

A Pituitary Homeobox 2 (Pitx2):*microRNA-200a-3p*: β -catenin Pathway Converts Mesenchymal Cells to Amelogenin-expressing Dental Epithelial Cells*

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Background: An efficient method is required for converting somatic cells to specific cell fates.

Results: A combination of Pitx2 and *miR-200a-3p* promotes dental epithelial gene expression in cells.

Conclusion: A two-step method efficiently converts mesenchymal cells to dental epithelial cells.

Significance: A new method to generate dental epithelial cells, which are difficult to isolate, is identified for use in regeneration and repair.

Pitx2, Wnt/ β -catenin signaling, and microRNAs (miRs) play a critical role in the regulation of dental stem cells during embryonic development. In this report, we have identified a Pitx2: β -catenin regulatory pathway involved in epithelial cell differentiation and conversion of mesenchymal cells to amelogenin expressing epithelial cells via *miR-200a*. Pitx2 and β -catenin are expressed in the labial incisor cervical loop or epithelial stem cell niche, with decreased expression in the differentiating ameloblast cells of the mouse lower incisor. Bioinformatics analyses reveal that *miR-200a-3p* expression is activated in the pre-ameloblast cells to enhance epithelial cell differentiation. We demonstrate that Pitx2 activates *miR-200a-3p* expression and *miR-200a-3p* reciprocally represses Pitx2 and β -catenin expression. Pitx2 and β -catenin interact to synergistically activate gene expression during odontogenesis and *miR-200a-3p* attenuates their expression and directs differentiation. To understand how this mechanism controls cell differentiation and cell fate, oral epithelial and odontoblast mesenchymal cells were reprogrammed by a two-step induction method using Pitx2 and *miR-200a-3p*. Conversion to amelogenin expressing dental epithelial cells involved an up-regulation of the stem cell marker *Sox2* and proliferation genes and decreased expression of mesenchymal markers. E-cadherin expression was increased as well as ameloblast specific factors. The combination of Pitx2, a regulator of dental stem cells and *miR-200a* converts mesenchymal cells to a fully differentiated dental epithelial cell type. This pathway and reprogramming can be used to reprogram mesenchymal or oral epithelial cells to dental epithelial (ameloblast) cells, which can be used in tissue repair and regeneration studies.

The epithelial stem cells that control growth of the rodent incisor are located in the cervical loop (a stem cell niche) at the posterior end of the incisor (1). The cervical loop consists of the inner enamel epithelium, outer enamel epithelium, and stellate reticulum cells in the core of the cervical loop and a thin layer of stratum intermedium cells (2, 3). The stem cells in the core of the cervical loop will divide and insert into the basal layer of epithelium, the outer enamel epithelium. They will proliferate as transit amplifying cells and then differentiate to ameloblast cells, which secrete enamel. The labial epithelial stem cells differentiate to ameloblasts, whereas odontoblasts originate from mesenchymal stem cells and secrete dentin on both sides of incisor. A signaling network has been proposed to regulate epithelial stem cell proliferation in the cervical loop stem cell niche (4). These signals originate from mesenchymal tissue derived from the neural crest cells to regulate epithelial stem cells in concert with epithelial signals.

The signaling molecules include fibroblast growth factor (FGF)8, expressed in pre-tooth epithelium and bone morphogenic protein (Bmp)4,³ expressed in intervening epithelium and their interaction and competing actions specify tooth formation (5, 6). FGF and Bmp regulate restricted expression of the homeobox transcription factor Pitx2 that is required for tooth development (7). Wnts comprise a large family of secreted ligands that activate several receptor-mediated pathways (8). The well known canonical Wnt/ β -catenin pathway activation causes β -catenin accumulation, nuclear translocation, and transcriptional activation by complexes of β -catenin, Lef/Tcf, and Pitx2 transcription factor family members (8–10). Activation of Wnt/ β -catenin signaling initiates the *de novo* formation of hair follicles, feather buds, mammary placodes, taste buds, and teeth (4, 11–17). Wnt/ β -catenin signaling is required for multiple stages of tooth development and dental epithelial cell proliferation and differentiation (14). The Lef-1 transcription factor regulates genes involved in cell proliferation and differ-

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³ The abbreviations used are: Bmp, bone morphogenic protein; LaCL, labial incisor cervical loop; EMT, epithelial-mesenchymal transition; miR, microRNA; GRN, gene regulatory network; P0, postnatal day 0.

entiation. *Lef-1* deficiency causes arrested tooth development at the bud stage in mice, and the dental epithelial cells fail to survive (18, 19).

miRs are non-coding small RNAs that regulate gene function post-transcriptionally. Animal miRs are imperfectly paired to the 3'-UTR of target mRNA and inhibit protein production either through destabilization of mRNA or inhibition of translation (20). Tooth development, including epithelium stem cell differentiation, is tightly controlled by miRs and a loss of mature miRs results in the development of supernumerary incisors in the *Dicer* conditional knock-out mouse (21, 22). miRs control stem cell differentiation in the incisor, and miR depletion causes an expansion and increased proliferation of dental stem cells (21).

The *miR-200* family regulates the epithelial-mesenchymal transition (EMT) associated with tumor cell migration, invasion, adhesion, and metastasis (23). The *miR-200* family targets and represses the expression of genes involved in this process. These genes include *Zeb1*, *Zeb2*, and *Jagged1* (23–29). The *miR-200* family is selectively expressed in differentiating dental epithelial cells and have low levels of expression in the dental stem cell niche (21, 22, 30). The *miR-200* family is comprised of five members, *miR-429-200a-200b* in one cluster and *miR-200c-141* in another cluster located on different chromosomes. We recently reported a *Pitx2:miR-200c/141:Noggin* pathway regulated Bmp signaling and epithelial cell differentiation during odontogenesis (31). Thus, *Pitx2* and *miR-200* appear to control the fate of dental stem cells.

There are many protocols used for regeneration therapies to develop fully functioning organs including teeth. Current tooth bioengineering relies on the sequential and reciprocal interactions between neural crest-derived mesenchymal cells and stomadial epithelium, *in vitro* differentiation of dental epithelial progenitor cells through epithelial-mesenchymal interactions and tooth organ germ bioengineering from molar tooth germ-derived epithelial and mesenchymal cells (3, 32–37). However, for replacement of a functional tooth, these tissues are difficult to obtain and maintain in culture. Mesenchymal stem cells derived from bone marrow and dental pulp stem cells are used to make dental cells and tissues, repair dental structures, and regenerate bone (38–42). Stem cells have great promise in tissue bioengineering studies, but they are difficult to obtain. Additionally, more efficient methods are needed for generating dental cells.

The discovery that fibroblast cells can be converted to induced pluripotent cells by induction of a mixture of transcription factors has led to the development of cell reprogramming for tissue engineering (43). miRs have also evolved as regulators of gene programs that control cell differentiation and cell fate decisions (44). miRs modulate these functions through positive and negative feedback loops to reinforce cellular decisions (45).

Because dental stem cells are difficult to obtain, culture and propagate as well as producing human epithelial-mesenchymal tooth-forming tissues, we propose a new method using a combination of transcription factor and miRs in a sequential addition to both oral epithelial cells and odontoblast mesenchymal cells to produce amelogenin producing dental epithelial cells.

EXPERIMENTAL PROCEDURES

Expression and Reporter Constructs—The expression plasmids containing the cytomegalovirus (CMV) promoter linked to the *mmu-miR-200a* and *mmu-miR-21* precursor were constructed in pSilencer 4.1 (Ambion). *Pitx2*, and β -catenin S37A expression plasmids were constructed in pcDNA 3.1 MycHisC (Invitrogen) as described previously (46–49). *Pitx2* 3'-UTR and *Pitx2* mutant 3'-UTR generated by mutagenesis (QuikChange site-directed mutagenesis kit, Agilent Technologies) were directionally cloned into the pGL3 CXCR4 1P (Addgene, plasmid 11310). The 7 \times TopFlash reporter plasmid was constructed into luciferase vector by inserting seven Lef/Tcf binding sites upstream of the minimal thymidine kinase promoter. The FopFlash reporter, which has the Lef/Tcf binding sites mutated, was also constructed in the luciferase vector (50). The *Pitx2c 3kb* and *Lef-1* promoters have been reported previously (9, 51). All constructs were confirmed by DNA sequencing.

Cell Culture, Transient Transfections, Luciferase, and β -Galactosidase Assays—HEK 293 FT, MDPC, and LS-8 cells were cultured in DMEM supplemented with 5 or 10% FBS and penicillin/streptomycin and transfected by electroporation or Lipofectin. The procedures for transient transfections and luciferase and β -galactosidase assays were described previously (46). Transfected cells were incubated for 48 h. LiCl was added to the appropriate cells at a final concentration of 10 mM 23 h before harvest. The pcDNA3.1 empty vector, pLL3.7, or pSilencer 4.1 negative control vectors were added to equalize the total amount of cotransfected expression vectors. SV-40 or CMV β -galactosidase reporter plasmids were co-transfected in all experiments as a control for transfection efficiency. All plasmids were double-banded CsCl-purified.

Generation of MDPC and LS-8 miR-200a Stable Cell Lines—Fragments containing the *miR-200a* precursor were amplified by PCR and directionally cloned into the EcoRI site of pLL3.7. Lentivirus was generated by cotransfection of the above construct with packaging plasmids into HEK293T cells, as described previously (31). Blastomycin (5 μ g/ml) was added to cells for 5 days, and surviving cells were propagated and subcultured. MDPC and LS-8 cells were transduced and subsequently FACS-sorted for green fluorescent protein (GFP), which is co-expressed on a single transcript with the miR.

Chromatin Immunoprecipitation (ChIP) Analysis—The ChIP analysis was performed as described (52) using the ChIP assay kit (Upstate Biotechnology, Inc.) with the following modifications. LS-8 cells were fed for 24 h, harvested, and plated in 60-mm dishes. Cells were cross-linked with 1% formaldehyde for 10 min at 37 °C the next day. Samples were incubated with PITX2ABC rabbit polyclonal antibody (Capra Science) overnight at 4 °C. Immune complexes were washed consecutively for 5 min with each of the following solutions: low salt immune complex wash buffer and high salt immune complex wash buffer four times, and LiCl immune complex wash buffer and TE buffer twice. An aliquot of the immunoprecipitated DNA from non-transfected cells was used for PCR (32 cycles). All reactions were done at an annealing temperature of 61 °C. Two primers for amplifying the *Pitx2* binding site in the *miR-200a*

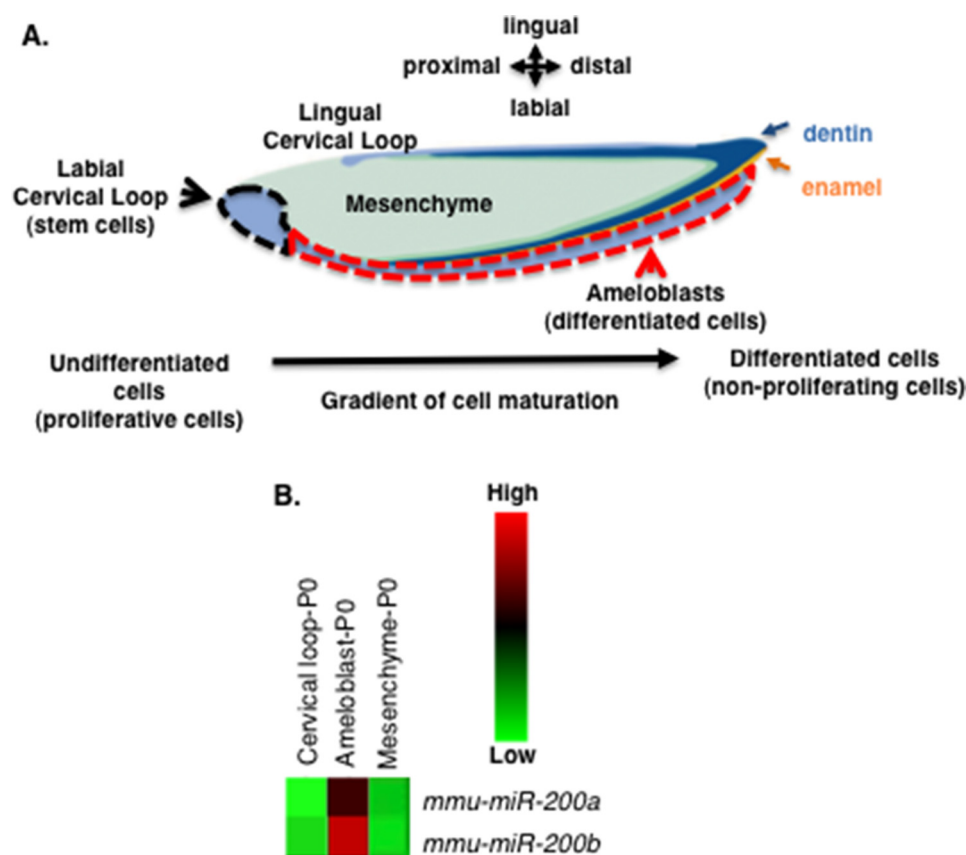


FIGURE 1. miR-200a-3p expression is associated with differentiating dental epithelial cells. *A*, schematic of the mouse lower incisor cell and tissue structures. The black dotted line denotes the labial cervical loop (LaCL, stem cell niche), the red dotted line denotes the pre-secretory, secretory, and mature differentiated epithelial tissues (pre-ameloblasts and ameloblasts). Green shaded region, mesenchyme; dark blue, dentin; orange, enamel. *B*, heat map of selected miR-200a-3p and miR-200b-3p expression in the isolated dental epithelial tissue compartment (LaCL versus ameloblasts) and dental mesenchyme (ameloblast versus mesenchyme). These tissues were isolated from P0 mice lower incisors, total RNA was harvested, and miRs were analyzed by microRNA arrays. Five separate biological samples were analyzed. *mmu*, *Mus musculus*.

promoter are as follows: sense, 5'-TTCTTGGCTCTGTATGGGAGA-3'; antisense, 5'-CCCCTCTTGCCTTTTTTCAG-3'. All of the PCR products were evaluated on a 1% agarose gel in 1× TBE for appropriate size (175 bp) and confirmed by sequencing. As controls, the primers were used without chromatin, and normal rabbit IgG was used replacing the PITX2 antibody to reveal nonspecific immunoprecipitation of the chromatin. Furthermore, the same set of experiments was done with control primers targeting the distal region of miR-200a promoter lacking putative Pitx2 binding sites. The control primers are as follows: sense, 5'-AGGCAACAGACACCTGC-ACT-3'; antisense, 5'-GAATGACTGTCTCCCCTCCA-3'.

