Conditional inactivation of *Tgfbr2* in cranial neural crest causes cleft palate and calvaria defects

Yoshihiro Ito[1], Jae Yong Yeo[1], Anna Chytil[2], Jun Han[1], Pablo Bringas, Jr[1], Akira Nakajima[1], Charles F. Shuler[1], Harold L. Moses[2] and Yang Chai[1,*]

[1]Center for Craniofacial Molecular Biology School of Dentistry University of Southern California, 2250 Alcazar Street, CSA 103, Los Angeles, CA 90033, USA
[2]Department of Cancer Biology, Vanderbilt University, 22 South Pierce Avenue, PRB Room 649, Nashville, TN 37232, USA
*Author for correspondence (e-mail: ychai@usc.edu)

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Summary

Cleft palate and skull malformations represent some of the most frequent congenital birth defects in the human population. Previous studies have shown that TGFβ signaling regulates the fate of the medial edge epithelium during palatal fusion and postnatal cranial suture closure during skull development. It is not understood, however, what the functional significance of TGFβ signaling is in regulating the fate of cranial neural crest (CNC) cells during craniofacial development. We show that mice with *Tgfbr2* conditional gene ablation in the CNC have complete cleft secondary palate, calvaria agenesis, and other skull defects with complete phenotype penetrance. Significantly, disruption of the TGFβ signaling does not adversely affect CNC migration. Cleft palate in *Tgfbr2* mutant mice results from a cell proliferation defect within the CNC-derived palatal mesenchyme. The midline epithelium of the mutant palatal shelf remains functionally competent to mediate palatal fusion once the palatal shelves are placed in close contact in vitro. Our data suggests that TGFβ IIR plays a crucial, cell-autonomous role in regulating the fate of CNC cells during palatogenesis. During skull development, disruption of TGFβ signaling in the CNC severely impairs cell proliferation in the dura mater, consequently resulting in calvaria agenesis. We provide in vivo evidence that TGFβ signaling within the CNC-derived dura mater provides essential inductive instruction for both the CNC- and mesoderm-derived calvarial bone development. This study demonstrates that TGFβ IIR plays an essential role in the development of the CNC and provides a model for the study of abnormal CNC development.

Key words: Cranial neural crest (CNC), Calvaria development, Palatogenesis, TGFβ type II receptor signaling

Introduction

The vertebrate neural crest is a pluripotent cell population derived from the lateral ridges of the neural plate during early stages of embryogenesis. During craniofacial development, cranial neural crest (CNC) cells migrate ventrolaterally as they populate the branchial arches. The proliferative activity of these crest cells produces the discrete swellings that demarcate each branchial arch. Following their migration, CNC cells contribute extensively to the formation of mesenchymal structures in the head and neck, of which palate and calvaria development are classic examples. The migration, proliferation and differentiation of CNC cells are regulated by growth factor signaling pathways and their downstream transcription factors before they become committed to an array of different phenotypes (Noden, 1983; Noden, 1991; Lumsden, 1988; Graham and Lumsden, 1993; Le Douarin et al., 1993; Echelard et al., 1994; Imai et al., 1996; Trainor and Krumlauf, 2000).

The mammalian palate develops from two primordia: the primary and the secondary palate. The primary palate represents only a small part of the adult hard palate and is the part anterior to the incisive fossa. The secondary palate is the primordium of the hard and soft palate in adults. Palate development is a multi-step process that involves palatal shelf growth, elevation, midline fusion of palatal shelves and the disappearance of the midline epithelial seam. The palatal structures are composed of the CNC-derived ectomesenchyme and pharyngeal ectoderm (Ferguson, 1988; Shuler, 1995; Wilkie and Morriss-Kay, 2001; Zhang et al., 2002).

TGFβ signaling plays a pivotal role in regulating palatogenesis. During mouse palatal development, both TGFβ1 and TGFβ3 are expressed in the medial edge epithelium (MEE) of the palatal shelves, whereas TGFβ2 expression is restricted to the CNC-derived mesenchyme beneath the MEE (Fitzpatrick et al., 1990; Pelton et al., 1990). Upon fusion of the palatal shelves and disappearance of the midline epithelial seam, the expression of TGFβ1 and TGFβ3 is lost, suggesting crucial functions of TGFβ signaling in regulating palatal fusion. Loss-of-function mutation of *Tgfb2* or *Tgfb3* results in cleft palate. *Tgfb2*-null mutant mice exhibit anteroposterior cleft of the secondary palate with only 23% phenotype penetrance (Sanford et al., 1997). Significantly, *Tgfb3*-null mutation results in 100% penetrance of cleft secondary palate (Kaartinen et al., 1995; Proetzel et al., 1995). The etiology of cleft palate in *Tgfb3*-null mutant mice is apparently due to a failure of fusion of palatal shelves, which has been rescued by

addition of exogenous TGFβ3 in an in vitro organ culture system (Brunet et al., 1995; Taya et al., 1999). Subsequent studies have shown that TGFβ3 is specifically required for the fusion of palatal shelves, probably by enhancing the transformation of MEE cells into the palatal mesenchyme and inducing apoptosis in the MEE (Sun et al., 1998; Martinez-Alvarez et al., 2000).

TGFβ IIR is expressed in both the MEE and CNC-derived palatal mesenchyme (Wang et al., 1995; Cui et al., 1998). The physiological function of TGFβ IIR in regulating palatogenesis is not known because *Tgfbr2*-null mutation results in early embryonic lethality, thus, making it impossible to investigate the functional significance of this signaling molecule in regulating palatogenesis (Oshima et al., 1996). Up until now, most of the palatogenesis studies, such as the ones involving TGFβ signaling, have mainly focused on the molecular regulation of the fate of MEE cells. Although CNC cells are critical for palatogenesis, very little is known about the molecular mechanism that regulates the fate of the CNC-derived palatal mesenchyme during palatogenesis.

The vertebrate skull includes both the neurocranium (such as the calvaria and base of skull) and viscerocranium (such as mandible, zygoma, maxilla, etc.). Calvaria formation is a complex and lengthy developmental process that is initiated during embryogenesis and is completed in adulthood. The size flexibility of the calvaria is crucial for accommodating the rapid growth of the brain. Both the mesoderm and CNC-derived ectomesenchyme contribute to the cranial skeletogenic mesenchyme, which gives rise to bony elements (such as frontal, parietal and occipital bones) collectively known as the calvaria (Wilkie and Morriss-Kay, 2001). Studies have shown that the dura mater, a dense fibrous membrane underneath the calvaria, and cranial sutures provides crucial regulatory signals for calvaria development. To date, studies suggest that cranial sutures function as signaling centers for bone growth and remain patent postnatally to accommodate cranium expansion. Premature closure of cranial sutures affects the growth of the calvaria and results in craniosynostosis (Wilkie and Morriss-Kay, 2001).

Multiple growth and transcription factors play pivotal roles in regulating the osteogenic ability of cranial sutures. In particular, TGFβ signaling stimulates osteogenic progenitor cell proliferation and can induce premature suture obliteration in cultured fetal rat calvaria, suggesting that TGFβ signaling plays an important regulatory role in postnatal calvaria development (Opperman et al., 2000). In addition, TGFβ signaling within the immature dura mater (in newborn and immature animals) possesses the ability to induce calvaria bone repair, while diminished TGFβ signaling within the mature dura mater fails to repair calvarial defect, suggesting that TGFβ signaling is a crucial regulator for calvarial ossification (Greenwald et al., 2000). TGFβ IIR is expressed in the dura mater and cranial sutures, presumably playing an important role during skull development (Pelton et al., 1990; Lawler et al., 1994; Wang et al., 1995). Collectively, these studies have demonstrated that TGFβ signaling has an important regulatory function for postnatal cranial suture patency and skull repair. However, it remains unclear what the physiological function of TGFβ signaling is in regulating the initiation and development of the calvaria during embryogenesis.

