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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 Bmper 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, Notch 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 noggin 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 noggin 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 RNase 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 noggin, which is a Bmp antagonist, is a direct target of miR-200c. Upstream regulation of miR-200c includes interaction

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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 Bmper and is activated by endogenous Pitx2. Bmper acts similarly to noggin through the endocytosis of Bmp4 and inhibition of Bmp4 signaling (Kelley et al., 2009). We further report that Bmper is expressed in the dental epithelium during tooth development, adding to the tissue-specific activity of Bmper. 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 *Tbx1* 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 the dental stem cells in the labial CL, such as *Lgr5*, *Abcg2*, *Bmi1*, *Oct3/4* (*Pou5f1* – Mouse Genome Informatics) and *Yap1*; however, their involvement in stem cell maintenance and proliferation is unknown (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 procedure 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., 2012). 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, β-actin-driven neomycin selection marker with a poly(A) signal, and the miR-200c/141 stem-loop flanked by loxP sites into the *miR-200c/141* locus. Germline-transmitted mice (lacZ-neo-flox) 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 $Pitx2^{Cre}$;$Dicer1^{loxP/loxP}$ ($Pitx2^{Cre}$/*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 observed by μCT in all three of the specimens that we scanned.

miRNA microarrays and qPCR

mRNA microarray analysis comparing gene expression in the incisor of postnatal day (P) 0 control and $Pitx2^{Cre}$/*Dicer1* has been described (Cao et al., 2010b). For miRNA comparison between the labial CL cells 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.

mRNA 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′-GCCTTCCTTC-TTGGGTATG-3′ and 5′-ACCACCAGACAGCACTGTG-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, 1:200) 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-GCTTCCAGGTTA-3′ and 5′-GGAAGTGTCCCAAATGACG-3′. The *Nog* 3′-UTR was cloned downstream of the luciferase gene in the pGL3 vector (Promega) using primers 5′- GCCCAGACACTTGATGGAT-3′ and 5′-TCCTGTTCTGCACTTCTTCCT-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′-TCAGTGGATCCTCTTGCTATGCACGTTTTCG-3′ and 5′-TGACTG-GATCCGCTTGCTTGCACGATAATCA-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 TAATTA 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′-TCATAGGATCCCCTATGGCAGGAGGACACAC-3′ and 5′-ATACTG-GATCCAGACAGACCACCGAATGGAC-3′.

LS-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 (Amen 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 Bmpr1a (pcDNA3.1) 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), Bmper (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 *miR-200c* promoter region containing the Pitx2 binding site (sense, 5′-AAATGATGGCTGTCCTCGTC-3′; antisense, 5′-AGTGGGAGAAGCCCAGGTAT-3′) or the p-Smad1 binding site (sense, 5′-CTGGTTTTGGCCTCAGTGAT-3′; antisense, 5′-TACCCAACCAGTCCACCTTC-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′-ATGTCTGCTGCTTCCACACA-3′; antisense, 5′-CTGCTGTGTCATGCTCCCTA-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′-TATACTGTACAAGTAGCTCAGTGATGGGCAAGTCAG-3′ and 5′-TTCTGGAATTCTCCCCTTTGCCAAGTGATAC-3′. The insert was ligated into pLL3.7 using *Bsr*GI and *Eco*RI. 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 *Pitx2*^{Cre}, *Wnt1*^{Cre} and *Krt14*^{Cre} has demonstrated that mature miRNAs play central roles in tooth

