TGF- β 3-induced Palatogenesis Requires Matrix Metalloproteinases

Laurence Blavier,^{*,†} Alisa Lazaryev,^{*} John Groffen,^{*} Nora Heisterkamp,^{*} Yves A. DeClerck,^{*,‡§} and Vesa Kaartinen^{||}

^{*}Division of Hematology-Oncology, Department of Pediatrics; [†]Department of Biochemistry and Molecular Biology; and ^{||}Developmental Biology Program, Department of Pathology, Childrens Hospital Los Angeles and the Keck School of Medicine of the University of Southern California, Los Angeles, California 90027

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Cleft lip and palate syndromes are among the most common congenital malformations in humans. Mammalian palatogenesis is a complex process involving highly regulated interactions between epithelial and mesenchymal cells of the palate to permit correct positioning of the palatal shelves, the remodeling of the extracellular matrix (ECM), and subsequent fusion of the palatal shelves. Here we show that several matrix metalloproteinases (MMPs), including a cell membrane-associated MMP (MT1-MMP) and tissue inhibitor of metalloproteinase-2 (TIMP-2) were highly expressed by the medial edge epithelium (MEE). MMP-13 was expressed both in MEE and in adjacent mesenchyme, whereas gelatinase A (MMP-2) was expressed by mesenchymal cells neighboring the MEE. Transforming growth factor (TGF)- β 3-deficient mice, which suffer from clefting of the secondary palate, showed complete absence of TIMP-2 in the midline and expressed significantly lower levels of MMP-13 and slightly reduced levels of MMP-2. In concordance with these findings, MMP-13 expression was strongly induced by TGF- β 3 in palatal fibroblasts. Finally, palatal shelves from prefusion wild-type mouse embryos cultured in the presence of a synthetic inhibitor of MMPs or excess of TIMP-2 failed to fuse and MEE cells did not transdifferentiate, phenocopying the defect of the TGF- β 3-deficient mice. Our observations indicate for the first time that the proteolytic degradation of the ECM by MMPs is a necessary step for palatal fusion.

INTRODUCTION

The formation of the palate is of critical importance to separate the oropharynx from the nasopharynx. A dysfunction in one of the regulators of this developmental process can lead to a cleft palate, one of the most common birth defects in humans (Chenevix-Trench *et al.*, 1992). In the mouse embryo, the entire process of palatal formation takes place between day 12 and 15 (E12 and E15) of development (Ferguson, 1988). The fusion itself occurs over a relatively short period of time during which the medial edge epithelia (MEE) of the shelves form a midline seam, which is then disrupted to allow mesenchymal continuity (Pourtois, 1966; Smiley and Koch, 1971). Complete fusion of the secondary palate requires disappearance of the MEE from the midline, as well as the breakdown of their basement membrane.

The molecular mechanisms controlling palatal fusion are complex and not fully understood. However, studies in the

mouse have pointed to primary and secondary causes of defective palatogenesis. In mice deficient for the epidermal growth factor receptor or the platelet-derived growth factor receptor, a cleft palate is often associated with a primary defect in the development of the first branchial arch (Shiota *et al.*, 1990; Brunet *et al.*, 1993; Robbins *et al.*, 1999). In these cases, delayed development of the lower jaw interferes with forward displacement of the tongue and prevents the elevation and subsequent fusion of the shelves (Robbins *et al.*, 1999). In transforming growth factor (TGF)- β 3-deficient mice a cleft palate develops in all mice due to the inability of the MEE to fuse (Kaartinen *et al.*, 1995; Proetzel *et al.*, 1995). In the developing mouse head, TGF- β 3 expression is precisely restricted to the MEE cells, and its expression temporally correlates with the initiation of palatal fusion (Fitzpatrick *et al.*, 1990; Pelton *et al.*, 1990; Gehris *et al.*, 1991). The fusion of palatal shelves from TGF- β 3 $-/-$ embryos placed in organ cultures can be restored by adding the mature form of TGF- β 3 into the medium, thus providing evidence for a direct role of TGF- β 3 in this process (Kaartinen *et al.*, 1997; Taya *et al.*, 1999). Recently, Sun *et al.* (1998) came to a similar conclusion by using chicken palate as an experimental model system.

[§] Corresponding author. E-mail address: declerck@hsc.usc.edu.

[†] Current address: MMP Unit, National Institute of Dental and Craniofacial Research, Bethesda, MD 20892.

Remodeling of the extracellular matrix (ECM) is an essential event in many biological processes involving cell migration, cell–cell interaction, proliferation, and differentiation. Under normal physiological conditions, the highly regulated turnover of the ECM leads to the growth of the embryo concomitant with a precisely controlled organogenesis. It is believed that matrix-degrading proteinases play an important role in tissue remodeling (Basbaum and Werb, 1996; Werb, 1997). Among those are the matrix metalloproteinases (MMPs), a complex family of proteinases secreted as proenzymes (Birkedal-Hansen *et al.*, 1993). MMPs function primarily at the cell surface or in the extracellular space and their proteolytic activity is controlled through zymogen activation and inhibition by endogenous proteinase inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs) (Denhardt *et al.*, 1993). It is generally believed that the balance between MMPs and TIMPs is among the critical determinants that control the integrity of the ECM and subsequently affect cell fate. Little is known about the role of ECM-degrading proteinases in palatal fusion, but the observation that degradation of the basement membrane adjacent to the MEE occurs simultaneously with epithelio-mesenchymal transdifferentiation (EMT) suggests that proteinases are involved (Shuler *et al.*, 1992; Kaartinen *et al.*, 1997).

