contact between the palatal shelves. This apical CSPG has a functional role in palatal shelf adhesion, as either the alteration of CSPG synthesis by β-D-Xyloside or its specific digestion by chondroitinase AC strikingly alters the *in vitro* adhesion of palatal shelves. We also demonstrate the absence of this apical CSPG in the clefted palates of transforming growth factor beta 3 (TGF-β_3) null mutant mice, and its induction, together with palatal shelf adhesion, when TGF-β_3 is added to *TGF-β_3* null mutant palatal shelves in culture. When chick palatal shelves (that do not adhere *in vivo* nor express TGF-β_3, nor CSPG in the MEE) are cultured *in vitro,* they do not express CSPG and partially adhere, but when TGF-β_3 is added to the media, they express CSPG and their adhesion increases strikingly. We therefore conclude that the expression of CSPG on the apical surface of MEE cells is a key factor in palatal shelf adhesion and that this expression is regulated by TGF-β_3.

Key Words: **palate fusion; chondroitin sulphate proteoglycan; cleft palate; *TGF-β_3* null mutant mouse; chick.**

INTRODUCTION

The secondary palate in mammals forms by the union of the two palatal shelves that arise from the medial aspects of the maxillary processes of the first branchial arch. Initially, they grow down both sides of the tongue [embryonic day 13 (E13) in mice] and then elevate (E14) and approach each other, and the epithelium that covers their tips, the medial edge epithelium (MEE), contacts in the midline at E14.5 and adheres, forming the midline epithelial seam (Ferguson, 1988; Shuler *et al.*,1991). Through programmed cell death (Mori *et al.*, 1994; Taniguchi *et al.*, 1995; Martínez-Álvarez *et al.*, 2000a), epithelial–mesenchymal transformation (Fitchett and Hay, 1989; Griffith and Hay, 1992; Martínez-Álvarez *et al.*, 2000a), and migration to the oral and nasal aspects of the palate (Carette and Ferguson, 1992), the midline epithelial cells disappear, allowing continuity of the mesenchyme in the fusion zone at E15. Eventually, the mesenchyme of the anterior two-thirds of the palate undergoes intramembranous ossification and becomes the hard palate.

During palate fusion in mammals, the adhesion of the opposing MEE is a critical event whose alteration causes cleft palate (Newall and Edwards, 1981a,b; Pratt *et al.*, 1984; Abbott and Pratt, 1987). However, this process does not take place in all species. Unlike in mammals, avian palatal shelves approach and contact, but do not adhere *in vivo* (Greene *et al.*, 1983), resulting in a natural cleft. Some reptiles show an avian-like pattern of palate development, although in alligators and crocodiles, palatal shelves adhere after their contact and the fusion progresses until a continu-

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0012-1606/02 $35.00

ous palate is formed (reviewed in Ferguson, 1988). There is a species and tissue specificity in this adhesion process, as it fails, for instance, when mouse MEE is placed in contact with alligator MEE, tongue, or superficial maxillary epithelium (Ferguson *et al.*, 1984). Temporal specificity in palatal shelf adhesion has also been reported, as precociously removed palatal shelves do not adhere in culture (Pourtois, 1966; Humphrey, 1970; Smiley and Koch, 1971).

The initial adhesion of palatal shelves seems to be correlated with the appearance of certain changes in the most superficial MEE cells that occur just prior to their contact. These cells bulge on the MEE surface soon after palatal shelf elevation (Martínez-Álvarez *et al.*, 2000b) and develop microvilli (DeAngelis and Nalbandian, 1968; Hayward, 1969; Souchon, 1975), filopodia and lamellipodia (Waterman *et al.*, 1973; Meller *et al.*, 1980; Schüpbach *et al.*, 1983; Taya *et al.*, 1999) on their apical surface, whose function might be to increase the adhesion area between opposing MEE (Martínez-Álvarez *et al.*, 2000a). Unlike the basal MEE cells, the superficial MEE cells have an extensive Golgi apparatus and numerous cytoplasmic small coated vesicles (DeAngelis and Nalbandian, 1968), all characteristics compatible with apical secretion processes. The presence of these ultrastructural features correlates with the most striking change observed on the MEE surface just prior to the contact of palatal shelves: the appearance of a coat mostly formed by glycoconjugates (Greene and Kochhar, 1974; Souchon, 1975; Meller and Barton, 1978), whose experimental disruption *in vitro* causes the inhibition of palatal shelf adhesion (Greene and Pratt, 1977). By analyzing the lectin binding capacity of this superficial coat, Zschäbitz *et al.* (1994) demonstrated biochemical differences between the matrix that covers the MEE and other palatal epithelia, and suggested its importance in cell recognition and adhesion during palate fusion. Glycoprotein molecules have also been observed in the intercellular matrix interposed between the edges of the lens plate (Van Rybroeck and Olson, 1981; Yao *et al.*, 1996) and the neural primordium (Smits Van Prooije *et al.*, 1986; Trasler and Morriss-Kay, 1991) during their apposition, and there is experimental evidence showing that disruption in the synthesis or enzymatic degradation of these proteoglycans seriously disrupts the fusion process (Morriss-Kay and Crutch, 1982; Morriss-Kay and Tuckett, 1989; Alonso *et al.*, 1998). Among the different proteoglycans that could form part of these glycoconjugates and have a role during palatal shelf adhesion, our recent demonstration of the presence of chondroitin sulphate proteoglycan (CSPG) covering the superficial bulging MEE cells (Martínez-Álvarez *et al.*, 2000b), strongly points to its direct participation in this mechanism.

Disrupted palatal shelf adhesion causes cleft palate in the transforming growth factor beta 3 (TGF-β_3) null mice (Proetzel *et al.*, 1995; Kaartinen *et al.*, 1995; Martínez-Álvarez *et al.*, 2000a). *TGF-β_3* is strongly expressed in the MEE cells of the still vertical palatal shelves, and this expression persists until the midline epithelial seam disappears (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990). TGF-β_3 is a critical molecule for palatal fusion: its inhibition by antibodies or antisense oligodeoxynucleotides prevents *in vitro* palatal shelf adhesion in mice (Brunet *et al.*, 1995) and mutation of the *TGF-β_3* gene in both mice (Proetzel *et al.*, 1995; Kaartinen *et al.*, 1995) and humans (Lidral *et al.*, 1998; Mitchell *et al.*, 2001) results in cleft palate. Palatal shelves from *TGF-β_3* null mutant mice grow, elevate, and approach normally, but their adhesion fails due to disruption of an incompletely characterized adhesion mechanism. A striking decrease in the number of superficial bulging cells and filopodia present on the MEE surfaces of *TGF-β_3* null mutant mouse palatal shelves has been reported recently (Taya *et al.*, 1999; Martínez-Álvarez *et al.*, 2000b). While the *TGF-β_3* null palatal shelves appear ultrastructurally to have a reduced surface coat (Taya *et al.*, 1999), no information exists on the status of the MEE glycoconjugates. In other systems, TGF-βs regulate the synthesis of certain glycoconjugated components of the extracellular matrix (Ignotz and Massagué, 1986, 1987; Chimal-Monroy and Díaz de León, 1999), and TGF-βs have been shown to regulate the expression of chondroitin sulphate and dermatan sulphate proteoglycans in different tissues, epithelial and mesenchymal cells, and even in the palate (Bassols and Massagué, 1988; Sharpe and Ferguson, 1988; D'Angelo and Greene, 1991).

To investigate whether the presence of CSPG on the mouse MEE surface has a role in palatal shelf adhesion, we first established the timing of its expression during palate development and then altered its activity through the *in vitro* addition of β-D-Xyloside or chondroitinase AC. To investigate the influence of TGF-β_3 on the production of CSPG by MEE cells, we analyzed its expression in *TGF-β_3* null palates and explored whether the addition of TGF-β_3 to either *TGF-β_3* null mutant mouse or chick palate cultures stimulates both the expression of CSPG and palatal shelf adhesion. Our results demonstrate the presence of CSPG on the MEE surface only immediately prior to palatal shelf adhesion and that this CSPG is required for that adhesion to occur. We also show that *TGF-β_3* null palates lack CSPG on their MEE surfaces and that addition of TGF-β_3 stimulates the production of this proteoglycan by the superficial MEE cells, in both mice and chick, together with palatal shelf adhesion. We therefore conclude that the cleft palate produced by the absence of TGF-β_3 might be caused, at least in part, by disruption of the synthesis/secretion of CSPG by the superficial MEE cells, thus impeding the appropriate adhesion between palatal shelves.

MATERIALS AND METHODS

Animals

Albino Swiss (CD1) or C57BL/6J *TGF-β_3* heterozygous mice (Jackson Laboratories) were mated and the day of detecting the vaginal plug was designated day 0. Time-mated pregnant mice were killed by an overdose of chloroform, and the embryos were re-

moved by Caesarian section, placed in sterile cold 1/1 Dulbecco's modified Eagle's medium (DMEM/F12) (Sigma), and decapitated. Embryos to be used for culture experiments were removed under sterile conditions. Once heads were obtained, the jaw and tongue were removed. In C57BL/6J *TGFβ*$_3$ heterozygous mice, genotyping was performed as described in Proetzel *et al.* (1995).

Fertilized White Leghorn chick eggs (Granja Santa Isabel, Córdoba, Spain) were incubated at 37.5°C and 80% humidity for 8.5 days [stage 35 (35HH) of Hamburger and Hamilton, 1951]. Embryos were removed under sterile conditions and decapitated, and the heads were placed in sterile cold DMEM/F12.