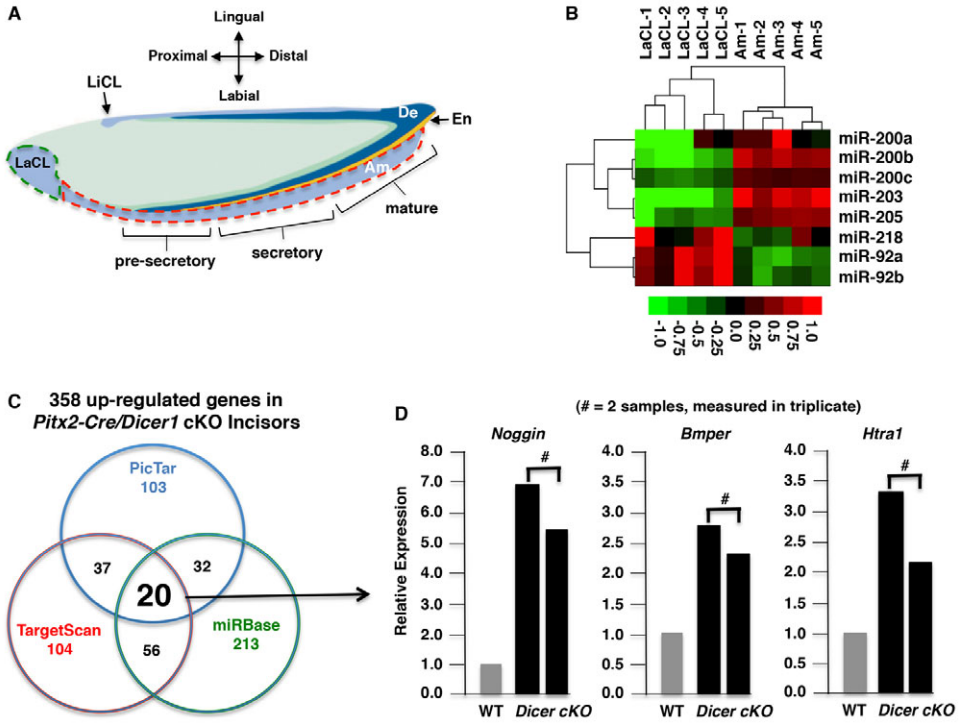

Fig. 1. miRNA and mRNA microarray analyses reveal differentially expressed miRNAs and their putative target mRNAs in the mouse incisor. (**A**) Depiction of the mouse lower incisor showing the location of the labial and lingual cervical loops (laCL and liCL, respectively; green dashed line) and the ameloblast (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 *Pitx2*^{Cre}*/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 *Pitx2*^{Cre}*/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 *Pitx2^{Cre}/Dicer1* conditional knockout (cKO) mice (Cao et al., 2010b). These analyses revealed 358 transcripts that were upregulated greater than 2-fold in *Pitx2^{Cre}/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 (*Nog*), *Bmper* and *Htra1*, and qPCR confirmed the upregulation (Fig. 1D). We focused on the miR-200c:*Nog* interaction for several reasons: *Nog* expression was increased over 6-fold in *Pitx2^{Cre}/Dicer1* cKO mice; the overexpression of *Nog* has previously been shown to inhibit ameloblast differentiation (Hu et al., 2012; Plikus et al., 2005); and the 3′-UTR of *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 *Pitx2^{Cre}/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 *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 *Nog* abolished this repression (Fig. 2C). Furthermore, transfection of miR-200c decreased endogenous *Nog* expression in LS-8 cells (Fig. 2D).

We also characterized the miR-203:*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 *PITX2* (supplementary material Fig. S1A). miR-203 targeted *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