In a previous study on the temporo-spatial expression of TIMP-2 during embryogenesis, we observed a high level of TIMP-2 in the connective tissues surrounding the nasopharynx and the oropharynx of E10.5 to E18.5 mouse embryos (Blavier and DeClerck, 1997). This observation led us to hypothesize that TIMP-2 plays a role during craniofacial development, and that this process involves tissue remodeling by MMPs. In this study, we provide evidence that MMPs are required for successful palatal fusion and that their expression is in part controlled by TGF- β 3 in this process.

MATERIALS AND METHODS

Animals and Tissues

TGF- β 3-deficient mice were generated in a C57BL/6 background (Kaartinen *et al.*, 1995). Mutant and wild-type females were allowed to mate during a 6-h period and pregnancies were timed. The presence of a vaginal plug defined day 0 and hour 0 of development. At 14.5 d post coitum (p.c.), pregnant females were sacrificed by cervical dislocation, and fetuses were removed from the amniotic sacs. Heads and bodies were collected separately. Of each fetus, the head was embedded in paraffin for histological analyses and in situ hybridization, and the corresponding remaining body was used for DNA extraction and genotypic analysis by Southern blot.

Histological Analyses

Mouse embryonic heads were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C, dehydrated through a series of ethanol solutions of increased concentrations, cleared in xylene, and embedded in paraffin. Serial 6- μ m-thick sections were cut in the coronal plane starting from the back of the eyeballs to the tip of the snout. Sections were spread onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and dried overnight at 42°C. At ~100- μ m intervals, sections were stained with Mayer's hematoxylin and eosin for routine histology, whereas the remaining sections were used for in situ hybridization.

In Situ Hybridization

In situ hybridizations were performed as previously described (Blavier and DeClerck, 1997). Briefly, paraffin sections were dewaxed in xylene, rehydrated through a series of ethanol solutions of decreased concentrations, treated with proteinase K (5 μ g/ml), post-fixed in 4% paraformaldehyde in PBS, acetylated in triethanolamine hydrochloride/acetic anhydride, washed in PBS, dehydrated, and air dried. The sections were incubated overnight at 50°C in the hybridization buffer containing a single-stranded riboprobe radio-labeled with [α -³²P]UTP (PerkinElmer Life Science Products, Boston, MA). The following murine-specific cDNA probes were used: TIMP-1 (from Dr. D. Denhardt, Rutgers University, Piscataway, NJ); TIMP-2 (from Dr. R. Khokha, Ontario Cancer Institute, Toronto, Ontario, Canada); TIMP-3 (from Dr. D. Edward, University of Calgary, Alberta, Canada); TIMP-4 (from Dr. S. Apte, Cleveland Clinic Foundation, Cleveland, OH); MMP-2 and MMP-9 (from Dr. Z. Werb, University of California, San Francisco, San Francisco, CA); MT1-MMP (from Dr. M. Seiki, University of Tokyo, Tokyo, Japan); MMP-7 (from Dr. L. Matrisian, Vanderbilt University, Nashville, TN); MMP-3 and MMP-13 (from Dr. H. Nagase, Imperial College of Medicine, London, United Kingdom); and TGF- β 3 (Pelton *et al.*, 1990). For each antisense probe tested, a sense probe was also generated as negative control. After hybridization, the sections were washed extensively, dehydrated, and air dried. The slides were then dipped in photographic emulsion (Hypercoat LM-1; Amersham Pharmacia Biotech, Arlington Heights, IL) and exposed for 3 to 5 d at 4°C. After exposure, the slides were developed and counter-stained with Mayer's hematoxylin.

Organ Cultures

E14.0 embryos were dissected under a stereomicroscope. Of each embryo, the head was separated from the body, and the mandibles removed to expose the palate. The elevated palatal shelves were then dissected from the maxilla in Hank's buffer (Life Technologies, Gaithersburg, MD), and placed in corresponding pairs on Millipore filters with their medial edges in contact with each other. These filters were placed on the grid of an organ culture dish containing 1 ml of chemically defined serum-free BGJb medium (Life Technologies), supplemented with ascorbic acid (50 μ g/ml) and glutamine (200 μ g/ml). When indicated, BB-3103 (British Biotech Pharmaceuticals, Oxford, United Kingdom) or recombinant TIMP-2 was added to the culture medium. BB-3103 is a soluble hydroxamate-based inhibitor of MMPs with broad specificity that has been used in several cell culture studies (Foda *et al.*, 1999; Jill *et al.*, 1999; Theret *et al.*, 1999). The medium was changed every day and cultures were maintained for 63 h at 37°C in a humidified atmosphere containing 5% CO₂. At the end of the experiment, the filters were carefully removed and processed for paraffin embedding. Serial sections were obtained, stained with hematoxylin and eosin, and examined for the presence or absence of MEE cells in the midline. For each organ culture, 20 sections were examined for fusion of the palatal shelves. A score of 0 (complete fusion) was given when the section showed a complete absence of MEE. A score of 1 (incomplete fusion) was recorded when discontinuous islands of MEE cells were seen, and a score of 2 (absence of fusion) was given in the presence of a continuous MEE consisting of two epithelial layers.

