Genetic interactions between Pax9 and Msx1 regulate lip development and several stages of tooth morphogenesis

Mitsushiro Nakatomi,1, Xiu-Ping Wang,1, Darren Key,1, Jennifer J. Lund, Annick Turbe-Doan, Ralf Kist, Andrew Aw, Yiping Chen, Richard L. Maas, Heiko Peters

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A B S T R A C T
Developmental abnormalities of craniofacial structures and teeth often occur sporadically and the underlying genetic defects are not well understood, in part due to unknown gene–gene interactions. Pax9 and Msx1 are co-expressed during craniofacial development, and mice that are single homozygous mutant for either gene exhibit cleft palate and an early arrest of tooth formation. Whereas in vitro assays have demonstrated that protein–protein interactions between Pax9 and Msx1 can occur, it is unclear if Pax9 and Msx1 interact genetically in vivo during development. To address this question, we compounded the Pax9 and Msx1 mutations and observed that double homozygous mutants exhibit an incompletely penetrant cleft lip phenotype. Moreover, in double heterozygous mutants, the lower incisors were consistently missing and we find that transgenic Bmp4 expression partly rescues this phenotype. Reduced expression of Shh and Bmp2 indicates that a smaller “incisor field” forms in Pax9+/−;Msx1+/− mutants, and dental epithelial growth is substantially reduced after the bud to cap stage transition. This defect is preceded by drastically reduced mesenchymal expression of Fgf3 and Fgf10, two genes that encode known stimulators of epithelial growth during odontogenesis. Consistent with this result, cell proliferation is reduced in both the dental epithelium and mesenchyme of double heterozygous mutants. Furthermore, the developing incisors lack mesenchymal Notch1 expression at the bud stage and exhibit abnormal ameloblast differentiation on both labial and lingual surfaces. Thus, Msx1 and Pax9 interact synergistically throughout lower incisor development and affect multiple signaling pathways that influence incisor size and symmetry. The data also suggest that a combined reduction of Pax9 and Msx1 gene dosage in humans may increase the risk for orofacial clefting and oligodontia.

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Introduction
The early development of mammalian craniofacial structures such as jaws, secondary palate and teeth is controlled by reciprocal interactions between ectodermal epithelium and neural crest-derived mesenchyme. These interactions are tightly controlled and involve dynamic changes in expression patterns of signaling molecules, growth factors and their receptors, and transcription factors (reviewed in Chai and Maxson, 2006). The developing mouse tooth is a widely used system to investigate the functions of individual genes in this process and has been highly informative to define the molecular networks that control different steps during tooth morphogenesis (Pispa and Thesleff, 2003; Tucker and Sharpe, 2004). Several homologous genes are expressed in overlapping patterns during tooth morphogenesis and their simultaneous inactivation often results in a more severe phenotype when compared to that of the single gene knockout mice. For example, consistent with functional redundancy among closely related transcription factors, double mutants lacking either Msx1 and Msx2, Dlx1 and Dlx2, Lhx6 and Lhx7, or Gli2 and Gli3 exhibit phenotypes that occur earlier or that are enhanced in severity relative to those in the respective single mutants (Bei and Maas, 1998; Thomas et al., 1997; Denaxa et al., 2009; Hardcastle et al., 1998). In contrast, it is unknown to what extent genes encoding transcription factors that belong to different classes and that recognize different DNA sequences functionally interact during craniofacial and tooth development.

Pax9 and Msx1 encode transcription factors with different DNA binding motifs, a paired domain and a homeodomain, respectively, and are co-expressed during mouse craniofacial and tooth development (Mackenzie et al., 1991; Neubuser et al., 1995). Targeted gene inactivation of each gene in mice has shown that both genes are essential for tooth and secondary palate development (Satokata and Maas, 1994; Peters et al., 1998). In homozygous Pax9 or Msx1 mutants, all teeth fail to form and morphogenesis of the first molar, which has
been examined in most detail, arrests after the dental epithelium has formed a bud. At this stage, both genes are necessary to maintain the mesenchymal expression of Bmp4 (Chen et al., 1996; Peters et al., 1998), which is required for progression of the molar rudiment from the bud stage to the cap stage (Jernvall et al., 1998; Bei et al., 2000). In addition, heterozygous mutations in human MSX1 or PAX9 underlie dominantly inherited oligodontia (congenital absence of at least six permanent teeth, excluding third molars), and are infrequently associated with missing primary teeth and orofacial clefting (Vastardis et al., 1996; Stockton et al., 2000; for recent reviews see Vieira, 2008; Nieminen, 2009). In contrast to humans, heterozygous Pax9 or Msx1 loss-of-function mutations in mice do not affect secondary palate and tooth development, indicating that different gene dosages are required in the two species. In support of this, a reduction of functional Pax9 gene dosage to levels below heterozygosity is associated with oligodontia as the predominant phenotype in a Pax9 hypomorphic mouse model (Kist et al., 2005).

