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TISSUE-SPECIFIC STEM CELLS

STEM CELLS

The *miR-200* family is required for ectodermal organ development through the regulation of the epithelial stem cell niche

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Abstract

The murine lower incisor ectodermal organ contains a single epithelial stem cell (SC) niche that provides epithelial progenitor cells to the continuously growing rodent incisor. The dental stem cell niche gives rise to several cell types and we demonstrate that the *miR-200* family regulates these cell fates. The *miR-200* family is highly enriched in the differentiated dental epithelium and absent in the stem cell niche. In this study, we inhibited the *miR-200* family in developing murine embryos using new technology, resulting in an expanded epithelial stem cell niche and lack of cell differentiation. Inhibition of individual miRs within the *miR-200* cluster resulted in differential developmental and cell morphology defects. *miR-200* inhibition increased the expression of dental epithelial stem cell markers, expanded the stem cell niche and decreased progenitor cell differentiation. RNA-seq. identified *miR-200* regulatory pathways involved in cell differentiation and compartmentalization of the stem cell niche. The *miR-200* family regulates signaling pathways required for cell differentiation and cell cycle progression. The inhibition of *miR-200* decreased the size of the lower incisor due to increased autophagy and cell death. New *miR-200* targets demonstrate gene networks and pathways controlling cell differentiation and maintenance of the stem cell niche. This is the first report demonstrating how the *miR-200* family is required for in vivo progenitor cell proliferation and differentiation.

KEYWORDS
ectoderm, epigenetics, microRNA, progenitor cells, stem cell expansion, stem cell plasticity, tissue-specific stem cells, transgenic mouse

1 | INTRODUCTION

The regulation of gene expression by noncoding RNAs, including microRNAs (miRs), have been shown to contribute to the development of different organs, including teeth.[1-4] At each stage, a precise gene expression profile is required in both the epithelial and mesenchymal tissues for organogenesis to proceed. Several major signaling pathways, including Wnt, Bmp, Hedgehog, TGF-β, Eda, and Hippo signaling pathways are required to orchestrate tooth development by controlling the expression of target genes,

including transcription factors, within the dental epithelium and mesenchyme.[5-9] However, other mechanisms to regulate gene expression such as noncoding RNAs and epigenetics have begun to be examined for their roles in ectodermal organ development.

Our group has overcome the technical challenges of studying miRs and has developed a Plasmid based *miR* Inhibitor System (PMIS).[10] The inhibitor encodes a 122 nt structured transcript which targets miRs for suppression by seed sequence homology, allowing a single inhibitor to functionally inhibit miRs with identical seed sequences. The PMIS was engineered to bind RISC factors associated with mature miR processing to create a high affinity binding of the miR to the inhibitor, increase efficiency and specificity. The PMIS system can be used to establish stable cell and mouse lines for functional studies in vitro and in vivo.[10-12]

Murine lower incisor organization makes it an attractive model for studying how miRs govern stem cell differentiation. Dental epithelial stem cells (DESCs) are compartmentalized within the labial cervical loop (LaCL) and begin to differentiate as they migrate out of this stem cell niche compartment (top panel, Figure 1). Individual stem cell niches use distinct combinations of stem cell factors and signaling molecules to control stem cell self-renewal and proliferation. Stem cells must reside in the niche to maintain long-term self-renewal, but how they are confined remains unresolved. The DESC population is self-maintaining throughout the lifetime of the animal.[13,14] DESCs express high levels of the transcription factors *Sox2*, *Bmi1* and *Pitx2*, and have been shown to give rise to all the different types of cells composing the lower incisor.[15] DESCs differentiate at the transient amplifying zone, a stretch of cells that separates the LaCL from the rest of the lower incisor.[16,17] Many studies have shed light on the spatio-temporal cues that regulate the differentiation of the dental epithelium, but the nature of these cues is not entirely understood. We demonstrate in this report how dental epithelial stem cells are maintained in their niche through *miR-200* regulation of stem cell factors, signaling pathways and autophagy mechanisms.