Establishment of Palatal Mesenchymal Cell Cultures and Induction with TGF- β 3

Fetuses (E14.0) were dissected as described above ("organ cultures"). Tips of elevated palatal shelves were removed as pairs and dissociated with 0.25% trypsin/0.1% EDTA in PBS for 10 min at 37°C as described (Nugent *et al.*, 1998). Digested samples were briefly triturated, filtrated through 70- μ m mesh, and cells were seeded on 30-mm dishes and grown to confluence in Opti-MEM (Life Technologies), containing 5% fetal calf serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin (37°C, 5% CO₂). For TGF- β 3

Figure 1. (A) Histological analysis of a coronal section performed on a 14.5-d p.c. embryonic head, showing the palatal shelves in the process of fusion. (B) Enlargement of the same photomicrograph showing the interrupted medial edge epithelium (arrow) undergoing epithelial-mesenchymal transdifferentiation. n, nasopharynx; o, oropharynx; t, tongue; ps, palatal shelf. Bars, A, 300 μ m; B, 100 μ m.

induction, semiconfluent cultures were washed once with PBS and incubated for 24 h in Opti-MEM without fetal calf serum. TGF- β 3 (10 ng/ml) was added and 24 h later the culture medium was harvested for Western blot analysis. The remaining cells were used for RNA isolation.

Northern and Western Blot Assays

Total RNAs were isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Aliquots of 10 μ g were run in 1.2% guanidine thiocyanate-agarose gels and blotted onto Hybond-N filters (PerkinElmer Life Science Products). Northern hybridizations were carried out according to standard procedures using 32 P-labeled murine cDNA fragments for MMP-2, MT1-MMP, MMP-13, and TIMP-2 as probes. For Western blot assays the harvested culture media was concentrated 10-fold by using Centricon ultrafiltration cartridges with a cut off M_r of 10,000 (Amicon, Beverly, MA). Equal aliquots (20 μ g) of the conditioned media were analyzed by Western blotting according to standard procedures by using a polyclonal antibody against mouse MMP-13 (kindly provided by Dr. C. Lopez-Otin, University of Oviedo, Oviedo, Spain) at a 1:2000 dilution.

RESULTS

Shortly after the vertically oriented palatal shelves have elevated to the horizontal position, their medial borders become closely apposed and begin to fuse. This event initially occurs in the middle region of the shelves. It is followed by the fusion of the posterior halves of the palatal shelves, whereas the final part to close is in the region of the incisive canals (Kaufman, 1992). Changes in the ECM occur and MEE cells undergo EMT shortly after the apposing palatal shelves contact each other and the midline epithelial seam is formed. To ensure that the expression analysis of TIMPs and MMPs was performed at that time point, we first examined sections located 100 μ m apart in each embryonic head by using routine histology (see MATERIALS AND METHODS). Sections showing palatal shelves in close contact or with discontinuous midline were identified (Figure 1), and 6- μ m sections situated between those sections were then processed for in situ hybridization for TIMPs and MMPs expression.

Expression of TIMPs during Palatal Fusion

The analysis of the expression of TIMP-1, TIMP-2, TIMP-3, and TIMP-4 during the formation of the secondary palate in E14.5 embryos is shown in Figure 2. Data revealed some interesting and significant differences in the expression of these inhibitors. TIMP-1 mRNA was selectively expressed in the osteogenic tissues of the mandible, the maxilla, and the periorbital region and was entirely absent from epithelial and mesenchymal tissues (Figure 2A). TIMP-3 mRNA was present in the mesenchyme around the nasal epithelium but not in the palate (Figure 2B), whereas no specific signal was detected with a probe for TIMP-4 (Figure 2D). TIMP-2 mRNA was diffusely expressed in the mesenchymal tissues of the palate, the nose, and the tongue as we have previously reported (Blavier and DeClerck, 1997). Moreover, it was very intensely and precisely expressed at the site of contact between the palatal shelves (Figure 2C). No signal above background was obtained with all sense probes tested (our unpublished results).

Expression of MMPs during Palatal Fusion

Because TIMP-2 is a known regulator of MMP activity, we decided to examine the expression of several MMPs in these sections, including MMP-3 (stromelysin), MMP-7 (matrilysin), MMP-9 (gelatinase B), MMP-13 (collagenase-3), and in particular MMP-2 (gelatinase A) and MT1-MMP (membrane type, MMP-14), with which TIMP-2 is known to preferentially interact (Butler *et al.*, 1998; Shofuda *et al.*, 1998) (Figure 3). No signals were detected for MMP-3 and MMP-7 (our unpublished results), and MMP-9 was selectively expressed in the ossification centers in the maxillary region (Figure 3A). MT1-MMP mRNA was expressed at the point of contact between the two palatal shelves and displayed a diffuse signal in the surrounding mesenchymal tissue, similar to what we observed for TIMP-2 (Figure 3B). MMP-2 was expressed in the osteogenic mesenchyme of the mandible and in the mesenchymal tissue around the oropharynx and in the palate, but the midline seam did not show an equally intense signal as seen with TIMP-2 (Figure 3C). Most interestingly, mRNA for MMP-13 was abundantly and almost

Figure 2. Localization of mRNA expression of TIMPs during palatal fusion. Coronal sections of 14.5-d p.c. embryonic heads were analyzed by in situ hybridization with antisense riboprobes for TIMP-1 (A), TIMP-3 (B), TIMP-2 (C), and TIMP-4 (D). Photomicrographs were taken under dark-field illumination, the transcripts are seen as bright grains. The arrow points to the hybridization signal at the midline seam (C). (Note: the pigmented epithelium of the eye appears bright under dark-field, this is not a positive signal.) t, tongue; ps, palatal shelf. Bar, A, 200 μ m.

exclusively present at the site of palatal fusion both in epithelial and mesenchymal cells (Figure 3D). It was also expressed to a lesser degree in the osteogenic tissue of the mandible.

