The Mn1 transcription factor acts upstream of Tbx22 and preferentially regulates posterior palate growth in mice

Wenjin Liu1, Yu Lan1, Erwin Pauws2, Magda A. Meester-Smoor3, Philip Stanier2, Ellen C. Zwarthoff3 and Rulong Jiang1,1*

The mammalian secondary palate exhibits morphological, pathological and molecular heterogeneity along the anteroposterior axis. Although the cell proliferation rates are similar in the anterior and posterior regions during palatal outgrowth, previous studies have identified several signaling pathways and transcription factors that specifically regulate the growth of the anterior palate. By contrast, no factor has been shown to preferentially regulate posterior palatal growth. Here, we show that mice lacking the transcription factor Mn1 have defects in posterior but not anterior palatal growth. We show that Mn1 mRNA exhibits differential expression along the anteroposterior axis of the developing secondary palate, with preferential expression in the middle and posterior regions during palatal outgrowth. Extensive analyses of palatal gene expression in wild-type and Mn1+/− mutant mice identified Tbx22, the mouse homolog of the human X-linked cleft palate gene, as a putative downstream target of Mn1 transcriptional activation. Tbx22 exhibits a similar pattern of expression with that of Mn1 along the anteroposterior axis of the developing palatal shelves and its expression is specifically downregulated in Mn1−/− mutants. Moreover, we show that Mn1 activated reporter gene expression driven by either the human or mouse Tbx22 gene promoters in co-transfected NIH3T3 cells. Overexpression of Mn1 in NIH3T3 cells also increased endogenous Tbx22 mRNA expression in a dose-dependent manner. These data indicate that Mn1 and Tbx22 function in a novel molecular pathway regulating mammalian palate development.

KEY WORDS: Cleft palate, Mn1, Palate development, Anterior-posterior patterning, Tbx22, Mouse

INTRODUCTION

The meningioma 1 (MNI) gene was first identified as the gene disrupted by a balanced chromosomal translocation that caused meningioma, a benign brain tumor (Lekanne Deprez et al., 1995). MNI encodes a protein of 1319 amino acids, with no homology to any known functional domains, but the gene is evolutionarily conserved from Drosophila to human (Lekanne Deprez et al., 1995). Although its relation to meningioma remains unclear, as no mutations or deletions of the MNI gene have been found in other individuals with meningioma, the MNI gene has been shown to play important roles in acute myeloid leukemia (AML) pathogenesis (reviewed by Grosveld, 2007). MNI is the target of a recurrent chromosomal translocation, t(12;22)(p13;q12), which is associated with human AML (Buijs et al., 1995). The translocation fuses the MNI gene with the TEL gene that encodes an ETS family DNA-binding transcription factor. Both in vitro and in vivo studies showed that the MNI-TEL fusion protein is oncogenic and that the transforming activity of the fusion protein depended on the N-terminal 500 amino acid residues of the MNI protein (Buijs et al., 2000; Kawagoe and Grosveld, 2005a; Kawagoe and Grosveld, 2005b; Carella et al., 2006). In addition, MNI is overexpressed in many individuals with AML associated with other chromosomal abnormalities and in some individuals with AML without karyotype abnormalities (Ross et al., 2004; Valk et al., 2004; Du et al., 2005; Heuser et al., 2006) (reviewed by Grosveld, 2007). Moreover, overexpression of MNI in the bone marrow caused malignant myeloid disease in mice (Carella et al., 2007). Although the molecular mechanisms involving MNI in AML pathogenesis remains to be elucidated, several biochemical studies have showed that MNI can function as a transcriptional co-activator of the nuclear hormone receptors for retinoic acid or vitamin D (van Wely et al., 2003; Sutton et al., 2005). MNI has also been shown to interact with the transcriptional co-activators p300/CBP and RAC3, and to mediate transcriptional activation via CACCC-rich DNA sequences (van Wely et al., 2003; Meester-Smoor et al., 2007). Thus, in addition to involvement in AML, MNI may interact with other transcription factors to regulate cell proliferation and cell differentiation during mammalian development.