To investigate the role of TGFβ signaling in regulating the fate of CNC cells during palate and calvaria development, we performed tissue-specific *Tgfbr2* gene ablation using *Cre/loxP* recombination exclusively in the cranial neural crest lineage. Our study shows that loss of *Tgfbr2* in the CNC cells results in cleft secondary palate and calvaria defects with 100% phenotype penetrance. Specifically, conditional *Tgfbr2* mutation inhibits cyclin D1 expression and affects CNC cell proliferation in the palatal mesenchyme. The midline epithelium of the mutant palatal shelf remains functionally competent to mediate palatal fusion once the palatal shelves are placed in close contact in vitro. Disruption of TGFβ signaling in the CNC severely impairs cell proliferation in the dura mater, consequently resulting in calvaria agenesis. We provide the first in vivo evidence that TGFβ signaling within the CNC-derived dura mater provides essential inductive instruction for both the CNC- and mesoderm-derived calvarial bone development.

Materials and methods

Two-component genetic system for marking the progeny of CNC cells

Both *Wnt1-Cre* transgenic line and *R26R* conditional reporter allele have been described previously (Danielian et al., 1998; Soriano, 1999). Mating *Wnt1-Cre* and *R26R* mice generated transgenic mice with progenies of neural crest cells labeled with β-gal because once *Wnt1-Cre* expression commences in premigrating neural crest cells, the β-galactosidase is indelible. Detection of β-galactosidase (*lacZ*) activity in both whole embryos and tissue sections was carried out as previously described (Chai et al., 2000).

Generation of *Tgfbr2$^{fl/fl}$;Wnt1-Cre mutant* mice and histological analysis

All mouse embryos used in this study were maintained on C57BL6/J background. Mating *Tgfbr2$^{fl/+}$;Wnt1-Cre* with *Tgfbr2$^{fl/fl}$* mice generated *Tgfbr2$^{fl/fl}$;Wnt1-Cre* null alleles that were genotyped using PCR primers as previously described (Chytil et al., 2002). All samples were fixed in 10% buffered formalin and processed into serial paraffin wax-embedded sections using routine procedures. For general morphology, deparaffinized sections were stained with Hematoxylin and Eosin using standard procedures.

Analysis of cell proliferation, death and density

DNA synthesis activity within the palate or skull was monitored by intraperitoneal BrdU (5-bromo-2′-deoxy-uridine, Sigma) injection (100 μg/g body weight) at E12.5, E13.4 and E14.5. One hour after the injection, mice were sacrificed and embryos were fixed in Carnoy's fixative solution and processed. Serial sections of the specimen were cut at 5 μm intervals. Detection of BrdU labeled cells was carried out by using a BrdU Labeling and Detection kit and following manufacturer's protocol (Boehringer Mannheim). BrdU-positive and total number of cells within the palatal mesenchyme or MEE of palatal shelf were counted from five randomly selected sections per sample. Five palate samples were evaluated from each experimental group. TUNEL assay was performed using the In Situ Cell Death Detection (fluorescein) kit (Roche Molecular Biochemicals) by following the manufacturer's protocol. Cell density analysis was performed by counting the number of cells per unit area from 20 randomly selected sections per experimental group. Student's *t*-test was applied for statistical analysis. A P value of less than 0.05 was considered statistically significant.

Palatal shelf organ cultures

Timed-pregnant mice were sacrificed on postcoital day 13.5 (E13.5). Genotyping was carried out as described above. The palatal shelves

were microdissected and cultured in serumless chemically defined medium as previously described (Shuler et al., 1991). After 3 days in culture, palates were harvested, fixed in 10% buffered formalin and processed.

Western analysis

The total protein concentration in the palates was determined by comparison with BSA standards. Seventy-five micrograms total protein from each sample was loaded in each well on a 12% polyacrylamide gel. Western analysis was carried out as previously described (Chai et al., 1999). Antibodies used: anti-cyclin D1 and anti-CDK4 (BD Biosciences), anti-Msx1 (kindly provided by P. Denny, USC) and anti-β-actin (Santa Cruz Biotechnology).

Whole-mount skeletal staining

The three-dimensional architecture of the skeleton was examined using a modified whole-mount Alcian blue-Alizarin Red S staining protocol (details available upon request).

Immunohistochemistry

Sectioned immunohistochemistry was performed with an Immunostaining kit (Zymed) according to manufacturer's directions. The following antibodies were used for this experiment: anti-BrdU (Sigma), anti-cyclin D1 (BD Biosciences) and anti-p21 (Santa Cruz Biotechnology). Positive staining was shown in orange-red for immunohistochemistry. The slides were counterstained with Hematoxylin.

Results

Fate of cranial neural crest during palatogenesis

To date, little is known about the fate of the CNC-derived palatal mesenchyme or the molecular mechanism that regulates the specification of these progenitor cells during palate development. We provide in vivo analysis of the dynamic distribution of CNC cells during palatogenesis by using the *Wnt1-Cre;R26R* animal model for indelibly marking the progenies of CNC cells (Chai et al., 2000). During extension of the palatal shelf, CNC-derived cells (blue) are mixed with non-CNC-derived cells (pink, mesenchymally derived) at both anterior and posterior regions of the developing palate at E13.5 (Fig. 1A-D). Between E13.5 and E14.5, rapid growth of the palatal shelves brings the two processes into horizontal apposition above the tongue. Subsequently, the opposing palatal shelves fuse following the disappearance of midline epithelial cells at around E14.5. At this point, the anterior region of the secondary palate shows disruption of the midline epithelium at the fusion site (Fig. 1E,F). The palatal