Epithelial and Mesenchymal Expression of MMPs and TIMP-2

A closer look at the expression of TIMP-2, MT1-MMP, MMP-2, and MMP-13 at the cellular level is shown in Figure 4. Interestingly, TIMP-2 and MT1-MMP colocalized at the midline seam and were intensively expressed in the MEE cells, MT1-MMP displaying some expression also in the adjacent mesenchymal cells (Figure 4, A and C). MMP-2 expression was restricted to the mesenchymal cells (Figure 4B), whereas MMP-13 showed intense expression both in MEE and in adjacent mesenchymal cells in the midline seam (Figure 4D). These data indicate that epithelial and mesenchymal cells both contribute to the expression of MMPs at the medial edge.

Comparison of TIMP and MMP Expression at the MEE in Wild-Type and TGF- β 3-deficient Embryos

Because our results suggested that MMPs and TIMP-2 play an active role in palatal fusion, one would anticipate that

their expression would be regulated by morphogenic growth factors that are involved in palatal fusion. Considering the known role of TGF- β 3 in this process, we first examined whether its expression colocalized with MMPs and TIMP-2 in the proximity of the MEE. This analysis revealed that TGF- β 3 transcripts were intensely present at the site of fusion of the palatal shelves, as previously reported (Pelton *et al.*, 1990), colocalizing precisely with the signals given by TIMP-2, MT1-MMP, and MMP-13 (Figure 5, A, C, G, and I). This colocalization of TGF- β 3, TIMP-2, MT1-MMP, and MMP-13 at the level of the MEE cells raised the possibility that during palatal morphogenesis the expression of these MMPs and TIMP-2 is under the regulatory control of TGF- β 3. To test this hypothesis, we compared the expression of TIMP-2, MMP-2, MT1-MMP, and MMP-13 in wild-type and TGF- β 3 $-/-$ mouse embryos, the latter of which show defective palatogenesis resulting in a bilateral cleft of the secondary palate (Kaartinen *et al.*, 1995). As shown in Figure 5, A and B, TGF- β 3 mRNA is expressed in both control and mutant at a comparable level. This is because the strategy used to create a TGF- β 3 null mutation leads to the formation of a truncated, but stable mRNA detectable with the isoform-specific probe. We observed a decrease in the expression of MMP-2 at the medial edge, no

Figure 3. Localization of mRNA expression of MMPs during palatal fusion. Coronal sections of 14.5-d p.c. embryonic heads were analyzed by in situ hybridization with antisense riboprobes for MMP-9 (A), MT1-MMP (B), MMP-2 (C), and MMP-13 (D). The arrows point to the positive hybridization signal at the midline seam (B-D). Photomicrographs were taken under dark-field illumination. t, tongue; ps, palatal shelf. Bar, A, 200 μ m.

Figure 4. Comparison of cellular expression of TIMP-2, MMP-2, MT1-MMP, and MMP-13 at the site of palatal fusion. High magnification of the midline seam showing TIMP-2 (A) and MT1-MMP (C) expression by the MEE cells (arrows in A and C), MMP-2 (B) expression by the adjacent mesenchymal cells (arrow, B), and MMP-13 expression both in the MEE cells and adjacent mesenchymal cells (arrows, D). Photographs were taken under bright-field illumination, the transcripts are seen as dark grains. Bar, A, 10 μ m.

Figure 5. Comparison of TGF- β 3, TIMP-2, MMP-2, MT1-MMP, and MMP-13 expression by in situ hybridization in wild-type (A, C, E, G, and I) and TGF- β 3-deficient embryos (B, D, F, H, and J). Arrows point to the hybridization signal in the midline seam. Photomicrographs were taken under dark-field illumination. n, nasopharynx; o, oropharynx; t, tongue; ps, palatal shelf. Dotted line in D and J indicates the surface of the tongue. Bar, A, 100 μ m

change in expression of MT1-MMP, and a much more significant reduction in MMP-13 expression at the level of contact between the palatal shelves in the mutant compared with the wild-type (Figure 5, E–J). Significantly, there was a complete absence of TIMP-2 expression by the MEE in TGF- β 3-deficient embryos compared with the wild-type (Figure 5, C and D). These data, which show significant changes in MMPs and TIMP-2 expression in the palate of TGF- β 3^{-/-} mice, strongly suggest that MMPs play an active role in palatogenesis.

TGF- β 3 Is a Potent Inducer of MMP-13 in Palatal Mesenchymal Cells

As shown above, MMP-13 is expressed in the wild-type midline seam, both in epithelial and mesenchymal cells (Figures 3D and 4D). Interestingly, MMP-13 expression was

Figure 6. Expression of MMP-13 in isolated palatal fibroblasts is modulated by TGF- β 3. TGF- β 3 induces MMP-13 expression both on an RNA (A) and a protein level (B) in palatal fibroblasts, whereas TIMP-2, MMP-2, and MT1-MMP do not display a similar response (C). Serum-starved cells were induced with 10 ng/ml TGF- β 3 and cells and medium were harvested for Northern (10 μ g of total RNA per lane) and Western (20 μ g of total protein per lane) analyses, respectively.

dramatically reduced in TGF- β 3-deficient mice, which suggested that MMP-13 expression is directly induced by TGF- β 3 during palatal fusion (Figure 5, I and J). Because palatal epithelial cells did not maintain a stable phenotype in vitro, we could only test our hypothesis on palatal mesenchymal cells. We established palatal mesenchymal cell cultures from the tips of prefusion palatal shelves and studied MMP and TIMP-2 expression both in wild-type and TGF- β 3^{-/-} samples with and without TGF- β 3 stimulation (Figure 6, A and B). We observed a 10-fold increase in MMP-13 RNA level and a fourfold increase in protein level by TGF- β 3. Interestingly, in these palatal mesenchymal cells, TIMP-2, MMP-2, and MT1-MMP did not show increased expression as a response to TGF- β 3 stimulation in vitro (Figure 6C). Thus, TGF- β 3 effectively stimulates MMP-13 expression both in wild-type and TGF- β 3^{-/-} palatal fibroblasts.