Mouse and Chick Palate Cultures

Palatal shelves from E13.5 CD1, *TGF-*$\beta_3^{+/+}$, and *TGF-*$\beta_3^{-/-}$ mouse or 35HH chick heads were dissected under sterile conditions in DMEM/F12. Isolated or paired mouse palatal shelves and isolated chick palatal shelves were then placed on 2 × 2-mm Millipore filters (0.8-μm pore size) and cultured in Trowell's tissue culture, as described in Brunet *et al.* (1993). Cultures were incubated for 12 (isolated shelves), 24, or 36 (paired shelves) h at 37°C in a 5% CO_2 incubator. Chick paired palatal shelves were cultured for 45 h in 0.5% agar gels, as described in Sun *et al.* (1998a), using DMEM/F12 + 1% ascorbic acid as culture medium. Recombinant TGF-β_3 (10 ng/ml; Sigma) was added to the culture medium in those experiments requiring this condition. In all control and treated cultures, medium was replaced after 24 h.

In Vitro Inhibition of Chondroitin Sulphate Activity

To inhibit *in vitro* the biological activity of CSPG, we used two strategies. First, we disrupted the synthesis of CSPG by using the sulphated proteoglycan synthesis inhibitor P nitrophenyl β-D-Xylopyranoside (Sigma) (β-D-Xyloside). Second, we specifically digested CSPG by adding chondroitinase AC to the culture medium.

E13.5 mouse paired palatal shelves were cultured in DMEM/F12 in the presence of β-D-Xyloside (25 μl/ml of 10.8 mg/ml of sterile Hank's; Sigma). Medium was replaced after 24 h. Cultures were maintained for 36 h to allow palatal shelf fusion. Sterile Hank's or a similar amount of α-D-Xyloside (active anomere of β-D-Xyloside) was added to the medium in control cultures. All cultures were fixed in Carnoy fixative for 1 h and processed for histology.

A total of 20 μl/ml of a solution of 9 U.I. Chondroitinase AC (Sigma) in 450 μl of PBS was used to enzymatically digest CSPG. We also used 10 U.I. of Heparinase type II (Sigma) in 100 μl of PBS to specifically digest heparan sulphate in some culture experiments. Control cultures had the same amount of heat-inactivated enzymes added.

Table 1 shows the number of cultures studied per experiment.

TABLE 1
Palatal Shelf Adhesion in Mouse and Chick Palate Cultures

Type of palate culture	No. cultures studied	Average length of adhered/fused MEE
Controls	33	
Hank's	10	
α-D-Xyloside treated	10	2530 ± 327 μm (100%)
Inactivated enzymes treated	13	
β-D-Xyloside treated	38	943 ± 427 μm (37%)[a]
Chondroitinase AC treated	10	143 ± 105 μm (5%)[a]
Heparinase II treated	7	1600 ± 812 μm (63%)[a]
TGF-$\beta_3^{+/+}$	33	2801 ± 395 μm (100%)
TGF-$\beta_3^{-/-}$	12	970 ± 559 μm (34%)[a]
TGF-β_3 treated, TGF-$\beta_3^{-/-}$	10	2133 ± 379 μm (77%)[a]
Chick (controls)	17	400 ± 413 μm (100%)
TGF-β_3 treated, Chick	17	1500 ± 968 μm (375%)[a]

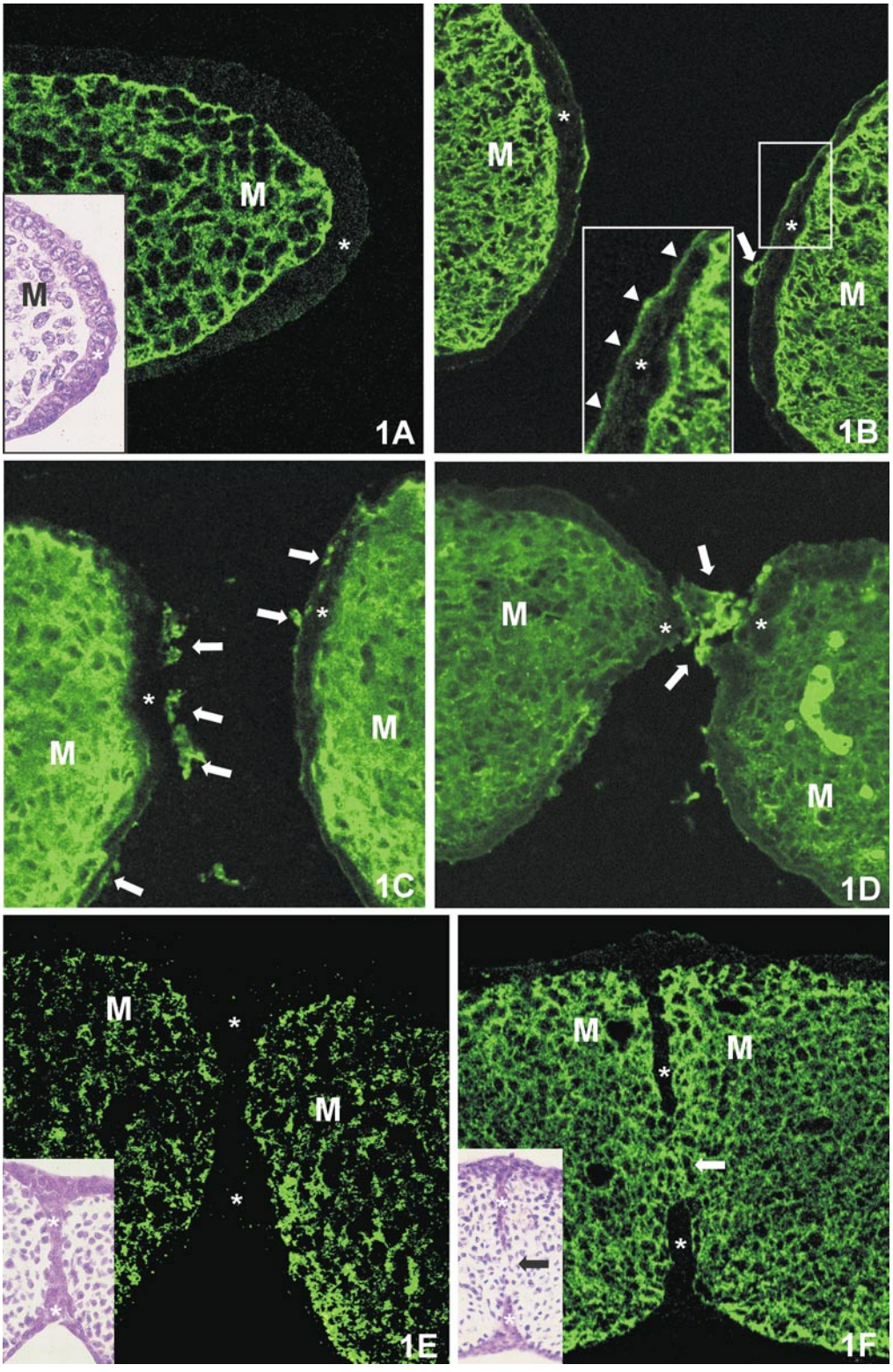
Note. Measurements are mean ± standard error. In all cases $P < 0.001$ by two-tailed Student's *t* test.

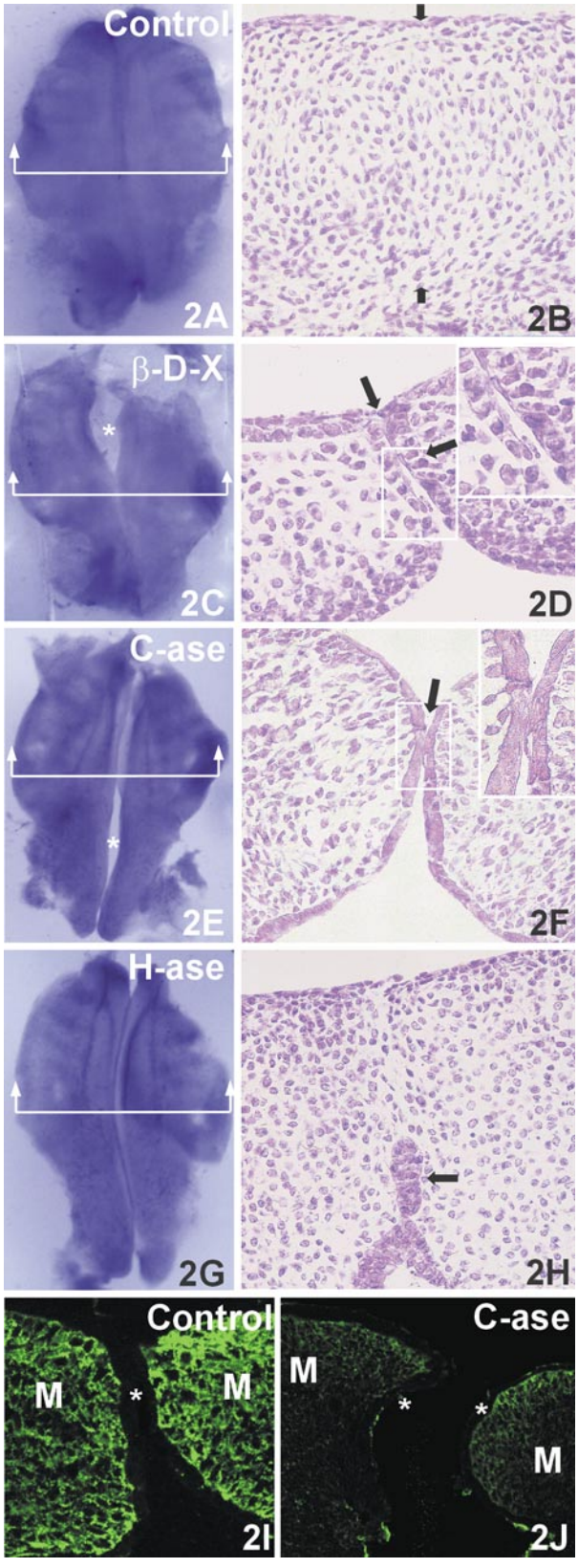
[a] Percentage of adhesion/fusion in each condition related to the adhesion/fusion obtained in control or TGF-$\beta_3^{+/+}$ palate cultures (considered 100%).