To further investigate the roles of MNI in oncogenesis and development, Meester-Smoor et al. (Meester-Smoor et al., 2005) generated mice with a targeted deletion in the orthologous Mni1 gene. Although the mutant mice did not exhibit any increased incidence of tumor formation, all Mni1−/− homozygous mutant mice died shortly after birth and exhibited severe craniofacial developmental defects, including cleft palate, and some Mni1−/− heterozygous mutant mice also had cleft palate (Meester-Smoor et al., 2005). In mice, as in humans, the secondary palate develops from bilateral outgrowth on the oral side of the developing maxillary processes. The palatal processes initially grow vertically flanking the developing tongue. At a specific developmental time, the bilateral palatal shelves reorient to the horizontal position above the tongue, grow toward and fuse with each other at the midline to form the intact roof of the oral cavity (Ferguson, 1988). Cleft palate may result from disturbances in the growth, elevation or fusion of the palatal shelves. Gene inactivation studies in mice have demonstrated that many genes play essential roles in palatal shelf growth, including Bmp4, Bmpr1a, Fgf10, Fgfr2b, Msx1, Osr2, Shox2 and Tgfbr2, indicating that multiple molecular pathways interact to regulate palate development (Zhang et al., 2002; Han et al., 2003; Ito et al., 2003; Lan et al., 2004; Rice et al., 2004; Alappat et al.,

1Department of Biomedical Genetics and Center for Oral Biology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642, USA. 2Institute of Child Health, University College London, London WC1N 1EH, UK. 3Department of Pathology, Josephine Nefkens Institute, Erasmus MC, Rotterdam, The Netherlands.

*Author for correspondence (e-mail: rulong_jiang@urmc.rochester.edu)

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Moreover, as palate development occurs concurrently with significant growth and morphogenesis of the craniofacial complex, gross defects in structures outside of the palatal shelves may sometime hinder palatal shelf elevation or contact, resulting in cleft palate (reviewed by Chai and Maxson, 2006). To understand the roles of Mn1 in palate development, we have characterized its expression patterns during normal palate development and have identified a primary role for Mn1 in differential regulation of palatal growth along the anteroposterior axis.

The mammalian secondary palate is divided anatomically into the anterior bony region (hard palate) and the posterior muscular region (soft palate) (Sperber, 2002). Cleft palate defects affecting the entire palate (complete cleft of the secondary palate) or either the anterior or posterior regions (incomplete cleft of the secondary palate) have been well documented (reviewed by Hilliard et al., 2005). Consistent with the morphological and pathological differences in the anterior and posterior palate, recent studies have clearly demonstrated that there is molecular heterogeneity along the anteroposterior axis of the developing secondary palate (reviewed by Hilliard et al., 2005; Li and Ding, 2007). During early palate development, expression of several crucial signaling molecules and transcription factors, including Bmp4, Fgf10, Mox1 and Shox2, is highly restricted along the anteroposterior axis. Expression of Bmp4 and Mox1 is restricted to the most anterior 25%, whereas Fgf10 and Shox2 are expressed in the anterior half of the developing palatal shelves, up to the level of the first molar tooth buds, prior to palatal fusion (Zhang et al., 2002; Alappat et al., 2005; Yu et al., 2005; Hilliard et al., 2005; Li and Ding, 2007). Fgf10 signals through the Fgf1b2 receptor to regulate palatal epithelial cell proliferation and survival (Rice et al., 2004; Alappat et al., 2005). Bmp4 and Mox1 appeared to function in a positive-feedback loop to regulate mesenchymal proliferation in the anterior palate (Zhang et al., 2002). Interestingly, exogenous Bmp4 induced Mox1 expression and cell proliferation in the anterior, but not in the posterior, palatal mesenchyme in explant culture assays (Zhang et al., 2002; Hilliard et al., 2005). Shox2 is also required for growth of the anterior palate, and mice that lack Shox2 exhibited incomplete cleft within the anterior palate while the mutant posterior palate fused normally (Yu et al., 2005). Although the anterior and posterior palatal regions exhibit similar growth rates during palatal outgrowth (Zhang et al., 2002; Li and Ding, 2007), no factor has been reported to regulate preferentially the growth of the posterior palate. The Mox2 homeobox gene has been reported as being expressed in the posterior but not in the anterior palatal shelves in certain strains of mice (Jin and Ding, 2006; Li and Ding, 2007). Some, but not all, Mox2 mutant mice exhibited cleft palate, but they did not have defects in palatal shelf growth and their cleft palate defect appeared to result from postfusion rupture (Jin and Ding, 2006). In this report, we show that Mn1 preferentially regulates the growth of the posterior palate in mice. In addition, we show that palatal expression of Tbx22, mutations of which cause X-linked cleft palate and ankyloglossia (CPX) in humans (Braybrook et al., 2001; Braybrook et al., 2002), is specifically regulated by Mn1. These data provide novel insights into the molecular mechanisms that regulate the regional growth and patterning of the secondary palate.