Inhibition of Palatal Fusion In Vitro

To further establish the role of MMPs in palatal fusion, we studied the effect of a synthetic inhibitor of MMPs as well as TIMP-2 on the fusion of explants from prefusion palatal shelves obtained from E14.0 wild-type embryos and maintained for 63 h in organ culture. The synthetic MMP-inhibitor used here, BB-3103, did not show cytotoxic effects on palatal cells at concentrations up to 10 μ M (our unpublished results). It has been shown that palatal explants fuse in vitro after disruption of the basement membrane and that the MEE cells transdifferentiate into mesenchymal cells (Fitchett and Hay, 1989; Shuler *et al.*, 1992; Kaartinen *et al.*, 1997). Moreover, persistence of MEE cells in the midline region in organ cultures corresponds to palatal clefting in vivo (Kaartinen *et al.*, 1997; Taya *et al.*, 1999). As shown in Figure 7, in the absence of inhibitor, all shelves had fused as dem-

Figure 7. Synthetic inhibitor of MMPs prevents the fusion of palatal shelves in culture. Palatal shelves from wild-type E14.0 embryos were dissected and cultured as pairs at the air-medium interface in organ culture dishes as described in MATERIALS AND METHODS. This figure shows representative sections of the palatal shelves after 63 h of culture under control conditions (A and B), and in the presence of 1 μ M (C and D) and 10 μ M (E and F) BB-3103. Bars, A, C, and E, 50 μ m; B, D, and F, 10 μ m.

Figure 8. Inhibition of palatal fusion and EMT *in vitro* by BB-3103 and TIMP-2. At the end of the organ culture, serial sections were examined for the presence of MEE cells in the midline (as shown in Figure 7). Sections were scored 0 for the absence of MEE cells, 1 for discontinuous MEE, and 2 for the presence of a continuous double layer of MEE (see MATERIALS AND METHODS). Each dot represents the mean score of 20 sections examined for each organ cultured under the indicated conditions. The number of organ cultures examined was 17 for control, eight in the presence of 1 μ M BB-3103, nine in the presence of 10 μ M BB-3103, and five in the presence of 10 μ g/ml TIMP-2.

DISCUSSION

taneous remodeling of the extracellular matrix, including degradation of the basement membrane. Although the mechanisms to remove epithelial cells from the midline seam have been intensely studied during the last decade (Fitchett and Hay, 1989; Shuler *et al.*, 1991; Carette and Ferguson, 1992; Shuler *et al.*, 1992), matrix remodeling taking place during palatal fusion has received much less attention.

It has recently been reported that MMPs are involved in the development of the lower jaw and Meckel's cartilage, under the control of the TGF- β family and epidermal growth factor receptor (Miettinen *et al.*, 1995, 1999). In the present study we provide evidence for the first time that MMPs are directly involved in palatal fusion and the EMT associated with this process. This evidence is based on 1) the observation that MMP-2, MT1-MMP, MMP-13, and TIMP-2 are highly expressed in the palate of E14.0–14.5 embryos at the time the epithelial fusion occurs; 2) the observation that TIMP-2 and MMP-13 are down-regulated in TGF- β $-/-$ embryos presenting with a cleft palate at birth; 3) the demonstration that TGF- β strongly induces MMP-13 expression in cultured palatal fibroblasts isolated from the tips of elevated palatal shelves; and 4) the demonstration that a synthetic inhibitor specific for MMPs as well as recombinant TIMP-2 prevent palatal fusion and EMT.

MMP-13 (collagenase-3) is particularly interesting in the context of palatal fusion, because it displays exceptionally wide substrate specificity. In addition to native fibrillar collagens I, II, and III, it shows very high gelatinase activity, degrading type IV, X, and XIV collagens, tenascin, fibronectin, and aggrecan core protein (Freije *et al.*, 1994; Fosang *et al.*, 1996; Knäuper *et al.*, 1996, 1997; Mitchell *et al.*, 1996). It has been suggested that because of its wide specificity, the physiological expression of MMP-13 is limited to situations in which rapid and effective remodeling of collagenous ECM takes place. In fact, the only normal human tissues shown to express MMP-13 are developing fetal bone and gingival wounds. Although it has been suggested that in rodents MMP-13 replaces the role of MMP-1 (which is apparently absent in rodents) it has been shown that MMP-13 expression during normal mouse development is restricted to areas of endochondral and intramembranous bone formation (Gack *et al.*, 1995; Mattot *et al.*, 1995). Moreover, MMP-13 is expressed during many pathological conditions associated with excessive degradation of the ECM, such as osteoarthritis, chronic cutaneous ulcers, intestinal ulcerations, and malignant tumors. Our present study shows that in addition to bone development, MMP-13 is highly induced in the midline seam (both in medial edge epithelial and mesenchymal cells) during palatal fusion and is likely to play an essential role in this process.