Mouse Tissue Isolation and Real-time PCR Analyses—The PITX2C cDNA was cloned into the K14 promoter construct (53). We placed the hrGFP (humanized Renilla GFP) gene in the cassette to observe expression in live cells and have observed good expression of PITX2C in transgenic mice by PCR. The PITX2C GFP DNA was excised from the plasmid and used for pronuclear injection. Donor female mice (FVB/NCr), stud male (FVB/NCr), vasectomized male (ICR) and recipient female (ICR) were used in the experiments. Multiple founders were analyzed for transgene expression and crossed to BL6 mice and reevaluated for expression (54). All animals were housed in the Program of Animal Resources of the Institute of Biosciences and Technology and were handled in accordance

with the principles and procedure of the Guide for the Care and Use of Laboratory Animals. The Texas A&M Health Science Center, Institutional Animal Care and Use Committee approved all experimental procedures. Wild type (C57BL/6) and K14-PITX2C transgenic mouse mandible, maxilla, and palate tissues were harvested at various developmental time points, and total RNA was prepared for analyses of gene and miR expression. These tissues were harvested using modified procedures for isolating epithelial stem cells and tissue (21, 31, 55–58). After removing the skin from mouse heads, the hemi-mandibles and maxilla were isolated by removing muscle, tendons, ligaments, and bones using a scalpel under a dissecting microscope. The incisors and molars were further dissected from bone. The incisors and molars were incubated in dispase II and collagenase I for 30 min at 37 °C to separate epithelium from mesenchyme. Total RNAs were prepared using the RNeasy minikit from Qiagen. The amount and integrity of the RNA samples were assessed by measurement at 260 and 280 nm and gel analyses. LS-8 (59) or MDPC (60) cells transfected with pLL-scramble or pLL-miR-200a precursor stable cell lines were harvested 48 h after seed. Total RNA was reverse transcribed into cDNA by the iScript Select cDNA synthesis kit (Bio-Rad). Realtime PCR was carried out in a total reaction of 25 μl containing 12.5 μl of iQ SYBR Green Supermix, 0.1 μM forward primer, 0.1 μM reverse primer, 0.25 μl of cDNA template in the MyiQ Singlecolor real-

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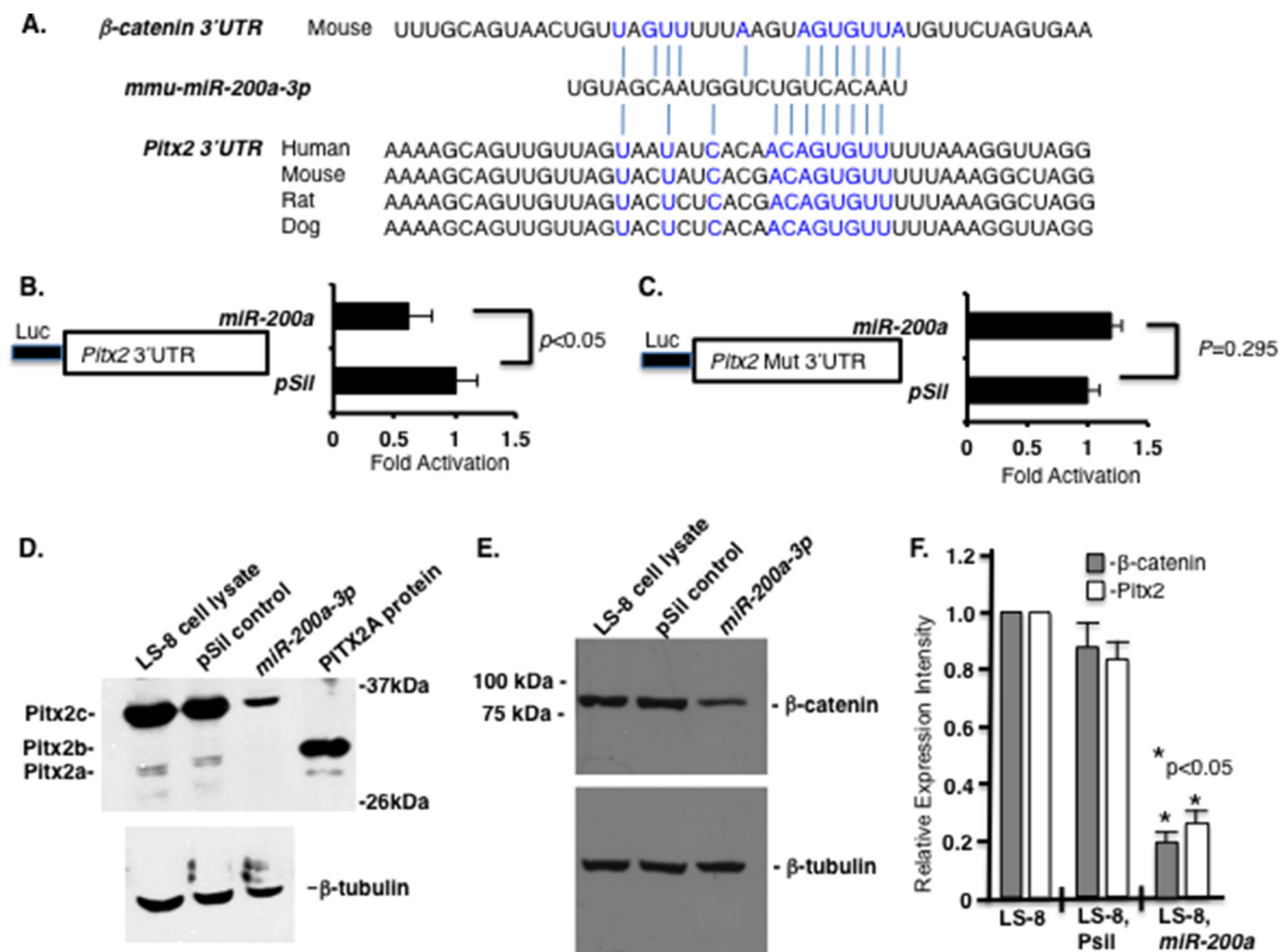


FIGURE 2. miR-200a-3p directly targets the *Pitx2* and β -catenin 3'-UTR and represses *Pitx2* and β -catenin expression. *A*, *miR-200a-3p* is evolutionarily conserved among several vertebrate species, and the *miR-200a* target sites in the *Pitx2* and β -catenin 3'-UTR are shown. *B*, *Pitx2* 3'-UTR luciferase construct transfected with either *miR-200a* or empty vector in LS-8 cells. Luciferase (*Luc*) activity was measured using the Dual-Luciferase system to control for transfection efficiency and normalization ($n = 3$; $p < 0.05$). *C*, as a control, the *miR-200a* target site was mutated in the *Pitx2* 3'-UTR and showed no inhibition when co-transfected with *miR-200a* or empty vector ($n = 3$; $p = 0.295$). *D*, *miR-200a-3p* represses endogenous *Pitx2a* and *Pitx2c* isoform expression. Western blot of endogenous *Pitx2* in *miR-200a* precursor transfected LS-8 cells 48 h post-transfection. β -Tubulin is shown as a loading control. The *Pitx2b* isoform was not detected in LS-8 cells. *E*, *miR-200a* represses endogenous β -catenin expression. Western blot of β -catenin protein in control or *miR-200a* precursor transfected LS-8 cells 48 h post-transfection. β -Tubulin is shown as a loading control. LS-8 cell lysate (empty vector, mock) and pSil-neg vector served as controls. *F*, quantitation of β -catenin and *Pitx2* endogenous expression from three Western blots using different LS-8 cell lysates expressing *miR-200a* or controls (empty vector or no vector; $n = 3$; $p < 0.05$). *mmu*, *Mus musculus*.

time detection system and analyzed by the MyiQ optical system software (version 2.0, Bio-Rad). β -Actin served as a reference gene for normalization of E-cadherin mRNA levels. *Leif-1* primer sequences were 5'-GCAGCTATCAACCAGATCC-3' (forward) and 5'-GATGTAGGCAGCTGTATTC-3' (reverse); E-cadherin primer sequences were 5'-GCTTCAGTTCGGAGGTCTAC-3' (forward) and 5'-AGATGCCGCTTCACTTGTGAT (reverse); cyclin D2 primer sequences were 5'-GAGCTGCTGGCCAA-GATCAC (forward) and 5'-GACTTGGATCCGGCGTTATG (reverse) (50). Dog E-cadherin primer sequence were 5'-AGTGA-CTCGCAATGATGTGG-3' (forward) and 5'-GAACCGCTTC-TTCATAGTC-3' (reverse) and dog GAPDH primers sequence 5'-CATCACTGCCACCCAGAAG-3' (forward) and 5'-CAGT-GAGCTTCCCGTTCAG-3' (reverse) (27). The thermal cycling profile consisted of 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 18 s. Samples were run in triplicate. No-template control was run in each experiment. Melting

curve analyses were performed to confirm amplification specificity of the PCR products, and all PCR products were sequenced to confirm their identity.

Western Blot Assays—Expression of endogenous *Pitx2* and β -catenin proteins were demonstrated using the PITX2ABC antibody (Cappa Science) and β -catenin antibodies (Millipore). Approximately 10–40 μ g of transfected cell lysates or tissues were analyzed in Western blots. Following SDS gel electrophoresis, the proteins were transferred to PVDF filters (Millipore), immunoblotted, and detected using specific antibodies and ECL reagents from GE Healthcare.

Immunofluorescent Staining—The pLL-Scramble, pLL-*Pitx2*, pLL-*miR-200a*, and pLL-*Pitx2-miR-200a* cells were plated onto fibronectin-coated chamber slides (BD Biosciences) and stained at day 12 or day 3, respectively. For E-cadherin and amelogenin staining, cells were washed with PBS, fixed in ice-cold acetone for 10 min at 4 °C, and air-dried. Fixed cells were washed with PBS for 2–5 min and incubated in 10% normal goat