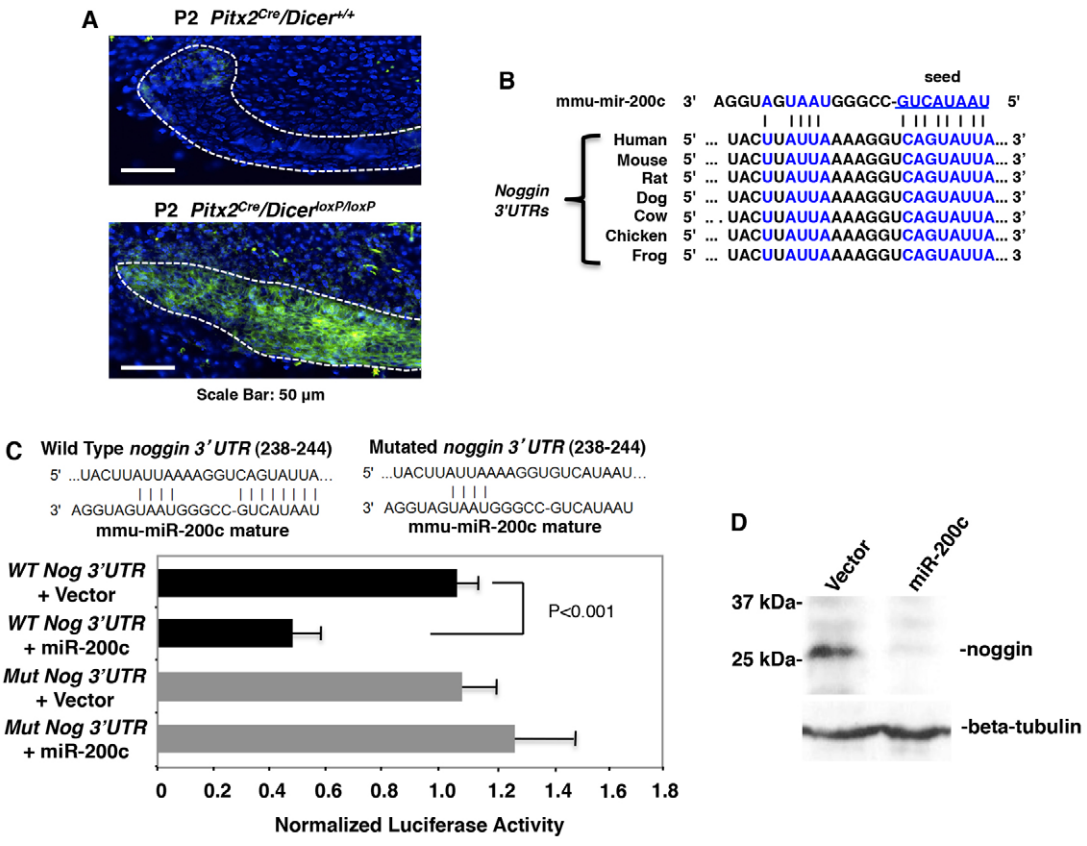

Fig. 2. Characterization of the miR-200c:noggin regulatory pathway. (**A**) Noggin (green) was present at higher levels in the labial CL (outlined) of *Pitx2^{Cre}/Dicer1* (bottom) and *miR-200c/141* null (not shown) incisors than in controls (top) at P2. (**B**) The target sequence of miR-200c in the 3′-UTR of *Nog* is highly conserved in vertebrates, with 100% conservation of the seed sequence (underlined). (**C**) Normalized luciferase activity of the 3′-UTR *Nog*-luciferase reporter (WT *Nog 3′UTR*) with empty plasmid (Vector) or CMV-miR-200c (miR-200c) shows loss of luciferase activity with expression of miR-200c. There is no loss of luciferase activity when the miR-200c seed sequence is mutated (Mut *Nog 3′UTR*). Error bars indicate s.e.; five independent experiments (n=5). (**D**) Western blot analysis shows a decrease in noggin levels when miR-200c is overexpressed in LS-8 oral epithelial-like cells. Beta-tubulin provided a loading control.

Bmper 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-PITX2* incisors (Fig. 3A). A conserved Pitx2 binding site was identified ~4 kb upstream of pri-miR-200c (Fig. 3B).

The importance of Pitx2 during craniofacial and tooth development has been well documented (Lu et al., 1999; Venugopalan et al., 2011), and Pitx2 is localized to the dental epithelium (Hjalt et al., 2000). To test the interaction of Pitx2 with this putative binding site, we performed chromatin immunoprecipitation (ChIP) assays in LS-8 cells, which express Pitx2 (Green et al., 2001). Endogenous Pitx2 bound to the *miR-200c/141* promoter, whereas anti-IgG antisera did not immunoprecipitate this chromatin region (Fig. 3C). As a further control, sequence upstream of the Pitx2 binding site, using either anti-PITX2 or IgG antisera in the ChIP assay, was not amplified (Fig. 3D). ChIP using anti-PITX2 antibody showed an ~8-fold enrichment of chromatin containing the putative Pitx2 binding site (Fig. 3E).

To confirm that Pitx2 regulates miR-200c expression through this Pitx2 binding site, we ligated a 2 kb gene fragment spanning the identified Pitx2 binding element into the pTK-Luc vector upstream of the minimal *TK* promoter (TK-Luc). Co-transfection of the reporter and *PITX2A* expression plasmids showed a ~6-fold increase in luciferase activity compared with co-transfection with empty vector (Fig. 3F). The activation was impaired when the Pitx2

