Developmental Biology 353 (2011) 344–353

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Osr2 acts downstream of Pax9 and interacts with both Msx1 and Pax9 to pattern the tooth developmental field

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ARTICLE INFO

Article history:
Received for publication 2 November 2010
Revised 7 March 2011
Accepted 10 March 2011
Available online 17 March 2011

Keywords:
Msx1
Odd-skipped
Odontogenic
Osr2
Pax9
Tooth development

ABSTRACT

Mammalian tooth development depends on activation of odontogenic potential in the presumptive dental mesenchyme by the Msx1 and Pax9 transcription factors. We recently reported that the zinc finger transcription factor Osr2 was expressed in a lingual-to-buccal gradient pattern surrounding the developing mouse molar tooth germs and mice lacking *Osr2* developed supernumerary teeth lingual to their molars. We report here generation of a gene-targeted mouse strain that allows conditional inactivation of *Pax9* and subsequent activation of expression of *Osr2* in the developing tooth mesenchyme from the *Pax9* locus. Expression of *Osr2* from one copy of the *Pax9* gene did not disrupt normal tooth development but was sufficient to suppress supernumerary tooth formation in the $Osr2^{-/-}$ mutant mice. We found that endogenous *Osr2* mRNA expression was significantly downregulated in the developing tooth mesenchyme in $Pax9^{del/del}$ mice. Mice lacking both *Osr2* and *Pax9* exhibited early tooth developmental arrest with significantly reduced *Bmp4* and *Msx1* mRNA expression in the developing tooth mesenchyme, similar to that in $Pax9^{del/del}$ mutants but in contrast to the rescue of tooth morphogenesis in $Msx1^{-/-}Osr2^{-/-}$ double mutant mice. Furthermore, we found that Osr2 formed stable protein complexes with the Msx1 protein and interacted weakly with the Pax9 protein in co-transfected cells. These data indicate that Osr2 acts downstream of Pax9 and patterns the mesenchymal odontogenic field through protein–protein interactions with Msx1 and Pax9 during early tooth development.

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Introduction

Tooth development has long been used as a model system for studying the molecular mechanisms of organogenesis (Pispa and Thesleff, 2003; Thesleff et al., 1995). Morphologically, tooth development begins with formation of a thickened stripe of the oral epithelium, termed the dental lamina, at sites of the future dental arches in the maxilla and mandible, which occurs at around the 11th day of gestation (E11) in mice. The dental lamina cells proliferate and bud into the underlying neural crest-derived ectomesenchyme at specific sites and induce the mesenchyme to condense around the epithelial buds from E12 to E13. Subsequently, the epithelium folds and extends farther into the mesenchyme, wrapping itself around the condensing mesenchyme to form "cap" (E14) and then "bell"-shaped tooth germs (at about E16). As development proceeds, epithelial cells in contact with the dental mesenchyme differentiate into enamel-producing ameloblasts and their adjacent mesenchymal cells differentiate into dentin-producing odontoblasts. Thus, formation of each individual tooth, from initiation through morphogenesis to ultimate cytodifferentiation, involves extensive interactions between the dental epithelium and the underlying mesenchyme.

Tissue recombination experiments showed that the early oral epithelium provides the instructive signals for tooth initiation. The mouse E9–E11 rostral mandibular epithelium elicited tooth formation when combined with non-dental second branchial arch mesenchyme (Lumsden, 1988; Mina and Kollar, 1987). However, no teeth formed when the E9–E11 presumptive dental epithelium was recombined with developing limb bud mesenchyme, indicating that only the neural crest derived mesenchyme is odontogenic competent (Lumsden, 1988). As development proceeds, however, the tooth inductive potential rapidly shifts to the dental mesenchyme (Lumsden, 1988; Mina and Kollar, 1987). It was demonstrated that the dental mesenchyme from E13 mouse embryos induced tooth formation from various non-dental epithelia, including limb epithelium, causing enamel organ morphogenesis and amelogenesis (Kollar and Fisher, 1980; Lumsden, 1988; Mina and Kollar, 1987; Ruch et al., 1973; Ruch et al., 1984). Thus, whereas tooth initiation depends on a site-specific epithelium, activation of tooth inductive potential in the neural crest

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doi:10.1016/j.ydbio.2011.03.012

derived presumptive dental mesenchyme is essential for subsequent tooth morphogenesis (Lumsden, 1988).