CSPG Immunohistochemistry

TGF-$\beta_3^{+/+}$ (and CD1) and *TGF-*$\beta_3^{-/-}$ mouse embryonic heads were carefully washed with saline solution to avoid tissue and blood contamination and then fixed in Carnoy fixative for 2 h at room temperature. Mouse and chick isolated palatal shelf cultures and *TGF-*$\beta_3^{+/+}$ (and CD1) and *TGF-*$\beta_3^{-/-}$ mouse palate cultures from different experimental conditions were fixed in Carnoy fixative for 1 h. No less than four different specimens per experimental condition were analyzed. Carnoy fixative was used because it maintains the carbohydrate epitope recognized by the CS-56 antibody used. Specimens were dehydrated in a graded ethanol series and embedded in paraffin following standard procedures. Sections (8-μm-thick) were preincubated in 1% PBS–BSA for 30 min and incubated overnight with the anti-chondroitin sulphate monoclonal antibody CS-56 (Sigma), which recognizes the glycosaminogly-

FIG. 1. E14.5 mouse palatal shelves immunolabeled with the anti-CSPG monoclonal antibody CS-56. (A) When palatal shelves are still far from each other, there is no anti-CSPG immunostaining on the MEE surface. Insert shows an adjacent section stained with hematoxylin and eosin. (B) As palatal shelves approach each other, an anti-CSPG positive material appears on the MEE surface, and the superficial bulging cells are surrounded by abundant CSPG (arrow). The boxed area is shown in the insert at higher magnification. Note the presence of anti-CSPG positive material on the MEE surface (arrowheads). (C) This apical CSPG (arrows) greatly increases when contact between palatal shelves becomes imminent. (D) Abundant CSPG containing material accumulates at the point of contact between palatal shelves (arrows). (E) However, when palatal shelf adhesion has occurred, no CSPG is observed in the midline epithelial seam. The insert shows an adjacent section stained with hematoxylin and eosin. (F) Islands of totally anti-CSPG negative epithelium remain in the midline when palatal mesenchyme becomes confluent (arrow). Notice that CSPG is never localized between mouse MEE cells. The insert shows an adjacent section stained with hematoxylin and eosin. Asterisk, medial edge epithelium. M, mesenchyme.





can moiety of native chondroitin sulfate proteoglycan (Avnur and Geiger, 1984). Sections were then reincubated in a fluorescein-conjugated goat anti-mouse IgM (Vector) for 30 min. Some sections were counterstained with propidium iodide (Molecular Probes) (1/12,000 dilution) for 30 min and all were mounted in Aquamount (GURR).

Samples were analyzed by using either an argon (488/514 λ) or helium/neon (543 λ) laser ZEISS LSM T-300 Scan Confocal Microscope. Digitalized images were recorded and overlapped when required.

Histological Preparation

Embryonic heads and palate cultures from different experimental conditions were fixed in 4% paraformaldehyde or Carnoy fixative, dehydrated in a graded ethanol series, and embedded in paraffin. Transversal sections (8-μm-thick) were hematoxylin and eosin-stained following standard procedures. Sections were studied by using a Nikon Optiphot light microscope and photographed with a Nikon FX 35A camera. To measure the length of the adhered opposing MEE in all palate cultures, a measuring grid inserted in a 20× ocular lens was used. The length of the adhered MEE in every 10 (mouse) or 20 (chick) sections taken from the middle 100 (mouse) or 200 (chick) sections of each palate culture was added. The average length of adhered MEE for each group was then calculated. The final values are expressed as the arithmetic mean ± standard error. For comparison of the average measurements between experimental and control samples, a two-tailed Student's *t* test for independent samples was applied and *P* values below 0.001 were interpreted as indicating statistical significance.

RESULTS

Expression Pattern of CSPG in the Medial Edge Epithelium

Immunolabeling of E14 (not shown) and E14.5 mouse palates with the anti-CSPG CS-56 monoclonal antibody

FIG. 2. Thirty-six-hour paired palate cultures untreated (A, B, and I) and treated with β-D-Xyloside (C and D), chondroitinase AC (E, F, and J) or heparinase II (G and H). (B, D, F, and H) Histological sections taken from the cultures shown in (A, C, E, and G), respectively, at the level indicated by the line. (I and J) Confocal images of anti-CSPG immunolabeled sections. (A and B) Paired palatal shelves cultured for 36 h always adhere/fuse. Arrows in (B) indicate the area of fusion. (C and D) However, when β-D-Xyloside is added to the culture medium, palatal shelf adhesion is greatly diminished (area between arrows in D), if not totally impeded (asterisk in C). (E and F) Similar results are obtained when chondroitinase AC is added to the culture medium, although the adhered area is frequently smaller (arrow in F). (G and H) When heparinase type II is added to the culture medium, palatal shelf adhesion is somewhat better and an epithelial seam forms (arrow in H). (I and J) Palate cultures treated with chondroitinase AC show a great decrease in the anti-CSPG immunolabeling in both the MEE (asterisk in J) and mesenchyme (M) when compared with controls (I). Opposing MEE (asterisk) have partially adhered in (I) and are separated in (J). The boxed areas in (D) and (F) are shown in the top right corner at higher magnification.

showed positive staining of the palatal mesenchyme in all embryos studied (Figs. 1A–1F). At E14, palatal shelves were either vertical alongside the tongue or had elevated and were positioned horizontal above the tongue. No anti-CSPG-positive material was observed among MEE cells nor on the MEE surface at this time point (not shown). At E14.5, the palatal shelves approach each other and have contacted in the middle third of the palate in most cases, forming the midline epithelial seam. No anti-CSPG-positive staining was observed on the MEE surface when palatal shelves are still far from each other (Fig. 1A). However, anti-CSPG-positive material covered most of the MEE surface when palatal shelves became closer (Figs. 1B and 1C), and CSPG-positive staining greatly increased when contact between palatal shelves was imminent (Fig. 1D). Positive staining was never noticed between basal MEE cells, but was observed surrounding the bulging superficial MEE cells (Fig. 1B).

In the zones where contact between palatal shelves had occurred, the midline epithelial seam showed totally negative anti-CSPG staining (Fig. 1E). However, as the midline epithelial seam disrupted, anti-CSPG-positive mesenchyme occupied the midline, leaving only small islands of totally negative anti-CSPG labeling (Fig. 1F).

These results indicate that a CSPG-containing material is located transiently on the MEE surface only prior to palatal shelf adhesion, and that this material is absent among basal MEE cells.

In Vitro Altered Chondroitin Sulphate Expression Interferes Palatal Shelf Adhesion

Palatal shelves cultured for 36 h in control conditions always fuse (Figs. 2A and 2B). However, the addition of the sulphated proteoglycan synthesis inhibitor P nitrophenyl β-D-Xylopyranoside (β-D-Xyloside) to the culture medium resulted in a complete absence of fusion of the palatal shelves, which remained either separated or minimally adhered after 36 h of culture (Fig. 2C). Histological sections taken from these cultures showed either no adhesion or minimal contact between opposing MEE (Fig. 2D, and Table 1). Similar results were obtained when the CSPG-degrading enzyme chondroitinase AC was added to the culture medium, although it seemed to alter palatal shelf adhesion more strikingly (Figs. 2E and 2F, and Table 1). Palatal shelf adhesion and fusion was somewhat altered when the heparan sulphate-degrading enzyme heparinase type II was added (Figs. 2G and 2H, and Table 1). The two-tailed Student's *t* test applied to the length of adhered/fused MEE in all specimens of each group showed $P < 0.001$ in all cases, thus indicating its statistical significance.

Immunolabeling of 36-h cultured palates with the anti-CSPG CS-56 monoclonal antibody showed a greatly reduced expression of CSPG in both the mesenchyme and the MEE surface in those cultures treated either with chondroitinase AC (Fig. 2J) or β-D-Xyloside (not shown) when compared with control (Fig. 2I) or heparinase-treated (not shown) palate cultures.

These observations indicate that CSPG is required on the opposing MEE to achieve palatal shelf adhesion *in vitro*.