By profiling the expression of miRs within the stem cell niche and in the differentiating dental epithelial tissues, several miR families have emerged as likely candidates for regulating DESC differentiation. The *miR-200* family encodes five mature miRs that compose two different functional groups, which are defined by seed sequence homology (Supporting Information Figure S1A). *miR-200a/141* and *miR-200b/c/429* share seed sequences and thus target the same set of mRNAs.[18] Interestingly, even though the seed regions of each functional group only differ by a single nucleotide, they may regulate predominantly nonoverlapping sets of genes.[19] Each group has also been shown to target transcription factors known to be required for ectoderm organ development including *Sox2*, *Klf4* (*miR-200c*), and *Pitx2* (*miR-200a*).[3,4,20] We have previously reported that the *miR-200* family is not expressed in the DESCs and labial cervical loop region[2,21]; however, the *miR-200* family are expressed and associated with differentiating dental epithelial cells. By constructing *PMIS-miR-200a* and *PMIS-miR-200c* inhibition mouse lines, we examined in vivo how inhibiting either functional group independently, or, both at once by crossing the lines, affected stem cell maintenance and differentiation.

Significance statement

Current microRNA (miR) inhibition methods cannot be used to study in vivo developmental stem cell processes. CRISPR-Cas genome editing cannot specifically knockout a miR within a cluster. Furthermore, not all miRs, especially within introns can be targeted by the CRISPR method without affecting gene expression. Embryonic Stem (ES) cells have been profiled for miR expression; however, cell-based assays using oligonucleotides targeting miRs are not specific and are toxic. The authors developed a highly specific, effective miR inhibitor that can be used to knockdown miRs during embryonic development to determine their effect on stem cells, cell proliferation, and differentiation. The authors show that the *miR-200* family acts to compartmentalize an ectodermal stem cell niche by regulating progenitor cell differentiation during development.

2 | MATERIALS AND METHODS

2.1 | Animals

Mice were housed and handled in accordance with guidelines established by the University of Iowa Institutional Animal Care and Use committee. All experimental techniques were approved by the University of Iowa IACUC guidelines. The construction of *PMIS* inhibitor mice was previously described.[10] To obtain double inhibitor mice, we crossed the *PMIS-miR-200a* and *PMIS-miR-200b* mouse lines. E0.5 was designated upon finding a vaginal plug.

2.2 | RNA sequencing

Poly(A) sequencing libraries were prepared using the Illumina TruSeq-stranded-mRNA protocol, after RNA quality was assessed using an Agilent Technologies 2100 Bioanalyzer. Adenylated mRNAs were isolated using oligo-(dt) magnetic beads (two rounds) and fragmented using elevated temperature in a divalent cation buffer. After library construction and quality control, sequencing was performed on the NovaSeq 6000 sequencing system by Illumina. The bioinformatics pipeline included: Cutadapt (removes adaptor contamination), HISAT2 (read mapping), StringTie (assembly), and then transcriptomes were merged using perl scripts and gffcompare. StringTie