Extensive gene expression analyses and mouse genetic studies have provided significant insight into the molecular network controlling early tooth development. Prior to tooth initiation, several signaling molecules of the bone morphogenetic protein (Bmp) and fibroblast growth factor (Fgf) families are expressed in the presumptive dental epithelium and are responsible for inducing the expression of several transcription factors, including Msx1 and Pax9, in the presumptive dental mesenchyme (Dassule and McMahon, 1998; Lyons et al., 1989; Mandler and Neubuser, 2001; Neubuser et al., 1997; Vainio et al., 1993; Wozney et al., 1988). Mice lacking either *Msx1* or *Pax9* had tooth developmental arrest at the early bud stage (Peters et al., 1998; Satokata and Maas, 1994). Expression of Bmp4, a critical mesenchymal odontogenic signal, was dramatically reduced in the developing tooth mesenchyme by E13.5 in either $Msx1^{-/-}$ or $Pax9^{-/-}$ mutant mice (Chen et al., 1996; Peters et al., 1998). Maintenance of *Msx1* mRNA in the developing tooth mesenchyme also depends on Pax9 function (Ogawa et al., 2006; Peters et al., 1998). In vitro biochemical studies showed that Pax9 was able to activate reporter gene expression driven by either the mouse *Bmp4* or *Msx1* gene promoter sequences and that Msx1 and Pax9 activated the *Bmp4* promoter synergistically (Ogawa et al., 2006). Moreover, addition of recombinant Bmp4 protein rescued development of $Msx1^{-/-}$ mutant mandibular molar tooth germs to the late bell stage in explant cultures (Bei et al., 2000; Chen et al., 1996). Furthermore, mice with tissue-specific inactivation of *Bmpr1a*, which encodes a type-I receptor for Bmp family ligands, in the oral epithelium exhibited tooth developmental arrest at the bud stage (Andl et al., 2004; Liu et al., 2005). These data indicate that the Msx1 and Pax9 transcription factors play essential roles during early tooth development by activating mesenchymal odontogenic signals, including Bmp4, which signal back to the epithelium to drive tooth morphogenesis beyond the bud stage.

We reported recently that mice homozygous for a targeted null mutation in the *Osr2* gene had supernumerary tooth formation lingual to the molars (Zhang et al., 2009). *Osr2* encodes a zinc finger protein with extensive sequence similarity to the *Drosophila* Odd-skipped family transcription factors that regulate multiple developmental processes during embryogenesis and tissue morphogenesis (Coulter and Wieschaus, 1988; Green et al., 2002; Hao et al., 2003; Hart et al., 1996; Lan et al., 2001; Saulier-Le Drean et al., 1998; Wang and Coulter, 1996). We found that *Osr2* mRNA exhibited a lingual-to-buccal gradient, complementary to that of *Bmp4*, in the mesenchyme surrounding the early developing tooth buds during normal tooth development and that *Bmp4* mRNA expression was upregulated and expanded into the lingual side of the developing tooth mesenchyme in the $Osr2^{-/-}$ mice (Zhang et al., 2009). Furthermore, whereas $Msx1^{-/-}$ mutant mice had tooth developmental arrest at the bud stage accompanied by loss of *Bmp4* expression from the developing tooth mesenchyme, $Msx1^{-/-}Osr2^{-/-}$ double mutant mice showed restoration of *Bmp4* mRNA expression in the tooth mesenchyme and rescue of first molar tooth morphogenesis (Zhang et al., 2009). These data suggest that Osr2 is a negative regulator of mesenchymal odontogenic potential and patterns the tooth developmental field at least in part by antagonizing the Msx1–Bmp4 pathway.

Since mice lacking either Msx1 or Pax9 had tooth developmental arrest at the early bud stage accompanied by loss of *Bmp4* expression (Chen et al., 1996; Peters et al., 1998), the rescue of molar tooth development and restoration of *Bmp4* expression in the developing tooth mesenchyme in the $Msx1^{-/-}Osr2^{-/-}$ double mutant mice suggests that Osr2 may also interact with Pax9 during tooth development. To address this possibility, we generated a new line of *Pax9* null mutant mice and found that Pax9 functions upstream of Osr2 and is required for tooth development beyond the early bud stage even in the absence of Osr2.