In this study, we provide genetic evidence for a critical interaction between Pax9 and Msx1 in craniofacial and lower incisor development. Whereas cleft lip formation is incompletely penetrant in Pax9−/−;Msx1−/− double homozygous mutants, lower incisors are consistently missing in Pax9+/-;Msx1−/− double heterozygous mice. Our data demonstrate that the concomitant reduction of Pax9 and Msx1 gene dosages affect lower incisor development during initiation, morphogenesis and differentiation, and these processes are sensitive to Bmp4 gene dosage. Moreover, the regulation of Fgf3 and Fgf10 expression in the dental papilla of lower incisors is one critical downstream function of combined Pax9 and Msx1 function that controls the normal growth of these teeth.

Materials and methods

Mouse mutants and phenotype analysis

Generation and genotyping of Pax9 and Msx1 knockout mice have been described previously (Peters et al., 1998; Satokata and Maas, 1994). Three different genetic backgrounds were used in this study. First, for scoring of cleft lip and skull abnormalities, transgenic rescue experiments and whole-mount in situ hybridization, we crossed BMP4 transgenic mice (Zhang et al., 2000), which were on a mixed C57BL/6;CBA/J genetic background, to Pax9/Msx1 double heterozygous mice to generate triple mutants with a mixed C57BL/6;CBA/J;CD1 genetic background. Second, for histological analysis and gene expression studies on sections, Pax9/Msx1 double heterozygous mice on a CD1 background (−/−N10) or on a mixed C57BL/6;FVB;BALB/c background were used. In both genetic backgrounds the phenotype of missing incisors was fully penetrant. Mice on a mixed C57BL/6;FVB;BALB/c background were also used for BrdU incorporation and apoptosis assays. Staging of embryos was done by taking the morning of vaginal plug detection as embryonic day 0.5 (E0.5). A careful staging of embryos between E12.0 and E12.5 used for whole-mount RNA in situ hybridization was carried out by comparing the number of Shh-positive mystacial vibrissae rudiments, which develop in a highly coordinated caudo-rostral pattern between E12.0 and E13.5 (Van Exan and Hardy, 1984). Heterozygous and homozygous mutants carrying the BMP4 transgene, which was previously shown to partially rescue early molar development of Msx1 homozygous mutant embryos (Zhao et al., 2000) were distinguished by DNA dot-blot analysis as previously described (Zhang et al., 2000). Skeletal elements were stained using Alcian Blue for cartilage and Alizarin Red for bone (Peters et al., 1998). For histological analysis, tissues were fixed with 4% paraformaldehyde, followed by standard paraffin embedding, sectioning, and hematoxylin and eosin staining.

**RNA in situ hybridization and Pax9 immunostaining**

RNA in situ hybridization on whole-mounts was performed as previously described (Spore and Schughart, 1998). Non-radioactive, mouse antisense RNA probes were generated using 640 bp Shh, 240 bp Bmp2, 1550 bp Bmp4, 1300 bp Fgf3, 580 bp Fgf10 and 625 bp p21 fragments (Nakatomi et al., 2006; details available upon request).
respectively, by in vitro transcription labeling with digoxigenin-UTP according to the manufacturer’s protocol (Roche, IN, USA). In addition, mRNA riboprobes for mouse Ameloblastin, Follistatin, Lef1, Msx1, Notch1, Pax9, p21, Runx2, Wnt-5a, and rat Shh for in situ hybridization on sections only were used as described (Aberg et al., 2004; Wang et al., 2009). Detection of Pax9 protein on paraffin sections using a rat monoclonal anti-Pax9 antibody and Fast Red as a color substrate was performed as described previously (Gerber et al., 2002).

Cell proliferation assay and TUNEL staining

Timed pregnant mice were injected intraperitoneally with 1 ml/100 g body weight of BrdU solution according to the manufacturer’s protocol (Invitrogen, CA, USA). After 2 h, mouse heads were dissected and fixed in 4% PFA/PBS, processed for paraffin sections (7 µm), and BrdU incorporated cells were detected using a BrdU detection kit (Invitrogen, CA, USA). Images of every second section from the center of incisor teeth to the periphery were photographed, and five sections were counted for each tooth from three different mice of each genotype. A BrdU index was calculated by counting BrdU incorporated cells divided by total cells (ascertained as hematoxylin-stained nuclei).

The significance between wild type and Pax9+/−;Msx1+/− mutant incisors was evaluated by Student’s t-test. Apoptotic cells were identified on paraffin sections using TUNEL staining with the In Situ Cell Death Detection Kit (Roche, IN, USA).