**MATERIALS AND METHODS**

**Mice**

Mice carrying the targeted mutation in Mn1 have been described previously (Meester-Smoor et al., 2005). Mn1+/– mice were maintained in the FVB congenic background and were intercrossed to generate homozygous mutant embryos for experimental analysis. Wild-type C57BL/6J and CD-1 mice were also used for in situ hybridization analysis of Mn1 and Tbx22 mRNA expression during palate development.

**In situ hybridization and histological analyses**

Embryos at different stages were dissected, fixed in 4% paraformaldehyde (PFA) in PBS overnight at 4°C. Whole-mount in situ hybridization was performed as described previously (Lan et al., 2001). For section in situ hybridization, PFA-fixed embryos were dehydrated through graded alcohols and embedded in paraffin, sectioned at 7 μm, followed by prehybridization processing and by hybridization with digoxigenin-labeled cRNA probes as described previously (Zhang et al., 1999).

For histology, embryos were collected at predetermined stages, fixed in either Bouin’s fixative or 4% PFA overnight, dehydrated through graded ethanol, embedded in paraffin wax and sectioned at 7 μm, followed by staining with Hematoxylin and Eosin.

**Analyses of cell proliferation and cell apoptosis**

Cell proliferation was measured by BrdU incorporation assays or detection of Ki67. For BrdU incorporation assays, timed mating was set up between Mn1+/– heterozygous mice and pregnant female mice were injected intraperitoneally with BrdU (5-bromo-2-deoxy-uridine, Roche) labeling reagent at gestational day 12 or 13, with a dose of 15 μl/g body weight. One hour after injection, embryos were dissected, fixed in Carnoy’s fixative, dehydrated through graded ethanol, embedded in paraffin wax and sectioned at 5 μm. Sections from anterior, middle or posterior regions of the developing palatal shelves were selected for detection of BrdU-labeled cells by using the BrdU labeling and detection kit (Roche) following the manufacturer’s protocol. Following BrdU detection, sections were counterstained with nuclear fast red (Vector Laboratories) to label all cellular nuclei. The total number of cells and the number of BrdU-positive cells in the palatal epithelium and mesenchyme on each of five consecutive sections were counted. Cell proliferation index was calculated as the percentage of the total cells being BrdU-positive. ANOVA was applied for statistical analyses and a P value less than 0.01 was considered statistically significant.

For detection of Ki67, paraffin sections from selected palatal regions of staged mouse embryos were stained with an antibody against Ki67 as described previously (Casey et al., 2006). Cell apoptosis was detected by TUNEL assays. Paraffin sections from selected palatal regions of staged mouse embryos were analyzed by using the DeadEnd Fluorometric TUNEL System (Promega) following the manufacturer’s instructions.

**Expression vectors and promoter-luciferase constructs**

The Mn1 expression vector was constructed by subcloning an Mn1 cDNA fragment containing the full-length protein-coding region into the pcDNA3TOPO (Invitrogen) expression vector. The human Tbx22 promoter-luciferase reporter vectors (pGL3-TBX22 hP0 and pGL3-TBX22 hP1) have been described previously (Andreou et al., 2007). The mouse Tbx22 promoter-luciferase reporter vectors were similarly constructed by PCR amplifying the mouse Tbx22 promoter regions (see Fig. S3 in the supplementary material) followed by subcloning into the pGL3-basic vector (Promega). All subcloned fragments were sequence verified.

