The Pitx2:miR-200c/141:noggin pathway regulates Bmp signaling and ameloblast differentiation

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SUMMARY
The mouse incisor is a remarkable tooth that grows throughout the animal’s lifetime. This continuous renewal is fueled by adult epithelial stem cells that give rise to ameloblasts, which generate enamel, and little is known about the function of microRNAs in this process. Here, we describe the role of a novel Pitx2:miR-200c/141:noggin regulatory pathway in dental epithelial cell differentiation. miR-200c repressed noggin, an antagonist of Bmp signaling. Pitx2 expression caused an upregulation of miR-200c and chromatin immunoprecipitation assays revealed endogenous Pitx2 binding to the miR-200c/141 promoter. A positive-feedback loop was discovered between miR-200c and Bmp signaling. miR-200c/141 induced expression of E-cadherin and the dental epithelial cell differentiation marker amelogenin. In addition, miR-203 expression was activated by endogenous Pitx2 and targeted the Bmp antagonist Bmpr to further regulate Bmp signaling. miR-200c/141 knockout mice showed defects in enamel formation, with decreased E-cadherin and amelogenin expression and increased noggin expression. Our in vivo and in vitro studies reveal a multistep transcriptional program involving the Pitx2:miR-200c/141:noggin regulatory pathway that is important in epithelial cell differentiation and tooth development.

KEY WORDS: Bmp, Noggin, Pitx2, Stem cells, Tooth development, miR-200 family, miR-200c, miR-141, miR-203

INTRODUCTION
The mouse incisor presents an attractive model with which to study organogenesis and epithelial stem cell differentiation. First, it grows continuously throughout the life of the animal. Second, enamel is deposited asymmetrically and exclusively on the labial (i.e. toward the lip) surface, enabling preferential abrasion on the enamel-free surface and the maintenance of incisor length. The epithelial stem cells that control growth of the mouse incisor are located in niches called the labial and lingual cervical loop (CL) at the proximal end of the incisor (Harada et al., 1999); however, only the labial CL produces ameloblasts that generate enamel. Although it is well accepted that dental epithelial stem cells are housed in the labial CL, their precise location is still a matter of debate (Harada et al., 1999; Seidel et al., 2010; Tummers and Thesleff, 2003; Tummers and Thesleff, 2008).

The Fgf, Bmp, Shh, Wnt and Eda signaling pathways in ectoderm-derived epithelium and neural crest-derived mesenchyme regulate numerous aspects of tooth development and renewal. In particular, the role of the Bmp signaling pathway has been well characterized. Several Bmps are expressed in the developing tooth at various stages (Mustonen et al., 2002; Vainio et al., 1993). Specifically, Bmp4 is expressed on the labial side of the incisor and induces ameloblast differentiation (Wang et al., 2007; Wang et al., 2004). Disruption of Bmp activity by nogggin overexpression blocks molar development and late stage incisor epithelial cell differentiation (Plikus et al., 2005). During initiation of tooth formation, Bmp signaling in the epithelium antagonizes Fgf pathways, and this interaction is thought to determine the sites of tooth formation (Bei and Maas, 1998; Neubüser et al., 1997; St Amand et al., 2000). Inactivation of the Bmp receptor Bmpr1a in epithelium or mesenchyme causes arrest of tooth development soon after placode formation (Andl et al., 2004; Li et al., 2011b; Thomas et al., 1998). In addition, misexpression of the Bmp antagonist follistatin under the control of the Krt14 promoter disrupts ameloblast differentiation in the incisor, whereas a lack of follistatin leads to ectopic enamel formation on the lingual surface (Plikus et al., 2005; Wang et al., 2007; Wang et al., 2004). A lack of nogggin results in fusion of the upper incisor (Hu et al., 2012). Although the function of Bmp and other signaling pathways is well documented in tooth development and renewal, little is known about the role of microRNAs (miRNAs) in these processes.

Small RNAs, and miRNAs in particular, have important effects on development and disease through the modulation of specific signaling pathways (Lewis and Steel, 2010; Michon, 2011; Park et al., 2010; Zhang et al., 2010). miRNAs are endogenously expressed, short (~21 nucleotides) non-coding RNA molecules that regulate gene expression (Fabian et al., 2010; Winter et al., 2009). miRNAs interact with the 3′-UTR of target mRNAs to inhibit protein synthesis and/or decrease mRNA stability (Chekulaeva and Filipowicz, 2009; Eulalio et al., 2008). Primary (pri) miRNAs are processed by RNome III enzymes such as drosha and dicer to make mature miRNAs (Doi et al., 2003; Lee et al., 2003). The inactivation of Dicer1 in epithelia has demonstrated the importance of mature miRNAs in tooth development and epithelial stem cell differentiation (Cao et al., 2010b; Michon et al., 2010). However, the specific miRNAs involved have yet to be characterized.