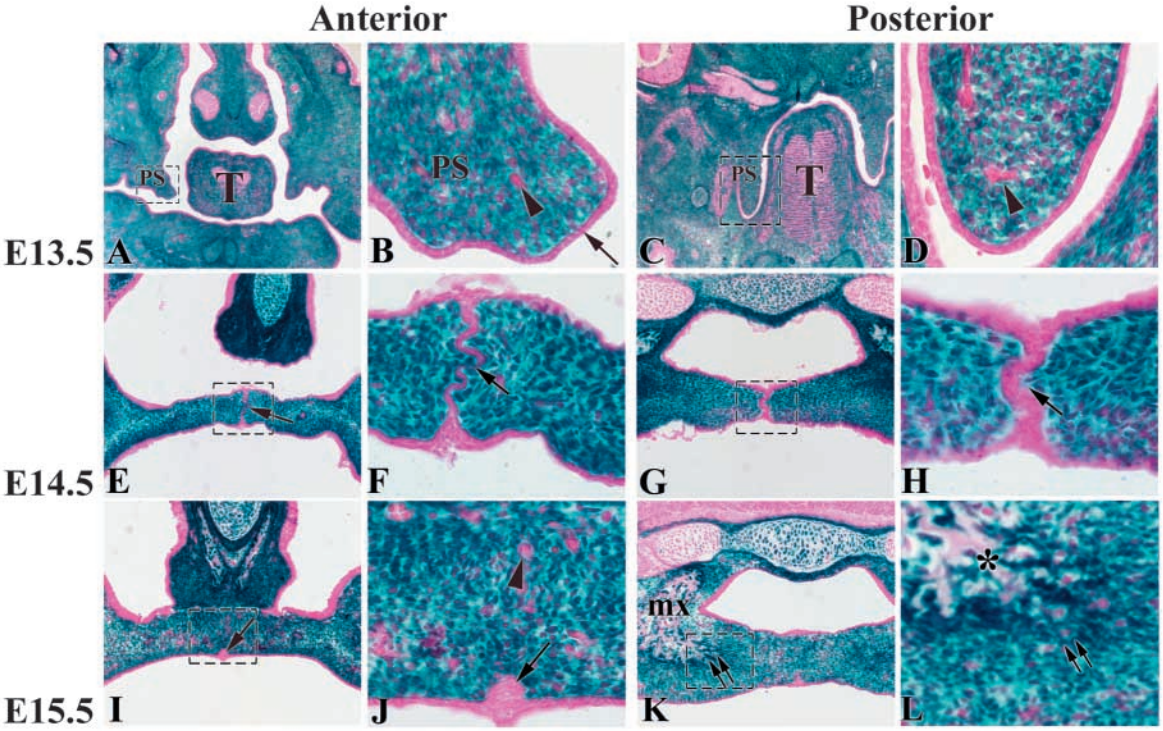

Fig. 1. Contribution of CNC cells during palatogenesis as seen in *Wnt1-Cre;R26R* mice. (A,B) At E13.5, the anterior region of the palatal shelf (PS) projects downwards along the side of tongue (T). CNC-derived cells (blue) contribute significantly to the palatal mesenchyme, although there are few non-CNC cells (arrowhead) present in the palate. The palatal epithelium is free of β-gal-positive cells, accurately reflecting their embryonic origin and validating the specificity of the two-component genetic system for marking the progenies of CNC cells. The boxed areas in A,C,E,G,I,K are enlarged in B,D,F,H,J,L, respectively. (C,D) Posterior portion of the palatal shelf is populated with both CNC- and non-CNC-derived cells at E13.5. (E,F) At E14.5, anterior portion of the palate is fused. There is disruption of the midline epithelium (arrow). Notice there are very few non-CNC cells (pink) at the fusion site. (G,H) Posterior palatal shelves have begun the fusion process with the remaining intact midline epithelium (arrow) at E14.5. (I,J) At E15.5, palatal fusion is complete with the disappearance of midline epithelium. Arrowhead indicates non-CNC-derived palatal mesenchymal cells. Arrow indicates remaining of the midline epithelium at the junction with the oral epithelium. (K,L) Aggregated CNC cells (double arrow) are present to initiate palatal bone formation at E15.5. MX, maxilla; *, the forming palatal bone.

mesenchyme is mainly populated with CNC-derived cells, especially in the region adjacent to the midline epithelium, indicating the important biological function of the CNC cell during palatal fusion (Fig. 1F). In the posterior portion of the secondary palate, the opposing palatal shelves have fused, leaving a remnant of a continuous midline epithelium at the fusion site (Fig. 1G,H). At E15.5, palatal fusion is complete and the palatal mesenchyme is mainly populated with CNC-derived cells (Fig. 1I,J). After fusion, CNC-derived cells have begun to form an aggregated cell mass to initiate palatal bone formation in the palatal mesenchyme (Fig. 1K,L).

TGFβ IIR is specially required in the CNC-derived ectomesenchyme during palatogenesis

Although TGFβ IIR is strongly expressed in the CNC-derived palatal mesenchyme, mice deficient for the *Tgfbr2* gene die on embryonic day 10.5 (E10.5) as the result of defects of yolk sac hematopoiesis and vasculogenesis (Wang et al., 1995; Oshima et al., 1996). To circumvent this early lethality and to investigate the specific function of TGFβ IIR in regulating CNC cells during palatogenesis, we crossed a *Tgfbr2* conditional allele with the *Wnt1-Cre* transgenic mouse line and generated *Tgfbr2$^{fl/fl}$;Wnt1-Cre* embryos, in which the CNC-derived palatal mesenchyme was homozygous for the *Tgfbr2$^{fl/fl}$* null allele. Genetically, in the presence of *Cre* recombinase, the second exon of the *Tgfbr2* gene is removed, resulting in a null allele as previously described (Chytil et al., 2002). The *Wnt1* transgene drives *Cre* expression specifically in the neural crest lineage (Chai et al., 2000). In control (normal) embryos, one or both active *Tgfbr2* allele(s) was retained.

The complete failure of mouse secondary palate fusion was first detected in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos at E14.5 when normal palatal fusion had just occurred (Fig. 2A,B). We compared cross-sections of E14.5 *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryonic heads with the ones of *Tgfbr2$^{+/fl}$;Wnt1-Cre* or *Tgfbr2$^{fl/fl}$* littermate embryos. There was decreased cellular density (P<0.05) in the elevated palatal shelf mesenchyme of *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos (4298±275 cells/mm^2) when compared with the normal developing palate (5174±168 cells/mm^2), in which fusion occurred with the partial disappearance of the midline epithelium (Fig. 2C-F). At E16.5, both of the palatal shelves had elevated into horizontal position but failed to fuse at the midline in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos, while completed palatal fusion was observed in the control samples (Fig. 2G,H). The CNC-derived palatal mesenchyme began to form an aggregated cell mass as a

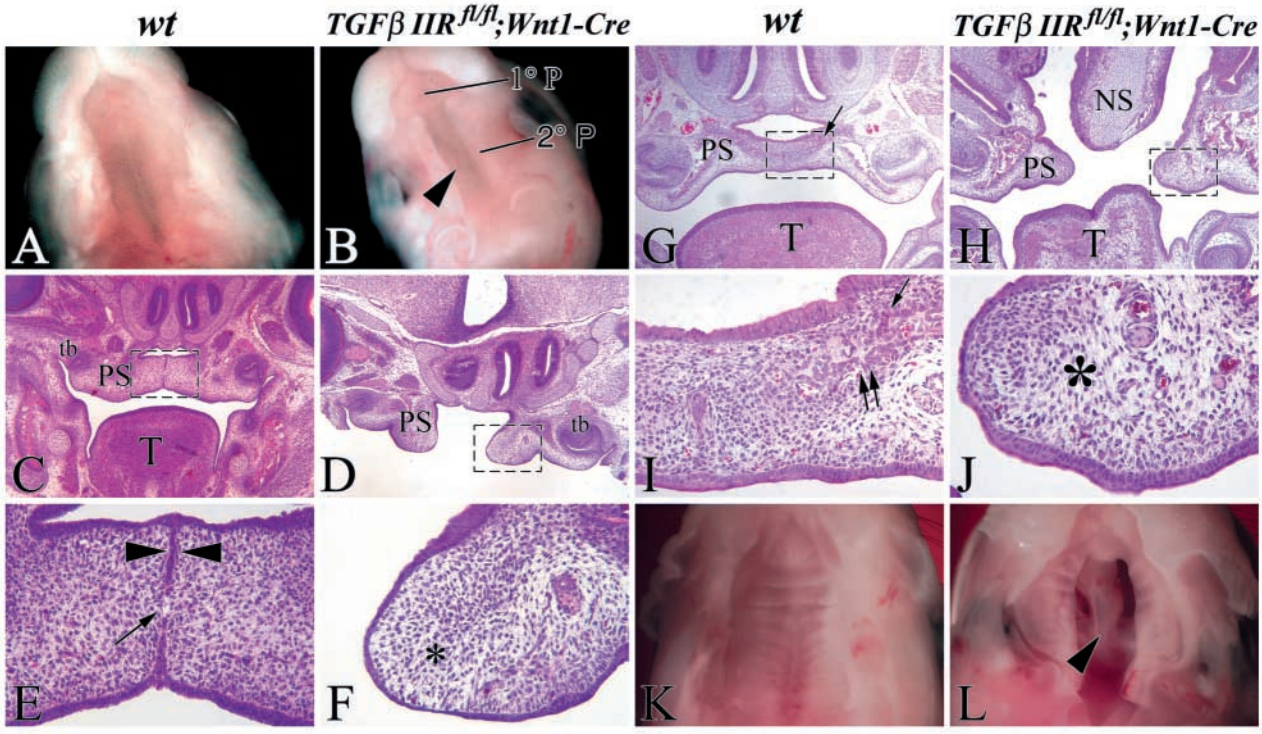

Fig. 2. *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutation causes complete cleft secondary palate. (A,C) At E14.5, palatal fusion is well under way in the wild-type embryo. PS, palate; T, tongue; tb, tooth bud. (B,D) At E14.5, *Tgfbr2$^{fl/fl}$;Wnt1-Cre* embryos show cleft secondary (2°P) palate (arrowhead), while the primary (1°P) palate is normal. PS, palatal shelf. Boxed areas in C,D are enlarged in E,F, respectively. (E) At E14.5, palate fusion is in progress and there is remnant of midline epithelium (arrowhead) at the fusion site (arrow, breakdown of epithelial seam). Cellular density of the palatal mesenchyme=5174±168 cells/mm^2. (F) E14.5 *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mouse palatal shelf shows reduction in cell density within the palatal mesenchyme (asterisk). Cellular density of the palatal mesenchyme=4298±275 cells/mm^2, a reduction of 17% when compared with the wild-type samples. (G,I) At E16.5, palatal fusion is complete in the wild-type embryos. Aggregated cell mass (double arrow) is clearly visible within the palatal mesenchyme (arrow, the initiation of palatal bone formation). (H,J) In *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples, there is failure of palatal fusion and significant reduction of cellular density within the palatal mesenchyme (asterisk) at E16.5. NS, nasal septum. Boxed areas in G,H are enlarged in I,J, respectively. (K) At birth, craniofacial structures are well developed in the wild-type embryos and both the primary and secondary palate have completely fused and developed properly. (L) In *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos, a complete cleft secondary palate is visible at birth. The development of primary palate is normal.