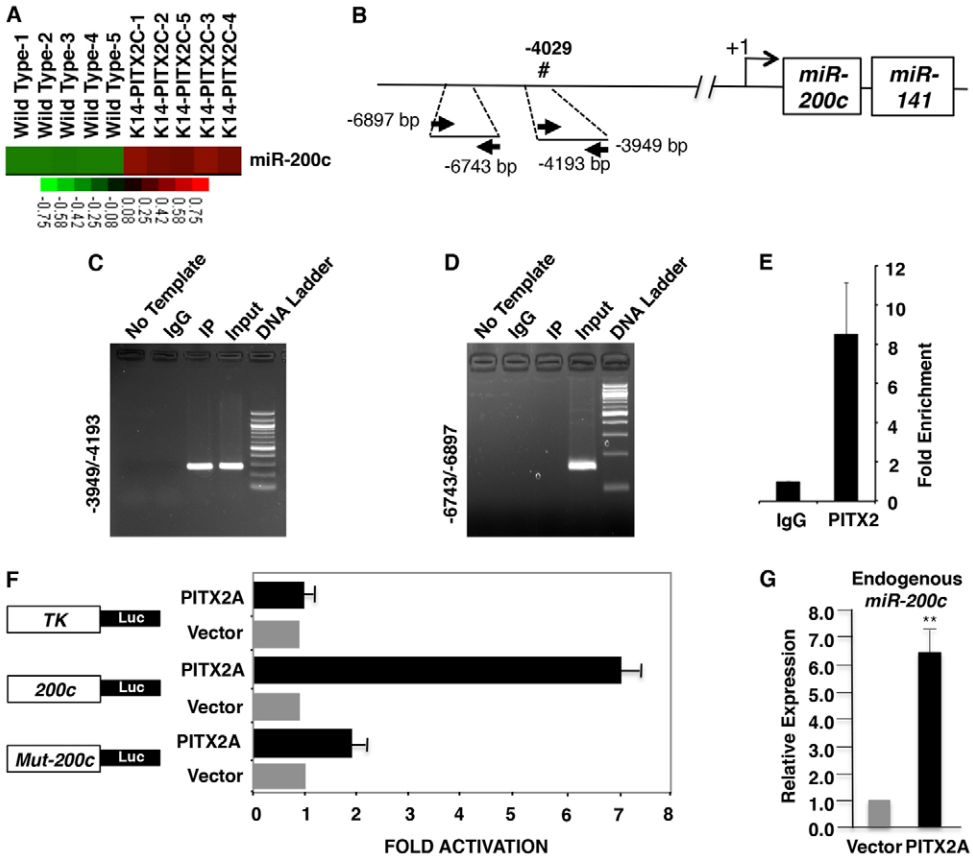

Fig. 3. Pitx2 directly binds to and activates expression of the *miR-200c/141* cluster. (**A**) Heat map showing an increase in miR-200c expression from an miRNA microarray experiment comparing wild-type (control) and *Krt14-PITX2* overexpression mouse incisors. (**B**) A Pitx2 binding site (TAATCC) 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 ameloblast cells 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.,