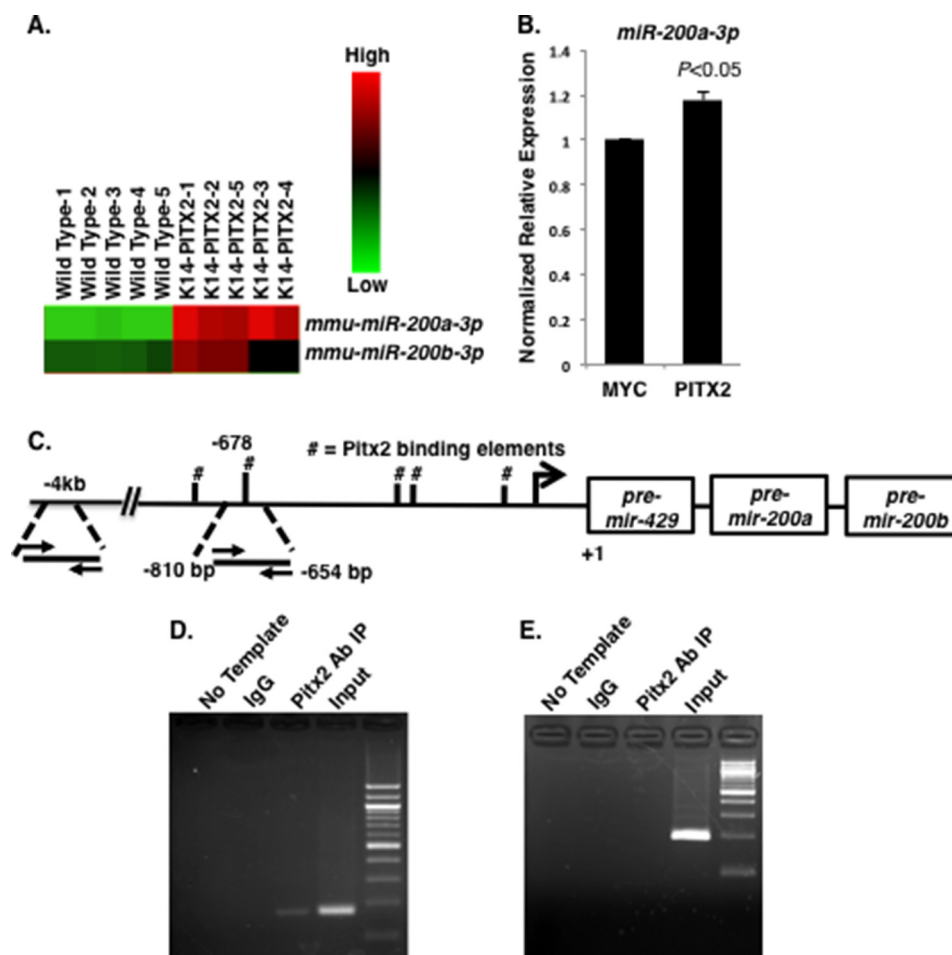


FIGURE 3. Endogenous Pitx2 binds to and activates the *miR-200a* promoter. *A*, heat map of *miR-200a-3p* and *miR-200b-3p* expression in wild type and K-14-PITX2 overexpression mouse lower incisor epithelial tissue. *B*, real time PCR of endogenous *miR-200a-3p* expression in LS-8 cells transfected with empty vector (Myc) or PITX2 ($n = 3$). *C*, schematic representation and location of the Pitx2-binding site in the *mmu-miR-429-200a-200b* promoter. #, the Pitx2 binding elements (TAATCC). *D*, ChIP of endogenous Pitx2 binding to the Pitx2 element ~678 bp upstream of *pre-miR-429-200a-200b* transcript in LS-8 cells. Rabbit antisera were used as a control IP and Pitx2ABC antisera from CAPP A SCIENCE was used to IP Pitx2 binding to the chromatin. The input chromatin is shown as a positive control for the ChIP. *E*, control ChIP using the Pitx2 antisera and primers to a 4-kb upstream region of the *pre-miR-429-200a-200b* transcript. This chromatin does not contain a Pitx2-binding site and was not immunoprecipitated using Pitx2 antisera; the primers did amplify the input chromatin.

serum-PBS 30 min at room temperature, and then cells were incubated with rat anti-E-cadherin or anti-amelogenin antibodies at 4 °C overnight. After overnight incubation, cells were rinsed by washing in PBS for 3–5 min, and cells were incubated with Alexa Fluor 488 goat anti-rat IgG (H+L) (Molecular Probes, labeling & detection by Invitrogen) 30 min at 37 °C. Cells were washed with PBS for 3–5 min and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). Images were acquired using a Nikon ECLIPSE 80i microscope and NIS-Elements AR software (version 3.2).

RNA Sequencing—Total RNA was isolated from LS-8 and MDPC-23 cell lines after transduction with virus and fluorescence-activated cell sorted (FACS), using the miRNeasy mini kit (Qiagen) following the manufacturer's instructions. RNA quality and concentration was determined using the Agilent 321 Bioanalyzer (Agilent Technologies). Using 1 μ g of RNA and the TruSeq Stranded Total RNA Library Prep kit (Illumina), reverse transcription with bar-coded primers, complementary DNA amplification, and 100 \times 100 paired ended

sequencing with Illumina HiSeq 2000 were performed. Each sample had one biological duplicate that was barcoded separately and sequenced in an independent lane. Quality control of the obtained reads and mapping to the mouse reference genome (GRCm38/mm10) were performed using the combination of the Galaxy web-based analysis suite (61–63) and in-house Perl scripts. Mapped reads were analyzed using Cufflink tool set (64) to identify significant changes in gene expression. The low expression transcripts (<10 reads in all samples) were filtered out, and p values were adjusted using a threshold for false discovery rate ≤ 0.001 . Differentially expressed transcripts were identified using threshold of fold change ≥ 2 and false discovery rate ≤ 0.001 . The differentially expressed genes were further used for hierarchical clustering performed using Cluster (version 3.0), and Java Treeview was used for visualization (65, 66). Gene ontology category enrichment was assessed using GOrilla. Q-RT-PCR analysis for the genes indicated was compared with the RNA sequencing results. Amplification of VPS29 and actin was used to normalize the values.

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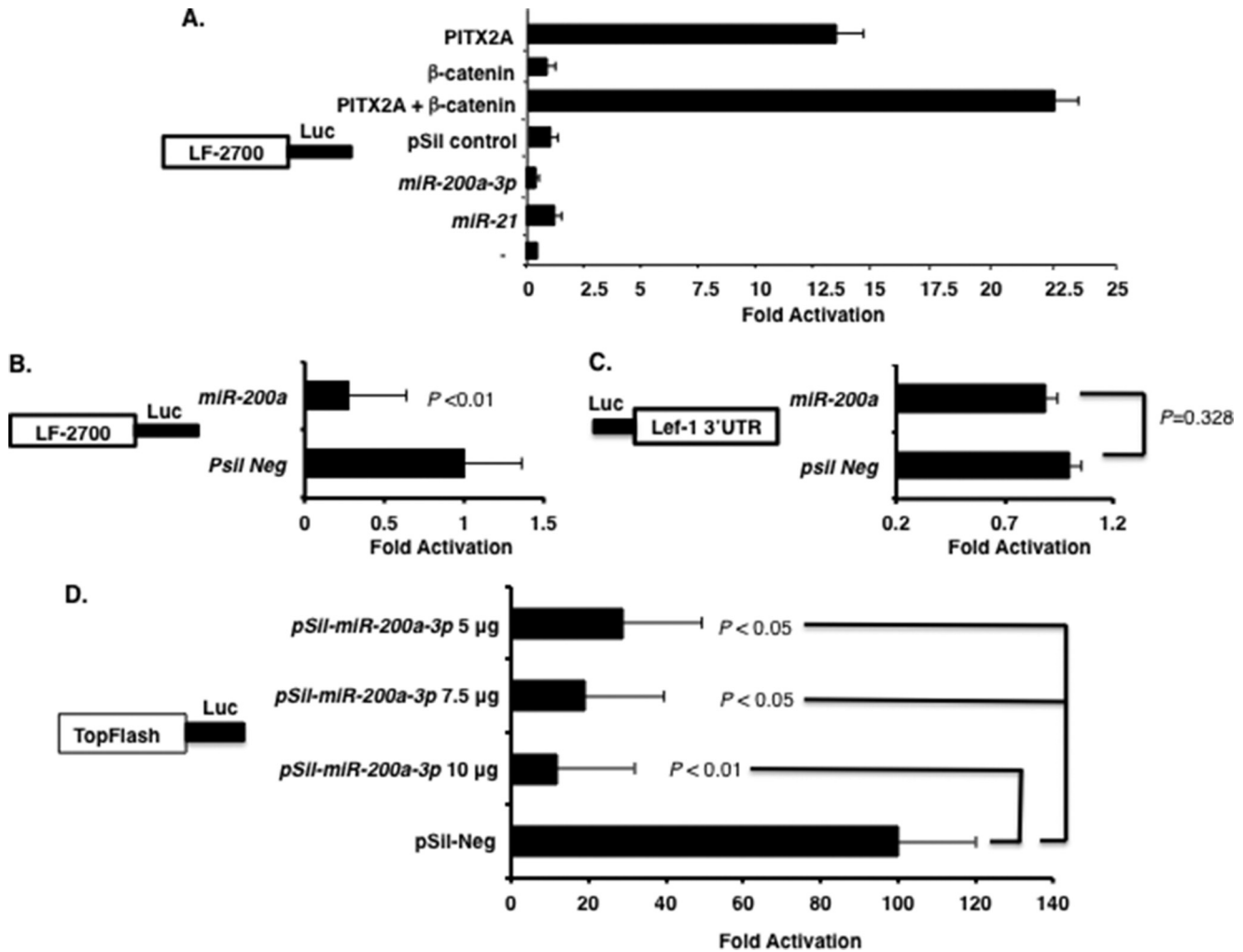


FIGURE 4. miR-200a-3p indirectly regulates the LEF-1 promoter and TopFlash reporter. *A*, *miR-200a* targets endogenous Pitx2 and β -catenin, which activate the LEF-1 promoter in LS-8 cells. The LEF-1 2.7-kb mouse promoter and Pitx2, β -catenin, *pre-miR-200a*, pSII-empty vector, and pre-miR-21 were transfected in LS-8 cells. To control for transfection efficiency, all transfections included the SV-40 β -galactosidase reporter (0.5 μ g). Cells were incubated for 48 h and then assayed for luciferase (*Luc*) and β -galactosidase activities as described previously (10). The activities are shown as mean fold activation compared with the luciferase plasmid with empty vector and normalized to β -galactosidase activity \pm S.E. from three independent experiments. *B*, *miR-200a* targets endogenous *Pitx2* and represses Lef-1 activity in LS-8 cells. The LEF-1 promoter was transfected with *miR-200a* or pSII vector only in LS-8 cells, which endogenously express Pitx2 and β -catenin. Luciferase activity was assayed, and *miR-200a* transfection was compared with vector only as in *A*. *C*, as a control, the Lef-1 3'-UTR luciferase construct was transfected with *miR-200a* or empty vector to demonstrate that *miR-200a* does not directly regulate Lef-1 expression. *D*, the TopFlash reporter (contains seven Lef-1 binding elements (50)) was co-transfected with increasing amounts of *miR-200a-3p* plasmid in LS-8 cells. Luciferase activity was measured as described in *A*.

RESULTS

Bioinformatics analyses of isolated murine dental epithelium and mesenchyme at different developmental stages identified gene and miR signatures. These data were then integrated into gene ontology category enrichment maps and used to identify processes that are common and different for dental epithelial cells compared with other cells. These data identified several new molecular mechanisms that control dental epithelial cell proliferation and differentiation. Pitx2 is the first transcriptional marker for tooth development, and the *miR-200* family is required for dental epithelial cell differentiation (6, 21, 31, 67, 68). We first performed a functional analyses of a new Pitx2- β -catenin-*miR-200a* regulatory pathway identified in the bioinformatics analyses and then used this new mechanism to convert both oral epithelial and dental mesenchymal cells to a dental epithelial cell fate. We reported a method to convert oral

epithelium and dental mesenchymal cells to dental epithelial cells, which express many genes that are hallmarks of these cells, including the expression of the ameloblast (differentiated dental epithelium)-specific marker, amelogenin.

Dental epithelial and mesenchymal tissue from P0 mouse incisors were isolated and screened for gene and miR expression to understand gene regulatory networks (GRNs) and how dental stem cells yield fully differentiated epithelial cells or ameloblasts. The incisor labile cervical loop (LaCL) contains dental stem cells that give rise to the differentiated ameloblasts (Fig. 1A). The epithelial compartment was divided into the LaCL or undifferentiated cells (*black dashed line*) and the differentiating cells (*red dashed line*), whereas the dental mesenchyme was separated from the epithelium, and total RNA was isolated for gene and miR expression analyses. A miR expression profile was identified showing that *miR-200a* and *miR-200b* were asso-