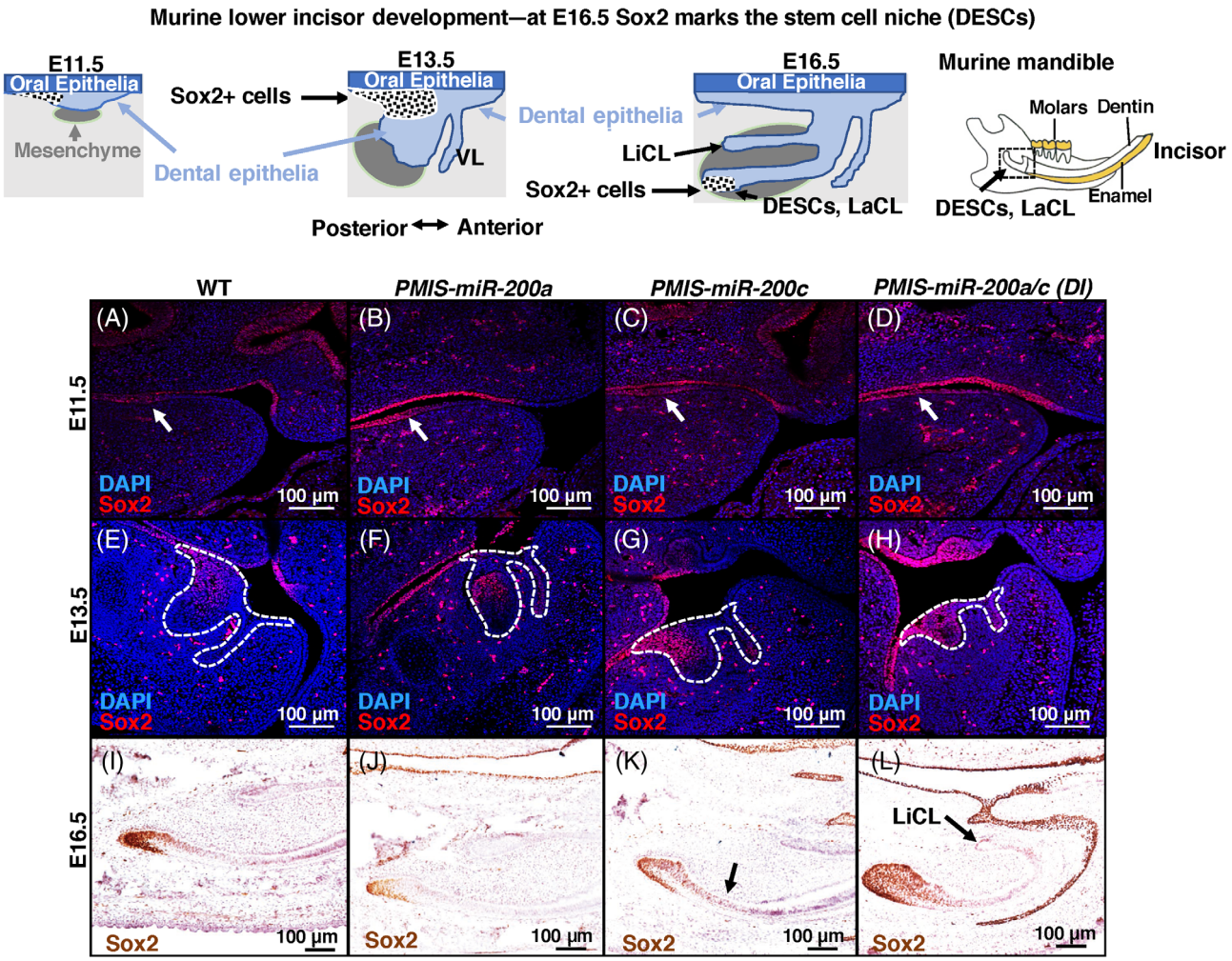


FIGURE 1 The *miR-200* family regulates *Sox2* expression in the stem cell niche. A depiction of lower incisor early development is shown in the top panel. The dental epithelial cells invaginate from a dental placode into the mandible and Sox2+ cells become localized to the LaCL by E16.5. The LaCL contains the dental epithelial stem cells (DESCs) which produce progeny to populate the cell layers of the continuously growing rodent incisor. A-D, *Sox2* is expressed in the oral epithelium and dental placodes of WT and inhibitor mice at E11.5. E-H, At E13.5, Sox2 is expressed in the oral epithelium as well as in a subset of posterior lower incisor tooth bud cells. The inhibition of *miR-200c* and the entire *miR-200* family results in a smaller tooth bud. When the entire *miR-200* family is inhibited, every cell in the lower incisor tooth bud is *Sox2+* at this stage. I-L, At E16.5, in WT and *PMIS-miR-200a* embryos, *Sox2* expression is compartmentalized within the labial cervical loop and only expressed by DESCs. When the *miR-200c* functional group is inhibited, *Sox2* expression is decompartmentalized, and transient amplifying cells as well as stellate reticulum cells are *Sox2+*. In *PMIS-miR-200a/c* lower incisors the entire, expanded cervical loop region is *Sox2+*. *Sox2* is expressed in the transient amplifying (TA) region as stratum intermedium (SI), stellate reticulum (SR), and lingual cervical loop (LiCL)