Here, we describe the central role of a Pitx2:miR-200c/141:noggin regulatory pathway in tooth development. We show that nogggin, which is a Bmp antagonist, is a direct target of miR-200c. Upstream regulation of miR-200c includes interaction
of Pitx2 with the shared promoter of miR-200c and miR-141 (Mir200c and Mir141 – Mouse Genome Informatics; collectively miR-200c/141), and subsequent activation of miR-200c. A second upstream regulator of miR-200c was identified to be Bmp signaling, thereby indicating a positive-feedback loop between miR-200c and Bmp signaling. Furthermore, miR-203 targets the Bmp antagonist Bmpr and is activated by endogenous Pitx2. Bmpr acts similarly to noggin through the endocytosis of Bmp4 and inhibition of Bmp4 signaling (Kelley et al., 2009). We further report that Bmpr is expressed in the dental epithelium during tooth development, adding to the tissue-specific activity of Bmpr. Finally, deletion of miR-200c/141 in mice resulted in enamel defects due to downregulation of the cell adhesion protein E-cadherin (E-cad; Cadh1 – Mouse Genome Informatics) and of amelogenin (Amel; Amelx – Mouse Genome Informatics), an essential protein in enamel formation, confirming our in vitro results in dental epithelial cells.

Sox2 and Thbx1 are dental stem cell markers as they are predominantly expressed in the CLs of the mouse incisors and/or affect progenitor cell proliferation (Cao et al., 2010a; Catón et al., 2009; Juuri et al., 2012; Mitsiadis et al., 2008). Other genes also mark predominantly expressed in the CLs of the mouse incisors and/or miR-200c/141 in the regulation of stem cells and ameloblasts during dental development (reviewed in Li et al., 2011a; Suomalainen and Thesleff, 2010). Recently, E-cad was shown to regulate dental epithelial stem cell proliferation and migration in the mouse incisor CL (Li et al., 2012). Our in vivo and in vitro studies demonstrate a central role for miR-200c/141 in the regulation of stem cells and ameloblasts during dental epithelial cell differentiation and tooth development.

MATERIALS AND METHODS

Animals

Animals were housed in the Program of Animal Resources of the Institute of Biosciences and Technology at the Texas A&M Health Science Center or in the animal facilities at UCSF. Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and animals were handled in accordance with the principles and procedures of the Guide for the Care and Use of Laboratory Animals. Procedures for the generation of miR-200c/141 knockout mice were described previously (Park et al., 2010b). Briefly, the miR-200c/141 knockout construct was generated by a previously described ‘knockout-first’ approach (Testa et al., 2004). Two homology arms at the 5′ and 3′ ends of the targeting vector mediated gene-specific targeting by homologous recombination. Targeting led to insertions of a promoterless lacZ reporter with an IRES and a poly(A) signal, and the miR-200c/141 stem-loop locus. Germline-transmitted mice (lacZ-KO) were crossed with germline deleter Cre (under the control of the beta-actin promoter) mice to produce mice with a reporter-tagged null allele [lacZ knockout allele (lacZ-KO)]. The Pitx2Cre-Dicer1LoxStop1/loxStop1 (Pitx2Cre/Dicer1) and Krt14-PITX2C transgenic mice have been described previously (Cao et al., 2010b; Venugopalan et al., 2008). We analyzed 12 miR-200c knockout mice and they all showed enamel defects by microcomputed tomography (µCT) or histology. The defect in tooth eruption (third molars) was confirmed by standard hematoxylin and eosin (H&E) staining, and by microcomputed tomography (µCT) or histology. The defect in tooth eruption (third molars) was confirmed by standard hematoxylin and eosin (H&E) staining, and by microcomputed tomography (µCT) or histology.

miRNA microarrays and qPCR

miRNA microarray analysis comparing gene expression in the incisor of postnatal day (P) 0 control and Pitx2Cre-Dicer1 has been described (Cao et al., 2010b). For miRNA comparison between the labial CLs and pre- ameloblast and ameloblast cells, these two regions from the P0 mouse lower incisors were manually dissected under a dissection microscope. Total RNA including miRNA from these tissues was prepared using the mirNeasy Mini Kit (Qiagen). Lower incisors from P0 control and Krt14-PITX2C mouse incisors were also dissected and total RNA was prepared using the mirNeasy Mini Kit. miRNA microarray analysis was performed by LC Sciences using µParaflo Microfluidic Biochip version 14, which detects miRNA transcripts listed in Sanger miRBase release 14.0. Quantitative real-time PCR (qPCR) analysis of miRNAs was performed using TaqMan microRNA assay probes (Applied Biosystems), and U6B probe (Applied Biosystems) was used as a reference for normalization. Microarray data are available at GEO under accession number GSE48583.

miRNA microarray and qPCR gene expression analyses

CodeLink Mouse Whole Genome chips (Applied Microarrays) were used for DNA microarray analyses. Total RNAs were reverse transcribed using oligo(dT) primers according to the manufacturer’s instructions (iScript Select cDNA Synthesis Kit, Bio-Rad). cDNA levels were normalized by PCR amplification with primers to beta-actin (5′-GCCCTTCTCTTGGTATGAG-3′ and 5′-ACCCAGACACAGCATGTTG-3′). Primer sequences are listed in supplementary material Table S1.

Histology

Embryos were fixed with 4% paraformaldehyde in PBS for 4 hours or overnight. Following fixation, samples were dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned (7 μm). Standard Hematoxylin and Eosin (H&E) staining was used to examine tissue morphology. µCT was performed on a MicroXCT-200 (Xradia) through the Micro-CT Imaging Facility at UCSF.