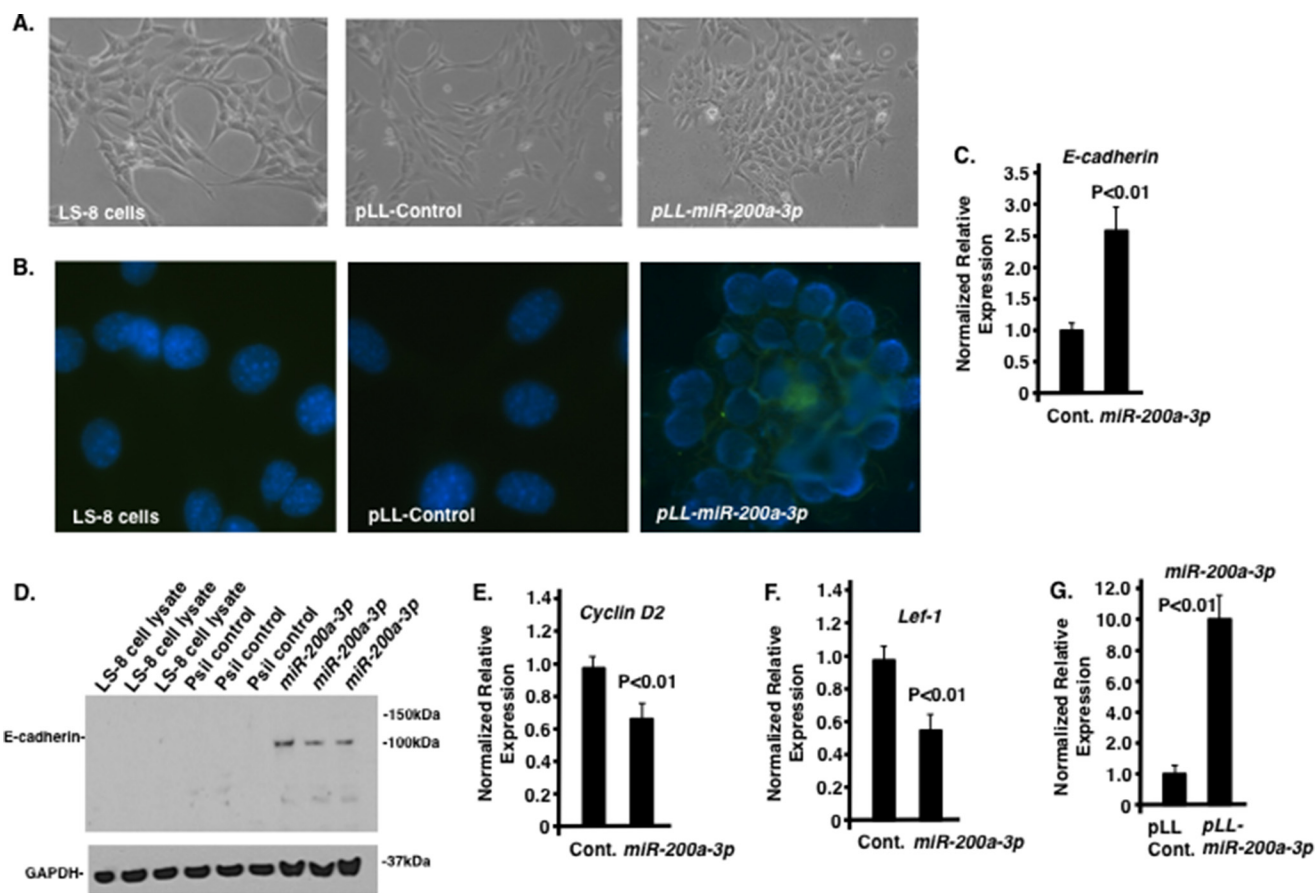


FIGURE 5. Effect of *miR-200a-3p* expression in LS-8 cells. *A*, morphology change of LS-8 cells transduced with pLL-control and pLL-*miR-200a* lentivirus constructs. *B*, E-cadherin staining (green fluorescence) of LS-8, pLL control, and pLL-*miR-200a* transduced cells. DAPI staining used to detect nuclei. *C* and *D*, quantitation by real time PCR and Western blot of E-cadherin in LS-8 cells transfected with pLL control (Cont.) vector and pLL-*miR-200a*, respectively. *E* and *F*, quantitation by real time PCR of cyclin D2 and *Lef-1* in LS-8 cells transfected with pLL control vector and pLL-*miR-200a*, respectively. *G*, as a control, *miR-200a* expression was increased in pLL-*miR-200a-3p*-transduced LS-8 cells.

ciated with differentiating epithelial cells (pre-ameloblast cells) (Fig. 1*B*). These miRs were not expressed in the dental mesenchyme, suggesting that *miR-200a* and *miR-200b* may promote epithelial cell differentiation. However, their molecular mechanism in dental epithelial cell differentiation was not known.

Analyses of *miR-200a-3p* binding elements identified *Pitx2* and β -catenin as potential targets through sequences in their 3'-UTRs (Fig. 2*A*). To demonstrate a functional *miR-200a-3p* regulation of *Pitx2*, the *Pitx2* 3'-UTR was cloned into the luciferase vector and co-transfected in LS-8 oral epithelial cells with *miR-200-3p* or empty vector. *miR-200a-3p* repressed luciferase activity from the vector containing the *Pitx2* 3'-UTR, but not when the *miR-200a-3p* target site was mutated in the *Pitx2* 3'-UTR construct (Fig. 2, *B* and *C*). Transfected *miR-200a-3p* decreased endogenous *Pitx2* protein expression (*Pitx2* isoforms) in LS-8 cells (Fig. 2*D*) and also decreased β -catenin protein expression in these cells (Fig. 2*E*) (69). Quantitation of multiple Western blots demonstrates significantly decreased *Pitx2* and β -catenin protein expression in *miR-200a* transfected LS-8 oral epithelial cells (Fig. 2*F*). These data demonstrate that both *Pitx2* and β -catenin are targeted by *miR-200a-3p*.

Mouse incisor P1 epithelial tissue from wild type (WT) and *K14-PITX2C* overexpression mice was analyzed for miR expression by miR arrays. *PITX2C* overexpression increased

miR-200a and *miR-200b* expression in the dental epithelium (Fig. 3*A*). Sequence analyses of the 5'-flanking region of the *miR-429-200a-200b* cluster identified a *Pitx2*-binding element (5'-TAATCC-3'). We next asked whether *Pitx2* regulated *miR-200a-3p* expression. Transfection of *Pitx2C* in LS-8 cells activated endogenous *miR-200a-3p* expression (Fig. 3*B*). Endogenous *Pitx2* binding to the *miR-200a* promoter was characterized by ChIP in LS-8 cells. *Pitx2* bound to a *Pitx2* element in the 5'-flanking region of the *miR-200a* chromatin (Fig. 3*C*) but not to an adjacent sequence. This sequence does not contain a *Pitx2* binding site (Fig. 3, *D* and *E*, respectively). Thus, *Pitx2* activates *miR-200a-3p* expression and *miR-200a-3p* acts to repress *Pitx2* expression. This tight regulation of *Pitx2* and *miR-200a* expression may be one mechanism to control dental epithelial differentiation.

Wnt/ β -catenin expression is required for tooth development at many stages and regulates incisor epithelial cell proliferation and differentiation (14). *Pitx2* activates *Lef-1* expression and in concert with β -catenin synergistically activates the *LEF-1* promoter (Fig. 4*A*) (9). When LS-8 cells (which endogenously express *Pitx2* and β -catenin) were cotransfected with the *LEF-1* promoter and *miR-200a*, luciferase activity from the *LEF-1* promoter decreased compared with miR empty vector and *miR-21* (used as controls) (Fig. 4*A*). A direct comparison of *LEF-1* pro-

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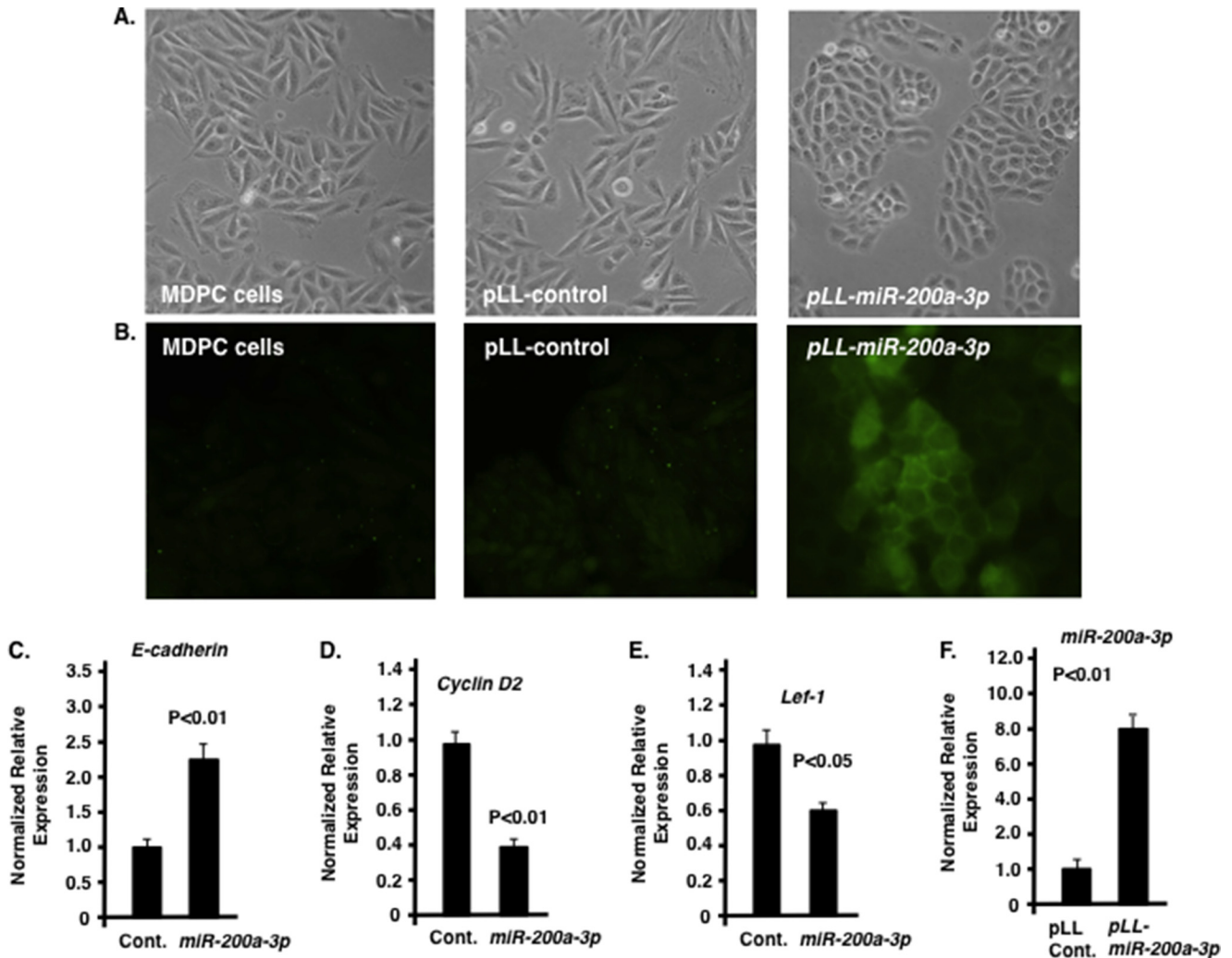


FIGURE 6. **Effect of miR-200a-3p expression in MDPC cells.** A, morphology change of MDPC cells transduced with pLL-control and pLL-miR-200a-3p. B, E-cadherin staining (green fluorescence) of MDPC, pLL control, and pLL-miR-200a transduced cells. C–E, quantitation by real time PCR of E-cadherin, cyclin D2, and Lef-1 in MDPC cells transduced with pLL control (Cont.) vector or pLL-miR-200a. F, as a control, miR-200a expression was increased in pLL-miR-200a-3p-transduced MDPC cells.

motor activity with *miR-200a* expression revealed a 4-fold decrease in luciferase activity compared with empty vector (Fig. 4B). As a control, we show that *miR-200a* does not regulate the *Lef-1* 3'-UTR luciferase construct (Fig. 4C). Thus, *miR-200a-3p* indirectly regulates *Lef-1* expression by inhibiting endogenous *Pitx2* and β -catenin, which both activate endogenous *Lef-1* expression. Titration of *miR-200a-3p* in LS-8 cells transfected with the TopFlash reporter decreased its luciferase activity compared with empty vector alone (Fig. 4D). The FopFlash reporter (*LEF-1* binding sites mutated) was not affected by *miR-200a-3p* expression (data not shown). Thus, *Pitx2* and β -catenin interact (9, 10) to regulate *Lef-1* expression and *miR-200a-3p* represses *Pitx2* and β -catenin expression and indirectly represses *Lef-1* expression and Wnt/ β -catenin signaling mechanisms.

Pitx2 is highly expressed in the LaCL and regulates progenitor cell proliferation and differentiation during incisor development along with *Lef-1* (7, 14, 18, 31). Because *miR-200a* caused an MET response when overexpressed in cells (27), we asked whether *miR-200a-3p* could cause the LS-8 oral epithelial-like

cells to convert to dental epithelial cells that express E-cadherin. Furthermore, E-cadherin, which promotes epithelial cell differentiation also decreases β -catenin transcriptional activity (70). However, this E-cadherin repression of β -catenin can be counteracted by *Pitx2* activation of *Lef-1* expression. Thus, *miR-200a* induces E-cadherin expression and MET, whereas *Pitx2* acts to increase *Lef-1* expression and cell proliferation.

LS-8 cells transduced with lentiviral scrambled or *miR-200a-3p* constructs were assayed for gene expression and cell morphology changes after 2 weeks in culture. The LS-8 cells grow similar to fibroblast cells in culture (59) and when transduced with scrambled control vector (Fig. 5A). After *miR-200a* transduction (pLL-miR-200a) these cells form clusters of cells that more closely resemble epithelial cells (Fig. 5A). Immunocytochemistry using an E-cadherin antibody showed increased E-cadherin expression in *miR-200a* transduced cells (Fig. 5B). Gene expression analyses of the *miR-200a-3p* transduced LS-8 cells showed increased E-cadherin (both transcripts and protein, Fig. 5, C and D) and *miR-200a* expression and decreased cyclin D2 and *Lef-1* expression compared with scrambled control cells (Fig. 5, E–G).