prelude to palatal bone development in the control sample (Fig. 2I), while CNC cell condensation was not observed in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryo (Fig. 2J). At birth, complete cleft secondary palate was observed in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice with 100% (36/36 newborn pups) phenotype penetrance (Fig. 2L).

Previous studies have shown that TGFβ signaling plays a pivotal role in regulating the fate of the medial edge epithelium (MEE) during palatal fusion (Pelton et al., 1990; Pelton et al., 1991; Fitzpatrick et al., 1990; Kaartinen et al., 1995; Sun et al., 1998; Martinez-Alvarez et al., 2000). To determine whether the *Tgfbr2$^{fl/fl}$* mutant MEE had any altered cellular function and was competent to mediate palatal fusion, we first evaluated cell proliferation and apoptosis activity in the MEE and found no difference between the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant and wild-type control samples at E12.5, E13.5 and E14.5 (data not shown), thereby suggesting that altered TGFβ signaling in the CNC-derived palatal mesenchyme did not adversely affect the fate of MEE cells during palatal fusion.

Next, we hypothesized that the failure of palatal fusion in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice was due to insufficient extension of the palatal shelves towards the midline. To test our hypothesis, we performed palatal fusion analysis by using a palatal shelf organ culture model. At E13.5, the developing palatal shelves were pointing downwards on both sides of the tongue. Each isolated pair of palatal shelves was placed in culture with the two segments just touching at the medial edge and kept in the original anteroposterior orientation, thus preventing any variability in growth rates from adversely affecting palatal development. During the 3 day culture period, both wild-type and *Tgfbr2$^{fl/fl}$* mutant palatal specimens fused. All cultured wild-type palatal shelves (*n*=32 pairs) showed complete fusion with normal disappearance of the MEE and development of a confluent palatal mesenchyme (Fig. 3A,C). Furthermore, osteoid-like structure was present in the cultured palatal shelf, suggesting that palatal bone formation was initiated in vitro (Fig. 3C, insert). Although all cultured *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant palatal shelves also showed fusion (*n*=9 pairs), some fused palates (4/9, 44%) had residual epithelium (arrow) at the midline, indicating a possible delay in the fusion process (Fig. 3B,D). Nevertheless, the MEE cells were competent to facilitate palatal fusion in *Tgfbr2$^{fl/fl}$* mutant samples once the palatal shelves were placed in close contact. In addition, osteoid-like structure was present (Fig. 3D, insert), suggesting that there was normal palatal bone formation in the cultured *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant palatal shelf.

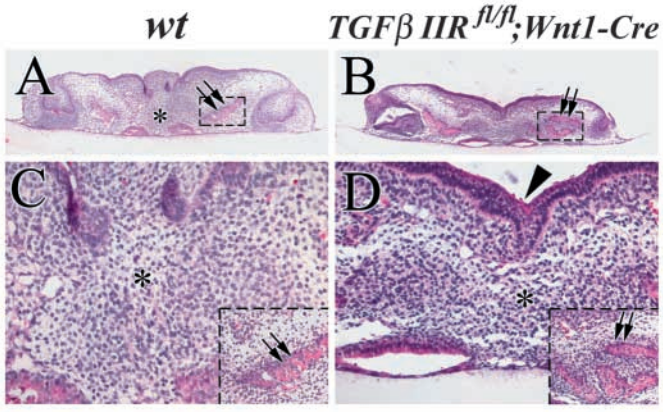

Fig. 3. *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant palatal shelves are able to fuse in vitro. (A,C) Wild-type E13.5 palatal shelves were cultured for 3 days. During this time, all palates fused (*n*=32), with complete disappearance of midline epithelium (asterisk). Boxed area in A is enlarged and shown as an insert in C. Double arrow indicates osteoid-like structure in the palate. (B,D) Cultured *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant palatal shelves also show fusion. Some fused palates, however, have residual epithelium (arrowhead) at the midline, indicating a possible delay in the fusion process. Boxed area in B is enlarged and shown as an insert in D. Double arrow indicates osteoid-like structure in the palate.

Conditional inactivation of *Tgfbr2* does not affect CNC migration but perturbs palatal mesenchymal cell proliferation

In order to test whether a CNC migration defect might have contributed to the deficiency of the CNC-derived palatal mesenchyme, and was responsible for the failure of palatal fusion, we crossed the *Tgfbr2* conditional allele with *R26R* transgenic mice and generated embryos with *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mutation. All of these embryos had identical malformations (such as complete cleft secondary palate) to the ones seen in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice. Whole-mount and sectioned β-gal staining showed no difference in migration or distribution of CNC cells within the first branchial arch and the frontonasal prominence between *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mutant and the wild-type control embryos from E8.5 to E11.5 (Fig. 4A-F and data not shown). At E14.5, palatal fusion was well under way in the wild-type embryos with CNC-derived cells populating the majority of the palatal mesenchyme (Fig. 4G). In *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mutant embryos, the palatal shelves were populated with the CNC-derived mesenchyme, without any indication of a deficiency in CNC migration (Fig. 4H). Taken together, our data suggest that there is no CNC migration defect that might have resulted in inadequate palatal shelf extension and failure of palate fusion in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice. We infer that TGFβ signaling is specifically required in the CNC-derived mesenchyme prior to palatal fusion.

To explore the mechanism responsible for causing the failure of palatal shelf extension in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos, we investigated whether there was a decrease in cell proliferation, an increase in apoptosis, or a combination of both in the CNC-derived palatal mesenchyme. Cell proliferation activity within the CNC-derived palatal mesenchyme, as measured by BrdU incorporation, appeared to be identical between control and *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos at E12.5 and E13.5 (Fig. 5A-D). However, at E14.5, there was a significant reduction ($P<0.01$) in the cell proliferation rate within the CNC-derived palatal mesenchyme of the *Tgfbr2$^{fl/fl}$* mutant embryos (18±2.9%) when compared with the ones of the wild-type control (29.6±5.9%) (Fig. 5E,F; Fig. 6). To rule out the possibility that palatal fusion itself was responsible for maintaining proliferation in the CNC-derived palatal mesenchyme, we had analyzed BrdU labeling indices in the palatal shelves both prior to and right after fusion (Fig. 5E and insert in E). The cell proliferation rate remained identical (30-32%) in the CNC-derived palatal mesenchyme of the wild-type embryos before and after the fusion process. Furthermore, by