as well as edgeR were then used to estimate expression levels of the transcripts. RNA-sequencing and analyses were performed by LC Sciences (Houston, Texas).

2.3 | BrdU labeling

BrdU was injected interperitoneally in pregnant females and allowed to incubate for 2 hours. After incubation, females were sacrificed, and embryos were prepared for paraffin section as previously described. Sections were processed according to the IF protocol described below.

2.4 | RNA isolation from tissue

Fresh tissue was dissected and RNA was isolated by flash freezing in liquid nitrogen and pulverization. The pulverized tissue was processed using the miRNeasy Mini kit by Qiagen to isolate total RNA and miR. RNA quality was assessed by gel electrophoresis prior to RT applications.

2.5 | RT-qPCR for mRNA and miRNA

For mRNA, 0.5-1 μg of total RNA was used in an RT reaction with the 5x primescript RT kit (Takara Bio). cDNA was diluted 1:5 and 1 μL was

used per qPCR reaction. qPCR reactions were normalized to *b-actin* levels. For miRNA, RT was performed using the Qiagen miScript kit. Resultant cDNA was diluted 1:5 and 1 μL was used per qPCR reaction. qPCR probes for specific genes were provided by Qiagen, as well as the normalization set for murine U6.

Gene	Forward primer	Reverse primer
β-actin	ctcttccagccttccttc	atctccttctgcatcctgtc
GLI2	tggccgcttcagatgacagatgttg	cgttagccgaatgtcagccgtgaag
GLI3	ggccatccacatggaatatc	tgaagagctgctacgggaat
PMIS-miR-200a	ctaaacatcgttaccaatcagacagtg	gtcagctcttagtattcatgagatg
PMIS-miR-200c	ctaatccatcattacccatcagacagtatta	gtcagctcttagtattcatgagatg

2.6 | Paraffin embedding and sectioning

After dissection embryos were fixed from 0.5 to 4 hours, 4% PFA, at room temperature, and dehydrated through an ethanol gradient. After dehydration, Tissues were precleared with xylene and incubated with three changes of paraffin, and then finally placed into blocks. In order to produce slides, 7 μm sections were cut from paraffin blocks, floated out in a 45°C water bath, and mounted (VWR Vistavision slides). Prior to staining, slides were deparaffinized an rehydrated through a reverse ethanol gradient. H&E staining has been previously described.[3]

2.7 | Immunohistochemistry

IHC was carried out using the protocol highlighted in the Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (ABCAM ab64264). Briefly, after rehydration, slides were subjected to antigen retrieval by boiling in citrate buffer (antigen unmasking solution, citric acid based, Vector) and allowed to slowly cool to room temperature. Slides were washed in PBS and blocked (protein, and then hydrogen peroxide block), and then incubated with primary antibody overnight (4°C). Slides were washed and then incubated secondary (anti-rabbit/anti-mouse biotinylated) and tertiary antibodies (streptavidin-HRP) to label targets with HRP, incubated for 30 seconds to 1 minute with DAB substrate. Slides were counterstained with hematoxylin and coverslips were added before imaging.