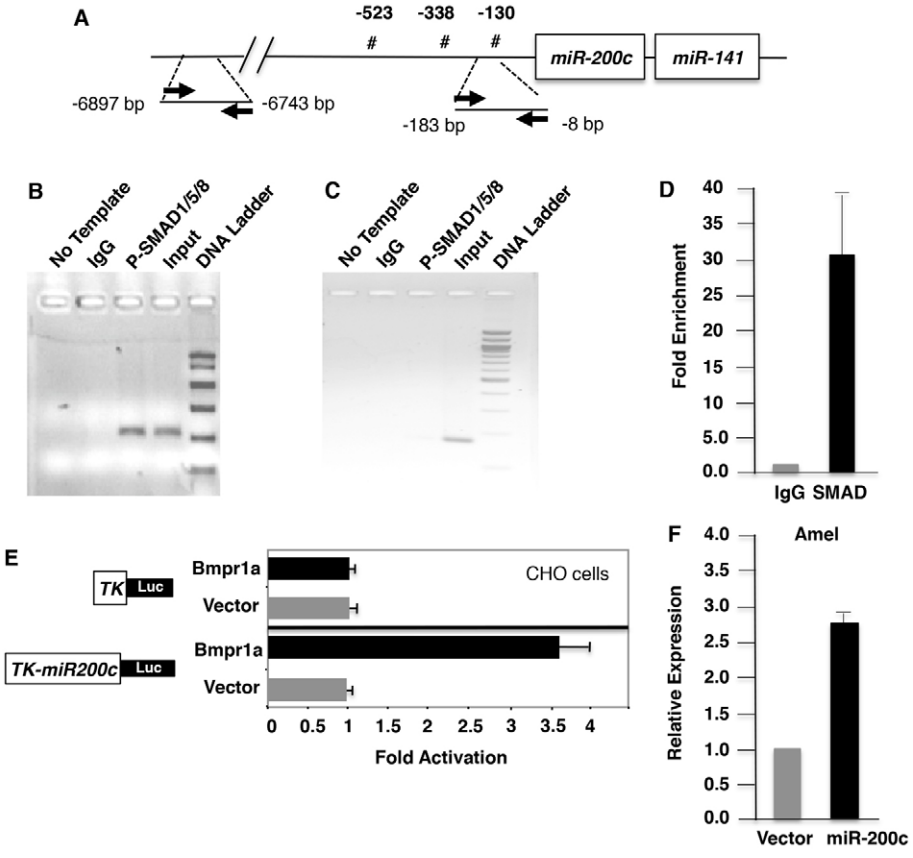

Fig. 4. Endogenous p-Smad1/5/8 binds to the *miR-200c* promoter and Bmp signaling activates the *miR-200c* promoter. (**A**) Several putative SMAD binding sites were identified in the *miR-200c/141* promoter region at positions −523, −338 and −130 bp (relative to the transcription start site of pre-miR-200c/141). Primers (arrows) to amplify the most proximal SMAD binding site region (−8 to −183), as well as a control region (−6897 to −6743) for ChIP experiments, are indicated. (**B**) ChIP analysis in LS-8 cells demonstrated the interaction of endogenous Smad1/5/8 with the SMAD binding site in the promoter region of *miR-200c/141*. (**C**) Control ChIP experiments showed no amplification or enrichment of the 154 bp DNA fragment with anti-Smad1/5/8 antibody. (**D**) A 175 bp DNA fragment was amplified and enriched ~30-fold following ChIP using anti-Smad1/5/8 antibody but not IgG. (**E**) Luciferase reporter assays in transfected CHO cells. The *miR-200c/141* promoter is activated when co-transfected with constitutively active Bmpr1a. (**F**) qPCR analysis of amelogenin (*Amel*) in LS-8 cells transfected with miR-200c. All error bars indicate s.e.

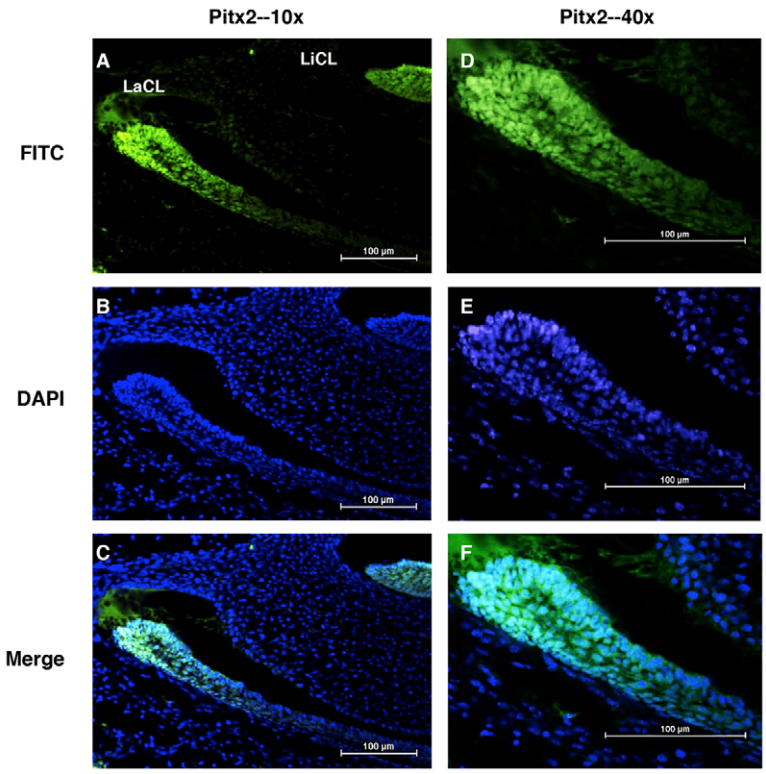

Fig. 5. Pitx2 expression in the E16.5 mouse incisor. (**A-C**) Pitx2 staining of embryonic day (E) 16.5 mouse incisor showing increased Pitx2 expression (green) in the labial cervical loop (LaCL) and lingual cervical loop (LiCL), which contain dental stem cells. DAPI staining (blue) was used to visualize the nuclei and provide contrast. (**D-F**) Higher magnification of A-C. Original magnifications are indicated.