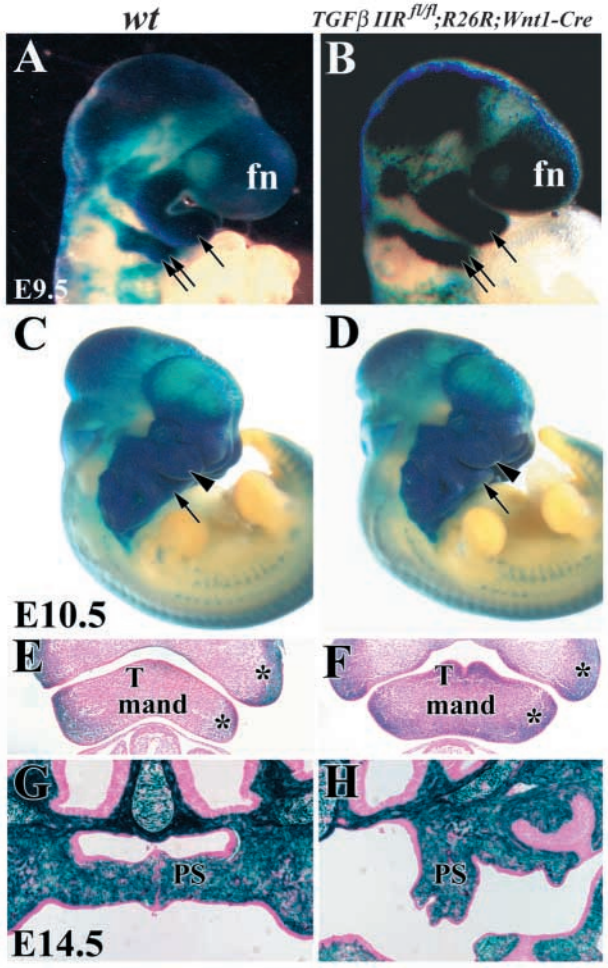

Fig. 4. Conditional null mutation of *Tgfbr2* signaling in the CNC-derived ectomesenchyme does not adversely affect the neural crest migration during early craniofacial development. (A) At E9.5, CNC cells (blue staining, *Wnt1cre;R26R*) have migrated into the frontonasal process (fn), and the first (arrow) and second (double arrow) branchial arches of the wild-type embryo. (B) Normal distribution of CNC cells is observed in the *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mutant embryos. (C,E) At E10.5, both mandibular (arrow) and maxillary (arrowhead) prominences are populated with CNC-derived cells in the wild-type embryo. (D,F) Identical CNC cell distribution is observed in both mandibular and maxillary prominences in the *Tgfbr2$^{fl/fl}$;R26R;Wnt1cre* mutant embryos. mand, mandibular prominence; *, CNC-derived cells; T, tongue bud with contributing CNC cells. (G) At E14.5, palatal fusion is well under way with CNC-derived cells populating the palatal shelf (PS) in the wild-type embryo. (H) *Tgfbr2$^{fl/fl}$;R26R;Wnt1cre* mutant embryos show identical pattern of CNC cells populating the palatal shelf (PS), indicating that there is no CNC migration defect.

processing single slide for β-gal and BrdU double staining, we found that the cell proliferation defect was exclusively associated with the CNC-derived palatal mesenchyme (not with non-CNC-derived mesenchyme) in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos (Fig. 2G,H). We concluded that TGFβ signaling specifically controls cell cycle progression in the CNC-derived palatal mesenchyme prior to palatal fusion. As *Wnt1-Cre* did not cause *Tgfbr2* deletion in the non-CNC-derived palatal mesenchyme, it was not possible to determine whether TGFβ IIR played a significant role in regulating the non-CNC-derived mesenchymal cell proliferation in the developing palate.

In order to understand the mechanism of TGFβ signaling in regulating the progression of the CNC-derived palatal mesenchymal cell cycle, we investigated possible alteration of cell cycle regulator expression in the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant embryos. Cyclin D1, a member of the cyclin D family, functions to regulate phosphorylation of the retinoblastoma gene products, thereby activating E2F transcription to facilitate cell cycle progression. We show that the expression of cyclin D1 was comparable in the palatal mesenchyme between the *Tgfbr2$^{fl/fl}$* mutant and the control samples at E12.5 and E13.5 (Fig. 5I-L). Significantly, cyclin D1 expression was greatly reduced in the palatal mesenchyme of the *Tgfbr2$^{fl/fl}$* mutant embryos at E14.5 when compared with the ones of wild-type control (Fig. 5M,N). The reduction of cyclin D1 expression was further confirmed by western and microarray analyses (Fig. 5 and data not shown). To rule out the possibility that palatal fusion itself was responsible for maintaining cyclin D1 expression in the CNC-derived palatal mesenchyme, we analyzed cyclin D1 expression in the palatal shelves both prior to and immediately after fusion (Fig. 5M and insert in M). Cyclin D1 expression remained in a similar pattern pre- and post-palatal fusion. We concluded that palatal fusion at E14.5 did not play a role in maintaining cyclin D1 expression in the CNC-derived palatal mesenchyme. We have also examined the expression of other cell cycle regulators (such as CDK4, CDK6, CDK inhibitors p21 and p18^{INK4c}) and found no significant difference between the wild-type and the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples (Fig. 5O,P, and data not shown). In addition, we have analyzed whether increased cell death might have contributed to compromised palatal shelf development in the mutant samples. TUNEL assay showed no difference in cellular apoptotic activity in the CNC-derived palatal mesenchyme between the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant and wild-type embryos (data not shown).

TGFβ signaling is known to regulate the expression of transcription factors which in turn may regulate the fate of CNC cells by controlling the progression of cell cycle (Moses and Serra, 1996; Han et al., 2003). Exogenous TGFβ can repress the transcriptional activity of the *Msx1* gene in the palatal mesenchyme in vitro (Nugent and Greene, 1998). We examined the expression level of Msx1 in the developing palate by western analysis. Msx1 expression level was identical between the wild type and the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant samples at E13.5 (Fig. 5Q). Significantly, Msx1 expression level was significantly elevated (2.5 times) in the palate of the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutants when compared with the Msx1 expression level in the controls (Fig. 5).

TGFβ signaling in the CNC-derived dura mater is required for calvaria development

During skull development, TGFβ ligand and its type II receptor are colocalized within the craniofacial mesenchyme and may regulate its differentiation (Fitzpatrick et al., 1990; Pelton et al., 1990; Lawler et al., 1994). A high level of TGFβ IIR mRNA expression is apparent in the meninges surrounding and covering the developing brain, suggesting an important functional role of this receptor in regulating the dura mater