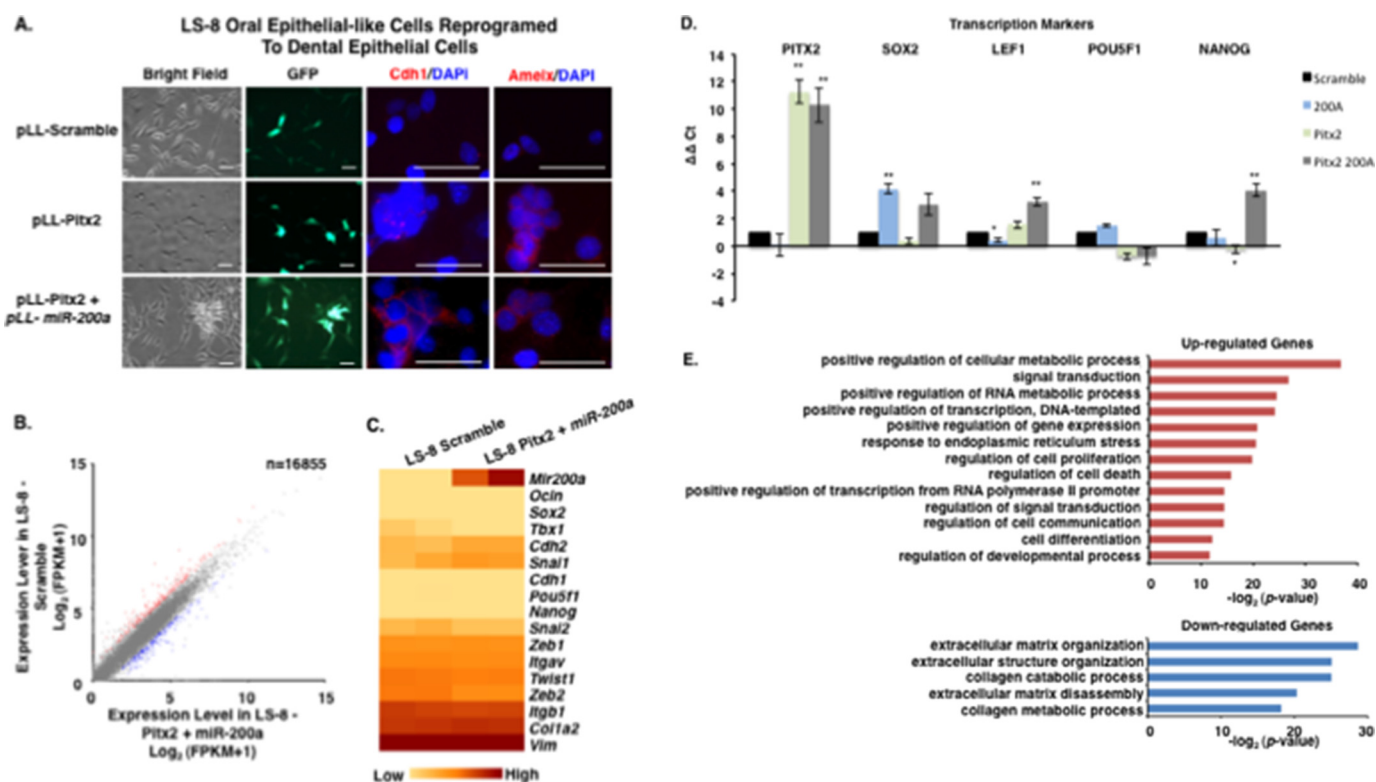


FIGURE 7. Reprogrammed LS-8 oral epithelial cells express amelogenin and dental epithelial factors. *A*, LS-8 oral epithelial cells are transduced with *pLL-Pitx2* and a combination of *pLL-Pitx2* and *pLL-miR-200a* or lentiviral vector expressing a *pLL-scrambled* RNA control. Cells were FACS sorted, and GFP and immunofluorescence microscopy analysis of changes in the expression levels of Cdh1 and Amelx were observed after 8 weeks. Pitx2-transduced cells express low levels of E-cadherin (Cdh1) and amelogenin (Amelx). Pitx2 and *miR-200a*-transduced cells express both Cdh1 and Amelx and form tight junctions between cells. GFP expression shows the cells were transduced with the lentiviral vector. *B*, RNA-seq analysis of gene expression in response to Pitx2-*miR-200a* overexpression in LS-8 cells. Significantly up- and down-regulated genes that have at least a 2-fold of expression level change were labeled *red* and *blue*, respectively. All expression levels were estimated by FPKM. *C*, heat map showing the expression dynamics of selected EMT genes upon Pitx2-*miR-200a* overexpression. Epithelial specific and mesenchymal specific genes were hierarchically clustered, respectively. *D*, real time PCR of selected transcription factors associated with dental epithelial proliferation and differentiation. Endogenous *Sox2*, *Lef-1*, and *Nanog* were increased in the Pitx2-*miR-200a*-transduced LS-8 cells. *Pitx2* was overexpressed as expected. (*Pitx2* cDNA is not regulated by *miR-200a*.) *Pou5f1* (Oct4) was not changed significantly ($n > 3$; *, $p < 0.05$, **, $p < 0.01$). *E*, gene ontology (GO) analysis significantly up- and down-regulated genes. Top enriched GO terms ($-\log_2 p$ value > 10) are highlighted for both up-regulated (*red*) and down-regulated (*blue*) genes that are related to EMT and morphogenic functions. FPKM, fragments per kilobase of transcript per million mapped reads.

The MDPC odontoblast mesenchymal cells (60) were also transduced with scrambled and *miR-200a-3p* lentiviral constructs and the cells expressing *miR-200a-3p* showed an epithelial cell morphology (Fig. 6*A*). The MDPC cells transduced with *miR-200a-3p* expressed high levels of E-cadherin in culture (Fig. 6*B*). The transduced MDPC cells have increased E-cadherin and *miR-200a-3p* expression and decreased cyclin D2 and *Lef-1* expression similar to the *miR-200a* transduced LS-8 cells (Fig. 6, *C–F*). Interestingly, *miR-200a-3p* overexpression alone was not sufficient to induce a GRN associated with dental epithelial cells (data not shown). Bioinformatics analyses of both LS-8 and MDPC cells transformed with *miR-200a* did not activate the dental epithelial cell program identified in mouse dental epithelial tissue.

Several combinations of transcription factors and miRs were analyzed for their effect to reprogram these cells. However, Pitx2 and *miR-200a* were the best candidates. We first transduced both cell types with Pitx2, and cells were treated with blastomycin to select stable transduced cells. After 4 weeks, the cells were transduced with *miR-200a* that express GFP, and stable cells were initially selected using blastomycin treatment, and after culture for 1 week, the cells were FACS sorted using

GFP as the marker. Two weeks later, the cells were analyzed for morphology and gene expression.

E-cadherin and amelogenin are well known dental epithelial differentiation markers and characterize fully differentiated ameloblast cells (71–76). LS-8 cells after Pitx2 transduction express amelogenin (Amelx) but not E-cadherin (CDH1) (Fig. 7*A*); it has been shown that increased *Lef-1* expression reduces E-cadherin expression (70). However, after transduction with Pitx2 and *miR-200a*, both E-cadherin and amelogenin were expressed (Fig. 7*A*, bottom panel). Gene expression involved in MET in the Pitx2 and *miR-200a* transduced LS-8 cells was analyzed by RNA-seq and confirmed by quantitative PCR (Fig. 7, *B–E*). Real time PCR confirmed the up-regulation of *Pitx2*, *Sox2*, *Lef-1*, and *Nanog* in converted LS-8 cells (Fig. 7*D*) and genes that regulate transcription and signal transduction from the RNA-seq experiment (Fig. 7*E*). Conversely, in LS-8 reprogrammed cells E-cadherin repression markers and mesenchymal markers were not changed significantly compared with scrambled control cells (Fig. 8, *A* and *B*). RNA-seq identified multiple genes associated with collagen and extracellular matrix processes that were down-regulated (Fig. 7*E*). These data are consistent with conversion of LS-8 oral epithelial cells

Pitx2 Regulates miR-200a-3p

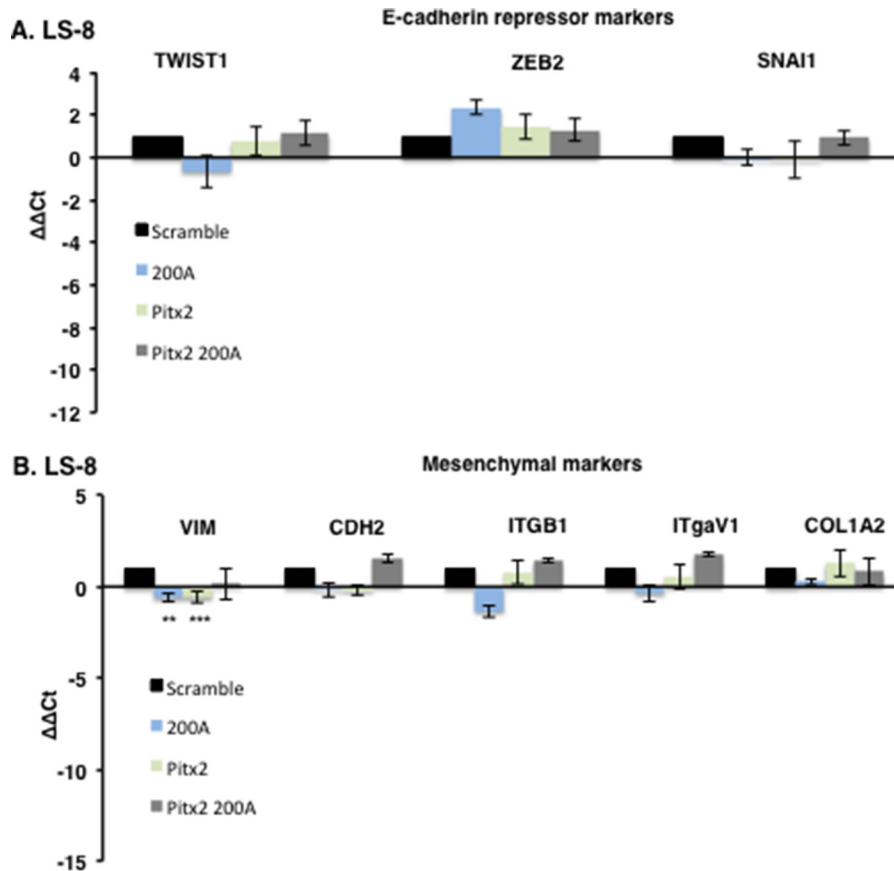


FIGURE 8. FACS sorted transduced LS-8 cells were analyzed for specific gene expression. A, the E-cadherin repressor genes *Twist1*, *Zeb2*, and *Snail1* were all increased in the Pitx2-miR-200a-transduced cells ($n = 3$). B, mesenchymal markers were not increased above scrambled control cells, and the expression levels were low ($>C_t 30$; $n = 3$).

TABLE 1

Selected murine P0 dental epithelial markers were identified by RNA-seq and DNA microarrays and compared with dental mesenchyme gene expression

RNA-seq experiments identified gene expression changes of the epithelial cell markers in converted oral epithelial (epi.) cells and dental mesenchyme (mes.) cells. Shown are selected murine dental epithelial genes in P0 mouse dental epithelium compared with mesenchymal cells; converted oral epithelial (LS-8) cells compared with non-transformed oral epithelial (LS-8) cells; and converted dental mesenchymal (MDPC) cells compared with non-transformed dental mesenchymal (MDPC) cells. exp., expression.

Gene	Expression levels	P0 dental mes. vs. P0 dental epi.	Converted cells vs. oral epi. cells	Converted cells vs. dental mes. cells
<i>Tbx5</i>	High exp.	(-) 15-fold	(+) 2-fold or less	(+) 4.1-fold
<i>Satb1</i>	Med. exp.	(-) 2-fold	(+) 2-fold or less	(+) 10-fold
<i>Pitx1</i>	High exp.	(-) 7-fold	(+) 2-fold or less	(+) 18-fold
<i>Lhx6</i>	Med. exp.	(-) 8.5-fold	(+) 2-fold or less	(+) 2.1-fold
<i>Isl1</i>	High exp.	(-) 6.3-fold	(+) 2-fold or less	(+) 10.4-fold
<i>Dach1</i>	Med. exp.	(-) 2.7-fold	(+) 2-fold or less	(+) 7.9-fold
<i>Atf2</i>	Med. exp.	(-) 4.1-fold	(+) 2-fold or less	(+) 2.8-fold
<i>Lef1</i>	Med. exp.	(-) 2.4-fold	(+) 2-fold or less	(+) 15.4-fold
<i>Hmx1</i>	Low exp.	(-) 3.2-fold	(+) 2-fold or less	(+) 10.8-fold
<i>Tbx1</i>	High exp.	(-) 5.5-fold	(+) 2-fold or less	(+) 2-fold or less
<i>Foxj3</i>	High exp.	(-) 10-fold	(+) 2-fold or less	(+) 6.2-fold
<i>Irf6</i>	High exp.	(-) 7-fold	(+) 2-fold or less	(+) 2-fold or less
<i>Cacna1g</i>	High exp.	(-) 9-fold	(+) 1.4-fold	(+) 2-fold or less
<i>Gadd45a</i>	High exp.	(-) 2.6-fold	(+) 2.3-fold	(+) 2-fold or less
<i>Mmp20</i>	Low exp.	(-) 1.6-fold	(+) 2-fold or less	(+) 2.1-fold
<i>Enam</i>	High exp.	(-) 11-fold	(+) 2-fold or less	(+) 2-fold or less

to dental epithelial cells. A selected list of dental epithelial (pre-ameloblast and ameloblast) transcription factors and other genes are listed in Table 1. These genes are highly expressed and appear to be signature genes for differentiated dental epithelium. We compare the converted LS-8 oral epithelial cells and MDPC cells to this signature. Most of these factors are expressed in the oral epithelial cells, and we find modest

increases in their expression after Pitx2 and *miR-200a* expression. However, the converted MDPC dental mesenchymal cells demonstrate large increases in the expression of these dental epithelial genes.