2008; Gregory et al., 2008; Korpal et al., 2008; Park et al., 2008; Wellner et al., 2009). 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 miR-200c/141 was expressed in the labial and lingual CLs and ameloblasts (supplementary material Fig. S3A,B,D-F). Deletion of the miR-200c/141 transcripts was confirmed by real-time PCR (supplementary material Fig. S3C). Microcomputed tomography (μCT) analysis of *miR-200c/141* knockout mice revealed a defect in enamel mineralization, which occurred further distally in the lower incisor, and we also identified an eruption defect of the third molar (Fig. 7A,B, white arrowheads). The mutant incisors appeared smaller, with a defect in the surrounding bone. A coronal section through the region of the first molar confirmed the incisor enamel defect (Fig. 7A′,B′, white arrowheads); in addition, the dentin layer was thinner (blue arrowheads), the surrounding bone was less dense (yellow arrowheads) and the crown of the first molar was tipped towards the tongue (Fig. 7A′,B′).

Histological analysis of *miR-200c/141* mutant incisors at P1 revealed disorganized and poorly polarized cells in the labial CL, and the cells appeared to be detached from each other (Fig. 7C,D). We consistently found detachment of the inner enamel epithelium/stellate reticulum (IEE/SR) cells and detachment of the SR cells in the labial CL (Fig. 7C,D). Loss of *miR-200c/141* resulted in a decreased and defective enamel matrix, correlating with an abnormal ameloblast layer at the secretory stage (Fig. 7E-H). Furthermore, a presumptive matrix resorption defect was identified at the mature stage (Fig. 7I,J). These data suggest a defect in dental stem cell differentiation correlating with abnormal and defective enamel formation. The histological data were supported by immunofluorescence staining for E-cad, Amel and noggin, which showed the downregulation of E-cad in the labial CL and presecretory/secretory stages, downregulation of Amel in the presecretory/secretory stages, and the upregulation of noggin in the labial CL of the mutant mice compared with controls (Fig. 7K-P). These data are consistent with the finding of increased Amel (Fig. 4F) and E-cad expression in LS-8 cells overexpressing miR-200c (Fig. 6).