Immunohistochemistry and immunofluorescence

Craniofacial tissue sections (7 μm) were used for immunohistochemistry and immunofluorescence. Antigen retrieval was performed by autoclaving in 0.1 M Tris-HCl buffer (pH 9.0) for 5 minutes. Primary antibodies against noggin (Abcam ab16054, 1:300), E-cad (BD Biosciences 70177, 1:500), PITX2 (Capra Sciences PA-1023, 1:500) and Amel (Santa Cruz L0506, 200) were diluted in TBS (0.5% Tween in phosphate-buffered saline) containing 0.1% Triton X-100, 5% goat serum and 1% BSA, incubated overnight at 4°C and detected with a biotinylated goat anti-rabbit IgG conjugate (Vector Labs, 1:200) using the avidin-biotin complex (Vector Labs) and AEC Staining Kit (Sigma) or fluorescent secondary antibody (Invitrogen). The cell immunofluorescence assays used cells fixed with paraformaldehyde. Cells were blocked with 10% goat serum and incubated with E-cad antibody (BD Biosciences, 1:500) overnight at 4°C. FITC-conjugated secondary antibody was used for detection. DAPI was used for counterstaining.

DNA cloning, cell culture, transient transfection, luciferase assay and western blotting

A 288 bp genomic DNA fragment upstream of miR-200c was cloned into the pSilencer4.1 vector (Ambion) using primers 5′-AAGAAGGG-GCTTCAGGTTA-3′ and 5′-GGAAGTGTCCEAAATGACG-3′. The Nog 3′-UTR was cloned downstream of the luciferase gene in the pGL3 vector (Promega) using primers 5′-GCCCGACACTTGAGGAG-3′ and 5′-TCTGTTCGTGACTCTTCTC-3′. The PCR-driven overlap extension method was used to mutate the binding site of miR-200c in the 3′-UTR of Nog. A 2 kb DNA fragment including the Pitx2 binding site upstream of miR-200c was cloned into the pTK-Luc vector using primers 5′-TCTGCTTTCCATGCGCCACGG-3′ and 5′-TGACTG-GATTCGCTTGGGCACGATACTA-3′; this vector construct uses the minimal TK promoter (Amendt et al., 1999). The QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) was used to mutate the Pitx2 binding site from TAATCC to TAATGA 4029 bp upstream of pre-miR-200c. A 1 kb DNA fragment that includes the phospho (p)-Smad1 binding site upstream of miR-200c was cloned into pTK-Luc using primers 5′-TCACTGGACACTCTGCAGCA-3′ and 5′-ATACGT-GATCCAGACACACGGAGAATG-3′. L5-8 oral epithelial-like cells, which are derived from neonatal mouse molar tissue (Chen et al., 1992), were cultured and transfected by electroporation as described (Amey et al., 2007). Cultures were fed for 24 hours prior to transfection, resuspended in PBS and mixed with 2 μg miRNA (p-Silencer), PITX2 and Bmpnr1a (pDNA3.1) expression plasmids, 0.1 μg reporter plasmid (pGL3) and 0.5 μg β-galactosidase plasmid. Transfected cells were incubated for 48 hours in 60 mm culture dishes, lysed and assayed for reporter activities and protein content by the Bradford assay (Bio-Rad).
Luciferase was measured using reagents from Promega. β-galactosidase was measured using Galacto-Light Plus reagents (Tropix). All luciferase activities were normalized to β-galactosidase activity and are shown as mean-fold differences relative to empty luciferase plasmids. DNAs were double CsCl banded for purity and cells were transfected by electroporation. To measure endogenous protein, cell lysates (10 μg) were separated on a 12% SDS-polyacrylamide gel. Following SDS-PAGE, the proteins were transferred to PVDF filters (Millipore), immunoblotted using primary antibody against noggin (Abcam ab16054, 1:500), PITX2 (Capra Sciences PA-1023, 1:500), Bmpr (R&D Systems MAB2299, 1:500), p-Smad1/5/8 (Cell Signaling 9511L, 1:500), Smad1 (Cell Signaling D59D7, 1:500) and beta-tubulin (Santa Cruz L0811, 1:2000). ECL reagents (Amersham) were used for detection.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed as previously described using the ChIP Assay Kit (Upstate Biotechnology) with the following modifications (Amen et al., 2007; Diamond et al., 2006). Unstimulated LS-8 cells were fed for 24 hours, harvested and plated in 60 mm dishes. Cells were cross-linked with 1% formaldehyde for 10 minutes at 37°C. All PCR reactions were performed at an annealing temperature of 60°C. The following primers were used to amplify the promoter region containing the Pitx2 binding site (sense, 5′-AAATGAGTGGCTCTGTTCATGCTC-3′; antisense, 5′-AGTGCGG-AAGAACCGGATGATAC-3′) or the p-Smad1 binding site (sense, 5′-CTGTGGTGCTTGCAGAGTATGCTGC-3′; antisense, 5′-TACCCCAACAGTCCACCTTG-3′). All ChIP PCR products were confirmed by size and sequencing. As controls, the primers were used in PCR experiments without chromatin; normal rabbit IgG replaced the Pitx2 antibody to reveal nonspecific immunoprecipitation of chromatin. Three parallel real-time PCRs were also performed in triplicate using these primers to quantify the enrichment of DNA pulled down by the PITX2 antibody as compared with the DNA pulled down by the IgG control. Primers located 6.7 kb upstream of pre-miR-200c (sense, 5′-ATGTCTGCTGCTTCACACA-3′; antisense, 5′-CTGCTGTGCTTGCAGACG-3′) were used as controls because this fragment does not contain a Pitx2 binding site or p-Smad1 binding site.