MDPC mesenchymal cells after *miR-200a* transduction express E-cadherin (CDH1) but not amelogenin; however, after Pitx2 transduction, these cells express amelogenin but not

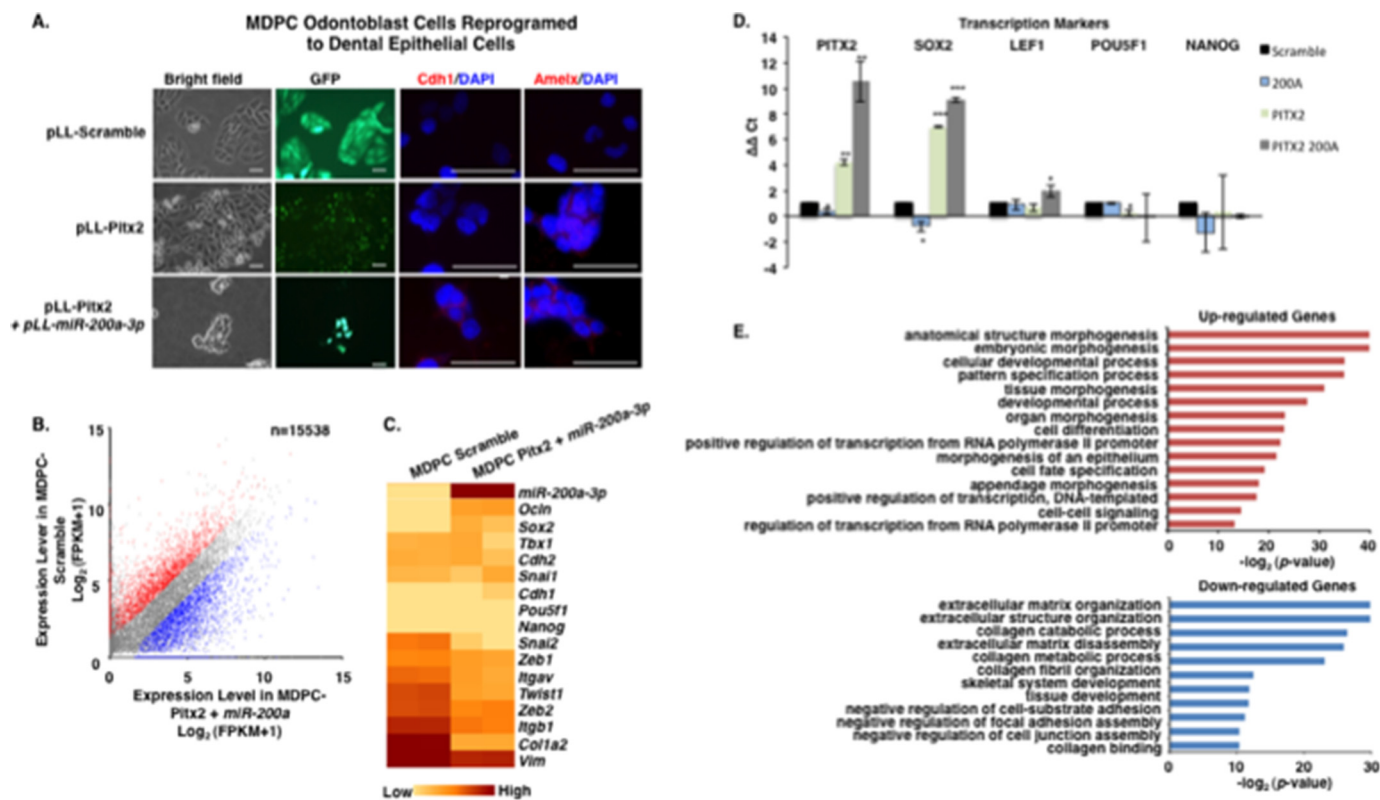


FIGURE 9. Reprogrammed MDPC odontoblast cells express amelogenin and dental epithelial factors. *A*, MDPC mesenchymal cells are transduced with Pitx2 and a combination of Pitx2 and *miR-200a* or lentiviral vector expressing a scrambled RNA control. Cells were FACS sorted, and GFP and immunofluorescence microscopy analysis of changes in the expression levels of Cdh1 and Amelx were observed after 8 weeks. Pitx2-transduced cells express low levels of E-cadherin (Cdh1) and amelogenin (Amelx). Pitx2 and *miR-200a*-transduced cells express both Cdh1 and Amelx and form tight junctions between cells. GFP expression shows the cells were transduced with the lentiviral vector. *B*, RNA-seq analysis of gene expression in response to Pitx2-*miR-200a* overexpression in MDPC cells. Significantly up- and down-regulated genes that have at least 2-fold expression level change were shaded red and blue, respectively. All expression levels were estimated by FPKM. *C*, heat map showing the expression dynamics of selected EMT genes upon Pitx2-*miR-200a* overexpression. Epithelial specific and mesenchymal specific genes were hierarchically clustered, respectively. *D*, real time PCR of selected transcription factors associated with dental epithelial proliferation and differentiation. Endogenous *Sox2* and *Lef-1* were increased in the Pitx2-*miR-200a* transduced LS-8 cells. *Pitx2* was overexpressed as expected (*Pitx2* cDNA not regulated by miR-200a). *Pou5f1* (Oct4) and *Nanog* were not significantly changed ($n > 3$; *, $p < 0.05$; **, $p < 0.01$). *E*, gene ontology (GO) analysis significantly up- and down-regulated genes. Top enriched GO terms ($-\log_2 p$ value > 10) are highlighted for both up-regulated (red) and down-regulated (blue) genes that are related to EMT and morphogenic functions.

E-cadherin (Fig. 9A). Transduction with Pitx2 and *miR-200a* stimulated both E-cadherin and amelogenin expression (Fig. 9A). Gene expression involved in MET in the Pitx2 and *miR-200a* transduced MDPC cells was analyzed by RNA-seq and confirmed by quantitative PCR (Fig. 9, B–E). Real time PCR confirmed the up-regulation of *Pitx2*, *Sox2*, and *Lef-1* in converted MDPC cells, and the RNA-seq experiments identified genes that regulate transcription and signal transduction (Fig. 9, D and E). As expected, in MDPC converted cells, E-cadherin expression was up-regulated, and mesenchymal markers were all down-regulated (Fig. 10, A and B). The significant gene expression changes demonstrate that these reprogrammed MDPC cells have undergone an epithelial conversion to a dental epithelial cell fate. RNA-seq identified multiple genes that were down-regulated and associated with collagen and extracellular matrix processes indicative of mesenchymal cells (Fig. 9E).

DISCUSSION

Recently, there has been an explosion of studies using dental stem cells and isolated dental epithelial-mesenchymal interactions to generate epithelial cells and tissue for tooth bioengi-

neering and regeneration (1, 59, 74, 77–89). Many of the genes required for epithelial cell proliferation and differentiation during tooth organogenesis and regeneration have been identified and are being used in research to make teeth (33–35, 90). There are an abundance of studies using dental stem cells, mesenchymal stem cells, bone marrow stem cells, and epithelial-mesenchymal interactions to differentiate epithelial cells capable of regenerating dental epithelium and dental structures (>100 manuscripts published). A common theme in these studies relies on the isolation of dental progenitor or stem cells to generate competent differentiated dental epithelial cells. These procedures are intrusive and provide limited amounts of material. A procedure to convert easily obtained oral epithelial cells or dental mesenchymal cells from patients would greatly facilitate tooth and tissue regeneration.

miRNAs have been identified as key regulators of progenitor cell differentiation and modulators of cell fate decisions (21, 31, 44, 45, 91). miRNAs regulate the fate of stem cells in many different tissues and organs through the specification or differentiation of cell types. miRNAs can target cell cycle regulators, promote differentiation by inactivating transcriptional repressors, and integrate with transcriptional and signaling networks in bone

Pitx2 Regulates miR-200a-3p

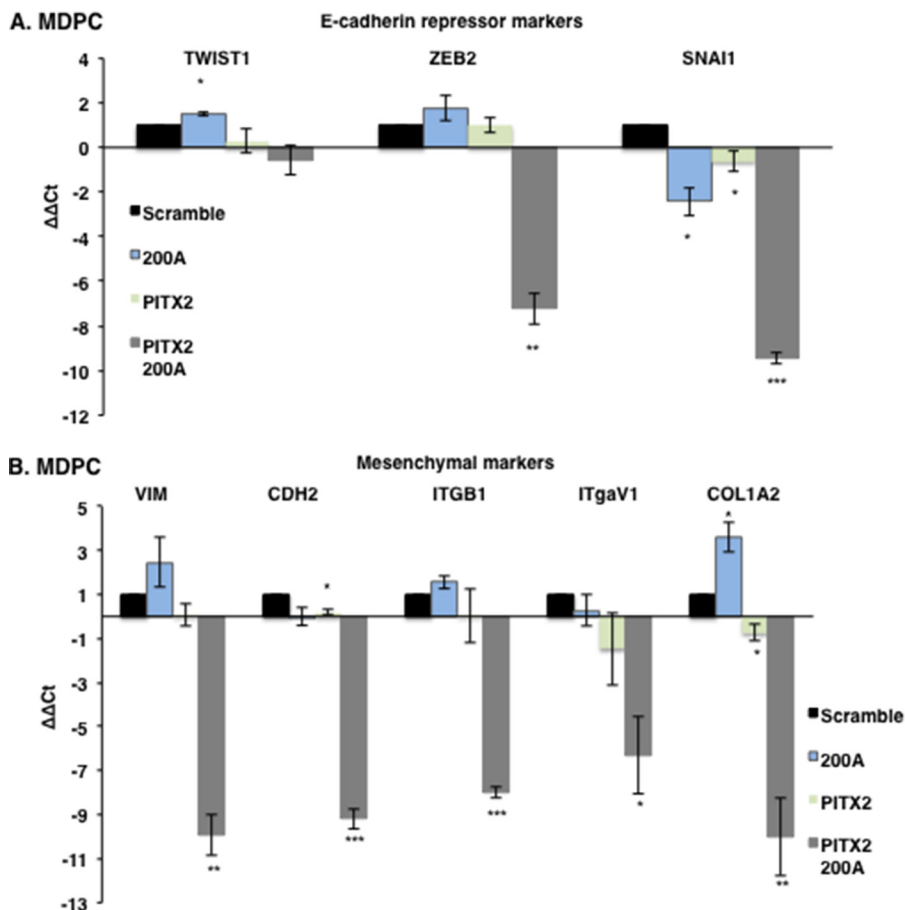


FIGURE 10. FACS sorted transduced MDPC cells were analyzed for specific gene expression. A, the E-cadherin repressor genes *Zeb2* and *Snail1* were significantly decreased in *Pitx2-miR-200a* transduced cells compared with scrambled control ($n = 3$). B, all measured mesenchymal markers were significantly down-regulated in *Pitx2-miR-200a* transduced cells compared with scrambled control ($n = 3$).

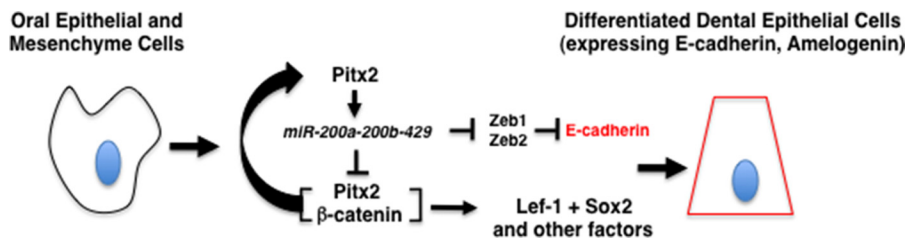


FIGURE 11. Model for the role of *Pitx2* and *miR-200a* in cell conversion. To convert oral epithelial or dental mesenchymal cells to differentiated dental epithelium, cells are transfected with *Pitx2*, which regulates endogenous *miR-200a* expression. It has been shown that *miR-200a* inhibits *Zeb1* and *Zeb2*, which repress E-cadherin expression. *miR-200a* feeds back to also repress *Pitx2* and β -catenin expression. *Pitx2* activates its own expression in concert with β -catenin providing a constant feed back loop to fine tune both *Pitx2* and *miR-200a* expression. However, overexpression of *Pitx2* cDNA drives *Lef-1* and other dental epithelial factors and promotes dental epithelial cell conversion with *miR-200a* overexpression.

formation, muscle differentiation, neurogenesis, and tooth and craniofacial morphogenesis (21, 31, 44, 45, 92). The use of miRs in cell reprogramming is a new field of research that has great promise for tooth regeneration.