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.,

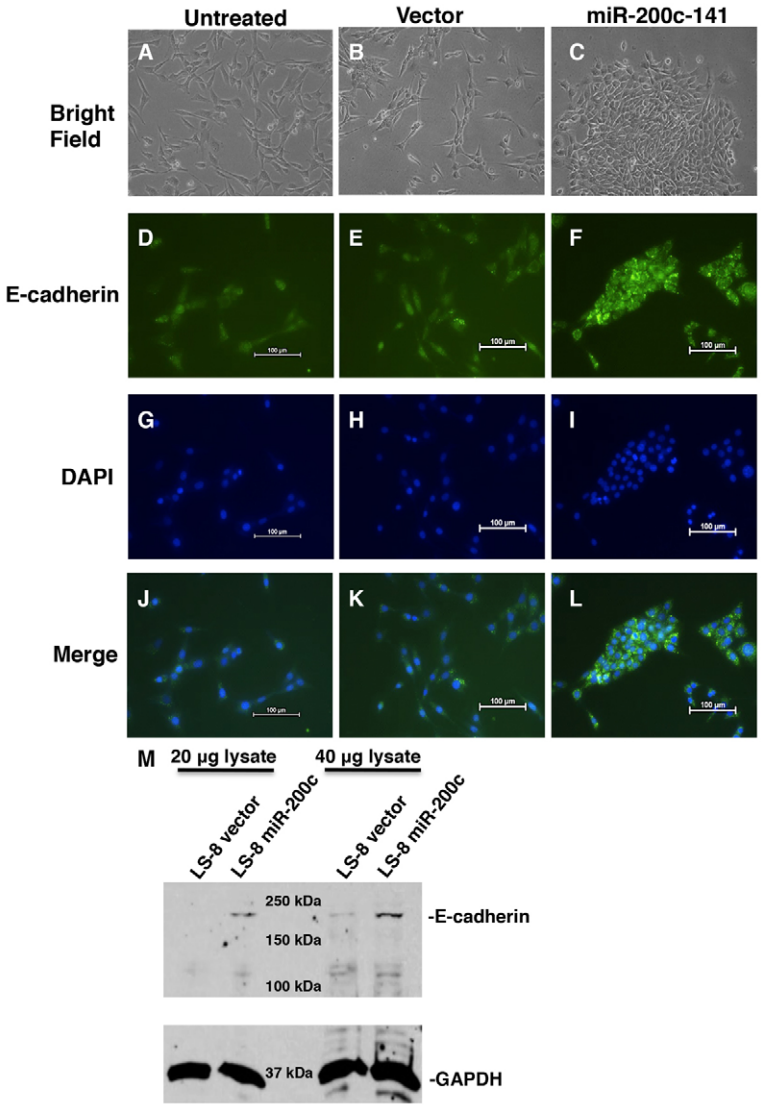

Fig. 6. miR-200c/141 overexpression in LS-8 cells induces E-cad expression and cell-cell adhesion. (**A-C**) Morphology of LS-8 cells that were left untreated (A), transduced with empty vector (B), or transduced with lentiviral miR-200c/141 (C). (**D-F**) Immunofluorescence staining shows increased levels of E-cad and cell-cell adherence with miR-200c/141 expression (F). (**G-I**) DAPI staining of LS-8 nuclei. (**J-L**) Overlay of E-cad and DAPI stains. (**M**) Western blot of E-cad expression in LS-8 cells transduced with empty vector or lentiviral miR-200c/141, shown at two concentrations of cell lysate. Gapdh provided a loading control.

2010). However, it is as yet unclear which specific miRNAs and target genes are important for these processes. Our *in vivo* and *in vitro* 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 *Bmper* 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*^{Cre}*/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*^{Cre}*/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

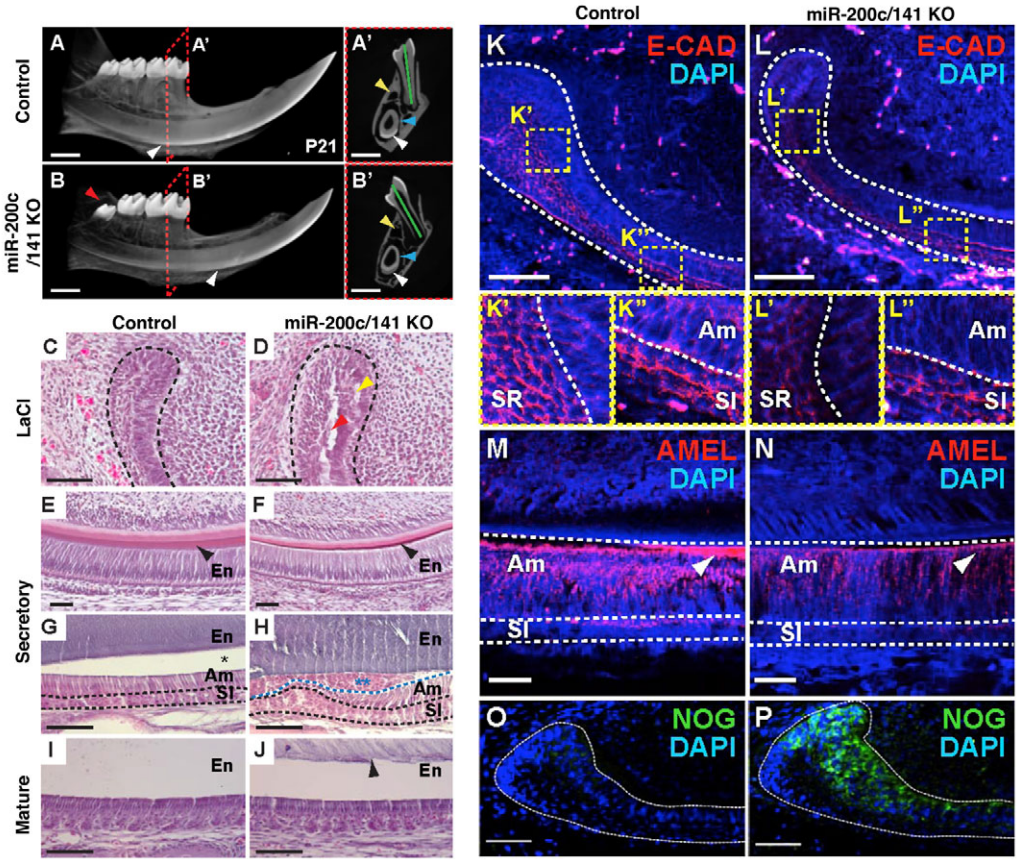

Fig. 7. *miR-200c/141* knockout mice exhibit tooth and bone defects. (**A**,**B**) µCT analysis of the hemi-mandible from control (wild-type) and *miR-200c/141* knockout (KO) mice show that enamel mineralization occurs further distally (white arrowheads) and that eruption of the third molar is compromised (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.