**Cell culture, transfection, and luciferase reporter assays**

NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For luciferase reporter assays, cells were plated in 24-well tissue culture plates (Corning) and co-transfected with 0.05 μg of a luciferase reporter vector, 0.05 μg of the pRL-Renilla luciferase expression vector (Promega) and increasing amounts of the Mn1 expression vector. Transfections were performed by using the Lipofectamine reagents (Invitrogen) in accordance with the manufacturer’s instructions. Cells were cultured for 48 hours after transfection and then assayed using the Dual-Luciferase Assay Kit (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity. All transfection experiments were carried out in triplicate and data were summarized from three repeat experiments.
Real-time RT-PCR
For detection of the effects of Mn1 on endogenous Tbx22 gene expression, NIH3T3 cells were plated in six-well tissue culture plates and transfected with increasing amounts of the Mn1 expression vector. Cells were cultured for 48 hours after transfection and total RNA was extracted using Trizol reagents (Invitrogen). First-strand cDNA was synthesized using SuperScript First-Strand Synthesis System (Invitrogen). Quantitative PCR amplifications were performed in an iCycler real-time PCR machine (Bio-Rad) using the SYBR GreenER qPCR Supermix (Invitrogen). Mn1 gene-specific PCR primers are 5′-AGATCCAGCTGAGAGACAA-3′ and 5′-TACTCATGGCCGCTTGTACT-3′. Tbx22 gene-specific PCR primers are 5′-GACCTGTCCTGATTGAGTCC-3′ and 5′-GCTGGTCTGAGTC-3′. Hprt gene-specific primers are 5′-TGCTGGTCTGAGTCC-3′ and 5′-CTGGGAAACCAACAGGGACCT-3′. For each sample, the relative levels of Mn1 and Tbx22 mRNAs were normalized to that of Hprt using the standard curve method.

RESULTS
Expression of Mn1 mRNA during early mouse embryogenesis and in the developing palate
Using whole-mount in situ hybridization, Mn1 mRNA expression was first detected at embryonic day E9.5 (Fig. 1A). At this stage, Mn1 mRNA was strongly expressed in the midbrain and hindbrain tissues and in the craniofacial mesenchyme. By E10.5, Mn1 mRNA was highly expressed in all of the prominences of the developing face, including frontonasal processes, maxillary processes, mandibular processes, and in the second branchial arch (Fig. 1B,D). Mn1 mRNA was also detected in a subset of mesenchymal cells in the developing somites and limb buds. This pattern of Mn1 mRNA expression persists at E11.5 (Fig. 1C,E). At this stage, palatal outgrowth had initiated on the oral sides of the maxillary processes and Mn1 mRNA was strongly expressed in the palatal primordia (Fig. 1E). In situ hybridization of sections of E12.5 embryos showed that Mn1 mRNA was highly expressed in the developing palatal mesenchyme and in the preossification mesenchymal cells in the mandible (Fig. 1F). Strong Mn1 mRNA expression persists in the brain, in particular in the ventricular zone (Fig. 1F).

The reported cleft palate phenotype of the Mn1 mutant mice prompted us to carry out a detailed analysis of Mn1 mRNA expression during palate development. Recent studies have demonstrated that several genes exhibit differential expression along the anteroposterior axis of the developing palatal shelves in mice (Zhang et al., 2002; Alappat et al., 2005; Yu et al., 2005; Hiliard et al., 2005; Li and Ding, 2007). The distinct gene expression patterns appear to divide the developing palatal shelves into three regions along the anteroposterior axis: (1) anterior palate that expresses high levels of Msox1 and Shox2 mRNAs (Zhang et al., 2002; Hiliard et al., 2005; Yu et al., 2005); (2) middle palate (roughly corresponding to the region flanked by the upper first molar tooth germs) (Alappat et al., 2005) that lacks Msox1 expression and exhibits anterior-to-posterior expansion of Shox2 mRNA expression from E12.5 to E14.5 (Li and Ding, 2007); and (3) posterior palate that expresses high levels of Msox2 mRNA but lacks Msox1 and Shox2 mRNA expression (Li and Ding, 2007). In situ hybridization of serial sections of the developing palatal shelves showed that Mn1 mRNA is differentially expressed along the anteroposterior axis of the developing palatal shelves, with high levels of expression in both mesenchyme and epithelium in the middle and posterior regions and very low levels in the anterior region of the palatal shelves during the vertical growth period from E12.5 to E13.5 as well as after palatal shelf elevation at E14.5 (Fig. 2).