Results

Pax9 and Msx1 interact synergistically during upper lip development

The overlapping craniofacial expression patterns of Msx1 and Pax9, the ability of their gene products to interact in vitro, and the similarities of dental abnormalities in humans and mice caused by mutations in MSX1 (Msx1) and PAX9 (Pax9) suggested that these genes might interact in vivo. To test this hypothesis, we first generated Pax9+/−;Msx1+/− mice. Apart from dental abnormalities (see below), these mice appeared healthy and were intercrossed to generate mutants with a complete loss of Pax9 and Msx1. Newborn Pax9+/−;Msx1−/− mice could be readily identified by midfacial hypoplasia (Figs. 1A and B) and 39% (7/18) of these mutants exhibited unilateral or bilateral cleft lip (Figs. 1C, D, and E), a defect that did not occur in Pax9 or Msx1 single homozygous mutants (N=150 for each mutant).
mainly results from (Figs. 2E, F, and L), whereas a ventral jaw, absence of the coronoid process is caused by absence of mutants, the alveolar bones are missing in all mutants. In the lower Pax9+/− in pure CD1 genetic backgrounds. defect in mixed C57BL/6J;CBA/J;CD1, mixed C57BL/6;FVB;BALB/c, and phenotype, which was consistently manifested as a severe growth (Figs. 2 G Msx1 premaxilla due to a missing nasal process results from absence of premaxilla (Figs. 2B, C, and I), whereas an anterior shortening of the an absence of the palatal processes in the posterior part of the lower incisor tissue were detectable in adult Pax9+/−;Msx1+/− mutant mice (Fig. 3K). Obvious developmental abnormalities other than oligodontia were not observed in Pax9+/−;Msx1+/− mutants. Since Msx1 and Pax9 are each required for the expression of Bmp4 in dental mesenchyme during early tooth formation (Chen et al., 1996; Peters et al., 1998), we examined if the formation of affected teeth could be rescued by transgenic expression of Bmp4. This transgene, a human BMP4 cDNA, is expressed in the dental mesenchyme and analysis of adult Pax9+/−;Msx1+−;BMP4tg/tg mutants on a C57BL/6J; CBA/J;CD1 genetic background showed that upper as well as lower third molars were present in all (n = 8) of these mice (Figs. 3C and F). Lower incisors were found in the majority (42/48, 87.5%) of animals but were hypoplastic (Fig. 3I). At a lower frequency (32/74; 43%), erupted lower incisors were found in Pax9+/−;Msx1+− mutants that carried only one transgenic BMP4 allele. In contrast, erupted lower incisors were not observed in Pax9+/−;Msx1+−;BMP4+−/− or Pax9+−/−; Msx1+/−;BMP4+−/− mice when these mutations were kept on a pure

Whereas this result identified an important interaction between Pax9 and Msx1 in upper lip development, skeletal staining of single and relevant compound mutant mice showed that abnormalities in the skull and jaws represent a combination of the defects seen in the single mutants. In addition to a cleft secondary palate (Figs. 2B, C, G, H, and I), the predominant phenotype caused by absence of Pax9 alone is an absence of the palatal processes in the posterior part of the premaxilla (Figs. 2B, C, and I), whereas an anterior shortening of the premaxilla due to a missing nasal process results from absence of Msx1 (Figs. 2 G–I). In addition, due to the absence of teeth in all mutants, the alveolar bones are missing in all mutants. In the lower jaw, absence of the coronoid process is caused by absence of Pax9 (Figs. 2E, F, and L), whereas a ventral flattening of the mandible mainly results from Msx1 deficiency (Figs. 2J, K, and L).

The cleft lip phenotype observed in Pax9−/−;Msx1−/− mice was incompletely penetrant in the mixed C57BL/6J;CBA/J;CD1 genetic background, and successive breeding onto a CD1 background suppressed the expression of this defect. Thus, to gain insight into the molecular pathways regulated by the genetic interaction between Pax9 and Msx1, we carried out a detailed analysis of the Pax9−/−;Msx1−/− lower incisor phenotype, which was consistently manifested as a severe growth defect in mixed C57BL/6J;CBA/J;CD1, mixed C57BL/6;FVB;BALB/c, and in pure CD1 genetic backgrounds.

Pax9+/−;Msx1+/− mutant mice exhibit oligodontia, which can be partially rescued by transgenic expression of BMP4

Whereas the dentition is normal in single heterozygous Pax9+/− or Msx1+/− mutant mice, all adult Pax9+/−;Msx1+/− mutants (n > 45 for each genetic background) lack lower incisors (Fig. 3H), and with incomplete penetrance in the C57BL/6J;CBA/J;CD1 genetic background, upper (8/14; 57%) and lower (6/14; 43%) third molars (Figs. 3B and E). Histological analysis revealed that no remnants of lower incisor tissue were detectable in adult Pax9+/−;Msx1+/− mutant mice (Fig. 3K). Obvious developmental abnormalities other than oligodontia were not observed in Pax9+/−;Msx1+/− mutants. Since Msx1 and Pax9 are each required for the expression of Bmp4 in dental mesenchyme during early tooth formation (Chen et al., 1996; Peters et al., 1998), we examined if the formation of affected teeth could be rescued by transgenic expression of Bmp4. This transgene, a human BMP4 cDNA, is expressed in the dental mesenchyme and analysis of adult Pax9+/−;Msx1+−;BMP4tg/tg mutants on a C57BL/6J; CBA/J;CD1 genetic background showed that upper as well as lower third molars were present in all (n = 8) of these mice (Figs. 3C and F). Lower incisors were found in the majority (42/48, 87.5%) of animals but were hypoplastic (Fig. 3I). At a lower frequency (32/74; 43%), erupted lower incisors were found in Pax9+/−;Msx1+− mutants that carried only one transgenic BMP4 allele. In contrast, erupted lower incisors were not observed in Pax9+/−;Msx1+−;BMP4+−/− or Pax9+−/−; Msx1+/−;BMP4+−/− mice when these mutations were kept on a pure