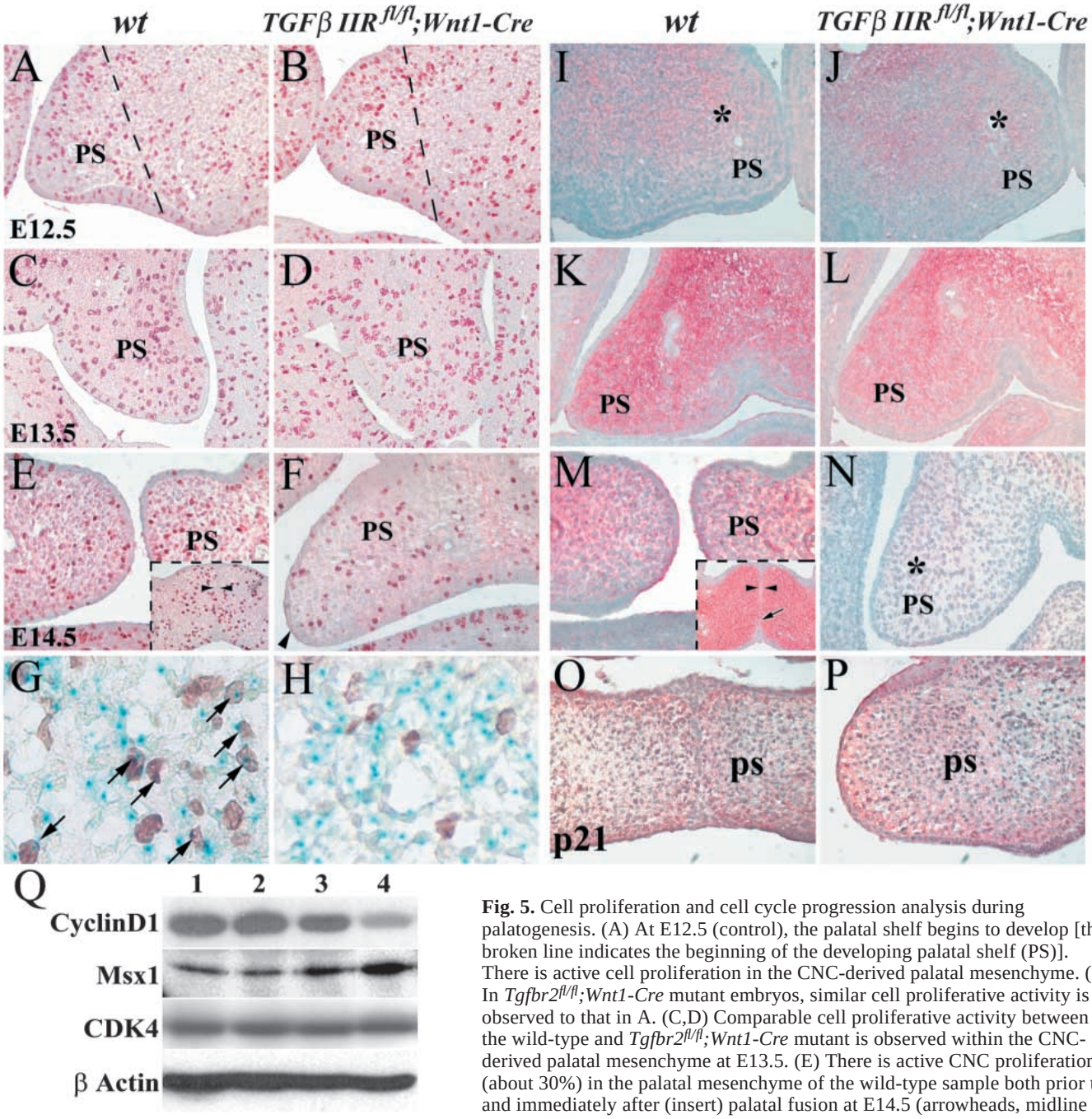

Fig. 5. Cell proliferation and cell cycle progression analysis during palatogenesis. (A) At E12.5 (control), the palatal shelf begins to develop [the broken line indicates the beginning of the developing palatal shelf (PS)]. There is active cell proliferation in the CNC-derived palatal mesenchyme. (B) In *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant embryos, similar cell proliferative activity is observed to that in A. (C,D) Comparable cell proliferative activity between the wild-type and *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant is observed within the CNC-derived palatal mesenchyme at E13.5. (E) There is active CNC proliferation (about 30%) in the palatal mesenchyme of the wild-type sample both prior to and immediately after (insert) palatal fusion at E14.5 (arrowheads, midline epithelial seam). (F) Significant reduction in CNC cell proliferation (18%) is clearly visible in the palatal mesenchyme of *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant samples. Arrowhead indicates midline epithelium. (G,H) Single slide stained for β-gal and then for BrdU to indicate whether or not the CNC-derived palatal mesenchyme is undergoing cell proliferation. In the wild-type samples (G), BrdU labeling (dark brown staining) significantly overlaps with β-gal-positive cells (arrow), indicating CNC-derived palatal mesenchymal cells are undergoing active cell proliferation at E14.5. In the *Tgfbr2*$^{fl/fl}$ mutant samples (H), BrdU-positive cells are not associated with β-gal-positive cells (blue), suggesting that the CNC-derived palatal mesenchyme fails to proliferate properly at E14.5. (I-L) Cyclin D1 expression (in red) is similar within the palatal mesenchyme in the wild-type and the *Tgfbr2*$^{fl/fl}$ mutant embryos at E12.5 (I,J) and E13.5 (K,L). (M,N) At E14.5, cyclin D1 is expressed extensively within the palatal mesenchyme in the wild-type sample (M), but is greatly reduced in the palatal mesenchyme of the *Tgfbr2*$^{fl/fl}$ mutant (N). The insert in M shows cyclin D1 expression in the palate immediately after fusion. Arrowheads indicate midline epithelial seam; arrow indicates gap in epithelial seam. (O,P) Normal p21 expression is shown in the palate of the wild-type and the *Tgfbr2*$^{fl/fl}$ mutant embryos at E14.5. Very low level of p21 expression is detected in the palatal mesenchyme (orange-red). (Q) Western analysis of cell cycle marker expression in the palate. Lane 1, E13.5 wild-type palate; lane 2, E13.5 *Tgfbr2*$^{fl/fl}$ mutant palate; lane 3, E14.5 wild type palate; lane 4, E14.5 *Tgfbr2*$^{fl/fl}$ mutant palate. Cyclin D1 expression is significantly reduced, while Msx1 expression is significantly elevated in the palate of E14.5 *Tgfbr2*$^{fl/fl}$ mutant embryos. CDK4 and β-actin expression remains consistent between the wild-type and the *Tgfbr2*$^{fl/fl}$ mutant samples.

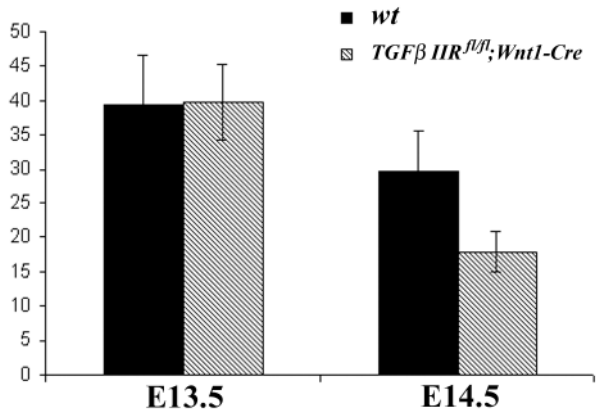

Fig. 6. Percentage of BrdU-labeled nuclei in the palatal mesenchyme of the wild-type and the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice. At E13.5 or E14.5, palate sample was serially sectioned for BrdU analysis. Five sections were randomly selected from each palate. The percentage of BrdU-labeled cells within the palatal mesenchyme was calculated from each section (E13.5: wild type, 39.2±7.3; mutant, 39.8±5.5; $P>0.05$) (E14.5: wild type, 29.6±5.9; mutant, 18.0±2.9; $P<0.01$). Five palates from each experimental group were analyzed.

development (Wang et al., 1995). Recently, it was shown that CNC cells contribute to the formation of the meninges, which underlies the entire calvaria (Jiang et al., 2002). Remaining unclear is the functional significance of TGFβ signaling in regulating the development of the dura mater as well as the consequence of an impaired dura formation in regulating the patterning of intramembranous bone development.

By analyzing the *Wnt1-Cre;R26R* embryos, we found that the CNC-derived dura mater covered the entire surface of the developing brain in the wild-type sample at E14.5 (Fig. 7A, blue). In *Tgfbr2$^{fl/fl}$;R26R;Wnt1-Cre* mutant embryos, dura development was severely impaired on the surface of the developing brain (Fig. 7B). Specifically, instead of having a well-defined dura that contained blood vessels as seen in the wild-type samples, the *Tgfbr2$^{fl/fl}$* mutant embryos showed a single cell layer, poorly developed dura mater (Fig. 7C,E). As shown in Fig. 4, there was no CNC migration defect in the *Tgfbr2$^{fl/fl}$* mutant embryos. This dura development defect resulted from severely impaired CNC cell proliferation activity in the *Tgfbr2$^{fl/fl}$* mutant embryos, while active CNC cell proliferation was observed in the dura of wild-type controls at E14.5 (Fig. 7D,F). Although there was only a poorly defined dura in the *Tgfbr2$^{fl/fl}$* mutants at E14.5, it suggested that CNC cells were able to contribute to early dura development. However, there was a specific requirement for TGFβ signaling during the continued dura development. As craniofacial development continued, the impaired TGFβ signaling in the CNC-derived dura mater failed to induce parietal bone formation (rostral region), while there was proper parietal bone development in the wild type samples at E16.5 (Fig. 7G,H). Eventually, the failure of inducing bone formation by the dura led to severely impaired calvaria development.