Wnt/ β -catenin signaling, Tcf/Lef-1, and Sox factors control stem cell renewal and differentiation in many tissues and organs, including teeth (14, 17, 93–95). Lef-1 is required for early tooth formation and cell proliferation (18, 19). *Pitx2* also controls dental progenitor cell proliferation and differentiation (31, 96). *Pitx2* physically interacts with β -catenin to activate *Lef-1* expression and *miR-200c* expression to inhibit *Noggin* and activate BMP to allow for dental epithelial cell differentiation (9, 10, 31). This unique signaling network and GRN sug-

gested that these factors might regulate dental epithelial cell fate. Our bioinformatics analyses demonstrate that Wnt signaling-associated factors involved in odontogenesis are increased in *Pitx2-miR-200* converted cells, including *Fzd9*, *Fzd8*, *Wnt11*, and *Fzd6*. Furthermore, we constructed a list of highly expressed factors expressed in P0 mouse dental epithelium, which we use as a signature to designate dental epithelium (Table 1). This signature only denotes the highly expressed genes and excludes other genes (not highly expressed) that are also associated with dental epithelium. The converted dental mesenchyme to dental epithelium cells all highly express these factors. Interestingly, the oral epithelial cells express these factors and thus are only moderately increased in the converted dental epithelial cells.

In this report, we have identified a new GRN where Pitx2 activates *miR-200a* expression, and this miR then feeds back to modulate *Pitx2* expression as dental progenitor cells differentiate. *miR-200a* represses β -catenin expression and this GRN then indirectly modulates *Lef-1* expression. *miR-200a* is a regulator of dental epithelial progenitor cell differentiation. However, *miR-200a* can also specify epithelial cell fates by repressing *Zeb* expression to allow for E-cadherin expression. This unique molecular mechanism guides tissue morphogenesis. Here, we hijacked this process by overexpressing Pitx2 as a cDNA (not regulated by *miR-200a*) to activate the dental epithelial GRN, including amelogenin and using *miR-200a* overexpression to establish the epithelial cell program.

Studies based in our laboratory with the current findings establish a new method to reprogram both oral epithelial and dental mesenchymal cells to a dental epithelial cell fate (Fig. 11). Because functional dental epithelial cell cultures are a limiting resource in tissue engineering, this technique should have great potential to help produce epithelial tissues for tooth and craniofacial repair and regeneration. Human oral epithelium can be obtained from patients and reprogrammed to yield sufficient amounts of dental epithelium for tooth replacement. We are currently using these findings to initiate tooth regeneration.

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REFERENCES

- Harada, H., Kettunen, P., Jung, H. S., Mustonen, T., Wang, Y. A., and Thesleff, I. (1999) Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *J. Cell Biol.* **147**, 105–120
- Tummers, M., and Thesleff, I. (2003) Root or crown: a developmental choice orchestrated by the differential regulation of the epithelial stem cell niche in the tooth of two rodent species. *Development* **130**, 1049–1057
- Tummers, M., and Thesleff, I. (2008) Observations on continuously growing roots of the sloth and the K14-Eda transgenic mice indicate that epithelial stem cells can give rise to both the ameloblast and root epithelium cell lineage creating distinct tooth patterns. *Evol. Dev.* **10**, 187–195
- Wang, X. P., Suomalainen, M., Felszeghy, S., Zelarayan, L. C., Alonso, M. T., Plikus, M. V., Maas, R. L., Chuong, C. M., Schimmang, T., and Thesleff, I. (2007) An integrated gene regulatory network controls stem cell proliferation in teeth. *PLoS Biol.* **5**, e159
- Neubüser, A., Peters, H., Balling, R., and Martin, G. R. (1997) Antagonistic interactions between FGF and BMP signaling pathways: a mechanism for positioning the sites of tooth formation. *Cell* **90**, 247–255
- St Amand, T. R., Zhang, Y., Semina, E. V., Zhao, X., Hu, Y., Nguyen, L., Murray, J. C., and Chen, Y. (2000) Antagonistic signals between BMP4 and FGF8 define the expression of Pitx1 and Pitx2 in mouse tooth-forming anlage. *Dev. Biol.* **217**, 323–332
- Lin, C. R., Kioussi, C., O'Connell, S., Briata, P., Szeto, D., Liu, F., Izpisua-Belmonte, J. C., and Rosenfeld, M. G. (1999) Pitx2 regulates lung asymmetry, cardiac positioning and pituitary and tooth morphogenesis. *Nature* **401**, 279–282
- Logan, C. Y., and Nusse, R. (2004) The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781–810
- Vadlamudi, U., Espinoza, H. M., Ganga, M., Martin, D. M., Liu, X., Engelhardt, J. F., and Amendt, B. A. (2005) PITX2, β -catenin, and LEF-1 interact to synergistically regulate the LEF-1 promoter. *J. Cell Sci.* **118**, 1129–1137
- Amen, M., Liu, X., Vadlamudi, U., Elizondo, G., Diamond, E., Engelhardt, J. F., and Amendt, B. A. (2007) PITX2 and β -catenin interactions regulate LeF-1 isoform expression. *Mol. Cell Biol.* **27**, 7560–7573
- Cha, K. B., Douglas, K. R., Potok, M. A., Liang, H., Jones, S. N., and Camper, S. A. (2004) WNT5A signaling affects pituitary gland shape. *Mech. Dev.* **121**, 183–194
- Gat, U., DasGupta, R., Degenstein, L., and Fuchs, E. (1998) *De Novo* hair follicle morphogenesis and hair tumors in mice expressing a truncated β -catenin in skin. *Cell* **95**, 605–614
- Hogan, B. L. (1999) Morphogenesis. *Cell* **96**, 225–233
- Liu, F., Chu, E. Y., Watt, B., Zhang, Y., Gallant, N. M., Andl, T., Yang, S. H., Lu, M. M., Piccolo, S., Schmidt-Ullrich, R., Taketo, M. M., Morrisey, E. E., Atit, R., Dlugosz, A. A., and Millar, S. E. (2008) Wnt/ β -catenin signaling directs multiple stages of tooth morphogenesis. *Dev. Biol.* **313**, 210–224
- Noramly, S., Freeman, A., and Morgan, B. A. (1999) β -Catenin signaling can initiate feather bud development. *Development* **126**, 3509–3521
- Thesleff, I., and Nieminen, P. (1996a) Tooth morphogenesis and cell differentiation. *Curr. Opin. Cell Biol.* **8**, 844–850
- Järvinen, E., Salazar-Ciudad, I., Birchmeier, W., Taketo, M. M., Jernvall, J., and Thesleff, I. (2006) Continuous tooth generation in mouse is induced by activated epithelial Wnt/ β -catenin signaling. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 18627–18632
- Kratochwil, K., Dull, M., Farinas, I., Galceran, J., and Grosschedl, R. (1996) Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* **10**, 1382–1394
- Sasaki, T., Ito, Y., Xu, X., Han, J., Bringas, P., Jr., Maeda, T., Slavkin, H. C., Grosschedl, R., and Chai, Y. (2005) LEF1 is a critical epithelial survival factor during tooth morphogenesis. *Dev. Biol.* **278**, 130–143
- Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297
- Cao, H., Wang, J., Li, X., Florez, S., Huang, Z., Venugopalan, S. R., Elangovan, S., Skobe, Z., Margolis, H. C., Martin, J. F., and Amendt, B. A. (2010) MicroRNAs play a critical role in tooth development. *J. Dent. Res.* **89**, 779–784
- Michon, F., Tummers, M., Kyryönen, M., Frilander, M. J., and Thesleff, I. (2010) Tooth morphogenesis and ameloblast differentiation are regulated by micro-RNAs. *Dev. Biol.* **340**, 355–368
- Mongroo, P. S., and Rustgi, A. K. (2010) The role of the miR-200 family in epithelial-mesenchymal transition. *Cancer Biol. Ther.* **10**, 219–222
- Brabletz, S., Bajdak, K., Meidhof, S., Burk, U., Niedermann, G., Firat, E., Wellner, U., Dimmler, A., Faller, G., Schubert, J., and Brabletz, T. (2011) The ZEB1/miR-200 feedback loop controls Notch signalling in cancer cells. *EMBO J.* **30**, 770–782
- Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., zur Hausen, A., Brunton, V. G., Morton, J., Sansom, O., Schuler, J., Stemmler, M. P., Herzberger, C., Hopt, U., Keck, T., Brabletz, S., and Brabletz, T. (2009) The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat. Cell Biol.* **11**, 1487–1495
- Korpala, M., Lee, E. S., Hu, G., and Kang, Y. (2008) The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J. Biol. Chem.* **283**, 14910–14914
- Gregory, P. A., Bert, A. G., Paterson, E. L., Barry, S. C., Tsykin, A., Farshid, G., Vadas, M. A., Khew-Goodall, Y., and Goodall, G. J. (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat. Cell Biol.* **10**, 593–601
- Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S., and Brabletz, T. (2008) A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. *EMBO Reports* **9**, 582–589
- Brabletz, S., and Brabletz, T. (2010) The ZEB/miR-200 feedback loop—a motor of cellular plasticity in development and cancer? *EMBO Rep.* **11**, 670–677
- Jheon, A. H., Li, C. Y., Wen, T., Michon, F., and Klein, O. D. (2011) Expression of MicroRNAs in the Stem Cell Niche of the Adult Mouse Incisor. *PLoS One* **6**, e24536