Fig. 3. Oligodontia in Pax9/−/Msx1/− double heterozygous mutant mice and in vivo rescue of tooth formation by transgenic BMP4. (A, D) Normal adult upper (A) and lower (D) molars. Missing upper (B) and lower (E) third molars in adult Pax9+/−;Msx1+/− mutant mice. (C, F) Rescued third molars in Pax9+/−;Msx1+/−;BMP4tg/tg mice. (G) Normal adult lower incisors. Upper incisors are present in all genotypes but are not shown. (H) Missing lower incisors in Pax9+/−;Msx1+/− mice and (I) partially rescued lower incisors in Pax9+/−;Msx1+/−;BMP4tg/tg mice. (J) Normal arrangement of dental tissues in the lower incisor. (K) No remnants of dental tissues are detectable in the lower jaw of adult Pax9+/−;Msx1+/− mutants and a tissue resembling bone marrow was observed instead. (L) In rescued lower incisors, the thickness of the dentin layer is reduced, and ameloblasts and enamel are missing. The dentin is connected to bone by a periodontal ligament, which is normally present only on the enamel-free lingual side. ab, alveolar bone; am, ameloblasts; de, dentin; en, enamel; od, odontoblasts; pl, periodontal ligament; pm, periodontal mesenchyme.
down-regulated in Msx1. Immunostaining at the bud stage revealed that Pax9 expression is not
suppressed in this genetic background. Consistent with a significant influence of the genetic background on lower incisor development, we observed that Msx1+/−;Pax9+/− mutant on a mixed C57BL/6×BALB/c background exhibited hypoplastic incisors, which erupted into the oral cavity but were chalky white and fragile, with hypoplastic cervical loops (data not shown).

Pax9+/−;Msx1+/− mutants form a small dental papilla and exhibit severe growth defects of the lower incisor epithelium

To determine at which stage lower incisor primordia in Pax9+/−;Msx1+/− mutants were morphologically affected, we carried out a histological analysis between the early bud (E12.5) and the bell (E15.5) stages (Figs. 4A–H). This analysis revealed that the dental papilla of Pax9+/−;Msx1+/− mutant lower incisor primordia is significantly smaller at E13.5 (Fig. 4F). At E14.5, the mutant lower incisors had progressed to the cap stage, but there was a pronounced epithelial growth defect affecting both upper (lingual) and lower (labial) incisor cervical loops, and a distinct enamel knot was not recognizable (Fig. 4G). At E15.5, wild type incisor germs attain the bell stage and a mesenchymal condensation is present next to each cervical loop (arrows in Fig. 4D). In contrast, in Pax9+/−;Msx1+/− mutants the paired condensations were absent and a single, mesenchymal protrusion developed instead that extended from the direct vicinity of the forming enamel knot (Fig. 5C) and, subsequently, is mainly associated with the mesenchyme adjacent to the upper and lower cervical loops (Fig. 5E). In contrast, Msx1 transcripts are abundant throughout the dental mesenchyme at E13.5 (Fig. 5I), and high expression levels persist in the distal compartment of the dental papilla (Fig. 5K). In Pax9+/−;Msx1+/− mutant incisors, the expression levels and expression domains of Pax9 (Figs. 5B, D, and F) and Msx1 (Figs. 5H, J, and L) in lower incisor mesenchyme resembled those in wild type incisors, though the domains were smaller after the growth retardation became more pronounced in the mutant. In contrast, the dental epithelial expression of Msx1 was qualitatively different between Msx1+/−;Pax9+/− mutants and wild type littermates. In E12.5 Msx1+/−;Pax9+/− mutant lower incisors, Msx1 was expressed in both labial and lingual side dental epithelium (Fig. 5H), whereas Msx1 is asymmetrically expressed in wild type incisor epithelium (Fig. 5G). At E13.5, when Msx1 was down-regulated in wild type incisor epithelium (Fig. 5I), Msx1 transcripts persisted in Msx1+/−;Pax9+/− mutant epithelium (Fig. 5J). Thus, while Msx1 expression is perturbed in Msx1+/−;Pax9+/− mutants, the double heterozygous phenotype cannot be explained simply on the basis of marked reductions in mesenchymal Pax9 or Msx1 expression.

Pax9+/−;Msx1+/− mutants form a smaller lower incisor field during tooth initiation

The reduced size of the dental papilla of Msx1+/−;Pax9+/− mutant lower incisors at E13.5 indicates that Pax9 and Msx1 interact prior to the bud stage. Since Pax9 and Msx1 are co-expressed at the time of tooth initiation (Neubuser et al., 1997; Mackenzie et al., 1991), we asked whether establishment of the lower incisor field was affected. This was done by analysis of Bmp2, p21 and Shh expression (Figs. 6A–J), which define the position and size of the dental primordia (Dassule