Mn1+− mutant mice exhibit palatal retardation and failure of palatal shelf elevation
To investigate which palatal developmental steps require Mn1 function, we carried out detailed histological analyses of Mn1−− mouse embryos throughout palate development. Mn1−− mutant embryos displayed normal palatal shelf outgrowth at E12.5 (data not shown). At E13.5, the palatal shelves of Mn1−− mutant embryos exhibited similar size and shape to those of wild-type embryos (Fig. 3A,B). By E14.5, the palatal shelves of wild-type embryos had already elevated to the horizontal position and initiated fusion by forming the midline epithelial seam (Fig. 3C), which was disintegrated by E15.5 to form the fused secondary palate (Fig. 3E).
By contrast, the bilateral palatal shelves of Mn1–/– mutant embryos failed to elevate and remained at the vertical position at E14.5 and at E15.5 (Fig. 3D,F).

Histological analyses of Mn1–/– mutant embryos at later stages revealed different fates of the palatal shelves along the anteroposterior axis. At E16.5, the middle and posterior palatal shelves in Mn1–/– mutant embryos were still vertically oriented and appeared greatly reduced in size (Fig. 4B). By E18.5, the posterior palatal shelves were further retarded and retracted to the maxillary processes (Fig. 4D), while the anterior palatal shelves were still clearly visible and often elevated on one or both sides (Fig. 4C).

**Mn1 is required for proper palatal shelf growth**

Impairment of palatal shelf elevation is often accompanied by and partially due to retarded palatal shelf growth, as has been reported in the Osr2–/– mutant mice (Lan et al., 2004). To investigate the cellular mechanisms of palatal shelf retardation and elevation failure in Mn1–/– mutant mice, we examined whether there are alterations in cell proliferation and cell survival during palate development in Mn1–/– mutant mice. No differences in cell apoptosis were found in the palatal shelves between wild-type and Mn1–/– mutant embryos at E12.5 and at E13.5 (data not shown). No significant alterations in cell proliferation were observed in the palatal shelves in Mn1–/– mutant embryos at E12.5 (data not shown). At E13.5, we detected a 57% reduction (P<0.01) in the posterior and a 49% reduction (P<0.01) in the middle regions of the palatal mesenchyme in Mn1–/– mutant embryos in comparison with their wild-type littermates (Fig. 5G). By contrast, the cell proliferation index was not significantly different in the anterior palatal mesenchyme in the same Mn1–/– mutant and wild-type embryos (Fig. 5G). Similarly, palatal epithelial cell proliferation was also significantly reduced in the middle and posterior palate but not in the anterior palate in Mn1–/– mutant embryos, in comparison with the wild-type littermates (Fig. 5H). The selective reduction in palatal cell proliferation in the middle and posterior regions of the palatal shelves in Mn1–/– mutant mice correlates with the differential expression of Mn1 mRNA along the anteroposterior axis during normal palate development.

To understand the dramatic retardation of the palatal shelves at later stages in Mn1–/– mutant embryos, we also examined cell proliferation and cell apoptosis in E14.5 and E15.5 embryos. The decrease in cell proliferation rate in the posterior and middle regions of the palatal shelves in the Mn1–/– mutant embryos continued through E15.5 (Fig. 6A-C and data not shown), indicating that Mn1 is an important regulator of palatal shelf growth before and after palatal shelf elevation. Moreover, by TUNEL assays, we detected increased apoptosis at E15.5 in the posterior regions of the palatal mesenchyme in Mn1–/– mutant embryos (Fig. 6D,E). These data indicate that the dramatic degeneration of the posterior palatal shelves observed by E18.5
Palatal shelf growth defect in Mn1−/− mutants is accompanied by a region-specific decrease in cyclin D2 expression

Several molecular pathways have been shown to regulate cell proliferation during palate development by regulating the expression of D-type cyclins. D-type cyclins are important cell cycle regulators that bind CDK4 or CDK6 to control cell cycle progression through the G1 phase (Matsushime et al., 1991; Xiong et al., 1991; Motokura et al., 1991; Morgan et al., 1997). Conditional inactivation of Tgfbr2 in cranial neural crest (CNC) cells downregulated cyclin D1 expression and reduced CNC cell proliferation in the palatal mesenchyme (Ito et al., 2003). Msx1 regulates neural crest cell proliferation and differentiation also through maintaining cyclin D1 expression (Hu et al., 2001). To understand the cellular mechanism by which Mn1 regulates palatal growth in the middle and posterior palatal shelves, we investigated possible alterations in the expression levels of D-type cyclins in Mn1−/− mutant embryos. No change in the levels of cyclin D1 (Ccnld1) expression was detected in either the epithelium or mesenchyme of the developing palate in Mn1−/− embryos in comparison with wild-type littermates (data not shown). By contrast, the expression of cyclin D2 (Ccnld2) is reduced in the middle and posterior regions of the palatal mesenchyme, as well as in the posterior palatal epithelium in Mn1−/− embryos at E13.5 (Fig. 7). These data suggest that Mn1 regulates palatal shelf growth, at least in part, through maintaining Ccnld2 expression in the middle and posterior palatal shelves.