**Lentiviral expression constructs and infection**

The pLL3.7 vector (Addgene, 11795) was used to make lentiviral miRNA expression constructs. miR-200c/141 was amplified using primers 5′-TATACTGTCAAGTGCTCGTATGCTGC-3′ and 5′-TTCTGAAATCTCTCCCTCCCTGTGATGATAC-3′. The insert was ligated into pLL3.7 using BsmGI and EcoRI. Second generation lentivirus plasmids (psPAX2 and pMD2.G, Addgene, 12260 and 12259) were used for lentivirus production. Briefly, 293FT cells were transfected with lentivirus plasmids using FuGene HD (Promega), medium collected after 28 hours, centrifuged and filtered to obtain virus. For lentivirus infection, LS-8 cells were subcultured and transferred to 6 cm dishes at 20% confluence. Virus was added immediately after plating and cultured for 2 weeks with medium change every 2-3 days.

**Statistical analysis**

Two-tailed unpaired Student’s t-test was used to determine the difference between two sets of values. Error bars indicate mean ± s.e. All experiments were repeated at least three times.

**RESULTS**

**mir-200c represses noggin in dental epithelial-like cells**

Inactivation of Dicer1 using Pitx2Cre, Wnt1Cre and Krt14Cre has demonstrated that mature miRNAs play central roles in tooth

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**Fig. 1. miRNA and mRNA microarray analyses reveal differentially expressed miRNAs and their putative target mRNAs in the mouse incisor.**

(A) Depiction of the mouse lower incisor showing the location of the labial and lingual cervical loops (LaCL and liCL, respectively; green dashed line) and the ameloblast (Am) region showing areas of presecretory, secretory and mature ameloblasts (red dashed line). En, enamel; De, dentin. These regions were dissected and used to determine mRNA and miRNA expression. (B) Heat map of selected miRNAs differentially expressed between the laCL and Am regions. (C) Using three programs (PicTar, miRBase and TargetScan), mRNAs that were previously identified to be upregulated in the incisor of Pitx2Cre/Dicer1 compared with control mice (Cao et al., 2010b) were analyzed for putative miRNA target sites. Only the miRNAs that were identified in our microarray analysis above were counted and 20 miRNA:mRNA combinations were identified by all three programs. (D) The upregulation of Bmper, Htra1 and Nog in Pitx2Cre/Dicer1 incisors was confirmed by qPCR. Error bars indicate s.e.; experiments were performed in triplicate with two different samples (#, the average of each sample is shown).
development and that these defects are associated with miRNA control of epithelial stem cell proliferation and differentiation (Cao et al., 2010b; Michon et al., 2010). We set out to discover which specific miRNAs are important in these processes by comparing the miRNA expression profiles of the labial CL (laCL) and ameloblast (Am) regions (Fig. 1A) in wild-type mouse incisors using microarray analysis. Numerous miRNAs were identified as differentially expressed between the labial CL and ameloblast regions. Subsets of these were identified as potential miRNAs targeting Bmp signaling antagonists, as discussed below (Fig. 1B).

To identify miRNA target genes important in tooth development, we utilized data generated from a comparison of mRNA expression in the incisors of control and \textit{Pitx2Cre/Dicer1} conditional knockout (cKO) mice (Cao et al., 2010b). These analyses revealed 358 transcripts that were upregulated greater than 2-fold in \textit{Pitx2Cre/Dicer1} cKO incisors. Three separate programs (PicTar, TargetScan, miRBase) were used to predict the presence of miRNA binding elements in these genes, and 20 miRNA:mRNA interactions were predicted to be important in the dental epithelium (Fig. 1C). Three of these upregulated genes encoded Bmp signaling antagonists, namely noggin (\textit{Nog}), \textit{Bmpcr} and \textit{Htra1}, and qPCR confirmed the upregulation (Fig. 1D). We focused on the miR-200c: \textit{Nog} interaction for several reasons: \textit{Nog} expression was increased over 6-fold in \textit{Pitx2Cre/Dicer1} cKO mice; the overexpression of \textit{Nog} has previously been shown to inhibit ameloblast differentiation (Hu et al., 2012; Plikus et al., 2005); and the 3′-UTR of \textit{Nog} was predicted to contain a conserved miR-200c binding site. Because miR-200c was one of the miRNAs shown to be differentially expressed in ameloblasts relative to the labial CL (Fig. 1B), we initiated studies of the miR-200c:noggin pathway.

Noggin was upregulated in P2 \textit{Pitx2Cre/Dicer1} cKO incisors compared with those of control mice (Fig. 2A). The wild-type incisors show low levels of noggin expression. To test whether the predicted miR-200c element in the 3′-UTR of \textit{Nog} was functional (Fig. 2B), we ligated this sequence downstream of the luciferase gene in the pGL3 plasmid and co-transfected this construct with a miR-200c expression plasmid into LS-8 dental epithelial-like cells (Fig. 2C). Luciferase activity was approximately halved with co-transfection of miR-200c compared with empty vector, and, importantly, mutation of the predicted miR-200c binding site in \textit{Nog} abolished this repression (Fig. 2C). Furthermore, transfection of miR-200c decreased endogenous \textit{Nog} expression in LS-8 cells (Fig. 2D).