![Fig. 4. Growth retardation of Pax9+/−;Msx1+/− mutant incisors after the bud to cap stage transition. Panels show histological staining of sagittal sections of normal (A–D) and mutant (E–H) lower incisor regions. Whereas the mutant dental rudiment appears normal at E12.5 (E), the size of the dental papilla (outlined by dotted line) is reduced at E13.5 (F). At E14.5, the cervical loops of Pax9+/−;Msx1+/− mutant incisors are clearly growth-retarded (G). At E15.5, mesenchymal condensations are normally established adjacent to each cervical loop (arrows in D). In contrast, these condensations are missing in Pax9+/−;Msx1+/− mutants and a uniform mesenchymal domain (outlined by dashed line) is formed (H). Abbreviations: ek, enamel knot; lcl, lower cervical loop; mc, Meckel’s cartilage; tb, tooth bud; to, tongue; ucl, upper cervical loop; vl, vestibular lamina; wt, wild type. Scale bars (all 100 µm) are presented for each developmental stage.](image-url)
regulated in wild type incisor epithelium at E13.5 (I) but expression remains in the small domains adjacent to the enamel knot (arrow in C) and the domains with maximal expression are smaller in the vicinity of the dental papilla (Fig. 6). Although the expression domains are smaller in Pax9+/-;Msx1+/- mutant lower incisors (Figs. 7A, K), the domains are expressed in both labial and lingual sides in Pax9+/-;Msx1+/- mutants (H). In addition, Msx1 is down-regulated in wild type incisor epithelium at E13.5 (I) but expression remains in Pax9+/-;Msx1+/- mutant epithelium (J). Abbreviations as in Fig. 4; scale bar indicates 100 µm and applies to all panels.

Growth retardation of Pax9+/-;Msx1+/- lower incisors is associated with a loss of mesenchymal Fgf3 and Fgf10 expression in the dental papilla

To investigate potential molecular causes for the lower incisor growth defects we analyzed the expression of Bmp4, Fgf3, and Fgf10 during tooth morphogenesis and differentiation (Harada et al., 2002; Wang et al., 2007). In addition, between E12.5 and E15.5, these genes exhibit expression domains similar to those of Pax9 and Msx1 in the dental mesenchyme. A reduced size of the Bmp4 expression domain was detected in Pax9+/-;Msx1+/- mutant lower incisors at E12.5 and E13.5, while an ectopic expression domain in the mesenchyme separating the incisor and vestibular lamina was detected at E12.5 (Figs. 7B and D). At E14.5, Bmp4 is also normally expressed in the enamel knot (Fig. 7E) and this domain was either missing (Fig. 7F) or severely reduced in Pax9+/-;Msx1+/- mutants. Expression of other enamel knot markers, such as Shh, p21, and Lef1 was also reduced in Pax9+/-;Msx1+/- mutants (Suppl. Fig. 2). In addition, the mesenchymal expression domain of Bmp4 in the mutants was significantly smaller compared to that of wild type lower incisors. At E15.5, two domains of up-regulated Bmp4 expression that normally exist adjacent to the growing tips of the dental epithelium (Fig. 7G) were lacking in Pax9+/-;Msx1+/- mutants (Fig. 7H; compare Fig. 4H). However, in contrast to earlier stages, at E15.5 Bmp4 appeared to be expressed in Pax9+/-;Msx1+/- mutant incisor mesenchyme at levels similar to those in wild type incisors.

In contrast to the transient down-regulation observed for Bmp4, the expression of Fgf3 was strongly affected in the mutants at all stages and was down-regulated to undetectable levels from E14.5 onwards (Figs. 7J, L, and P). Similar to Bmp4, mesenchymal Fgf10 expression was absent in Pax9+/-;Msx1+/- mutants (Fig. 7P). Moreover, expression levels of Fgf3 and Fgf10 during upper incisor and molar development were indistinguishable from those in control embryos (data not shown).

Previous studies have shown that the mitogenic activities of Fgf3 and Fgf10 stimulate epithelial growth during tooth morphogenesis (Harada et al., 2002; Wang et al., 2007); we therefore hypothesized that the severely reduced expression of Fgf3 and Fgf10 in Pax9+/-;Msx1+/- lower incisors might affect cell proliferation. To test this hypothesis, we carried out BrdU labeling assays (Figs. 8A–D, I). At
Incisor growth and differentiation in rodents is asymmetric, with the labial cervical loop longer than the lingual and responsible for generating functional ameloblasts (Fig. 9A). Pax9+/−;Msx1+/− mutant lower incisors, though severely hypoplastically, appeared symmetric and exhibited almost equivalent growth of both labial and lingual cervical loops (Fig. 9B). This symmetric growth was maintained at least until E18.5 and, in a C57BL/6;FVB;BALB/c genetic background was characterized by columnar ameloblasts on both sides of the Pax9+/−;Msx1+/− mutant incisors (Fig. 9B). The ectopic presence of functional ameloblasts at the lingual aspect of Pax9+/−;Msx1+/− mutant incisors was supported by the symmetric expression pattern of Ameloblastin (Fig. 9D). Normally, in E16.5 wild type incides, Follistatin transcripts are down-regulated in differentiating ameloblasts on the labial side, while expression remains in the undifferentiated inner dental epithelium on the lingual side (Fig. 9E; Wang et al., 2004). In contrast, in Pax9+/−;Msx1+/− mutants, Follistatin expression was down-regulated in both labial and lingual side dental epithelium, with only a thin layer of dental epithelium near the cervical loop regions retaining some follistatin transcripts (Fig. 9F). A partial loss of lower incisor symmetry was indicated as early as E14.5, when the lingual epithelium of Pax9+/−;Msx1+/− mutants exhibited Shh expression (Fig. 9H), whereas Shh is predominantly expressed in labial epithelium in wildtype incisors (Fig. 9G). Moreover, the symmetry defect in Pax9+/−;Msx1+/− mutant incisors is preceded by a strong reduction of mesenchymal expression of Notch1, while its expression was preserved in the Pax9+/−;Msx1+/− mutant incisor epithelium (Fig. 9I, J, and N). Interestingly, in contrast to the ectopic, ameloblast-specific gene expression in the lingual incisor epithelium of Pax9+/−;Msx1+/− mutant embryos, ameloblasts were completely missing in the rescued Pax9+/−;Msx1+/−;BMP4tg/+ lower incisors (Fig. 3L), while the corresponding control mice (Pax9+/−;Msx1+/−;BMP4−/+;BMP4−/−) had normal lower incisors (data not shown).

