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

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ABSTRACT

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.

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

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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/6J;CBA/J genetic background, to *Pax9*/*Msx1* double heterozygous mice to generate triple mutants with a mixed C57BL/6J;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 (Sporle 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),

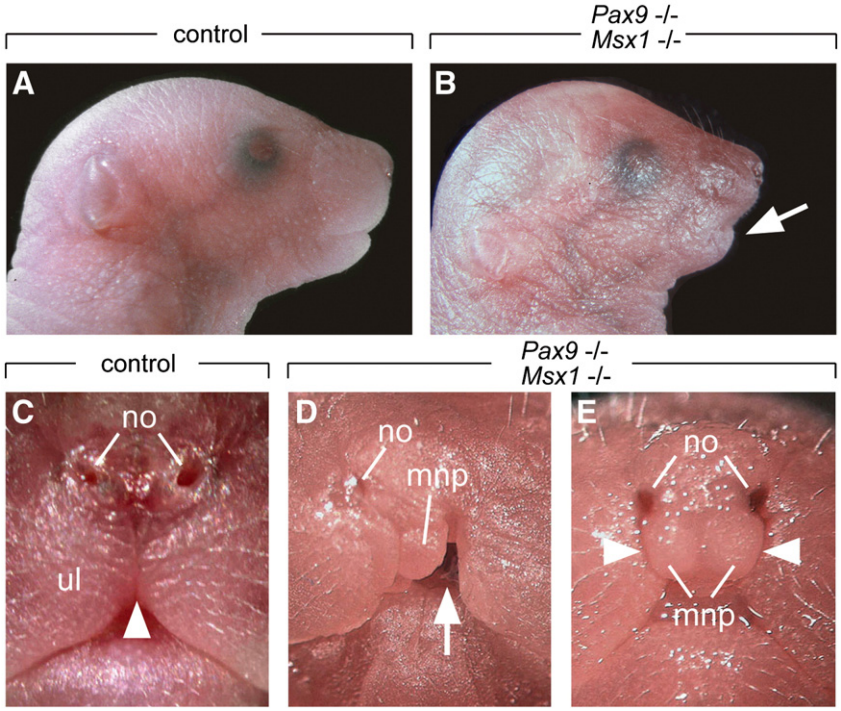

Fig. 1. Cleft lip in *Pax9;Msx1* double homozygous mutant mice. (A) Head of a normal, newborn mouse. (B) $Pax9^{-/-}$;$Msx1^{-/-}$ newborn mice exhibit reduced head size, low-set ears, and hypoplasia of the mandible (arrow). (C) In newborn control mice, a single midline furrow (arrowhead) divides the upper lip. (D) Unilateral cleft lip (arrow) of a $Pax9^{-/-}$;$Msx1^{-/-}$ mutant, leaving the left medial nasal process exposed. (E) Bilateral cleft lip (arrowheads), resulting in medial nasal processes forming a 'floating' primary palate. mnp, medial nasal process; no, nostrils; ul, upper lip.