In addition, our present results demonstrate a decrease in MMP-13 expression in TGF- β 3 ($-/-$) palates at E14.5 compared with wild-type controls. Interestingly, we could demonstrate that both in wild-type and TGF- β 3-deficient palatal fibroblasts MMP-13 expression could be strongly induced by TGF- β 3. Although the present manuscript was in preparation, Ravanti *et al.* (1999a) showed that TGF- β 1 stimulates a rapid expression of MMP-13 in human gingival fibroblasts. It was suggested that MMP-13 plays a unique role in maintaining a delicate balance between deposition and degradation of ECM during gingival wound repair, resulting in minimal scarring. In contrast to human gingival and murine

palatal fibroblasts, skin fibroblasts do not show a similar response to TGF- β stimulation (Ravanti *et al.*, 1999b). Thus, it appears that fibroblasts from the oral cavity, during both development and adulthood, share this unique capability to express MMP-13 when exposed to TGF- β s.

In addition to MMP-13, we could also detect the expression of TIMP-2, MMP-2, and MT1-MMP in the midline seam at the time of palatal fusion. The absence of TIMP-2 expression in TGF- β 3 $-/-$ mice associated with the absence of palatal fusion raises the question of the role of TIMP-2 in this process. However, in cultured palatal mesenchymal cells, TIMP-2, MT1-MMP, and MMP-2 expressions were not suppressed in TGF- β 3-deficient cells and were not induced by TGF- β 3, suggesting that during palatal fusion they are not direct targets for TGF- β 3 signal, but rather their expression is regulated by the fusion process and by epithelial-mesenchymal interaction. To explore this possibility would require the successful establishment of phenotypically stable epithelial cultures, which is currently not feasible. It is thus possible that the absence of TIMP-2 expression in TGF- β 3 mutants *in vivo* is a consequence of the fusion process. It has been shown that palatal fusion is associated with degradation of the basement membrane at the time of epithelial fusion (Shuler *et al.*, 1992; Kaartinen *et al.*, 1997). Furthermore, our data show that either a synthetic inhibitor of MMPs or TIMP-2 inhibits palatal fusion *in vitro*. Therefore, one would anticipate, in the absence of fusion *in vivo*, a shift of the MMPs/TIMP-2 balance in favor of TIMP-2 rather than, as observed in TGF- β 3 $-/-$ mice, a lack of TIMP-2 expression. However, this paradoxical suppression of TIMP-2 is likely to be explained by its dual function. It has been shown that TIMP-2 functions as an adapter molecule, of which the C-terminal domain binds to the C-terminal domain of proMMP-2 and the N-terminal domain binds to MT1-MMP. The formation of a trimolecular complex between TIMP-2, MT1-MMP, and proMMP-2 localizes proMMP-2 at the cell surface and promotes its activation by additional MT1-MMP (Butler *et al.*, 1998; Shofuda *et al.*, 1998). The observation that MT1-MMP and TIMP-2 are expressed by the MEE, and MMP-2 by the adjacent mesenchyme, also suggests that MMP-2 activation preferentially occurs at the surface of the MEE. Thus, a complete absence of expression of TIMP-2 in the MEE in TGF- β 3 $-/-$ mice likely prevents the activation of proMMP-2 by MT1-MMP. This effect, in association with a dramatic decrease in MMP-13 expression at the site of fusion, would result in decreased proteolytic activity, and subsequent failure of palatal fusion. Our data thus have pointed to two MMP-mediated pathways involved in palatal fusion, MMP-13 and the MMP-2/MT1-MMP/TIMP-2 pathway. Among these, MMP-13 is directly controlled by TGF- β 3. In contrary, the MT1-MMP/MMP-2/TIMP-2 pathway, at least in the mesenchyme, does not seem to be under the direct control of TGF- β 3. This functional redundancy may explain why neither MMP-2, TIMP-2, nor MT1-MMP-deficient mice develop a cleft palate (Itoh *et al.*, 1998; Caterina *et al.*, 1999; Holmbeck *et al.*, 1999).

Currently, it is thought that during palatal fusion most of the MEE cells are removed from the midline seam mainly by EMT. It has been shown that MMPs can directly induce EMT in mammary gland epithelial cells during neoplastic progression (Lochter *et al.*, 1997). Induction of MMP-3 was

shown to result in proteolytic removal of extracellular portions of E-cadherin, which led to a subsequent disappearance of E-cadherin and catenins from adherens junctions, and phenotypic transdifferentiation. Moreover, MMPs can either degrade or modify the basement membrane proteins and/or other ECM components. This will lead to altered cell-matrix interactions, which are known to play an important role in defining either an epithelioid or fibroblastoid phenotype (Sander *et al.*, 1998; Gimond *et al.*, 1999). Our present results demonstrate that inactivation of MMPs in vitro by using a synthetic MMP inhibitor, BB-3103 or TIMP-2, leads to a failed palatal fusion with no signs of EMT. Therefore, it is possible that MMPs, and in particular MMP-13, specifically expressed in the degrading midline seam during palatal fusion, could directly initiate EMT by influencing cell-cell and/or cell-matrix interactions. Furthermore, the observation that palatal fusion is inhibited by TIMP-2, which has no activity against members of the adamalysin metalloproteinase family (Amour *et al.*, 1998), further supports our claim for an involvement of the matrix metalloproteinases in palatal fusion.

In summary, palatal fusion is a complex process of craniofacial development involving a concerted action of many genes and their products. In the present study, we demonstrate that MMP-13 is precisely expressed as a response to TGF- β 3 stimulation in the degrading midline seam during palatal fusion, whereas MT1-MMP, MMP-2, and TIMP-2 appear indirectly controlled. The temporal and spatial well-defined expression of these proteins possibly plays a prominent role in palatal fusion.