**Discussion**

Previous work has shown that Pax9 and Msx1 are key regulators of tooth development, as Pax9 or Msx1 nullizygosity in mice results in a loss of the odontogenic potential of the dental mesenchyme and an arrest of tooth formation at the bud stage (Satokata and Maas, 1994; Peters et al., 1998). A drastic reduction of Pax9 gene dosage below that of Pax9 heterozygosity is associated with missing teeth, with lower incisors and third molars being the most susceptible tooth types (Kist et al., 2005). Here we showed that the same tooth types are affected in mice with concomitant heterozygosity of Pax9 and Msx1 and that the lower incisor defects involve altered gene expression patterns shortly after initiation, severe growth retardation during morphogenesis, and a loss of asymmetry during differentiation. These results therefore not only indicate a critical genetic interaction between Pax9 and Msx1 in tooth development, but also identify previously unknown functions of both genes at several different stages of odontogenesis.

Expression changes of epithelial markers are associated with early size reduction of Pax9+/−;Msx1+/− mutant lower incisors

The dental lamina is specified at E11.5 of mouse embryogenesis and expression of epithelial markers such as Bmp2, Shh and p21 subsequently identifies the sites of developing first molars and incisors (Dassule and McMahon, 1998; Cobourne et al., 2001, Gritti-Linde et al., 2002; Jernvall et al., 1998). In Pax9+/−;Msx1+/− mutant dental epithelium and by 24±2% (p<0.01) in the mutant dental mesenchyme (Figs. 8C, D, and I). In contrast, there was no obvious difference in apoptosis between Pax9+/−;Msx1+/− mutants and wild type incisors (Figs. 8E–H).

**Loss of labial-lingual asymmetry in lower incisors of Pax9+/−;Msx1+/− mutants**

E13.5, the BrdU index was reduced by 35±5% (mean±SEM, p<0.001) in the mutant dental epithelium, and by 23±1% (p=0.002) in the mutant dental mesenchyme (Figs. 8A, B, and I). At 14.5, the BrdU index was reduced by 25±2% (p=0.05) in the mutant
lower incisors, we found that these domains are either small or missing at E12.25 and E12.5, respectively. Thus, alterations in Pax9 and Msx1+−/− gene dosage influence the size of tooth primordia during initiation. Since Pax9 and Msx1 are co-expressed in dental mesenchyme, their interaction must therefore, directly or indirectly, affect the expression and/or activity of secreted factors that control the correct size and duration of a molecular response in the adjacent dental epithelium.

Several lines of evidence suggest that this control, at least in part, is mediated by the expression dependency of Bmp4 on Pax9 and Msx1. First, the expression of Bmp4 shifts from the epithelium to the mesenchyme at E11.5 and this expression is gradually lost in the developing molars of Pax9+−/−;Msx1+−/− single homozygous mutants (Vainio et al., 1993; Peters et al., 1998; Chen et al., 1996). Second, Bmp4 is sufficient to induce Shh expression in the epithelium of Msx1−/− deficient tooth rudiments (Zhang et al., 2000; Zhao et al., 2000). Third, a synergistic, Pax9/Msx1-mediated in vitro activation of transcription from the basal Bmp4 promoter was identified in a non-dental cell line (Ogawa et al., 2006). Finally, the lateral truncation of Shh expression domains in Pax9+−/−;Msx1+−/−− mutant lower incisors was partially rescued by transgenic Bmp4 expression as early as E12.25 (Fig. 2). Whereas we identified slightly reduced expression domains of Bmp4 at E12.5 and E13.5 (Fig. 7), whole-mount in situ hybridization of Bmp4 in Pax9+−/−;Msx1+−/−− mutant lower incisors failed to detect a laterally truncated domain of mesenchymal Bmp4 expression prior to E12.25. However, in addition to the limited sensitivity of this method to quantify small expression changes, we cannot rule out that the Pax9/Msx1 interaction in the dental mesenchyme may also affect other aspects of the Bmp4 pathway. For example, a Pax9/Msx1 interaction may be required to indirectly regulate Bmp4 protein stability or its efficient diffusion, both of which providing possible explanations for a spatially restricted response to Bmp4 signaling in the lower incisor epithelium of Pax9+−/−;Msx1+−/−− mutants.