We also characterized the miR-203: \textit{Bmper} interaction (supplementary material Fig. S1). Similar to miR-200c, miR-203 was differentially expressed in ameloblasts relative to the labial CL (Fig. 1B) and was responsive to \textit{PITX2} (supplementary material Fig. S1A). miR-203 targeted \textit{Bmper} through a conserved sequence in its 3′-UTR, which when mutated resulted in the loss of inhibition by miR-203 (supplementary material Fig. S1B). The inhibition of noggin expression was over 6-fold in \textit{Pitx2Cre/Dicer1} cKO mice compared with controls (Fig. 2A). The wild-type incisors show low levels of noggin expression. To test whether the predicted miR-200c element in the 3′-UTR of noggin (\textit{Nog}) is functional (Fig. 2B), we ligated this sequence downstream of the luciferase gene in the pGL3 plasmid and co-transfected this construct with a miR-200c expression plasmid into LS-8 dental epithelial-like cells (Fig. 2C). Luciferase activity was approximately halved with co-transfection of miR-200c compared with empty vector, and, importantly, mutation of the predicted miR-200c binding site in noggin abolished this repression (Fig. 2C). Furthermore, transfection of miR-200c decreased endogenous noggin expression in LS-8 cells (Fig. 2D).

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Bmp6 by miR-203 was further substantiated by western blot analysis of LS-8 cells transfected with a miR-203 expression plasmid (supplementary material Fig. S1C).

Endogenous Pitx2 binds to the miR-200c 5′ flanking chromatin and activates miR-200c expression

To understand the transcriptional regulation of miR-200c, we screened for potential conserved transcription factor binding sites upstream of pre-miR-200c using ECR Browser (http://ecrbrowser.dcode.org). Notably, miR-200c and miR-141 are adjacent to each other and share the same promoter region on chromosome 6. Although miR-141 was not differentially regulated in this miRNA screen, miR-141 was previously reported to be upregulated in ameloblasts compared with the labial CL, similarly to miR-200c (Jheon et al., 2011). However, miR-141 was expressed at significantly lower levels than miR-200c when tested by qPCR (data not shown). To determine whether PITX2 activates miR-200c transcription in vivo, we compared miRNA expression profiles between control and Krt14-PITX2 overexpression mouse incisors. This analysis revealed the upregulation of miR-200c in Krt14-

Fig. 3. Pitx2 directly binds to and activates expression of the miR-200c/141 cluster. (A) Heat map showing an increase in miR-200c expression from an miRNA microarray experiment comparing wild-type (control) and Krt14-PITX2 overexpression mouse incisors. (B) A Pitx2 binding site (TAATCC) was identified at position −4029 bp of the miR-200c/141 promoter (+1 bp was assigned at the start of pre-miR-200c). Primers (arrows) to amplify the putative Pitx2 binding site region (−4193 to −3949), as well as a control region (−6897 to −6743) for ChIP experiments, are indicated. (C) ChIP analysis in LS-8 cells demonstrated the interaction of endogenous Pitx2 with the putative Pitx2 binding site in the promoter region of miR-200c/141. (D) Control ChIP experiments showed no amplification or enrichment of the 154 bp DNA fragment with anti-PITX2 antibody. IP, immunoprecipitate. (E) A 244 bp DNA fragment was amplified and enriched ~8-fold following ChIP using anti-PITX2 antibody but not IgG. (F) Luciferase assays demonstrate activation of miR-200c/141 by Pitx2. Luciferase plasmids (Luc) containing the wild-type miR-200c promoter region (miR-200c-Luc) or a mutated Pitx2 binding site (Mut-Luc) were co-transfected with empty vector (Vector) or CMV-PITX2 (PITX2A). Four independent experiments (n=4). (G) Levels of endogenous miR-200c were measured by TaqMan qPCR in LS-8 cells transfected with CMV-PITX2. Three independent experiments (n=3); **P<0.01. All error bars indicate s.e.
binding site was mutated from TAATCC to TAATTA (Mut TK-Luc) (Fig. 3F). Furthermore, transfection of PITX2A increased endogenous miR-200c expression in LS-8 cells ~6-fold (Fig. 3G).

miR-200c and Bmp signaling form a regulatory positive-feedback loop

To investigate whether miR-200c regulates Bmp signaling, we constructed a Bmp reporter plasmid containing two Bmp-responsive elements (2×BRE) upstream of the minimal TK promoter and luciferase gene. Co-transfection of miR-200c and the BRE-containing plasmid into LS-8 cells resulted in a ~1.6-fold increase in reporter activity compared with empty vector (supplementary material Fig. S2A). Western blot analysis of miR-200c-transfected cell lysates revealed increased levels of p-Smad1/5/8 protein, a marker of Bmp signaling (supplementary material Fig. S2B). However, pan-Smad1 protein was not affected (supplementary material Fig. S2B). Notably, LS-8 cells express endogenous miR-200c and Pitx2 at low levels (data not shown). These cells are derived from neonatal mouse oral epithelial tissues and express dental epithelial differentiation factors (Chen et al., 1992).