TGF-β_3 Induces CSPG Expression by MEE Cells and Palatal Shelf Adhesion in Vitro

We immunolabeled E14.5 *TGF-$\beta_3^{+/+}$* and *TGF-$\beta_3^{-/-}$* mouse palate sections with the anti-CSPG CS-56 monoclonal antibody and stained cellular nuclei with propidium iodide to allow the observation of the entire MEE (Figs. 3A and 3B). E14.5 *TGF-$\beta_3^{-/-}$* almost contacting MEE showed a striking reduction of CSPG on their surfaces (Fig. 3B) when compared with controls (Fig. 3A). In order to determine the influence of TGF-β_3 on the production of CSPG by MEE cells, we cultured E13.5 *TGF-$\beta_3^{-/-}$* isolated palatal shelves for 12 h in the presence or absence of TGF-β_3. Since the MEE disappears when mouse isolated palatal shelves are cultured for 24 h or more (unpublished observation), we selected 12 h as the culture period for this experiment. To analyze differences in palatal shelf adhesion, paired palatal shelves were cultured for 24 h, since this period is sufficient for control palatal shelves to adhere and fuse. As expected, CSPG was never detected on the MEE surface in the *TGF-$\beta_3^{-/-}$* cultures (Fig. 3C). The addition of TGF-β_3 to both 12-h *TGF-$\beta_3^{-/-}$* isolated palatal shelf cultures and to 24-h *TGF-$\beta_3^{-/-}$* palate cultures resulted in an increase in both the expression of CSPG on the MEE surface and in palatal shelf adhesion that fairly mimicked the results obtained in the *TGF-$\beta_3^{+/+}$* palates (Figs. 3D–3J, and Table 1). *TGF-$\beta_3^{-/-}$* isolated palatal shelves cultured for 12 h in a medium containing 10 ng/ml TGF-β_3 clearly showed an anti-CSPG-positive material covering the MEE surface (Fig. 3D). In cultures of *TGF-$\beta_3^{+/+}$* paired palatal shelves, opposing MEE had almost completely adhered after 24 h (Fig. 3E), and as expected, the epithelial seam was totally anti-CSPG-negative (not shown). To analyze the presence of CSPG on the MEE, we selected those sections where adhesion between opposing MEE had partially occurred and noticed the presence of CSPG on the MEE surface (Fig. 3F). *TGF-$\beta_3^{-/-}$* paired palatal shelves cultured for 24 h had failed to adhere adequately (Fig. 3G, and Table 1) and showed no anti-CSPG-positive staining on the MEE surface (Fig. 3H). However, when TGF-β_3 was added to 24-h *TGF-$\beta_3^{-/-}$* palate cultures, an increase in palatal shelf adhesion occurred (Fig. 3I, and Table 1) together with the presence of CSPG on the MEE surface (Fig. 3J).

We finally investigated whether TGF-β_3 is able to stimulate the *in vitro* adhesion of chick palatal shelves and/or induce CSPG production by MEE cells. When cultured in agar gels for 45 h, paired chick palatal shelves adhered partially in most cases (Fig. 4A). The addition of TGF-β_3 to the culture medium strikingly increased palatal shelf adhesion, leading sometimes to palatal shelf fusion (Fig. 4B, and Table 1). To determine whether this effect of TGF-β_3 on chick palatal shelf adhesion correlates with the induction of

the synthesis of CSPG by MEE cells, we cultured isolated chick palatal shelves for 12 h with or without the addition of TGF-β_3 to the culture medium, and then immunolabeled the sections with the anti-CSPG CS-56 monoclonal antibody. Confocal visualization of the control cultures showed little or no anti-CSPG staining in the MEE (Fig. 4C). However, increased expression of CSPG both among (Fig. 4D) and over the MEE surface (Fig. 4E) was present in the TGF-β_3-treated cultures.

The two-tailed Student's *t* test applied to the length of adhered/fused MEE in all specimens of each group showed $P < 0.001$ in all cases, thus indicating its statistical significance.

Taken together, these results indicate that TGF-β_3 stimulates palatal shelf adhesion in both mouse and chick embryos while inducing the production of CSPG by MEE cells.

DISCUSSION

The CSPG-Containing Material That Covers the MEE Prior to Contact between Palatal Shelves Is Necessary for Palatal Shelf Adhesion

Our work demonstrates that the presence of CSPG covering the MEE apical surface prior to and during initial mouse palatal shelf adhesion is essential for palatal shelf adhesion. The presence of a glycoconjugate-rich cell coat, likely synthesized by MEE cells (Pratt and Hassell, 1975), was reported in earlier investigations to cover the MEE apical surfaces. This presence was demonstrated by using ruthenium red, a polyvalent cation that binds fairly specifically to polyanions such as glycosaminoglycans (GAGs) (Greene and Kochhar, 1974; Souchon, 1975), or by the preferential adherence of concanavalin-A to the MEE surface (Pratt and Gibson, 1973). This glycoconjugate surface coat was also shown to be required for the initial adherence of palatal MEE, as the addition to paired palatal shelf cultures of Diazo-oxo-norleucine, a glutamine analog that inhibits glutamine transfer reactions and thereby blocks glycosaminoglycans and glycoprotein synthesis, resulted in a striking inhibition of palatal shelf adhesion (Greene and Pratt, 1977).

Which glycoconjugates are responsible for palatal shelf adhesion was never determined. We demonstrate here the appearance of CSPG on the mouse MEE apical surface only immediately prior to palatal shelf adhesion and that this expression increases as the contact between palatal shelves becomes imminent. This temporal pattern of expression is in contrast with the observation of the appearance of the carbohydrate coat on the MEE surface 48 h prior to palatal shelf adhesion (Greene and Kochhar, 1974), although a thicker coat was observed shortly before fusion (Souchon, 1975). The CSPG detected here could be part of a more complex extracellular matrix covering the MEE surface, formed by different glycoconjugates (Brinkley *et al.*, 1992). The appearance of CSPG only at E14.5 suggests that the molecule containing it is secreted just prior to palatal adhesion and that this molecule is essential for such adhesion.

We also demonstrate that CSPG is absent among nonapical MEE cells during mouse palate development. This might be a characteristic of the mouse MEE. When the expression of CSPG was induced in *TGF-β_3* null mouse palate cultures through the addition of TGF-β_3, CSPG expression was only observed on the MEE surface and never among MEE cells. On the contrary, a great expression of CSPG was noticed among MEE cells (together with CSPG expression on the MEE surface) when this experiment was performed by using chick palates. This is in keeping with earlier investigations showing the absence of ruthenium red staining in intercellular areas in the mouse MEE (Greene and Kochhar, 1974; Souchon, 1975) but its presence in humans (Meller and Barton, 1978).

We have investigated whether CSPG is required for palatal shelf adhesion in mice. We specifically altered the synthesis of sulphated proteoglycans through the addition of the inhibitor P nitrophenyl β-D-Xylopiranoside (β-D-Xyloside) to paired palate cultures. This β-D-Xyloside acts as an artificial chain initiator of sulphated proteoglycans biosynthesis that stimulates the synthesis of short core-protein-free undersulphated chondroitin sulphate chains (Gibson *et al.*, 1979) and of heparan sulphate to a lesser extent (Sobue *et al.*, 1987). β-D-Xylosides have become a popular tool for studying the role of sulphated proteoglycans in a wide variety of biological processes. By using chondroitinase AC in separate experiments, we also specifically degraded CSPG in mouse paired palate cultures. After both treatments, immunolabeling with the CS-56 monoclonal antibody showed the absence of CSPG on the MEE surface and a striking reduction of the *in vitro* palatal shelf adhesion. In addition, our results indicate that treatment of palate cultures with heparinase type II alters palatal shelf adhesion less strikingly. This means that Heparan sulphate could also play a role in this process. These data confirm that palatal shelf adhesion depends on mechanisms localized in the apical surface of the MEE cells (Schüpbach *et al.*, 1983; Taya *et al.*, 1999) and that the synthesis and secretion of CSPG by the superficial MEE cells plays a crucial role in this adhesion. This statement has additional support in the simultaneous experimental stimulation by TGF-β_3 of the expression of the apical CSPG and palatal shelf adhesion in the chick, whose palatal shelves do not adhere *in vivo* (despite their contact during palatogenesis) and lack the apical CSPG. Moreover, abundant CSPG surrounds the superficial bulging MEE cells, which have been shown to be primarily involved in palatal shelf adhesion (Martínez-Álvarez *et al.*, 2000a,b). CSPG has been shown to be required in the adhesion of other epithelial edges, as during the fusion of the neural folds (Morris-Kay and Tuckett, 1989; Alonso *et al.*, 1998) or in the formation of the lens (Alonso *et al.*, 1996; Gato *et al.*, 2001). Interestingly, as with the CSPG immunostaining, the ruthenium red material observed on the MEE surface in earlier studies is absent when palatal shelf adhesion is accomplished (Souchon,