31. Cao, H., Jheon, A., Li, X., Sun, Z., Wang, J., Florez, S., Zhang, Z., McManus, M. T., Klein, O. D., and Amendt, B. A. (2013) The Pitx2:miR-200c/141: noggin pathway regulates Bmp signaling and ameloblast differentiation. *Development* **140**, 3348–3359
32. Morotomi, T., Kawano, S., Toyono, T., Kitamura, C., Terashita, M., Uchida, T., Toyoshima, K., and Harada, H. (2005) In vitro differentiation of dental epithelial progenitor cells through epithelial-mesenchymal interactions. *Arch. Oral Biol.* **50**, 695–705
33. Tucker, A., and Sharpe, P. (2004) The cutting-edge of mammalian development; how the embryo makes teeth. *Nat. Rev. Genet.* **5**, 499–508
34. Zhang, Y. D., Chen, Z., Song, Y. Q., Liu, C., and Chen, Y. (2005) Making a tooth: growth factors, transcription factors and stem cells. *Cell Res.* **15**, 301–316
35. Ikeda, E., Morita, R., Nakao, K., Ishida, K., Nakamura, T., Takano-Yamamoto, T., Ogawa, M., Mizuno, M., Kasugai, S., and Tsuji, T. (2009) Fully functional bioengineered tooth replacement as an organ replacement therapy. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 13475–13480
36. Sharpe, P. T., and Young, C. S. (2005) Test-tube teeth. *Sci. Am.* **293**, 34–41
37. Yen, A. H., and Sharpe, P. T. (2006) Regeneration of teeth using stem cell-based tissue engineering. *Expert. Opin. Biol. Ther.* **6**, 9–16
38. Shi, S., Bartold, P. M., Miura, M., Seo, B. M., Robey, P. G., and Gronthos, S. (2005) The efficacy of mesenchymal stem cells to regenerate and repair dental structures. *Orthod. Craniofac. Res.* **8**, 191–199
39. Menicanin, D., Mrozik, K. M., Wada, N., Marino, V., Shi, S., Bartold, P. M., and Gronthos, S. (2014) Periodontal-ligament-derived stem cells exhibit the capacity for long-term survival, self-renewal, and regeneration of multiple tissue types *in vivo*. *Stem Cells Dev.* **23**, 1001–1011
40. Menicanin, D., Bartold, P. M., Zannettino, A. C., and Gronthos, S. (2010) Identification of a common gene expression signature associated with immature clonal mesenchymal cell populations derived from bone marrow and dental tissues. *Stem Cells Dev.* **19**, 1501–1510
41. Gronthos, S., Arthur, A., Bartold, P. M., and Shi, S. (2011) A method to isolate and culture expand human dental pulp stem cells. *Methods Mol. Biol.* **698**, 107–121
42. Hynes, K., Menicanin, D., Mrozik, K., Gronthos, S., and Bartold, P. M. (2014) Generation of functional mesenchymal stem cells from different induced pluripotent stem cell lines. *Stem Cells Dev.* **23**, 1084–1096
43. Takahashi, K., and Yamanaka, S. (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676
44. Ivey, K. N., and Srivastava, D. (2010) MicroRNAs as regulators of differentiation and cell fate decisions. *Cell Stem Cell* **7**, 36–41
45. Martinez N. J., and Gregory, R. I. (2010) MicroRNA gene regulatory pathways in the establishment and maintenance of ESC identity. *Cell Stem Cell* **7**, 31–35
46. Amendt, B. A., Sutherland, L. B., and Russo, A. F. (1999) Multifunctional role of the Pitx2 homeodomain protein C-terminal tail. *Mol. Cell Biol.* **19**, 7001–7010
47. Cox, C. J., Espinoza, H. M., McWilliams, B., Chappell, K., Morton, L., Hjalt, T. A., Semina, E. V., and Amendt, B. A. (2002) Differential regulation of gene expression by PITX2 isoforms. *J. Biol. Chem.* **277**, 25001–25010
48. Amendt, B. A., Sutherland, L. B., Semina, E. V., and Russo, A. F. (1998) The molecular basis of Rieger syndrome: analysis of Pitx2 homeodomain protein activities. *J. Biol. Chem.* **273**, 20066–20072
49. Filali, M., Cheng, N., Abbott, D., Leontiev, V., and Engelhardt, J. F. (2002) Wnt-3A/ β -catenin signaling induces transcription from the LEF-1 promoter. *J. Biol. Chem.* **277**, 33398–33410
50. Zhang, Z., Wlodarczyk, B. J., Niederreither, K., Venugopalan, S., Florez, S., Finnell, R. H., and Amendt, B. A. (2011) Fuz regulates craniofacial development through tissue specific responses to signaling factors. *PLoS One* **6**, e24608
51. Cao, H., Florez, S., Amen, M., Huynh, T., Skobe, Z., Baldini, A., and Amendt, B. A. (2010) Tbx1 regulates progenitor cell proliferation in the dental epithelium by modulating Pitx2 activation of p21. *Dev. Biol.* **347**, 289–300
52. Amen, M., Espinoza, H. M., Cox, C., Liang, X., Wang, J., Link, T. M., Brennan, R. G., Martin, J. F., and Amendt, B. A. (2008) Chromatin-associated HMG-17 is a major regulator of homeodomain transcription factor activity modulated by Wnt/ β -catenin signaling. *Nucleic Acids Res.* **36**, 462–476
53. Cui, X. M., Shiomi, N., Chen, J., Saito, T., Yamamoto, T., Ito, Y., Bringas, P., Chai, Y., and Shuler, C. F. (2005) Overexpression of Smad2 in Tgf- β 3-null mutant mice rescues cleft palate. *Dev. Biol.* **278**, 193–202
54. Venugopalan, S. R., Amen, M. A., Wang, J., Wong, L., Cavender, A. C., D'Souza, R. N., Akerlund, M., Brody, S. L., Hjalt, T. A., and Amendt, B. A. (2008) Novel expression and transcriptional regulation of FoxJ1 during oro-facial morphogenesis. *Hum. Mol. Genet.* **17**, 3643–3654
55. Chavez, M. G., Yu, W., Biehs, B., Harada, H., Snead, M. L., Lee, J. S., Desai, T. A., and Klein, O. D. (2013) Characterization of dental epithelial stem cells from the mouse incisor with two-dimensional and three-dimensional platforms. *Tissue Eng. Part C Methods* **19**, 15–24
56. Chavez, M. G., Hu, J., Seidel, K., Li, C., Jheon, A., Naveau, A., Horst, O., and Klein, O. D. (2014) Isolation and culture of dental epithelial stem cells from the adult mouse incisor. *J. Vis. Exp.* 10.3791/51266
57. Kuang-Hsien Hu, J., Mushegyan, V., and Klein, O. D. (2014) On the cutting edge of organ renewal: Identification, regulation, and evolution of incisor stem cells. *Genesis* **52**, 79–92
58. Zhang, Z., Florez, S., Gutierrez-Hartmann, A., Martin, J. F., and Amendt, B. A. (2010) MicroRNAs regulate pituitary development, and microRNA 26b specifically targets lymphoid enhancer factor 1 (Lef-1), which modulates pituitary transcription factor 1 (Pit-1) expression. *J. Biol. Chem.* **285**, 34718–34728
59. Chen, L. S., Couwenhoven, R. I., Hsu, D., Luo, W., and Snead, M. L. (1992) Maintenance of amelogenin gene expression by transformed epithelial cells of mouse enamel organ. *Arch. Oral Biol.* **37**, 771–778
60. Hanks, C. T., Fang, D., Sun, Z., Edwards, C. A., and Butler, W. T. (1998) Dentin-specific proteins in MDP-23 cell line. *Eur. J. Oral Sci.* **106**, 260–266
61. Goecks, J., Nekrutenko, A., Taylor, J., and Galaxy Team (2010) Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* **11**, R86
62. Blankenberg, D., Von Kuster, G., Coraor, N., Ananda, G., Lazarus, R., Mangan, M., Nekrutenko, A., and Taylor, J. (2010) “Galaxy: a web-based genome analysis tool for experimentalists.” *Curr. Protoc. Mol. Biol.* 10.1002/0471142727.mb1910s89
63. Giardine, B., Riemer, C., Hardison, R. C., Burhans, R., Elnitski, L., Shah, P., Zhang, Y., Blankenberg, D., Albert, I., Taylor, J., Miller, W., Kent, W. J., and Nekrutenko, A. (2005) “Galaxy: a platform for interactive large-scale genome analysis.” *Genome Res.* **15**, 1451–1455
64. Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L., and Pachter, L. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578
65. Saldanha, A. J. (2004) Java Treeview: extensible visualization of microarray data. *Bioinformatics* **20**, 3246–3248
66. de Hoon, M. J., Imoto, S., Nolan, J., and Miyano, S. (2004) Open source clustering software. *Bioinformatics* **20**, 1453–1454
67. Mucchielli, M. L., Mitsiadis, T. A., Raffo, S., Brunet, J. F., Proust, J. P., and Goridis, C. (1997) Mouse Otx2/RIEG expression in the odontogenic epithelium precedes tooth initiation and requires mesenchyme-derived signals for its maintenance. *Dev. Biol.* **189**, 275–284
68. Hjalt, T. A., Semina, E. V., Amendt, B. A., and Murray, J. C. (2000) The Pitx2 protein in mouse development. *Dev. Dyn.* **218**, 195–200
69. Xia, H., Cheung, W. K., Sze, J., Lu, G., Jiang, S., Yao, H., Bian, X. W., Poon, W. S., Kung, H. F., and Lin, M. C. (2010) miR-200a regulates epithelial-mesenchymal to stem-like transition via ZEB2 and β -catenin signaling. *J. Biol. Chem.* **285**, 36995–37004
70. Stockinger, A., Eger, A., Wolf, J., Beug, H., and Foisner, R. (2001) E-cadherin regulates cell growth by modulating proliferation-dependent β -catenin transcriptional activity. *J. Cell Biol.* **154**, 1185–1196
71. Chen, S., Lewallen, M., and Xie, T. (2013) Adhesion in the stem cell niche: biological roles and regulation. *Development* **140**, 255–265
72. Li, C. Y., Cha, W., Luder, H. U., Charles, R. P., McMahon, M., Mitsiadis, T. A., and Klein, O. D. (2012) E-cadherin regulates the behavior and fate of epithelial stem cells and their progeny in the mouse incisor. *Dev. Biol.* **366**,

- 357–366
73. Karpowicz, P., Willaime-Morawek, S., Balenci, L., DeVeale, B., Inoue, T., and van der Kooy, D. (2009) E-Cadherin regulates neural stem cell self-renewal. *J. Neurosci.* **29**, 3885–3896
 74. DenBesten, P. K., Machule, D., Zhang, Y., Yan, Q., and Li, W. (2005) Characterization of human primary enamel organ epithelial cells *in vitro*. *Arch. Oral Biol.* **50**, 689–694
 75. Nakahori, Y., Takenaka, O., and Nakagome, Y. (1991) A human X-Y homologous region encodes “amelogenin.” *Genomics* **9**, 264–269
 76. Snead, M. L., Lau, E. C., Zeichner-David, M., Fincham, A. G., Woo, S. L., and Slavkin, H. C. (1985) DNA sequence for cloned cDNA for murine amelogenin reveal the amino acid sequence for enamel-specific protein. *Biochem. Biophys. Res. Commun.* **129**, 812–818
 77. Peters, H., and Balling, R. (1999) Teeth, where and how to make them. *TIG* **15**, 59–65
 78. Jernvall, J., and Thesleff, I. (2000) Reiterative signaling and patterning during mammalian tooth morphogenesis. *Mech. Dev.* **92**, 19–29
 79. Zeichner-David, M., Diekwisch, T., Fincham, A., Lau, E., MacDougall, M., Moradian-Oldak, J., Simmer, J., Snead, M., and Slavkin, H. C. (1995) Control of ameloblast differentiation. *Int. J. Dev. Biol.* **39**, 69–92
 80. Mustonen, T., Tümmers, M., Mikami, T., Itoh, N., Zhang, N., Gridley, T., and Thesleff, I. (2002) Lunatic fringe, FGF, and BMP regulate the Notch pathway during epithelial morphogenesis of teeth. *Dev. Biol.* **248**, 281–293
 81. Limeback, H. (1987) Enamel protein and collagen production by cells subcultured from porcine tooth bud explants. *Biochem. Cell Biol.* **65**, 698–709
 82. Farges, J. C., Couble, M. L., Joffre, A., Hartmann, D. J., and Magloire, H. (1991) Morphological and immunocytochemical characterization of cultured rat incisor cervical epithelial cells. *Arch. Oral Biol.* **36**, 737–745
 83. Tabata, M. J., Matsumura, T., Liu, J. G., Wakisaka, S., and Kurisu, K. (1996) Expression of cytokeratin 14 in ameloblast-lineage cells of the developing tooth of rat, both *in vivo* and *in vitro*. *Arch. Oral Biol.* **41**, 1019–1027
 84. Matsumura, T., Tabata, M. J., Wakisaka, S., Sakuda, M., and Kurisu, K. (1998) Ameloblast-lineage cells of rat tooth germs proliferate and scatter in response to hepatocyte growth factor in culture. *Int. J. Dev. Biol.* **42**, 1137–1142
 85. Tabata, M. J., Matsumura, T., Fujii, T., Abe, M., and Kurisu, K. (2003) Fibronectin accelerates the growth and differentiation of ameloblast lineage cells *in vitro*. *J. Histochem. Cytochem.* **51**, 1673–1679
 86. Kawano, S., Saito, M., Handa, K., Morotomi, T., Toyono, T., Seta, Y., Nakamura, N., Uchida, T., Toyoshima, K., Ohishi, M., and Harada, H. (2004) Characterization of dental epithelial progenitor cells derived from cervical-loop epithelium in a rat lower incisor. *J. Dent. Res.* **83**, 129–133
 87. Harada, H., Toyono, T., Toyoshima, K., Yamasaki, M., Itoh, N., Kato, S., Sekine, K., and Ohuchi, H. (2002) FGF10 maintains stem cell compartment in developing mouse incisors. *Development* **129**, 1533–1541
 88. Harada, H., and Ohshima, H. (2004) New perspectives on tooth development and the dental stem cell niche. *Arch. Histol. Cytol.* **67**, 1–11
 89. Maas, R., and Bei, M. (1997) The genetic control of early tooth development. *Crit. Rev. Oral. Biol. Med.* **8**, 4–39
 90. Thesleff, I., and Tümmers, M. (2009) Tooth organogenesis and regeneration. The Stem Cell Research Community, *StemBook*
 91. Yoo, A. S., Sun, A. X., Li, L., Shcheglovitov, A., Portmann, T., Li, Y., Lee-Messer, C., Dolmetsch, R. E., Tsien, R. W., and Crabtree, G. R. (2011) MicroRNA-mediated conversion of human fibroblasts to neurons. *Nature* **476**, 228–231
 92. Gay, I., Cavender, A., Peto, D., Sun, Z., Speer, A., Cao, H., and Amendt, B. A. (2014) Differentiation of human dental stem cells reveals a role for microRNA-218. *J. Periodontol. Res.* **49**, 110–120
 93. Cui, L., Guan, Y., Qu, Z., Zhang, J., Liao, B., Ma, B., Qian, J., Li, D., Li, W., Xu, G. T., and Jin, Y. (2013) WNT signaling determine tumorigenicity and function of ESC-derived retinal progenitors. *J. Clin. Invest.* **123**, 1647–1661
 94. Liu, F., and Millar, S. E. (2010) Wnt/ β -catenin signaling in oral tissue development and disease. *J. Dent. Res.* **89**, 318–330
 95. Bei, M. (2009) Molecular genetics of tooth development. *Curr. Opin. Genet. Dev.* **19**, 504–510
 96. Liu, W., Selever, J., Lu, M. F., and Martin, J. F. (2003) Genetic dissection of *Pitx2* in craniofacial development uncovers new functions in branchial arch morphogenesis, late aspects of tooth morphogenesis and cell migration. *Development* **130**, 6375–6385