Because Bmp signaling plays a role in dental epithelial cell differentiation, we asked whether Bmp signaling regulates miR-200c expression. Analysis of the 5’ flanking region of miR-200c/141 identified several potential SMAD binding sites (Fig. 4A). ChiP assays using an anti-p-Smad1/5/8 antibody revealed endogenous p-Smad1/5/8 binding to the most proximal SMAD site in the miR-200c promoter (Fig. 4B). As an additional control, we could not immunoprecipitate chromatin upstream of the identified SMAD binding sites in the miR-200c promoter using anti-p-Smad1/5/8 or IgG antisera (Fig. 4C). Endogenous p-Smad1/5/8 binding to this region was enriched ~30-fold, as assessed by qPCR (Fig. 4D). Furthermore, co-transfection of the constitutively activated Bmpr1a expression plasmid and miR-200c TK-Luc plasmids in CHO cells resulted in a ~3.5-fold increase in luciferase activity compared with empty vector (Fig. 4E). Bmpr1a encodes a type I Bmp receptor that is expressed in the dental and palate epithelium and mesenchyme at early stages of development and mediates phosphorylation of Smad1 (Bonilla-Claudio et al., 2012; He et al., 2010). Importantly, in LS-8 cells transfected with miR-200c, qPCR analysis indicated increased Amel expression, a gene known to be responsive to Bmp in the dental epithelium and a marker for differentiated dental epithelial cells (Fig. 4F) (Arakaki et al., 2012; Gluhak-Heinrich et al., 2010).

Pitx2 is highly expressed in the incisor cervical loop stem cell niche

Pitx2 is highly expressed in the CLs, with less expression in ameloblasts (Fig. 5A-F), and miR-200c expression was initiated in the labial CL and highly expressed in pre-ameloblasts (Fig. 1B). Pitx2 is expressed in differentiated ameloblasts at low levels (Hjalt et al., 2000; Mucchielli et al., 1997). These data suggest that, as the transient progenitor cells in the stellate reticulum of the CL intercalate into the enamel epithelium, they express miR-200c, which turns on the differentiation program of E-cad expression, cell adhesion and ameloblast differentiation. Thus, as the progenitor cells migrate to the distal tip of the growing incisor they begin their differentiation and Pitx2 expression decreases, while Bmp signaling coordinates the continued expression of miR-200c.

miR-200c expression increases cell adhesion in LS-8 dental epithelial-like cells

Previous reports on miR-200 family members, and specifically miR-200c/141, describe their role in the regulation of cell-cell adhesion in various cell types (Brabletz et al., 2011; Burk et al., 2012; Gluhak-Heinrich et al., 2010). Importantly, in LS-8 cells transfected with miR-200c, qPCR analysis indicated increased Amel expression, a gene known to be responsive to Bmp in the dental epithelium and a marker for differentiated dental epithelial cells (Fig. 4F) (Arakaki et al., 2012; Gluhak-Heinrich et al., 2010).
E-cad has been used extensively in the analysis of cell adhesion and induction of E-cad expression is tightly associated with epithelial cell sheet formation (Brabletz et al., 2011; Burk et al., 2008; Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008; Wellner et al., 2009). We analyzed whether miR-200c/141 could induce cell-cell adhesion in LS-8 cells. LS-8 cells express E-cad, Pitx2 and miR-200c/141 at low levels (data not shown). We infected LS-8 cells using a lentiviral vector containing miR-200c/141 and observed that expression of miR-200c/141 led to clusters of cells, whereas the untreated and empty vector-treated cells did not form clusters (Fig. 6A-C). E-cad is highly expressed upon overexpression of miR-200c/141 in LS-8 cells (Fig. 6F,L). E-cad expression is also shown by western blot (Fig. 6M). Thus, when dental epithelial-like cells are induced to express E-cad they become more connected, similar to the enamel epithelial cells of the incisor tooth organ.

**miR-200c/141 knockout mice have defects in enamel and in cell adhesion**

To follow up our in vitro data, we generated mice with global inactivation of miR-200c/141 by replacing miR-200c/141 with a promoterless lacZ reporter. β-galactosidase staining showed that expression of miR-200c/141 led to clusters of cells, whereas the untreated and empty vector-treated cells did not form clusters (Fig. 6A-C). E-cad is highly expressed upon overexpression of miR-200c/141 in LS-8 cells (Fig. 6F,L). E-cad expression is also shown by western blot (Fig. 6M). Thus, when dental epithelial-like cells are induced to express E-cad they become more connected, similar to the enamel epithelial cells of the incisor tooth organ.

To follow up our in vitro data, we generated mice with global inactivation of miR-200c/141 by replacing miR-200c/141 with a promoterless lacZ reporter. β-galactosidase staining showed that expression of miR-200c/141 led to clusters of cells, whereas the untreated and empty vector-treated cells did not form clusters (Fig. 6A-C). E-cad is highly expressed upon overexpression of miR-200c/141 in LS-8 cells (Fig. 6F,L). E-cad expression is also shown by western blot (Fig. 6M). Thus, when dental epithelial-like cells are induced to express E-cad they become more connected, similar to the enamel epithelial cells of the incisor tooth organ.