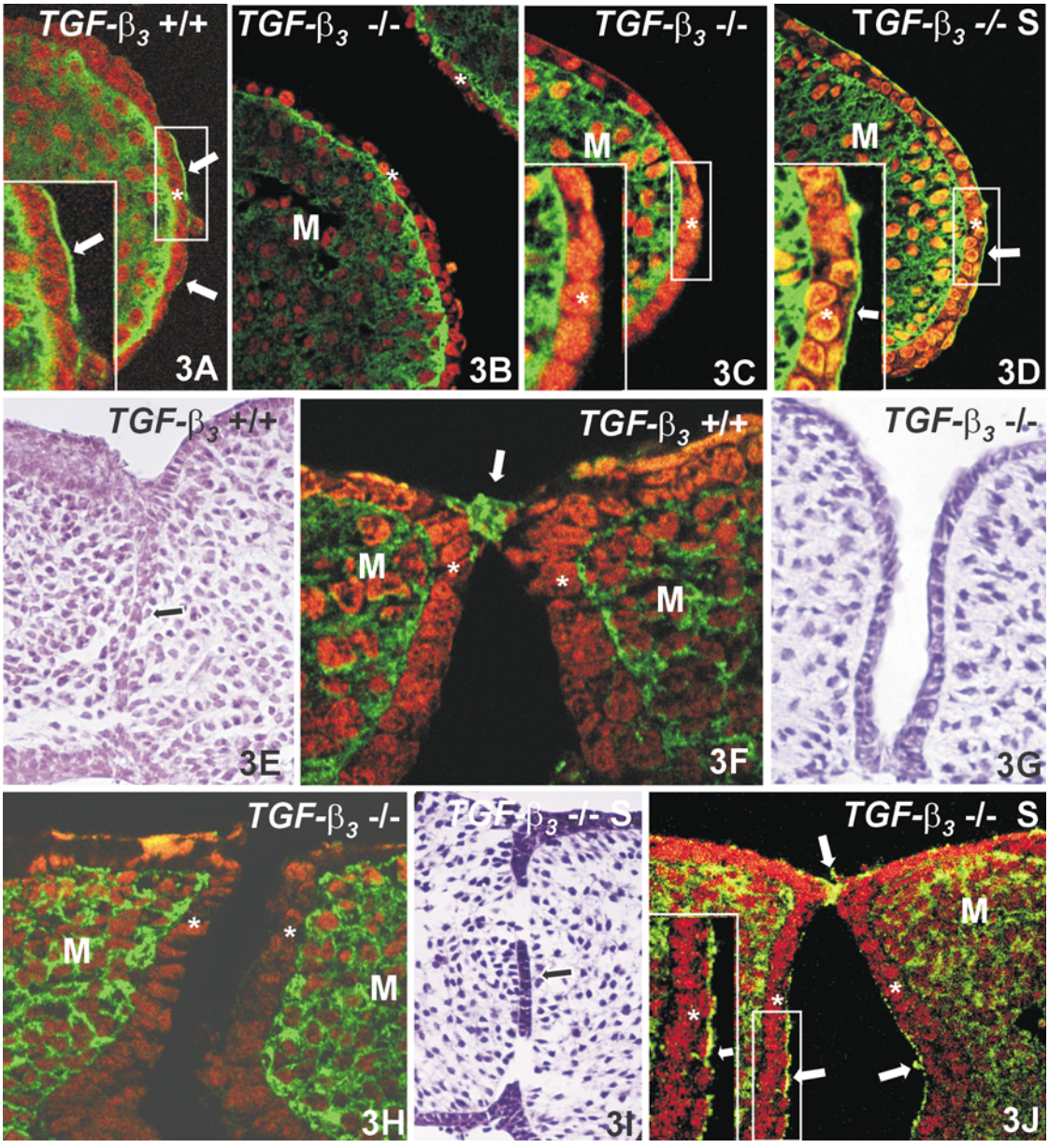


FIG. 3. Correlation between palatal shelf adhesion and presence of apical CSPG and TGF-β_3 in the mouse. (A–D, F, H, and J) Confocal images of anti-CSPG immunolabeled sections that have been stained with propidium iodide to better visualize nuclei. (E, G, and I) Hematoxylin and eosin-stained sections of palate cultures similar to those observed in (F, H, and J), respectively. (C and D) Twelve-hour cultures of E13.5 TGF-$\beta_3^{-/-}$ (C) and TGF-β_3 supplemented (S) TGF-$\beta_3^{-/-}$ (D) isolated mouse palatal shelves. (E–J) Twenty-four-hour TGF-$\beta_3^{+/+}$ (E and F), TGF-$\beta_3^{-/-}$ (G and H), and TGF-β_3 supplemented TGF-$\beta_3^{-/-}$ (I and J) paired palate cultures. (A) Approaching E14.5, TGF-$\beta_3^{+/+}$ palatal shelves have a layer of CSPG-containing material (arrows) covering the MEE surface. (B) There is not CSPG on the MEE surface of almost contacting E14.5 TGF-$\beta_3^{-/-}$ palatal shelves. (C) Twelve-hour E13.5 TGF-$\beta_3^{-/-}$ isolated palatal shelf cultures do not show anti-CSPG-positive immunostaining on the MEE surface. (D) When TGF-β_3 is added to TGF-$\beta_3^{-/-}$ palate cultures, an anti-CSPG-positive material is clearly observed on the MEE surface (arrow). (E and F) Opposing MEE of 24-h TGF-$\beta_3^{+/+}$ paired palate cultures adhere, and an epithelial seam is observed (arrow in E). In those sections where palatal shelf adhesion is not complete, an anti-CSPG-positive material is located at the place of contact between opposing MEE (arrow in F). (G and H) However, TGF-$\beta_3^{-/-}$ palatal shelves are mostly separated when cultured in pairs for 24 h (G), and no anti-CSPG-positive immunostaining is present on the MEE surface in these cultures (H). (I and J) The addition of TGF-β_3 to TGF-$\beta_3^{-/-}$ palate cultures rescues palatal shelf adhesion (arrow in I) and induces the presence of CSPG on the MEE surface (arrows in J). Asterisk, MEE. M, mesenchyme. The boxed areas in (A, C, D, and J) are shown in the lower left corners at higher magnification.

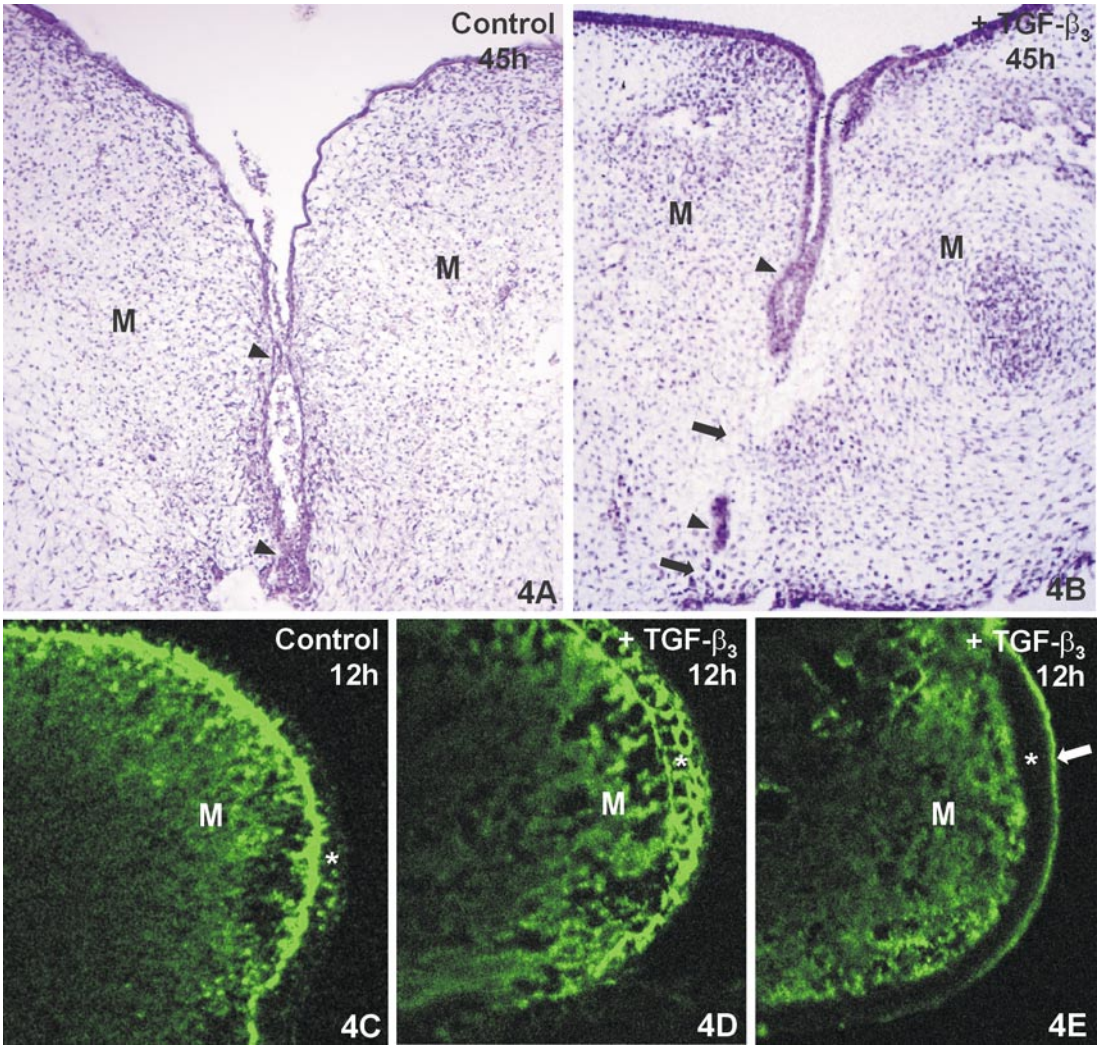


FIG. 4. Correlation between palatal shelf adhesion and presence of apical CSPG and TGF-β_3 in the chick. (A and B) Histological sections taken from untreated (A) and TGF-β_3-treated (B) 35HH chick paired palatal shelves cultured for 45 h. (C–E) Confocal images of anti-CSPG immunolabeled sections taken from 12-h 35HH chick isolated palatal shelf cultures, untreated (C) or TGF-β_3-treated (D and E). (A) Chick opposing MEE adhesion is partial (arrowheads) in most control cultures. (B) Opposing MEE adhesion increases when TGF-β_3 is added to the culture medium (arrowheads) and even palate fusion occurs (arrows). (C) There is almost no anti-CSPG-positive immunostaining among MEE cells nor on the MEE surface in untreated chick palatal shelf cultures. (D) However, when TGF-β_3 is added to the culture medium, a great amount of CSPG is observed amongst MEE cells. (E) A different confocal section from the specimen shown in (D) allows the observation of CSPG on the MEE surface. Asterisk, MEE. M, mesenchyme.