Effects of Mn1 deficiency on palatal gene expression

To investigate the molecular mechanisms involving Mn1 in palate development, we examined the expression patterns of other genes known to play important role in palate development, including Fgf10, Fgfr2, Osr2, Shh, Patch1, Pax9, Shox2 and Tgfβ3. No obvious differences in either levels or patterns of expression of these genes were found in the developing palatal shelves in wild-type and Mn1−/− mutant embryos (Fig. 8; see Fig. S1 in the supplementary material). In particular, the differential expression patterns of Fgf10, Shox2 and Meox2 mRNAs along the anteroposterior axis of the developing palatal shelves are maintained in the Mn1−/− mutant embryos (Fig. 8), indicating that there is no gross anteroposterior patterning defects in the developing palate in Mn1−/− mutant embryos.

Extensive expression analyses of other genes implicated in palate development showed that expression of Tbx22, which is homologous to the gene associated with CPX in humans, is specifically reduced in the developing palatal shelves in Mn1−/− mutant embryos (Fig. 9). Interestingly, expression of Tbx22 mRNA also exhibits a posterior preference in the developing palatal shelves, similar to the expression pattern of Mn1, during normal palate development in mice (Fig. 9A-C). Compared with
wild-type embryos, the expression levels of Tbx22 are dramatically reduced in the middle and posterior palatal shelves of Mn1–/– mutant embryos (Fig. 9D-F), indicating that Mn1 and Tbx22 function in the same molecular pathway to regulate mammalian palate development.

Mn1 is a transcriptional activator of Tbx22 gene expression

The similar expression patterns of Mn1 and Tbx22 mRNAs in the developing wild-type palatal shelves and the specific downregulation of Tbx22 mRNA in Mn1–/– mutant embryos suggest that Mn1 may directly regulate Tbx22 gene expression during palate development. We have previously shown that the human TBX22 gene is transcribed from two different promoters that are ~10 kb apart (Andreou et al., 2007). Quantitative RT-PCR assays of human embryonic RNA samples indicated that embryonic TBX22 mRNA expression was predominantly generated by the hP0 promoter (data not shown). Analyses of 5’ RACE (rapid amplification of cDNA ends) products from E12.5 mouse embryonic craniofacial RNA templates revealed that the mouse Tbx22 gene is also transcribed from at least two distinct promoters: a distal promoter corresponding to the human TBX22 hP0 and a proximal promoter corresponding to the human TBX22 hP1 (see Fig. S2 in the supplementary material). Interestingly, the previously determined core binding DNA sequence for the Mn1 transcription factor, CACCC, is present in various locations within 2 kb of the transcription start site for each of the mouse and human TBX22 promoters (see Fig. S3 in the supplementary material). To investigate whether Mn1 can activate transcription from the mouse and human TBX22 gene promoters, we constructed Tbx22 promoter-luciferase reporter plasmids (see Materials and methods) and tested the effects of Mn1 co-transfection on luciferase reporter expression in NIH3T3 cells. Transfection of each of the promoter-luciferase plasmids showed that the mouse mP0 and mP1 as well as human hP0 promoters each had promoter activity in NIH3T3 cells, with the hP0 promoter being threefold stronger than the mP0 or mP1 promoter (Fig. 10A). By contrast, the hP1-luciferase construct did not show promoter activity in NIH3T3 and three other cell lines tested (Fig. 10A) (Andreou et al., 2007) (and data not shown). Co-transfection with the Mn1 expression vector resulted in a dose-dependent increase in luciferase expression from each of the mP0-,- mP1- and hP0-luciferase constructs but not the hP1 promoter-luciferase construct (Fig. 10A). Moreover, transfection of NIH3T3 cells with the Mn1 expression vector resulted in a dose-dependent increase in endogenous Tbx22 mRNA expression (Fig. 10B,C). These data indicate that Mn1 functions as a transcriptional activator of Tbx22 gene expression.