Growth defects of Pax9+−/−;Msx1+−/−− mutant lower incisors involve loss of Fgf3 and Fgf10 expression in the dental papilla

Based on morphological criteria, the first recognizable defect of the Pax9+−/−;Msx1+−/−− lower incisor papilla is its reduced size at E13.5, a defect that is preceded by a smaller expression domain of Bmp4 and by an absence of Fgf3 expression in the dental mesenchyme at E12.5. Whereas in the mutants Fgf3 was weakly expressed at E13.5, Fgf3 expression was not detectable from E14.5 onwards and Fgf10 expression was strongly reduced at all stages analyzed. Fgf3 and Fgf10 have redundant functions during tooth development and not only regulate the proliferation of the mesenchymal cells of the dental papilla, but also act as mitogens in epithelial stem cells and transient amplifying (TA) cells in the lower incisor cervical loop (Harada et al., 2002; Wang et al., 2007). Consistent with these functions, lower incisor development is strongly affected in Fgf3−−/−:Fgf10−−/− compound mutant mice, whereas molars are less affected (Wang et al., 2007). In addition, recent work has identified important roles of FGF3 and FGF10 in the regulation of tooth size in humans (Tekin et al., 2007; Alsmadi et al., 2009; Milunsky et al., 2006; Shams et al., 2007). We thus conclude that the down-regulation of Fgf3 and Fgf10 expression in Pax9+−/−;Msx1+−/−− mutants is likely to be a major cause of the reduced cell proliferation and lower incisor growth defects.

Loss of lower incisor asymmetry

The specific growth rates and differentiation pathways of labial and lingual dental epithelia are key features of the continuously growing tissues in the mesenchyme adjacent to the cervical loops (G), whereas in the mutants these domains are missing (H). Note also the ectopic expression of Pax9+−/−;Msx1+−/−− in the mutants (R, T), except for an ectopic domain separating tooth bud and vestibular lamina (arrow in R). At E15.5, strong Bmp4 expression is normally seen in the mesenchyme adjacent to the cervical loops (G), whereas in the mutants these domains are missing (H). Note also the ectopic Bmp4 expression (arrow) outside the actual incisor region. Expression of Fgf3 in the incisor mesenchyme is moderately down-regulated at E12.5 (J) and E13.5 (L) and was not detectable at subsequent stages (N, P). Expression of Fgf10 was not affected in the mutants at E12.5 (R) and E13.5 (T), except for an ectopic domain separating tooth bud and vestibular lamina (arrow in R). Fgf10 expression was not detectable in Pax9+−/−;Msx1+−/−− mutants at E14.5 (V). At E15.5, up-regulated Fgf10 expression associated with the cervical loops is detectable in normal incisors (W) but was missing in Pax9+−/−;Msx1+−/−− mutants (X). Both Fgf3 and Fgf10 are expressed at normal levels during upper incisor development (not shown). Scale bars (all 100 µm) are presented for each developmental stage. Ek, enamel knot; lcl, lower cervical loop; tb, tooth bud; to, tongue; ucl, upper cervical loop; vl, vestibular lamina; wt, wild type.
rodent incisors. Whereas the labial cervical loop is large and generates enamel-secreting ameloblasts, the lingual cervical loop is small and does not produce functional ameloblasts. This asymmetry is controlled by a complex regulatory network involving the Follistatin-mediated inhibition of ameloblast differentiation at the lingual aspect (Wang et al., 2004) and by a Fgf3/Fgf10-mediated growth stimulation predominantly at the labial aspect (Wang et al., 2007). Although lower incisors are strongly growth-retarded in Msx1+/−;Pax9+/− mutants, the absent expression of Fgf3 and the weak but uniform expression of Fgf10 at E15.5 is consistent with similarly sized labial and lingual cervical loops. Moreover, the loss of asymmetric differentiation indicated by ectopic Ameloblastin expression in lingual dental epithelium is associated with an abnormal down-regulation of Follistatin expression in both labial and lingual dental epithelium in Msx1+/−;Pax9+/− mutants. Interestingly, in contrast to the ectopic formation of ameloblasts in the embryo, functional ameloblasts were entirely missing in the hypoplastic incisors of adult Msx1+/−;Pax9+/− mutants kept on a mixed C57BL/6×BALB/c background (data not shown). A likely explanation for the stage-specific difference of the phenotype may be that Pax9 and Msx1 continue to interact in the incisor mesenchyme of adult mice to regulate transcription Fgf3 and Fgf10. Thus, in certain genetic backgrounds the expression levels of Fgf3 and Fgf10 are apparently sufficient for early incisor growth, however, the levels could be insufficient to maintain an epithelial stem cell pool over a long period. Experimental strategies that inactivate Pax9 and Msx1 in adult mice will be required to directly test the involvement of Pax9 and Msx1 in the maintenance of dental stem cell compartments.

The first molecular events that establish lower incisor asymmetry are unknown, but may involve cell fate decisions at the bud stage. Notably, at E12.5, Msx1 is expressed in labial side dental epithelium, whereas Msx1+/−;Pax9+/− mutant lower incisors lose this asymmetric expression, with Msx1 expression in both labial and lingual dental epithelium. At E13.5, when Msx1 becomes down-regulated in wild type incisor epithelium, Msx1 transcripts persist in Msx1+/−;Pax9+/− mutant incisor epithelium. Although this may simply reflect the growth retardation of Msx1+/−;Pax9+/− mutant incisors, it will be interesting to determine whether the asymmetric epithelial expression of Msx1 contributes to the asymmetric growth and differentiation of mouse incisors.