At birth, the *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice showed severe skull defects, including a missing frontal and severely retarded parietal bone (with only the development of posterior border portion), as well as a smaller mandible and maxilla (Fig. 7I,J). The overall size of the skull of the *Tgfbr2$^{fl/fl}$* mutants was about 25% smaller than those of the wild-type littermates (Fig. 7K). As a result of compromised calvaria development, skeletal elements of the cranial base of the *Tgfbr2$^{fl/fl}$* mutant became visible when viewed from above (Fig. 7K). *Tgfbr2$^{fl/fl}$* mutation also affected the proper development of the mandible, with a dramatically reduced coronoid process and condyle, and a missing mandibular angle (Fig. 7L).

Discussion

The fate of CNC cells and the regulatory function of TGFβ IIR during palatogenesis

To date, most of the palate development studies have focused on the molecular regulation of the fate of midline epithelial cells during palatal fusion, while little is known about the molecular mechanism that controls the fate of CNC cells during palatogenesis (Kaartinen et al., 1997; Martinez-Alvarez et al., 2000). CNC fate determination is an important developmental event because successful migration, proliferation and differentiation of these pluripotent cells are crucial for normal craniofacial development. Here, we have investigated the molecular mechanism by which the *Tgfbr2* gene regulates CNC-cell migration, proliferation and, ultimately, the formation of an aggregated cell mass prior to palatal bone formation during palatogenesis. By systematically following the lineage of CNC cells as they contribute to palate formation, our study shows that CNC cells contribute to the vast majority of the palatal mesenchyme and may possess crucial roles to regulate the epithelial-mesenchymal interaction during the extension and fusion of the palatal shelves. Evidently, the mesoderm-derived cells also contribute to the formation of the palatal mesenchyme. The dynamic distribution and close association between the CNC- and non-CNC-derived palatal mesenchyme suggest that these two cell populations may interact constantly throughout various stages of palatal development.

Until now, the function of TGFβ signaling in regulating the CNC-derived palatal mesenchyme is not well understood. TGFβ subtype expression is conspicuous in the cranial neural crest-derived mesenchyme during early mouse craniofacial development (Heine et al., 1987; Massague, 1990). The presence of TGFβ and its cognate receptors is obvious in the mesenchyme during crucial epithelial-mesenchymal interactions related to the formation of the palate, tooth, and Meckel's cartilage (Nugent and Greene, 1998; Hall, 1992; Chai et al., 1994; Wang et al., 1995; Lumsden and Krumlauf, 1996; Ito et al., 2002). Although the TGFβ type II receptor is strongly expressed in the CNC-derived palatal mesenchyme, mice deficient in *Tgfbr2* die before the formation of the palate, making it impossible to investigate the functional significance of TGFβ signaling in regulating the fate of CNC cells during palatogenesis (Wang et al., 1995; Oshima et al., 1996). Our animal model of *Tgfbr2* conditional gene ablation in the neural crest cells offers a unique opportunity to investigate the functional mechanism of TGFβ signaling in regulating the fate of the CNC-derived palatal mesenchyme. Owing to the lack of a CNC migration defect in *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice, we conclude that TGFβ IIR is not crucial for the proper migration of CNC cells into the first branchial arch. The cell proliferation defect in the CNC-derived palatal mesenchyme of *Tgfbr2$^{fl/fl}$;Wnt1-Cre* mutant mice clearly indicates that

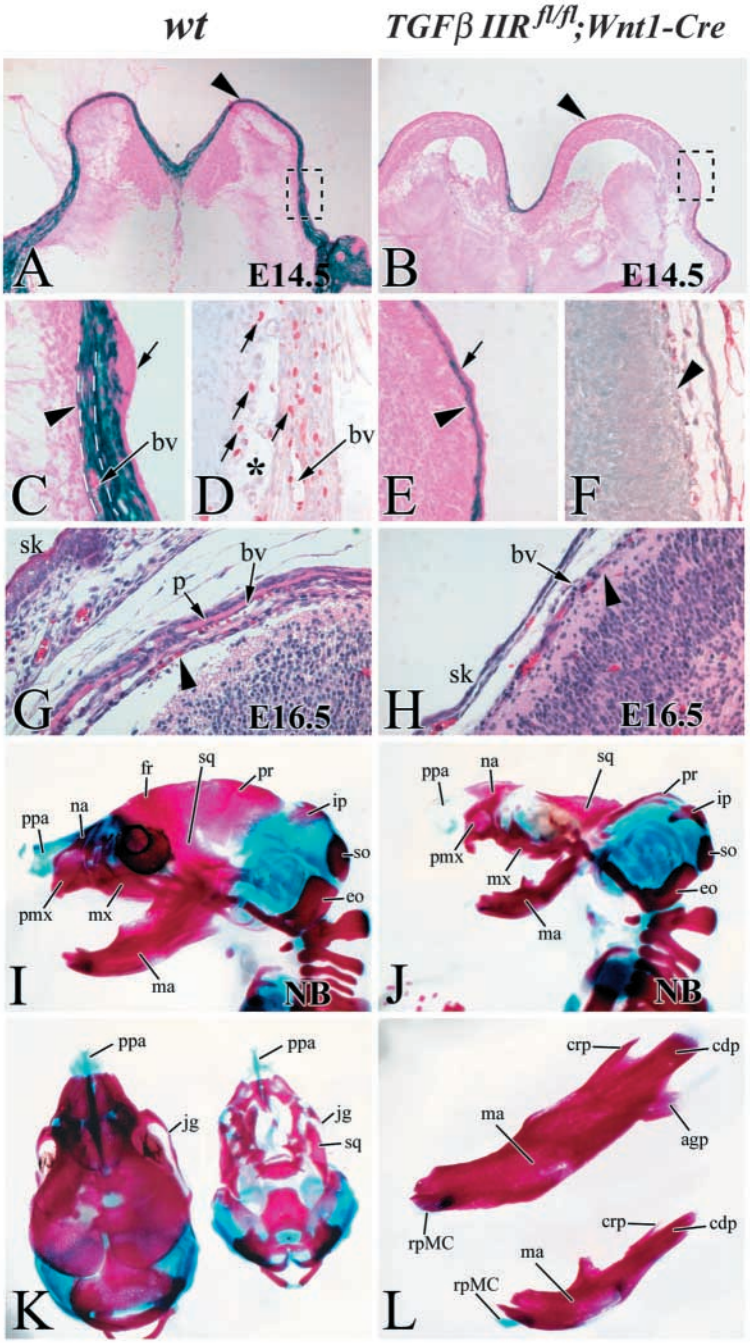

Fig. 7. Defects of the dura mater and skull in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice. (A) The well developed, CNC-derived meninges cover the entire surface of the developing brain in *Wnt1-Cre;R26R* embryo at E14.5. Arrowhead indicate the dura mater (blue). The boxed area is shown at higher magnification in C. (B) In the *Tgfbr2*$^{fl/fl}$ mutant sample, the dura mater development is severely impaired (arrowhead). The boxed area is shown at higher magnification in E. The dura mater (arrowhead, outlined by broken line) is well developed and contains blood vessels (bv) in the wild-type sample. The ectoderm (arrow) is free of *lacZ* expression. (D) Active cell proliferation is observed in the dura (arrows show BrdU-labeled cells). Asterisk indicates space resulting from tissue damage. A single-cell layer, poorly developed dura (arrowhead) in the *Tgfbr2*$^{fl/fl}$ mutant (arrow, ectoderm). (F) No detectable cell proliferation (as measured by BrdU labeling) in the poorly developed dura mater (arrowhead) in the *Tgfbr2*$^{fl/fl}$ mutant. (G) At E16.5, the parietal bone (p) is well developed with an underlying dura mater (arrowhead) containing blood vessels (bv). sk, skin. (H) In the *Tgfbr2*$^{fl/fl}$ mutant, the dura mater (arrowhead) is not completely formed. The blood vessels are poorly developed and there is no detectable parietal bone development. (I,J) Newborn skeletal preparations show severe skull defects in the *Tgfbr2*$^{fl/fl}$ mutant mice (J) compared with control (I). In K and L, wild-type skull and mandible are on the left and top of the figures, respectively. eo, exoccipital; fr, frontal; ip, interparietal; jg, jugal; ma, mandible; mx, maxilla; na, nasal; pmx, premaxilla; ppa, prominentia pars anterior; pr, parietal; rpMC, rostral process of Meckel's cartilage; so, supraoccipital; sq, squamosal.