Fig. 6. Analyses of cell proliferation and cell apoptosis in wild-type (WT) and Mn1–/– mutant embryos at E15.5. (A–C) Cell proliferation in palatal mesenchyme detected using immunohistochemical staining of the Ki67 protein. In comparison with wild-type embryos (A), cell proliferation in the middle palatal region in Mn1–/– mutant embryos (B) was greatly reduced. Error bars in C represent standard deviation and the asterisk indicates a significant difference (P<0.01) between the wild-type and mutant samples of mesenchyme and epithelium. (D,E) TUNEL assays of midpalatal sections of E15.5 palatal shelves of wild-type (D) and Mn1–/– mutant (E) embryos showed increased cell apoptosis in the palatal mesenchyme in the mutant. Small arrows indicate highly TUNEL-positive palatal mesenchymal cells and arrowheads indicate highly TUNEL-positive palatal epithelial cells.

Fig. 7. Expression of cyclin D2 was downregulated in Mn1–/– mutant palatal shelves at E13.5. (A,D) In the anterior region of the developing palate, expression of cyclin D2 was similarly weak in wild-type (A) and Mn1–/– mutant (D) embryos. (B,E) In the middle palate, cyclin D2 is highly expressed in both the epithelium and mesenchyme in the wild-type embryo (B) but its expression is much reduced in the Mn1–/– palatal mesenchyme (E). (C,F) In the posterior palate region, strong cyclin D2 expression was detected in both the epithelium and mesenchyme in the wild-type palate (C) but was much reduced in the Mn1–/– mutant embryo (F). Green arrows in C and F indicate the medial edge epithelium of the palatal shelves.
DISCUSSION

Cleft palate is a common birth defect in humans, occurring in approximately 1 in 1000 live births (Vanderas, 1987; Schutte and Murray, 1999; Gorlin et al., 2001). Approximately 50% of cleft palate cases are non-syndromic, although cleft palate has been associated with more than 300 syndromic developmental disorders. Several genes underlying syndromic forms of cleft palate have been identified, including IRF6 (Van der Woude and popliteal pterygium syndromes) (Kondo et al., 2002), MSX1 (cleft lip or palate with hypodontia) (van den Boogaard et al., 2000), P63 (ectodermal dysplasia and cleft lip or palate) (Celli et al., 1999; Ianakiev et al., 2000), PVRL1 (autosomal recessive cleft lip/palate with ectodermal dysplasia) (Suzuki et al., 2000) and TBX22 (CPX) (Braybrook et al., 2001). In addition, genetic studies in mice have revealed that mutations in more than 60 different genes each resulted in cleft palate and have uncovered specific molecular and cellular mechanisms controlling palate development (reviewed by Gritli-Linde, 2007). Interestingly, recent studies have demonstrated that palatal shelf growth is differentially regulated along the anteroposterior axis (reviewed by Hilliard et al., 2005). Zhang et al. (Zhang et al., 2002) first demonstrated that Msx1 and Bmp4 are both expressed in and required for the growth of the anterior region of the developing palatal shelves. In palatal explant culture assays, exogenous Bmp4 induced Msx1 mRNA expression and increased cell proliferation in the anterior but not in posterior palatal mesenchyme (Zhang et al., 2002; Hilliard et al., 2005). The Shox2 transcription factor is also specifically expressed in the anterior palate and mice lacking Shox2 exhibited growth defects in the anterior but not posterior regions of the developing palatal shelves (Yu et al., 2005). However, previous studies have also shown that the anterior and posterior palatal regions have similar cell proliferation rates during palatal outgrowth (Zhang et al., 2002; Li and Ding, 2007), suggesting that there must be factors that preferentially regulate posterior palatal growth. Our finding that Mn1 is differentially expressed along the anteroposterior axis and preferentially regulates middle and posterior palatal cell proliferation fills a longstanding gap in the understanding of the molecular mechanisms of palate development.