In addition to abnormal Msx1 expression in incisor epithelium, we found Notch1 to be markedly down-regulated in the dental mesenchyme of Msx1+/−;Pax9+/− mutant incisors at E12.5 and E13.5. Notch1 is expressed in the dental epithelium of developing molars at the dental lamina and bud stages, and in both dental epithelium and mesenchyme at the cap stage (Mitsiadis et al., 1995). In the developing mouse incisors, Notch signaling has been implicated in the regulation of epithelial cell fate in the cervical loops (Tummers et al., 2007). Notch1 seems to be expressed in the incisor mesenchyme earlier than in molar teeth. Interestingly, FGF10 has been shown to stimulate expression of lunatic fringe, a regulator of Notch signaling, and it has been proposed that FGF signaling is linked to cell fate...
decisions in incisor stem cells (Harada et al., 1999; Mustonen et al., 2002). Since Notch1 knockout mice die early during mid-gestation (Swiatek et al., 1994), conditional deletion of Notch1 in the dental mesenchyme will be required to investigate its role in the establishment of epithelial asymmetry in rodent incisors.

Genetic interactions in oligodontia, selective tooth agenesis and orofacial clefting

The causes for the majority of congenital, sporadic forms of missing teeth in humans are unknown, and gene–gene interactions may underlie a significant number of these cases. Based on the large number of studies showing that mutations in PAX9 or MSX1 cause non-syndromic oligodontia, both genes are promising candidates for an involvement in causative gene–gene interactions. A previous study found that concomitant polymorphisms in MSX1 and PAX9 were over-represented in a small group of human hypodontia patients (Vieira et al., 2004). Our findings provide direct evidence for a genetic interaction between heterozygous Pax9 and Msx1 loss-of-function mutations that result in oligodontia in mice. The missing lower incisors and background-dependent absence of third molars in Msx1−/−;Pax9+−/− mutant mice mimic sporadic hypodontia. Comprehensive mutation analyses including regulatory promoter and enhancer regions of PAX9 and MSX1 may help to uncover the genetic basis for some types of sporadic hypodontia in humans.

Msx1 and Pax9 are expressed similarly in both upper and lower incisors, as well as in molars, but our data shows that in Msx1−/−;Pax9−/− mutants the growth retardation and disruption of Pax9/ Msx1-dependent developmental pathways affects only some tooth types. In particular, while Pax9 and Msx1 expression are maintained at reduced levels in Msx1−/−;Pax9−/− lower incisors, Fgfr2 expression is completely lost after the bud stage. This finding suggests that Pax9 and Msx1 are involved in antagonizing a genetic pathway that inhibits Fgfr2 expression. While this pathway remains to be identified, it appears to be particularly potent in the dental field of lower incisors, thus providing a potential explanation for the increased dosage sensitivity of lower incisor development. Consistent with this view, different responses to molecular changes have been reported for various tooth types. For example, Fgfr2 loss-of-function mutations lead to defective maxillary incisors, whereas lower incisors and molars are unaffected (Lin et al., 2009). It has been suggested that different tissue origins may underlie these different genetic sensitivities. Maxillary incisors arise from the fronto-nasal process and neural crest-derived ectomesenchymal cells that migrate from caudal midbrain and rostral hindbrain, while mandibular incisors arise from the first branchial arch and neural crest-derived ectomesenchymal cells that migrate from the hindbrain (Cobourne and Mitsiadis, 2006). Specific patterns of homeobox-containing genes indicate that different identities of these cell populations are maintained during odontogenesis (Cobourne and Sharpe, 2003), and it seems plausible that Pax9 and Msx1 interact differently with distinct tooth-specific genetic programs.

Lastly, cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO) are among the most frequent developmental birth defects, with prevalences as high as 1/500 in some populations; studies suggest that more than 50% of cases are not associated with a syndrome (Murray, 2002). A combination of genetic interactions and environmental factors has been suggested to underlie most sporadic cases, but the identification of genes that play a major role in this multifactorial process is difficult (Jugessur and Murray, 2005; Vieira et al., 2005). Interestingly, CL/P and CPO are often associated with...
missing teeth, and recent analyses indicate that the incidence of missing teeth is significantly higher even outside the cleft region and in the mandibular jaw (Shapira et al., 2000; Slayton et al., 2003). These data suggest that upper lip, secondary palate and teeth share some genetic pathways during their development. In humans, CL/P has been identified in oligodontia patients with heterozygous MSX1 mutations (summarized in Vieira, 2008). In addition, specific polymorphisms in PAX9 or MSX1 have recently been associated with cleft lip in humans (Ichikawa et al., 2006; Modesto et al., 2006). CL/P is a relatively rare craniofacial abnormality in mice, but our data show that this phenotype manifests in Pax9−/−; Msx1−/− mouse mutants on a mixed C57BL/6/J:CBA/J:CD1 genetic background. However, the incomplete penetrance and variable expressivity (unilateral or bilateral cleft lip) indicate that additional genetic modifiers contribute to cleft lip formation in this digenic mouse model. Pax9/ Msx1-deficient mouse models may facilitate the identification of critical genetic modifiers of CL/P and help to elucidate the role of genetic interactions in non-syndromic craniofacial abnormalities.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdybio.2010.01.031.

References