**DISCUSSION**

Tooth development requires ectoderm-derived dental epithelial cells and neural crest-derived mesenchymal cells. Although the cellular and molecular pathways involved in tooth development are well characterized, and tooth renewal shares many of these same molecular pathways, the roles of specific miRNAs in these processes are unclear. The inactivation of mature miRNAs in the dental epithelium results in abnormal tooth development and stem cell differentiation and revealed a role for epithelial miRNAs in tooth development and renewal (Cao et al., 2010b; Michon et al.,
miR-200c regulates noggin

![Diagram of miR-200c regulation](image)

However, it is as yet unclear which specific miRNAs and target genes are important for these processes. Our in vitro and in vivo studies demonstrate that a Pitx2:miR-200c/141:noggin regulatory pathway is essential for tooth development and renewal. Pitx2, which is the earliest transcriptional marker in tooth development, is highly expressed in the labial CL, with decreased expression in ameloblasts, and induces a transcriptional program that involves miR-200c. Pitx2 activates miR-200c through the Pitx2 binding site in the promoter region of miR-200c/141. miR-200c represses translation of Nog through a conserved sequence in its 3’-UTR, thereby increasing Bmp signaling. Bmp signaling is also increased with repression of Bmpa by miR-203. Furthermore, miR-200c overexpression upregulated the expression of E-cad and Amel. Bmps induce high levels of expression of the enamel matrix protein ameloblastin in the dental epithelium (Arakaki et al., 2012; Gluhak-Heinrich et al., 2010); noggin inhibits Bmp signaling and strongly downregulates endogenous ameloblastin expression (Wang et al., 2004); and Krt14-Nog transgenic mice show repression of ameloblast differentiation and increased epithelial proliferation in both the labial and lingual CL (Wang et al., 2007). Noggin levels were increased in Pitx2<sup>C<sup>Cr</sup></sup>/?Dicer1 cKO mouse incisors, confirming the role of miRNAs, and specifically miR-200c, in its regulation. In addition to regulation by Pitx2, miR-200c is also regulated by Bmp signaling, indicating a positive-feedback loop between miR-200c and Bmp signaling.

Interestingly, Bmp signaling promotes the early stage of somatic cell reprogramming by inducing a mesenchyme-to-epithelial transition (MET) (Samavarchi-Tehrani et al., 2010). Bmp signaling induces a program of miRNA expression, which includes the miR-200 family and miR-205, that is associated with MET (Samavarchi-Tehrani et al., 2010). The increase in miR-200 family expression is also associated with embryonic stem cell (ESC) differentiation and, conversely, Zeb1 is expressed in normal undifferentiated ESCs and cancer cells (Bar et al., 2008; Ben-Porath et al., 2008; Wellner et al., 2009). Increased miR-200c/141 expression results in the repression of noggin, which increases Bmp signaling, and we suggest that this contributes to the transition of dental stem cells to differentiated epithelial cells.

Comparison of Pitx2<sup>C<sup>Cr</sup></sup>/?Dicer1 cKO mouse incisors with those of the miR-200c/141 knockout mouse reveals similar defects in dental epithelial cell differentiation (Cao et al., 2010b). In both mouse models the labial CL has expanded, presumably owing to increased progenitor cell proliferation, with a concomitant decrease in epithelial cell differentiation as demonstrated by the reduction in dental epithelial differentiation markers. Interestingly, the incisor phenotype observed in the global knockout of all mature miRNAs...
in the Pitx2Cre/Dicer1 cKO is similar to that of the miR-200c/141 knockout, suggesting that the miR-200 family plays a major role in incisor development and renewal. A major difference between the two mice is the expansion of the stem cells and production of multiple CL regions giving rise to multiple and branched incisors in the Pitx2Cre/Dicer1 cKO (Cao et al., 2010b).

Previous reports on miR-200 family members, and specifically miR-200c/141, describe their role in the regulation of cell-cell adhesion in various cell types (Brabletz et al., 2011; Burk et al., 2008; Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008; Wellner et al., 2009). Zeb1 and Zeb2 are transcriptional repressors of E-cad and targeting of these genes by the miR-200 family increases E-cad expression in epithelial cells. Interestingly, LS-8 dental epithelial cells formed adherent colonies upon overexpression of miR-200c/141, which correlated with an increase in E-cad levels. The regulation of E-cad by miR-200c is likely to be required for the adhesion of ameloblasts to the stratum intermediate (SI), which is an integral interaction for tooth development (Jheon et al., 2011). This is supported by studies in mice harboring a conditional inactivation of E-cad, which showed a compromise in the ameloblast-SI interface (Li et al., 2012). However, it is also likely that miR-200c plays a role in stem cell adhesion in the labial CL because conditional inactivation of E-cad also led to defects in attachment of the SR cells to the outer enamel epithelium (OEE), along with migration defects (Li et al., 2012). The labial CL consists of the IEE and OEE, as well as SR cells, which although epithelial-derived exhibit a mesenchyme-like morphology and are housed between the IEE and OEE (Harada et al., 1999; Tummers and Thesleff, 2003; Tummers and Thesleff, 2008). Notably, LS-8 cells were isolated from the oral/enamel epithelium of embryonic mouse teeth (Chen et al., 1992; Xu et al., 2006), and their lack of a dental epithelial phenotype in culture, along with the low expression of Amel and the absence of expression of many ameloblast-specific genes such as ameloblastin, enamelin and tuftelin (data not shown), suggests a predominantly SR-like cell population. These cells might not be fully differentiated as they lack a true dental epithelial