Mn1 specifically regulates Tbx22 gene expression during palate development

Mutations in the human TBX22 gene, including nonsense, frameshift, splice-site and missense changes, have been associated with CPX (Braybrook et al., 2001; Braybrook et al., 2005).
palatal expression of many genes, including that of Meox2, which is also preferentially expressed in the posterior palate during palatal outgrowth (Li and Ding, 2007) (Fig. 8), is not affected by Mnl deficiency indicates that Tbx22 is a specific downstream target of Mnl. Our data that Mnl activated, in a dose-dependent manner, expression of endogenous Tbx22 mRNA and the luciferase reporter gene driven by either the human or mouse Tbx22 promoter sequences in transfected NIH3T3 cells provide further support that Mnl activates transcription of the Tbx22 gene. These data link two spatially co-expressed transcription factors in a novel molecular pathway for the regulation of posterior palate development.

**Primary and secondary effects of Mnl deficiency on palate development**

It has previously been suggested that the cleft palate defect in the Mnl–/– mutant mice might be secondary to the severe cranial skeletal defects (Meester-Smoor et al., 2005). We found that Mnl is differentially expressed along the anteroposterior axis of the developing palatal shelves and required for proper palatal growth in the middle and posterior regions but not in the anterior region. The specific downregulation of Tbx22 mRNA expression in the Mnl–/– mutant palatal shelves also identifies a primary role for Mnl in palate development. However, Mnl–/– mutant mice exhibit complete cleft of the secondary palate, although no significant growth deficiency was found in the anterior palate at any stage of palate development. In addition, the palatal shelves failed to elevate, in particular in the middle and posterior regions, in the Mnl–/– mutant mice. Impairment of palatal shelf elevation is often caused by mechanical hindrance, such as aberrant palatal-tongue and palatal-mandible fusions in the Jag2–/– and Fgf10–/– mutant mice, respectively (Casey et al., 2006; Alappat et al., 2005). Defects in mandibular development or deformation of the tongue have also been suggested as causes of failure of palatal shelf elevation in several mutant mouse strains, including Ryk–/– and Foxf2–/– mutant mice (Halford et al., 2000; Wang et al., 2003). In addition, Pax9–/– mutant mice exhibited impairment of palatal shelf elevation that was attributable to deformation of the palatal shelves themselves (Peters et al., 1998). Mnl–/– mutant mice exhibited normal mandibular development (Meester-Smoor et al., 2005). However, the developing tongue did not properly descend to the floor of the mouth during the time of palatal shelf elevation in the Mnl–/– mutant mice, in comparison with that in the wild-type littermates (Fig. 3). Whereas Mnl mRNA is strongly expressed in the middle and posterior palatal mesenchyme, little Mnl expression was observed in the developing tongue (Fig. 2). Thus, the palatal elevation defect in the Mnl–/– mutant mice may be due to an intrinsic defect in the palatal shelves or secondary to as-yet unidentified defects in a mandibular component involved in tongue movement. Taken together, the cleft palate phenotype in Mnl–/– mutant mice probably results from a combination of the primary defects in palatal growth and secondary effects of other craniofacial abnormalities.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/23/3959/DC1

**References**


2002; Chaabouni et al., 2005). In addition, mutation-screening studies of individuals with isolated cleft palate showed that mutations in Tbx22 represent the most common single cause of cleft palate known (Marcano et al., 2004; Suphapeetiporn et al., 2007). These data suggest that TBX22 plays primary and crucial roles in palate development. Consistent with this hypothesis, Braybrook et al. (Braybrook et al., 2002) showed that Tbx22 mRNA is strongly expressed in the developing human palatal shelves prior to palatal elevation and fusion. Tbx22 mRNA expression was also detected in the base of the developing tongue (Braybrook et al., 2002; Bush et al., 2002; Herr et al., 2003). Although the exact roles of Tbx22 in palate development remain to be elucidated, these studies showed that palatal expression of Tbx22 mRNA was high during vertical palatal growth and declined after palatal shelf elevation in both human and mice, suggesting that Tbx22 may play a conserved role in palatal shelf growth. Welsh et al. (Welsh et al., 2007) showed, by whole-mount in situ hybridization, that Tbx22 mRNA is preferentially expressed in the posterior palate in E13.5 and E14.5 mouse embryos. We confirmed, by using section in situ hybridization, the differential expression of Tbx22 mRNA along the anteroposterior axis of the developing secondary palate (Fig. 9). Interestingly, Tbx22 mRNA expression was dramatically downregulated in the Mnl–/– mutant palatal shelves. The fact that


Devel. 215, 322-326.

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TBX22-hP0

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