1975). This suggests that CSPG is needed mainly for the recognition and establishment of the initial adhesion between opposing MEE.

The monoclonal antibody CS-56 used here is directed against an epitope present in the glycidic moiety of CSPG and it is common to most CSPG types (Avnur and Geiger, 1984). This implies that we can detect a broad number of molecules that have CSPG among their components. The resolution capacity of our study does not allow us to distinguish whether the CSPG detected on the MEE apical surface is part of a completely extracellular matrix or belongs to the extracellular domain of molecules with transmembrane components. However, since this CSPG plays a role in palatal shelf adhesion, it might belong to or be related with a cell adhesion molecule.

Different types of CSPG are involved in the maintenance of cell adhesion in other systems through their interaction with cadherins, CAMs, or integrins (Retzel *et al.*, 1996; Iida *et al.*, 1998; Stanford *et al.*, 1999; Li *et al.*, 2000). Recent investigations found mutations of *PVRL1*, encoding the

immunoglobulin related transmembrane cell–cell adhesion molecule nectin-1, in human cleft lip, with or without cleft palate (Suzuki *et al.*, 2000). This glycoprotein might be relevant for oral primordia adhesion, although only heparan sulphate has been identified in its glycosaminoglycan moiety (Terry-Allison *et al.*, 2001). The glycoproteins E- and N-Cadherin, N-CAM, and syndecan-1 have been observed prior to and during palatal shelf adhesion in mice (Brinkley *et al.*, 1992; Kerrigan *et al.*, 1998; Sun *et al.*, 1998b; Martínez-Álvarez *et al.*, 2000b). The location of these molecules in the preadhesion MEE, circumferentially placed in basal cells and basolaterally placed (or not existent, as in the case of syndecan) in the superficial cells, does not resemble the expression pattern of CSPG observed here, which is specifically located in the apical surface of the most superficial MEE cells. Moreover, both E-cadherin and syndecan (but not CSPG) are observed among MEE cells when the epithelial seam forms. These data suggest that the CSPG detected here does not belong to any of these molecules; however, it could be part of others still unidentified with a role in palatal shelf adhesion (Couchman *et al.*, 2001). In fact, the expression in the *TGF-β_3* null MEE of some of the previously mentioned molecules is greatly altered (Tudela *et al.*, 2002), coinciding with the absence of expression of apical CSPG and a failure of palatal shelf adhesion.

On the other hand, interaction between extracellular matrix proteoglycans and integrins present on the cell surface has been described (Woods *et al.*, 1998), and there is evidence of a role for integrins as cell surface receptors involved in extracellular matrix assembly (Darribére *et al.*, 2000). Our recent demonstration of the expression of vinculin (a linker between integrins and the actin cytoskeleton) on the apical surface of the most superficial MEE cells by the time of the initial contact between palatal shelves (Martínez-Álvarez *et al.*, 2000b) suggests the participation of integrins in palatal shelf adhesion. The critical presence of a CSPG-rich matrix on the apical surface of MEE cells at this time point may then cause epithelial adhesion through its interaction with integrins present in the opposing MEE surfaces.

More analyses are being carried out to determine which CSPG-containing molecule is responsible for palatal shelf adhesion. However, either as a component of a cell adhesion molecule or as part of a matrix that facilitates the union through integrins, our results evidence the necessary presence of this apical CSPG for palatal shelf adhesion.

The Role of TGF-β_3 during Palatal Shelf Adhesion

Both the presence in the MEE of TGF-β_3 mRNA (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990; Gehris *et al.*, 1994) and its receptor (Cui and Shuler, 2000) occur simultaneously with palatal shelf adhesion and fusion, and, as we demonstrate here, they also coincide with the expression of CSPG on the MEE surface. Inhibition of the biological activity of TGF-β_3 specifically alters palatal shelf adhesion both *in vitro* (Brunet *et al.*, 1995) and *in vivo* (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995), thus pointing to a very important role for TGF-β_3 in this process. In the *TGF-β_3* null homozygous mice, the palatal shelves grow, elevate, and contact normally, but do not adhere adequately, and their fusion fails. We demonstrate here that, concomitant with the failure of palatal shelf adhesion and the formation of cleft palate, *TGF-β_3* null mice lack the apical expression of CSPG in the MEE. Furthermore, the addition of TGF-β_3 to *TGF-β_3* null paired palate cultures stimulates both palatal shelf adhesion and the expression of this apical CSPG, and identical results are obtained when this experiment is carried out in chick palates, that neither present TGF-β_3 nor CSPG in the MEE and have a physiological cleft. Sun *et al.* (1998a), performing a similar chick palate adhesion assay, only reported a slight increase in the adhesion of opposing MEE when TGF-β_3 was added to the culture medium. However, in our study, this increase was almost fourfold compared with the adhesion observed in controls. TGF-β_3 seems to control directly the apical expression of CSPG by MEE cells and palatal shelf adhesion in different species, reinforcing its essential role in the induction of this process. The *in vivo* lack of adhesion between palatal shelves in those species bearing a physiological cleft palate could be due to a physiological downregulation of the transcription of the *TGF-β_3* gene, thus causing the absence of expression of apical CSPG and the reduction of the autoadhesion properties of the MEE. This downregulation could also be the origin of those cleft palates where an alteration of palatal shelf adhesion seems to be the primary cause, as in the 2,3,7,8-Tetraclorodibenzo-p-dioxin- (TCDD)-induced cleft palate, where (as in the *TGF-β_3* null mice) palatal shelves grow, elevate, and even contact, but a firm adhesion fails (Pratt *et al.*, 1984).

Different studies have demonstrated the ability of some members of the TGF-β superfamily to regulate extracellular matrix molecules in several cell types (Ignotz and Massagué, 1986; Locci *et al.*, 1999). In fact, TGF-β has been shown to activate the synthesis of proteoglycans, including CSPG, in different tissues and cell lines (Bassols and Massagué, 1988; Rapraeger, 1989; Chan and Anastassiades, 1998; Van Osch *et al.*, 1998; Brown *et al.*, 1999), and even in mouse embryonic palatal mesenchymal cells (D'Angelo and Greene, 1991). However, the mechanism(s) by which TGF-β_3 controls the production of apical CSPG by superficial MEE cells are unknown. TGF-β is able to regulate the expression of cell adhesion molecules (Chimal-Monroy and Díaz de León, 1999), where the apical CSPG is likely localized. It also modulates the synthesis of the glycidic chains of the proteoglycans (Inman and Colowick, 1985) and increases the molecular weight of GAG chains, including chondroitin sulphate, through the control of their elongation (Bassols and Massagué, 1988; Rapraeger, 1989). Likewise, TGF-β is able to regulate the synthesis of the protein core of proteoglycans, possibly by activating the transcription of the gene codifying for this protein (Bassols and Massagué, 1988). Finally, TGF-β has been shown to

modulate the equilibrium between metalloproteinases and their inhibitors, that controls the proteoglycan turnover in biological systems (Uría *et al.*, 1998). Interestingly, metalloproteinase-3 (MMP3) and its inhibitor TIMP3 are present in the mouse MEE immediately prior to palatal shelf adhesion (Morris-Wiman and Burch, 2000), thus suggesting their involvement in this process, and TGF-β_3 has been shown recently to control the activity of MMP-13 and TIMP-2 in the midline epithelial seam (Blavier *et al.*, 2001).

In conclusion, this work gives experimental evidence for the importance of the presence of CSPG on the apical surface of MEE cells in mouse palatal shelf adhesion and demonstrates its regulation by TGF-β_3.

ACKNOWLEDGMENTS

We thank Mrs. Carmen Benito, Mrs. Alicia Cerro, and Mrs. Dolores Arroyo (Universidad Complutense) for help with the histology, Dr. Fernando Setién (Centro de Técnicas Inmunológicas, Universidad Complutense) and Mr. Daniel Blanco for technical support, Dr. Sagrario Callejo (Universidad de Valladolid) for obtaining the confocal microscopy images, and Professor Puerta and Professor Viejo for departmental support. The authors also thank the anonymous referees for helpful advice. This work has been supported by a grant from the Comunidad de Madrid (Spain), Ref. 08.6/0042.1/2000.

REFERENCES

Abbott, B. D., and Pratt, R. M. (1987). Retinoids and epidermal growth factor alter embryonic mouse palatal epithelial and mesenchymal cell differentiation in organ culture. *J. Craniofac. Genet. Dev. Biol.* **7,** 219–240.