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REFERENCES

- Amour, A., Slocombe, P.M., Webster, A., Butler, M., Knight, C.G., Smith, B.J., Stephens, P.E., Shelley, C., Hutton, M., Knäuper, V., Docherty, A.J.P., and Murphy, G. (1998). TNF- α converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett.* 435, 39–44.
- Basbaum, C.B., and Werb, Z. (1996). Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. *Curr. Opin. Cell Biol.* 8, 731–738.
- Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., and Engler, J.A. (1993). Matrix metalloproteinases: a review. *Crit. Rev. Oral Biol. Med.* 4, 197–250.
- Blavier, L., and DeClerck, Y.A. (1997). Tissue inhibitor of metalloproteinase-2 is expressed in the interstitial matrix in adult mouse organs and during embryonic development. *Mol. Biol. Cell* 8, 1513–1527.
- Brunet, C.L., Sharpe, P.M., and Ferguson, M.W. (1993). The distribution of epidermal growth factor binding sites in the developing mouse palate. *Int. J. Dev. Biol.* 37, 451–458.
- Butler, G.S., Butler, M.J., Atkinson, S.J., Will, H., Tamura, T., Van Westrum, S.S., Crabbe, T., Clements, J., D'Ortho, M.P., and Murphy, G. (1998). The TIMP2 membrane type 1 metalloproteinase "receptor" regulates the concentration and efficient activation of progelatinase A - a kinetic study. *J. Biol. Chem.* 273, 871–880.
- Carette, M.J., and Ferguson, M.W. (1992). The fate of medial edge epithelial cells during palatal fusion in vitro: an analysis by DiI labeling and confocal microscopy. *Development* 114, 379–388.
- Caterina, J., Caterina, N., Yamada, S., Holmbeck, K., Longenecker, G., Shi, J., Netzel-Arnett, S., Engler, J., Yermovski, A., Windsor, J., and Birkedal-Hansen, H. (1999). Murine TIMP-2 gene-targeted mutation. *Ann. N.Y. Acad. Sci.* 878, 528–30, 528–530.
- Chenevix-Trench, G., Jones, K., Green, A.C., Duffy, D.L., and Martin, N.G. (1992). Cleft lip with or without cleft palate: associations with transforming growth factor alpha and retinoic acid receptor loci. *Am. J. Hum. Genet.* 51, 1377–1385.
- Denhardt, D.T., Feng, B., Edwards, D.R., Cocuzzi, E.T., and Malyankar, U.M. (1993). Tissue inhibitor of metalloproteinases (TIMP, aka EPA): structure, control of expression and biological functions. *Pharmacol. Ther.* 59, 329–341.
- Ferguson, M.W. (1988). Palate development. *Dev. Suppl.* 103, 41–60.
- 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., Kondaiah, P., and Akhurst, R.J. (1990). Differential expression of TGF beta isoforms in murine palatogenesis. *Development* 109, 585–595.
- Foda, H.D., Rollo, E.E., Brown, P., Pakbaz, H., Berisha, H., Said, S.I., and Zucker, S. (1999). Attenuation of oxidant-induced lung injury by the synthetic matrix metalloproteinase inhibitor BB-3103. *Ann. N.Y. Acad. Sci.* 878, 650–653.
- Fosang, A.J., Last, K., Knäuper, V., Murphy, G., and Neame, P.J. (1996). Degradation of cartilage aggrecan by collagenase-3 (MMP-13). *FEBS Lett.* 380, 17–20.
- Freije, J.M., Diez Itza, I., Balbin, M., Sanchez, L.M., Blasco, R., Tolivia, J., and Lopez Otin, C. (1994). Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. *J. Biol. Chem.* 269, 16766–16773.
- Gack, S., Vallon, R., Schmidt, J., Grigoriadis, A., Tuckermann, J., Schenkel, J., Weiher, H., Wagner, E.F., and Angel, P. (1995). Expression of interstitial collagenase during skeletal development of the mouse is restricted to osteoblast-like cells and hypertrophic chondrocytes. *Cell Growth Differ.* 6, 759–767.
- Gehris, A.L., D'Angelo, M., and Greene, R.M. (1991). Immunodepletion of the transforming growth factors beta 1 and beta 2 in the developing murine palate. *Int. J. Dev. Biol.* 35, 17–24.
- Gimond, C., van Der, F.A., van Delft, S., Brakebusch, C., Kuikman, I., Collard, J.G., Fassler, R., and Sonnenberg, A. (1999). Induction of cell scattering by expression of beta1 integrins in beta1-deficient epithelial cells requires activation of members of the rho family of GTPases and downregulation of cadherin and catenin function. *J. Cell Biol.* 147, 1325–1340.
- Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S.A., Mankani, M., Robey, P.G., Poole, A.R., Pidoux, I., Ward, J.M., and Birkedal-Hansen, H. (1999). MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell* 99, 81–92.
- Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H., and Itohara, S. (1998). Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res.* 58, 1048–1051.
- Jill, M.A., Cottell, D.C., Russell, K.J., FitzGerald, M.X., and O'Connor, C.M. (1999). Migration of neutrophils across human pulmonary endothelial cells is not blocked by matrix metalloproteinase or serine protease inhibitors. *Am. J. Respir. Cell. Mol. Biol.* 20, 1209–1219.
- Kaartinen, V., Cui, X.M., Heisterkamp, N., Groffen, J., and Shuler, C.F. (1997). Transforming growth factor-beta3 regulates transdiffer-