Fig. 7. miR-200c/141 knockout mice exhibit tooth and bone defects. (A,B) μCT analysis of the hemi-mandible from control (wild-type) and miR-200c/141 knockout (KO) mice show that enamel mineralization occurs further distally (white arrowheads) and that eruption of the third molar is compromised (red arrowhead) in the KO (n=3). (A’-B’). Coronal sections at the region of the first molar (red dotted lines in A,B) show an enamel defect (white arrowheads), a dentin defect (blue arrowheads), a decrease in alveolar bone (yellow arrowheads) and that the angle of the molar is shifted (green line) with the crown tipped towards the tongue in KO mice. (C-J) H&E staining at various stages of amelogenesis in the lower incisor. Cells in the labial CL (LaCL) appear detached; in part, in the KO mice (D) relative to controls (C); the yellow arrowhead indicates an IEE/SR detachment and the red arrowhead indicates SR detachment. At the secretory stage, a thinner layer of enamel matrix is secreted in KO mice (F) compared with controls (E) and the single row of ameloblasts is disrupted (H, double asterisks) compared with controls (G). The enamel matrix has detached from the ameloblasts due to processing (G, asterisk). At the mature stage, there is some retention of enamel matrix (J, arrowhead) in KO mice compared with controls (I). n=7. (K-P) Immunofluorescence staining for E-cad (K-L), Amel (M-N) and noggin (O-P) in the laCL and presecretory/secretory stages (white dashed lines). Lower levels of E-cad and Amel (white arrowheads) and higher levels of noggin are detected in KO mice compared with controls. Am, ameloblasts; En, enamel; SI, stratum intermedium; SR, stellate reticulum. Scale bars: 1 mm in A-B; 50 μm in C-J; 20 μm in K-P.
We have identified a Pitx2:miR-200c:noggin pathway in the regulation of dental stem cell differentiation. Pitx2 induces a transcriptional program involving miR-200c, which directly targets and inhibits noggin expression (Fig. 8B). Decreased noggin leads to increased Bmp signaling activity and epithelial cell differentiation. Notably, the increased Bmp activity feeds back to activate miR-200c/141 to maintain the differentiation pathway. The Pitx2:miR-200c:noggin regulatory pathway activates E-cad expression and promotes adhesion of the SR cells (Fig. 8B).

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Competing interests statement
The authors declare no competing financial interests.

Author contributions

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.089193/-/DC1

References


of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 9, 582-589.


**Fig. S1. Pitx2 endogenous activation of miR-203 and inhibition of Bmper by miR-203.** (A) miR-203 expression in multiple samples from control (wild-type) and Krt14-PITX2 overexpression mouse incisors. miR-203 is upregulated in the PITX2 overexpression mice. (B) The target sequence of miR-203 in the 3'UTR of Bmper and the mutated Bmper 3'UTR is shown. Normalized luciferase activity of the 3'UTR Bmper-luciferase reporter (WT Bmper 3'UTR) with empty plasmid (Vector) or CMV-miR-203 (miR-203) shows loss of luciferase activity with expression of miR-203. There is no loss of luciferase activity when the miR-203 seed sequence is mutated (Mut Bmper 3'UTR). Error bars indicate ± s.e.; five independent experiments (n=5); **P<0.01. (C) Western blot analysis shows a decrease in Bmper levels when miR-203 is overexpressed in LS-8 cells.

**Fig. S2. miR-200c activates Bmp signaling and p-Smad1/5/8.** (A) Luciferase activity of the Bmp reporter (2×BRE-Luc) co-transfected with empty vector (Vector) or CMV-miR-200c (miR-200c) in LS-8 cells. Error bars indicate ± s.e.; five independent experiments (n=5); **P<0.005. (B) Western blot analysis showing levels of phospho (p)-Smad1/5/8 upon 48-hour expression of miR-200c and pan-Smad1 in LS-8 cells.
Fig. S3. Expression of miR-200c and deletion of miR-200c/141 in mutant mice. (A,B) Localization of miR-200c by beta-galactosidase (beta-Gal) staining in the labial and lingual CL (laCL and liCL) in the ameloblasts (Am) and stratum intermedium (SI). (C) Confirmation of the loss of miR-200c expression in knockout mice by PCR. (D-F) Lower magnification of beta-Gal staining confirms specific expression in the LaCL, LiCL and ameloblasts. Error bars indicate ±s.e., three independent experiments (n=5); **P<0.01.

Table S1. RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Nog</td>
<td>CGGCCAGCACTATCTACACA</td>
<td>GCGTCTCGTTCAAGATCCTTC</td>
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<tr>
<td>Bmper</td>
<td>ATCAAAGTGCACTGGGAACC</td>
<td>AGGACAGAGGACTGGCTTGA</td>
</tr>
<tr>
<td>Htra1</td>
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<tr>
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<td>TCTGGAGTCTGGGCTAGGAA</td>
</tr>
<tr>
<td>Amel</td>
<td>TTTTGCTATGCCCTACCAC</td>
<td>GTATGAGGCTGAAGGTTGT</td>
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