TGFβ signaling is specifically required in the palatal mesenchyme prior to palatal fusion. We propose that TGFβ directly or indirectly regulates the expression of cell cycle regulators (such as cyclin D1) to control the progression of the cell cycle in CNC-derived palatal mesenchyme, and this regulation is crucial for proper palatal mesenchymal cell proliferation. Decreased palatal mesenchyme cell proliferation has resulted in compromised palatal shelf extension and failure of palatal fusion in *Tgfbr2*$^{fl/fl}$ mutant mice. It is important to note that our animal model does not address whether TGFβ IIR regulates the non-CNC-derived palatal mesenchymal cell proliferation, because *Wnt1-Cre* does not cause *Tgfbr2* deletion in this particular cell population. In addition, although cyclin D1 expression is significantly downregulated in the palatal mesenchyme of *Tgfbr2*$^{fl/fl}$ mutant mice, it is unlikely that a compromised cyclin D1 expression is directly responsible for causing the cleft palate defect in *Tgfbr2*$^{fl/fl}$ mutant mice because cyclin D1-null mutant mice do not have cleft palate (Fantl et al., 1995). Other cycle regulators (such as CDK inhibitors p21 or p18^{INK4c}) appear to be unaffected when we compared their expression patterns within the palatal mesenchyme between the wild-type and the *Tgfbr2*$^{fl/fl}$ mutant mice. Clearly, the method by which TGFβ signaling controls the progression of the CNC-derived palatal mesenchyme cell cycle during palatogenesis is complex; it will be the focus of our future studies.

Cell-autonomous requirement for TGFβ IIR in cranial neural crest during palatogenesis

Contrary to the successful fusion of our cultured *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant palatal shelves, cultured *Tgfb3*-null mutant palatal shelves fail to fuse, even when they are placed in close contact in vitro (Kaartinen et al., 1997). Despite clear adherence, the cultured *Tgfb3*-null mutant palatal shelves show persistent MEE cells and intact basement membrane. Significantly, supplementation of exogenous TGFβ3 facilitates the successful fusion of *Tgfb3*-null mutant palatal shelves in vitro with transformation of the MEE and degradation of the underlying basement membrane. Clearly, TGFβ3 is specifically required in regulating the fate of MEE cells during palatal fusion. The successful signaling of TGFβ3 requires an integral TGFβ receptor complex. Indeed, TGFβ IIR is also expressed in the MEE prior to palatal fusion (Cui et al., 1998). Our palatal organ culture experiment suggests that the basic TGFβ signaling cascade in MEE cells is intact despite the null mutation of *Tgfbr2* in the CNC-derived palatal mesenchyme. It also demonstrates that there is a cell-autonomous requirement for TGFβ signaling in the CNC-derived palatal mesenchyme during palatogenesis. In human clefting birth defects, failure of palatal fusion after proper palatal adhesion (such as the one in *Tgfb3*-null mutant mice) only represents a small percentage of the cleft palate cases, while failure of palatal shelf extension (such as the one in *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice) is associated with the majority of the cleft palate cases. Hence, the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant mice will serve as an important animal model for the investigation of the molecular etiology of human cleft palate.

Inductive signaling within the CNC-derived dura mater is critical for both the CNC- and non-CNC-derived calvarial bone development

Defects in the development of the dura mater and calvaria bone have significant implications. A recent study has shown that the mammalian frontal bones are neural crest derived (still controversial for avian) and that the parietal bones are of mesodermal origin. Furthermore, the dura mater that underlies the parietal bones is neural crest-derived and is sensitive to retinoic acid exposure during parietal bone ossification, suggesting that intramembranous ossification of this mesodermal bone requires interaction with the CNC-derived meninges (Jiang et al., 2002). Here, the defects of both frontal and parietal bones suggest that the CNC-derived dura mater is crucial for the induction of CNC-derived frontal bone and mesoderm-derived parietal bone formation. We hypothesize that the dura mater produces inductive signaling which interacts with the overlaying mesenchyme, whether neural crest or mesodermally derived, to control the initiation and patterning of frontal and parietal bones during calvaria development. Furthermore, our study indicates that TGFβ signaling plays a pivotal role in regulating the proliferation of the CNC-derived dura mater. Aberrant TGFβ signaling results in compromised dura mater development and consequently, in calvaria development defects.

TGFβ is known to regulate the fate of multipotential progenitor cells instructively by regulating the expression or function of tissue-specific transcription factors (Moses and Serra, 1996). For example, TGFβ downregulates the expression of homeobox gene *Msx1* and affects cell fate determination in limb development (Ganan et al., 1996). The expression patterns of TGFβ and *Msx1* have significant overlaps during palatal development and suggest an epistatic relationship between these genes when CNC-derived cells become committed to form the palatal mesenchyme (Pelton et al., 1990; Ferguson, 1994). Overexpression of TGFβ suppresses transcriptional activity of the *Msx1* gene in the palatal mesenchyme in vitro (Nugent and Greene, 1998). Similarly, TGFβ signaling may regulate the expression of the *Msx2* gene during calvaria development. TGFβ IIR and Msx2 are co-expressed in the CNC-derived meninges prior to calvaria formation. We have shown here that Msx1 expression is significantly elevated while cyclin D1 expression is greatly reduced in the palatal mesenchyme of the *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre* mutant embryos, suggesting that TGFβ may regulate the expression of the *Msx1* gene, which in turn controls the progression of the CNC cell cycle during palatogenesis. A recent in vitro study has shown that *Msx1* gene expression maintains cyclin D1 gene expression and controls cell cycle progression, thereby regulating terminal differentiation of progenitor cells during embryonic development (Hu et al., 2001). Our in vivo data suggests that the outcome of Msx1-regulated cyclin D1 expression might be tissue type-dependent. As suggested in the previous study, cyclin D1 is likely to be an indirect target of *Msx1* during embryonic development. Furthermore, our study supports the previously proposed model that reconciles the observed phenotype similarities between the *Msx1* loss- and gain-of-function mutations in the context of cell cycle regulation (Hu et al., 2001). In addition, mutations of the TGFβ IIR may also impinge on BMP signaling within the developing CNC and the CNC-derived mesenchyme, because there is significant overlap between the expression patterns of BMP and TGFβ during craniofacial development. TGFβ IIR can bind to BMPs, and the dominant-negative mutation of TGFβ IIR attenuates both BMP and TGFβ signaling (Massague, 1990; ten Dijke et al., 1994; Dumont and Arteaga, 2003). Potentially useful regionally restricted branchial arch and/or palatal mesenchyme markers (such as members of the homeobox-containing genes) need to be analyzed to dissect the TGFβ signaling cascade in regulating the fate of CNC cells during craniofacial morphogenesis.

The broad spectrum of phenotypic abnormalities suggests that TGFβ signaling is crucial for the transcriptional regulation of multiple regulatory signaling cascades during embryogenesis. We provide an animal model for investigating the molecular mechanism of cleft palate, calvaria agenesis and other CNC-related congenital malformations and demonstrate that TGFβ IIR signaling is specifically required in regulating the fate of CNC cells during craniofacial development. Future studies using this animal model will provide useful information on the mechanism of TGFβ IIR signaling in both normal and abnormal human development. In addition, genetic screening of the *Tgfbr2* mutation among individuals with secondary palate cleft and skull malformations may provide crucial information in linkage analysis to investigate the etiology of congenital malformations.

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