- entiation of medial edge epithelium during palatal fusion and associated degradation of the basement membrane. *Dev. Dyn.* 209, 255–260.
- 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.
- Kaufman, M.H. (1992). *The Atlas of Mouse Development*, London: Academic Press.
- Knäuper, V., Cowell, S., Smith, B., López-Otin, C., O'Shea, M., Morris, H., Zardi, L., and Murphy, G. (1997). The role of the C-terminal domain of human collagenase-3 (MMP-13) in the activation of procollagenase-3, substrate specificity, and tissue inhibitor of metalloproteinase interaction. *J. Biol. Chem.* 272, 7608–7616.
- Knäuper, V., Lopez Otin, C., Smith, B., Knight, G., and Murphy, G. (1996). Biochemical characterization of human collagenase-3. *J. Biol. Chem.* 271, 1544–1550.
- Lochter, A., Galosy, S., Muschler, J., Freedman, N., Werb, Z., and Bissell, M.J. (1997). Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J. Cell Biol.* 139, 1861–1872.
- Mattot, V., Raes, M.B., Henriot, P., Eeckhout, Y., Stehelin, D., Vandenbunder, B., and Desbiens, X. (1995). Expression of interstitial collagenase is restricted to skeletal tissue during mouse embryogenesis. *J. Cell Sci.* 108, 529–535.
- Miettinen, P.J., Berger, J.E., Meneses, J., Phung, Y., Pedersen, R.A., Werb, Z., and Derynck, R. (1995). Epithelial immaturity and multi-organ failure in mice lacking epidermal growth factor receptor. *Nature* 376, 337–341.
- Miettinen, P.J., Chin, J.R., Shum, L., Slavkin, H.C., Shuler, C.F., Derynck, R., and Werb, Z. (1999). Epidermal growth factor receptor function is necessary for normal craniofacial development and palate closure. *Nat. Genet.* 22, 69–73.
- Mitchell, P.G., Magna, H.A., Reeves, L.M., Lopresti-Morrow, L.L., Yocum, S.A., Rosner, P.J., Geoghegan, K.F., and Hambor, J.E. (1996). Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. *J. Clin. Invest.* 97, 761–768.
- Nugent, P., Ma, L., and Greene, R.M. (1998). Differential expression and biological activity of retinoic acid-induced TGF β isoforms in embryonic palate mesenchymal cells. *J. Cell Physiol.* 177, 36–46.
- Pelton, R.W., Hogan, B.L., Miller, D.A., and Moses, H.L. (1990). Differential expression of genes encoding TGF β s 1, 2, and 3 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. *J. Embryol. Exp. Morphol.* 16, 171–182.
- Proetzel, G., Pawlowski, S.A., Wiles, M.V., Yin, M., Boivin, G.P., Howles, P.N., Ding, J., Ferguson, M.W., and Doetschman, T. (1995). Transforming growth factor- β 3 is required for secondary palate fusion. *Nat. Genet.* 11, 409–414.
- Ravanti, L., Hakkinen, L., Larjava, H., Saarialho-Kere, U., Foschi, M., Han, J., and Kahari, V.M. (1999a). Transforming growth factor- β induces collagenase-3 expression by human gingival fibroblasts via p38 mitogen-activated protein kinase. *J. Biol. Chem.* 274, 37292–37300.
- Ravanti, L., Heino, J., Lopez-Otin, C., and Kahari, V.M. (1999b). Induction of collagenase-3 (MMP-13) expression in human skin fibroblasts by three-dimensional collagen is mediated by p38 mitogen-activated protein kinase. *J. Biol. Chem.* 274, 2446–2455.
- Robbins, J.R., McGuire, P.G., Wehrle-Haller, B., and Rogers, S.L. (1999). Diminished matrix metalloproteinase 2 (MMP-2) in ectomesenchyme-derived tissues of the Patch mutant mouse: regulation of MMP-2 by PDGF and effects on mesenchymal cell migration. *Dev. Biol.* 212, 255–263.
- Sander, E.E., van Delft, S., ten Klooster, J.P., Reid, T., van der Kammen, R.A., Michiels, F., and Collard, J.G. (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol.* 143, 1385–1398.
- Shiota, K., Fujita, S., Akiyama, T., and Mori, C. (1990). Expression of the epidermal growth factor receptor in developing fetal mouse palates: an immunohistochemical study. *Am. J. Anat.* 188, 401–408.
- Shofuda, K., Moriyama, K., Nishihashi, A., Higashi, S., Mizushima, H., Yasumitsu, H., Miki, K., Sato, H., Seiki, M., and Miyazaki, K. (1998). Role of tissue inhibitor of metalloproteinases-2 (TIMP-2) in regulation of pro-gelatinase A activation catalyzed by membrane-type matrix metalloproteinase-1 (MT1-MMP) in human cancer cells. *J. Biochem.* 124, 462–470.
- 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.
- Shuler, C.F., Halpern, D.E., Guo, Y., and Sank, A.C. (1992). Medial edge epithelium fate traced by cell lineage analysis during epithelial-mesenchymal transformation in vivo. *Dev. Biol.* 154, 318–330.
- Smiley, G.R., and Koch, W.E. (1971). Fine structure of mouse secondary palate development in vitro. *J. Dental Res.* 50, 1671–1677.
- Sun, D., Vanderburg, C.R., Odierna, G.S., and Hay, E.D. (1998). TGF β 3 promotes transformation of chicken palate medial edge epithelium to mesenchyme in vitro. *Development* 125, 95–105.
- Taya, Y., O'Kane, S., and Ferguson, M.W. (1999). Pathogenesis of cleft palate in TGF- β 3 knockout mice. *Development* 126, 3869–3879.
- Theret, N., Lehti, K., Musso, O., and Clement, B. (1999). MMP2 activation by collagen I and concanavalin A in cultured human hepatic stellate cells. *Hepatology* 30, 462–468.
- Werb, Z. (1997). ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 91, 439–442.