Alonso, I., Gato, A., Moro, J. A., Martin, C., Barbosa, M., Callejo, S., and Barbosa E. (1996). Role of sulfated proteoglycans in early lens development. *Int. J. Dev. Biol.* **Suppl. 1,** 241S–242S.

Alonso, I., Gato, A., Moro, J. A., Martín, P., and Barbosa, E. (1998). Neural tube defects induced by β-D-Xyloside: Evidence of a role for sulfated proteoglycans in neural fold fusion in rat embryos. *Eur. J. Anat.* **2,** 133–140.

Avnur, Z., and Geiger, B. (1984). Immunocytochemical localization of native chondroitin-sulfate in tissues and cultured cells using specific monoclonal antibody. *Cell* **38,** 811–822.

Bassols, A., and Massagué, J. (1988). Transforming growth factor β regulates the expression and structure of extracellular matrix chondroitin, dermatan sulfate proteoglycans. *J. Biol. Chem.* **263,** 3039–3045.

Blavier, L., Lazaryev, A., Groffen, J., Heisterkamp, N., DeClerck, Y. A., and Kaartinen, V. (2001). TGF-β_3-induced palatogenesis requires Matrix Metalloproteinases. *Mol. Biol. Cell* **12,** 1457–1466.

Brinkley, L., Morris-Wiman, J., and Bernfield, M. (1992). The distribution of syndecan during murine secondary palate morphogenesis. *J. Craniofac. Genet. Dev. Biol.* **12,** 82–89.

Brown, C. T., Nugent, M. A., Lau, F. W., and Trinkaus-Randall, V. (1999). Characterization of proteoglycans synthesised by cultured corneal fibroblasts in response to transforming growth factor β and foetal calf serum. *J. Biol. Chem.* **274,** 7111–7119.

Brunet, C. L., Sharpe, P. M., and Ferguson, M. W. J. (1993). The distribution of epidermal growth factor binding sites in the developing mouse palate. *Int. J. Dev. Biol.* **37,** 451–458.

Brunet, C. L., Sharpe, P. M., and Ferguson, M. W. J. (1995). Inhibition of TGF-β_3 (but not TGF-β_1 or TGF-β_2) activity prevents normal mouse embryonic palate fusion. *Int. J. Dev. Biol.* **39,** 345–355.

Carette, M. J. M., and Ferguson, M. W. J. (1992). The fate of medial edge epithelial cells during palatal fusion *in vitro*: An analysis by DiI labelling and confocal microscopy. *Development* **114,** 379–388.

Chan, C. K., and Anastassiades, T. P. (1998). Anionic glycoconjugates from differentiated cultures of bovine articular chondrocites: Modulation by TGF-beta. *In Vitro Cell. Dev. Biol. Anim.* **34,** 492–498.

Chimal-Monroy, J., and Díaz de León, L. (1999). Expression of N-cadherin, N-CAM, fibronectin and Tenascin is stimulated by TGF-β_1, β_2, β_3 and β_5 during the formation of precartilage condensations. *Int. J. Dev. Biol.* **43,** 59–67.

Couchman, J. R., Chen, L., and Woods, A. (2001). Syndecans and cell adhesion. *Int. Rev. Cytol.* **207,** 113–150.

Cui, X. M., and Shuler, C. F. (2000). The TGF-beta type III receptor is localised to the medial edge epithelium during palatal fusion. *Int. J. Dev. Biol.* **44,** 397–402.

D'Angelo, M., and Greene, R. M. (1991). Transforming growth factor-β modulation of the developing murine secondary palate. *Dev. Biol.* **145,** 374–378.

Darribére, T., Skalski, M., Cousin, H., Gaultier, A., Montmory, C., and Alfandari, D. (2000). Integrins: Regulators of embryogenesis. *Biol. Cell* **92,** 5–25.

DeAngelis, V., and Nalbandian, J. (1968). Ultrastructure of mouse and rat palatal processes prior to and during secondary palate formation. *Arch. Oral Biol.* **13,** 601–608.

Ferguson, M. W. J. (1988). Palate development. *Development* **103**(Suppl.), 41–60.

Ferguson, M. W. J., Honig, L. S., and Slavkin, H. C. (1984). Differentiation of cultured palatal shelves from alligator, chick, and mouse embryos. *Anat. Rec.* **209,** 231–249.

Fitchett, J. E., and Hay, E. D. (1989). Medial edge epithelium transforms to mesenchyme after embryonic palatal shelves fuse. *Dev. Biol.* **131,** 455–474.

Fitzpatrick, D. R., Denhez, F., and Kondaiah, P. (1990). Differential expression of TGF-β isoforms in murine palatogenesis. *Development* **109,** 585–595.

Gato, A., Martín, C., Alonso, I., Martínez-Álvarez, C., and Moro, J. A. (2001). Chondroitin sulphate proteoglycan is involved in lens vesicle morphogenesis in chick embryos. *Exp. Eye Res.* **73,** 469–478.

Gehris, A. L., Pisano, M. M., Nugent, P., and Greene, R. M. (1994). Regulation of TGF-β_3 gene expression in embryonic palatal tissue. *In Vitro Cell. Dev. Biol.* **30,** 671–679.

Gibson, K. D., Segen, B J., and Doller, H. J. (1979). Changes in chemical composition of chick embryos treated with β-xyloside and a lathyrogen. *Teratology* **19,** 345–356.

Greene, R. M., and Kochhar, D. M. (1974). Surface coat on the epithelium of developing palatine shelves in the mouse as revealed by electron microscopy. *J. Embryol. Exp. Morphol.* **31,** 683–692.

Greene, R. M., and Pratt, R. M. (1977). Inhibition by diazo-oxo-norleucine (DON) of rat palatal glycoprotein synthesis and epithelial cell adhesion in vitro. *Exp. Cell Res.* **105,** 27–37.

Greene, R. M., Shah, R. M., Lloyd, M. R., Crawford, B. J., Suen, R., Shanfeld, J. L., and Davidovitch, Z. (1983). Differentiation of the avian secondary palate. *J. Exp. Zool.* **225,** 43–52.

Griffith, C. M., and Hay, E. D. (1992). Epithelial–mesenchymal transformation during palatal fusion: Carboxyfluorescein traces cells at light and electron microscopic levels. *Development* **116,** 1087–1099.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Hayward, A. F. (1969). Ultrastructural changes in the epithelium during fusion of the palatal processes in rats. *Arch. Oral Biol.* **14,** 661–678.

Humphrey, T. (1970). Palatopharyngheal fusion in a human fetus and its relation to cleft formation. *Ala. J. Med. Sci.* **7,** 398–429.

Ignotz, R. A., and Massagué, J. (1986). Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. *J. Biol. Chem.* **261,** 4337–4345.

Ignotz, R. A., and Massagué, J. (1987). Cell adhesion protein receptors as targets for transforming growth factor-β action. *Cell* **51,** 189–197.

Iida, J., Meijne, A. M., Oegema, T. R., Jr., Yedmock, T. A., Kovach, N. L., Furcht, L. T., and McCarthy, J. B. (1998). A role of chondroitin sulfate glycosaminoglycan binding site in alpha4 beta1 integrin-mediated melanoma cell adhesion. *J. Biol. Chem.* **273,** 5955–5962.

Inman, W. H., and Colowick, S. P. (1985). Stimulation of glucose uptake by transforming growth factor β: Evidence for the requirement of epidermal growth factor-receptor activation. *Proc. Natl. Acad. Sci. USA* **82,** 1346–1349.

Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N., and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-β_3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* **11,** 415–421.

Kerrigan, J. J., McGill, J. T., Davies, J. A., Andrews, L., and Sandy, R. (1998). The role of cell adhesion molecules in craniofacial development. *J. R. Coll. Surg. Edinb.* **43,** 223–229.

Li, H., Lenng, T. C., Hoffman, S., Balsamo, J., and Lilien, J. (2000). Coordinate regulation of cadherin and integrin function by the chondroitin sulfate proteoglycan neurocan. *J. Cell Biol.* **149,** 1275–1288.

Lidral, A. C., Romitti, P. A., Basart, A. M., Doetschman, T., Leysens, N. J., Daack-Hirsch, S., Semina, E. V., Johnson, L. R., Machida, J., Burds, A., Parnell, T. J., Rubenstein, J. L. R., and Murray, J. C. (1998). Association of MSX1 and TGFβ_3 with nonsyndromic clefting in humans. *Am. J. Hum. Genet.* **63,** 557–568.

Locci, P., Baroni, T., Lilli, C., Martinese, D., Marinucci, L., Bellocchio, S., Calvitti, M., and Becchetti, E. (1999). TGFβ and TGFα antagonistic effect in vitro on extracellular matrix accumulation by chick skin fibroblasts at two distinct embryonic stages. *Int. J. Dev. Biol.* **43,** 157–165.

Martínez-Álvarez, C., Tudela, C., Perez-Miguelsanz, J., O'Kane, S., Puerta, J., and Ferguson, M. W. J. (2000a). Medial edge epithelial cell fate during palatal fusion. *Dev. Biol.* **220,** 343–357.

Martínez-Álvarez, C., Bonelli, R., Tudela, C., Gato, A., Mena, J., O'kane, S., and Ferguson, M. W. J. (2000b). Bulging medial edge epithelial cells and palatal fusion. *Int. J. Dev. Biol.* **44**, 331–335.

Meller, S. M., and Barton, H. L. (1978). Extracellular coat in developing human palatal processes: Electron microscopy and ruthenium red binding. *Anat. Rec.* **190,** 223–232.