Materials and methods

Generation of $Pax9^{fneo}$ mice

A *129/SvEv* strain mouse BAC clone containing the entire *Pax9* genomic region was isolated from the RPCI-22 BAC library (BACPAC Resources, Children's Hospital of Oakland, Oakland, CA). The targeting vector used the 4.6 kb *Sma*I–*Sma*I fragment containing the beginning of the 5′ untranslated region of exon 1 as the 5′ arm and the 2.8 kb *Bgl*II–*Hin*dIII fragment containing the exon 3 region as the 3′ arm (Fig. 1A). The genomic region from the middle of the 5′ untranslated to the middle of intron 2 was subcloned in between two directly repeated loxP sites and inserted back in between the 5′ and 3′ homology arms. An *FRT*-flanked *neo* expression cassette followed by the *Myc-Osr2A* cDNA fusion construct (Gao et al., 2009) was then inserted in the second intron just 3′ to the loxP sequence in the targeting vector. In addition, a diphtheria toxin expression cassette (DTA) was subcloned to the 3′ end of the 3′ homology arm for selection against random integration of the targeting vector. The targeting vector was linearized and electroporated into the *CJ7* mouse embryonic stem (ES) cells. ES cell culture and Southern hybridization screening of ES clones were carried out as previously described (Lan et al., 2004; Swiatek and Gridley, 1993). Two independently targeted ES cell clones were injected into blastocysts from C57BL/6J mice and the resultant chimeras bred with C57BL/6J females. F1 mice were genotyped by Southern hybridization analysis of tail DNA using the 5′ external probe (Fig. 1). Mice and embryos from subsequent generations were genotyped by PCR. PCR with *Pax9* 1F (5′-CCC ACG TTG CTG CTT AGA TT-3′) and *Pax9* 1R (5′-CGC ACT CCC AGA AAG AAA CT-3′) amplified a product of 186 bp from the wildtype *Pax9* allele and a product of 260 bp from the $Pax9^{fNeo}$ *allele*. Heterozygous F1 mice were backcrossed with C57BL/6J mice and N2 heterozygous mice were intercrossed for analysis of homozygous phenotype.

Other mouse strains

$Pax9^{del/+}$ mice were generated by crossing the $Pax9^{fNeo/+}$ mice to the *EIIa-Cre* transgenic mice (The Jackson Laboratory, Bar Harbor, ME). Genotyping PCR with *Pax9* 1F (5′-CCC ACG TTG CTG CTT AGA TT-3′) and *Pax9* 4R (5′-GTG CCC AGT CAT AGC CGA AT-3′) amplified a product of 600 bp from the $Pax9^{del}$ allele. $Pax9^{Osr2KI/+}$ mice were generated by crossing the $Pax9^{del/+}$ mice with the *FLPeR* mice (The Jackson Laboratory, Bar Harbor, ME). Genotyping PCR with *Pax9* 1F (5′-CCC ACG TTG CTG CTT AGA TT-3′) and *Pax9* 2R (5′-CGC CCA AGC TCT CCA TTT CAT TCA-3′) amplified a product of 450 bp from the $Pax9^{Osr2KI}$ allele. $Pax9^{del/+}Osr2^{+/-}$ mice were generated by crossing the $Pax9^{del/+}$ mice to the $Osr2^{+/-}$ mice (Lan et al., 2004). The $Osr2^{Osr2Aki}$ mouse strain has been described previously (Gao et al., 2009).

Antibodies

The monoclonal anti-MYC epitope antibody (clone 4A6) was purchased from Millipore Corporation and anti-β-actin antibody was from Santa Cruz Biotechnology, Inc. The monoclonal anti-FLAG M2 antibody was purchased from Sigma. The Alexa Fluor® 546 goat anti-mouse IgG was purchased from Invitrogen Inc.

Western blot analysis

Mouse embryonic facial tissues were lysed in RIPA buffer (Santa Cruz). The protein concentration of supernatants was determined with Bio-Rad Assay Reagent (Bio-Rad), using BSA as the standard. Extracts were diluted in SDS-loading buffer and analyzed by standard SDS-PAGE and western blot analysis.

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