in the *Pitx2*^{Cre}*/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 *Pitx2*^{Cre}*/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 intermedium (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 (Fig. 8A) (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

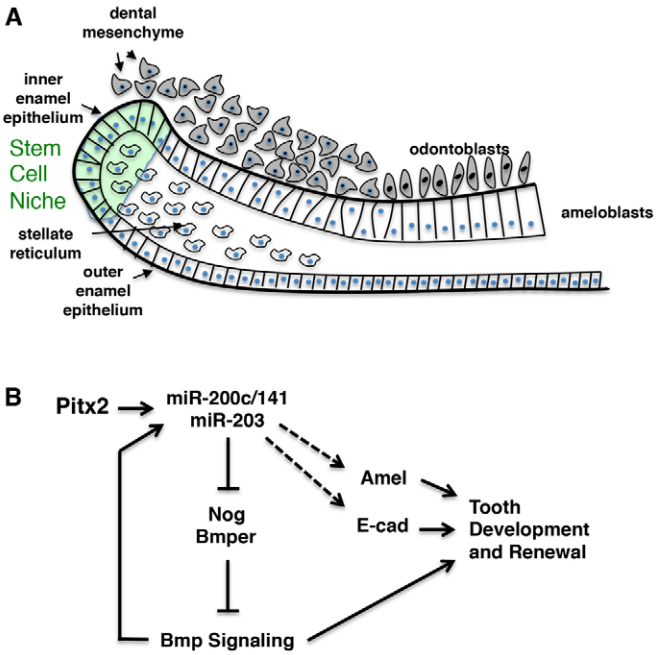

Fig. 8. A model for the role of miR-200c/141 in the adhesion of stem cells in the stellate reticulum to the outer enamel epithelium. (**A**) Depiction of the incisor labial cervical loop and associated cells. Putative epithelial dental stem cells are located in the stellate reticulum and the outer enamel epithelium (indicated in green). (**B**) Model of the Pitx2:miR-200c/141:noggin regulatory pathway in the regulation of tooth development and renewal. Pitx2 directly activates miR-200c/141 and miR-203, which directly inhibit noggin and Bmper, which inhibit Bmp signaling. Bmp feeds back to activate miR-200c/141 and miR-203 expression. miR-200c/141 and miR-203 indirectly regulate amelogenin (Amel) and E-cadherin (E-cad) expression. These factors regulate tooth development and renewal.

signature (low Amel, Pitx2, Tbx1 and E-cad and miR-200 family expression). These cells are converted to epithelial sheets by overexpressing miR-200c, which is highly expressed in epithelial cells such as MDCK cells. Thus, they might behave similarly to the SR cells in transitioning to polarized epithelial cells through the expression of miR-200c. We are currently working to establish a miRNA-regulated program to generate dental epithelial cells from other types of cells. Although it is clear that enamel epithelial stem cells are housed in the labial CL, it is as yet unknown whether these cells reside in the SR, in the OEE, or in both regions (Harada et al., 1999; Ohshima et al., 2005; Parsa et al., 2010; Seidel et al., 2010; Tummers and Thesleff, 2003; Tummers and Thesleff, 2008). One hypothesis is that miR-200c in the labial CL would increase Bmp and *E-cad* expression in the putative stem cells of the SR, induce cell adhesion and invasion into the OEE, which in turn differentiates to the IEE and ultimately ameloblasts (Fig. 8A) (Harada et al., 1999; Tummers and Thesleff, 2003). In *miR-200c/141* knockout mice, the expression of *E-cad* is reduced, which would lead to delayed migration of the IEE and ultimately to a delay in enamel formation. However, the function of miR-200c might not be limited to the labial CL. Although the effects observed at various ameloblast stages with the lack of miR-200c might be an indirect consequence of initial labial CL defects, it is clear that miR-200c directly affects dental epithelial cell differentiation. Further experiments will be required to better understand the functions of miR-200c in the labial CL and ameloblasts.

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
H.C., A.J., X.l., Z.S., J.W., S.F. and Z.Z. performed experiments, analyzed data and made figures; H.C., A.J., O.D.K. and B.A.A. designed experiments and wrote the manuscript; M.T.M., O.D.K. and B.A.A. provided reagents, mice and technical support; H.C., A.J., O.D.K. and B.A.A. designed protocols and analyzed data.

Supplementary material
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