Meller, S. M., De Paola, D. P., Barton, L. H., and Mandella, R. D. (1980). Secondary palatal development in the New Zealand white rabbit: A scanning electron microscopic study. *Anat. Rec.* **198,** 229–244.

Mori, C., Nakamura, N., Okamoto, Y., Osawa, M., and Shiota, K. (1994). Cytochemical identification of programmed cell death in the fusing fetal mouse palate by specific labelling of DNA fragmentation. *Anat. Embryol.* **190,** 21–28.

Mitchell, L. E., Murray, J. C., O'Brien, S., and Christensen, K. (2001). Evaluation of two putative susceptibility loci for oral clefts in the Danish population. *Am. J. Epidemiol.* **153,** 1007–1015.

Morriss-Kay, G. M., and Crutch, B. (1982). Culture of rat embryos with beta-D-xyloside: Evidence of a role for proteoglycans in neurulation. *J. Anat.* **134,** 491–506.

Morris-Kay, G. M., and Tuckett, F. (1989). Immunohistochemical localization of chondroitin sulphate proteoglycans and effects of chondroitinase ABC in 9-to-11-day rat embryos. *Development* **106,** 787–798.

Morris-Wiman, J., and Burch, H. (2000). Temporospatial distribution of matrix metalloproteinase and tissue inhibitors of matrix metalloproteinases during murine secondary palate morphogenesis. *Anat. Embryol.* **202,** 129–141.

Newall, D. R., and Edwards, J. R. G. (1981a). The effect of vitamin A on fusion of mouse palates. I. Retinyl palmitate and retinoic acid in vivo. *Teratology* **23,** 115–124.

Newall, D. R., and Edwards, J. R. G. (1981b). The effect of vitamin A on fusion of mouse palates. II. Retinyl palmitate, retinol, and retinoic acid in vitro. *Teratology* **23,** 125–130.

Pelton, R. W., Hogan, B. L., Miller, D. A., and Moses, H. L. (1990). Differential expression of genes encoding TGFs β1, β2, and β3 during murine palate formation. *Dev. Biol.* **141,** 456–460.

Pourtois, M. (1966). Onset of the acquired potentiality for fusion in the palatal shelves of rats. *Embryol. Exp. Morphol.* **16,** 171–182.

Pratt, R. M., Dencker, L., and Diewert, V. M. (1984). 2, 3, 4, 7, 8-tetrachlorodibenzo-p-dioxin-induced cleft palate in the mouse: Evidence for alterations in palatal shelf fusion. *Teratog. Carcinog. Mutagen.* **4,** 427–536.

Pratt, R. M., and Gibson, W. A. (1973). An immunofluorescent technique for observing the binding of concanavalin A frozen tissue sections. *J. Histochem. Cytochem.* **21,** 229–232.

Pratt, R. M., and Hassell, J. R. (1975). Appearance and distribution of carbohydrate-rich macromolecules on the epithelial surface of the developing rat palatal shelf. *Dev. Biol.* **45,** 192–198.

Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W. J., and Dotschman, T. (1995). Transforming growth factor-β_3 is required for secondary palate fusion. *Nat. Genet.* **11,** 409–414.

Rapraeger, A. (1989). Transforming growth factor (type beta) promotes the addition of chondroitin sulfate chains to the cell surface proteoglycan (syndecan) of mouse mammary epithelia. *J. Cell Biol.* **109,** 2509–2518.

Retzel, C., Gohring, W., and Rauch, U. (1996). Analysis of neurocan structures interacting with the neural cell adhesion molecule N-CAM. *Braz. J. Med. Biol. Res.* **29,** 1247–1249.

Schüpbach, P. M., Chamberlain, J. G., and Schroeder, H. E. (1983). Development of the secondary palate in the rat: A scanning electron microscopic study. *J. Craniofac. Genet. Dev. Biol.* **3,** 159–177.

Sharpe, P. M., and Ferguson, M. W. J. (1988). Mesenchymal influences on epithelial differentiation in developing systems. *J. Cell Sci.* **10,** 195–230.

Shuler, C. F., Guo, Y., Majumder, A., and Luo, R. Y. (1991). Molecular and morphologic changes during the epithelial–mesenchymal transformation of palatal shelf medial edge epithelium in vitro. *Int. J. Dev. Biol.* **35,** 463–472.

Smiley, G. R., and Koch, W. E. (1971). Fine structure of mouse secondary palate development in vitro. *J. Dent. Res.* **50,** 1671–1677.

Smits Van Prooije, A. E., Proelmann, R. E., Gesink, A. F., Van Groeningen, M. J., and Vermeig-Keers, C. (1986). The cell surface coat in neurulating mouse and rat embryos studied with lectins. *Anat. Embryol.* **175,** 111–117.

Sobue, M., Habuchi, H., Ito, K., Yonekura, H., Oguri, K., Sakurai, K., Kamohara, S., Ueno, Y., Noyori, R., and Suzuki, S. (1987). β-D-Xylosides and their analogues as artificial initiators of glycosaminoglycan chain synthesis. *Biochem. J.* **241,** 591–601.

Souchon, R. (1975). Surface coat of the palatal shelf epithelium during palatogenesis in mouse embryos. *Anat. Embryol.* **147,** 133–142.

Stanford, C. M., Solursh, M., and Keller, J. C. (1999). Significant role of adhesion properties of primary osteoblast-like cells in early adhesion events for chondroitin sulfate and dermatan sulfate surface molecules. *Exp. Eye Res.* **248,** 314–321.

Sun, D., Vanderburg, C. R., Odierna, G. S., and Hay, E. D. (1998a). TGF-β_3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro. *Development* **125,** 95–105.

Sun, D., McAlmon, K. R., Davies, J. A., Bernfield, M., and Hay, E. D. (1998b). Simultaneous loss of expression of syndecan-1 and E-cadherin in the embryonic palate during epithelial-mesenchymal transformation. *Int. J. Dev. Biol.* **42,** 733–736.

Suzuki, K., Hu, D., Bustos, T., Zlotogora, J., Richieri-Costa, A., Helms, J. A., and Spritz, R. A. (2000). Mutations of *PVRL1*, encoding a cell-cell adhesion molecule/herpesvirus receptor, in cleft lip/palate-ectodermal dysplasia. *Nat. Genet.* **25,** 427–430.

Taniguchi, K., Sato, N., and Uchiyama, Y. (1995). Apoptosis and heterophagy of medial edge epithelial cells of the secondary palatine shelves during fusion. *Arch. Histol. Cytol.* **58,** 191–203.

Taya, Y., O'Kane, S., and Ferguson, M. W. J. (1999). Pathogenesis of cleft palate in TGF-β_3 knockout mice. *Development* **126,** 3869–3879.

Terry-Allison, T., Montgomery, R. I., Warner, M. S., Geraghty, R. J., and Spear, P. G. (2001). Contributions of gD receptors and glycosaminglycan sulfation to cell fusion mediated by herpes simplex virus 1. *Virus Res.* **74,** 39–45.

Trasler, D. G., and Morriss-Kay, G. (1991). Immunohistochemical localization of chondroitin and heparan sulfate proteoglycans in pre-spina bifida splotch mouse embryos. *Teratology* **44,** 571–579.

Tudela, C., Formoso, M. A., Martínez, T., Pérez, R., Aparicio, M., Maestro, C., Del Río, A., Martínez, E., Ferguson, M. W. J., and Martínez-Álvarez, C. (2002). TGF-β_3 is required for the adhesion and intercalation of medial edge epithelial cells during palate fusion. *Int. J. Dev. Biol.* **46,** 333–336.

Uria, J. A., Jimenez, M. G., Balbin, M., and Lopez-Otin, C. (1998). Differential effects of transforming growth factor-β on the expression of collagenase-3 in human fibroblasts. *J. Biol. Chem.* **273,** 9769–9777.

Van Osch, G. J., Van der Veen, S. W., Buma, P., and Verwoerd-Verhoef, H. L. (1998). Effect of transforming growth factor-beta on proteoglycan synthesis by chondrocites in relation to differentiation stage and the presence of pericellular matrix. *Matrix Biol.* **17,** 413–424.

Van Rybroeck, J. J., and Olson, M. D. (1981). Surface coat material associated with the cells of the developing lens vesicle in the chick embryo. *Anat. Rec.* **201,** 261–271.

Waterman, R. E., Ross, L. M., and Meller, S. M. (1973). Alterations in the epithelial surface of A/Jax mouse palatal shelves prior to and during palatal fusion: A scanning electron microscopic study. *Anat. Rec.* **176,** 361–376.

Woods, A., Oh, E. S., and Couchman, J. R. (1998). Syndecan proteoglycans and cell adhesion. *Matrix Biol.* **17,** 461–463.

Yao, R., Alcala, J., and Maisel, H. (1996). Developmental changes in glycoconjugate composition during chick lens morphogenesis. *Exp. Eye Res.* **62,** 419–431.

Zschäbitz, A. R., Biesalski, H. K., Krahn, V., Gabius, H. J., Weiser, H., Khaw, A., Hemmes, C., and Stofft, E. (1994). Distribution patterns in glycoconjugate expression during the development of the rat palate. *Histochem. J.* **26,** 705–720.

Received for publication April 3, 2002
Revised July 16, 2002
Accepted July 17, 2002
Published online September 12, 2002