2.8 | Immunofluorescence

After paraffin sectioning, antigen retrieval was performed by boiling slides in citrate buffer from 12 to 20 minutes. Slides were washed (PBS) and blocked for 30+ minutes with 20% Donkey serum. Primary antibodies (see below) diluted in PBS were applied to the slide in a humidified chamber overnight, 4°C. The following day, slides were washed (3×, PBS), and secondary antibody was applied (1:300 dilution, Donkey anti Goat 594, Rabbit 488, or Mouse 488/594, Alexa Fluor). Slides were washed 3× (PBS) and counterstained with DAPI. Slides were washed 3× (PBS) and mounted (Aqua-mount, Lerner Labs). Images were taken using a Zeiss 700 confocal imaging system.

LS-8 cells[22] were seeded on cover slips, allowed to grow for 12 hours. Cells were then incubated in normal media (10%FBS) or media with 0.5% FBS and Chloroquine (5 μM) for 8 hours. Cells were fixed in 4%PFA, permeabilized with 0.2% triton and blocked with 10% goat serum. Slips were incubated overnight with LC3AB antibody (Cell Signaling Inc) at 1/2000 dilution at 4°. Slips were washed with PBS-tween ×4, and Goat anti rabbit A568 (Invitrogen) was incubated for an hour at RT. Cells were washed as above, treated with Mounting/Dapi (Vector labs) and placed on slides. Images were taken using a Zeiss 700 confocal microscope. Data shown is A568/Dapi merge.

Antibody	Company/number	Dilution
P63	Biocare Medical/CM163A	1:50
Cdh1	BD Transduction/610 181	1:200
BrdU	Abcam/ab6326	1:250
P21	BD Transduction	1:500
Lef-1	Cell Signaling/2230	1:100
LC3B	Cell Signaling/D11	1:50
DSPP	Santa Cruz/18 328	1:50
Desmoplakin	BioRad/DP-2.15	1:50
Irx1	Sigma Prestige/A043160	1:50
Sox2 (IF)	R&D Systems/AF2018	1:50
Sox2 (IHC)	Abcam/ ab92494	1:100
Pitx2	R&D Systems/AF7388	1:100
Tbx1	Zymed	1:100
Isl1	DSHB	1:10
GFP	Sigma	1:150
LC3AB	Cell Signaling	1:2000
GAPDH	Santa Cruz	1:8000
CC3	Cell Signaling/9661	1:500

2.9 | TUNEL staining

Seven micrometer paraffin sections baked to slides were rehydrated. The DeadEnd Fluorometric TUNEL system (Promega, G3250) protocol and kit was utilized to label dying cells. Images were taken with the Zeiss 700 confocal imaging system.

2.10 | 3-D reconstruction of lower incisors

Three-dimensional (3D) reconstruction of the lower incisors in WT, *PMIS-miR-200a*, *PMIS-miR-200c*, and *PMIS-miR-200a/c* lower incisors

was performed by serial sectioning the complete hemi-mandible and staining each section with H&E. Each section containing the lower incisor was then imaged (approximately 30 sections per lower incisor) using a Nikon Eclipse 80*i* microscope. The individual images were stacked using the stackreg function on ImageJ, and then loaded into Imaris to construct the 3D image.

2.11 | Statistical methods

P values were calculated using two tailed, unpaired *T* tests using at least three biological replicates for each group. Error bars are presented as ±SE of the mean in every figure. * indicates a *P* value of <.05, **<.01, ***<.001, ****<.0001.

3 | RESULTS

3.1 | The *miR-200* family is specifically inhibited in *PMIS-miR-200* embryos

The *miR-200* family is composed of five mature miRs which are in two clusters found on chromosome 1 and 12 in humans and chromosome 4 and 6 in mice. Two seed sequences are shared among the five mature miRs composing the family; these sequences differ by a single nucleotide and allow the miRs to target overlapping and non-overlapping pools of mRNAs (Supporting Information Figure S1A). Our group constructed mouse lines expressing the *PMIS* inhibitor for the *miR-200a/141* (*PMIS-miR-200a*) and *miR-200b/c/429* (*PMIS-miR-200c*) functional groups. Crossing the mice produced embryos expressing both inhibitor systems (*PMIS-miR-200a/c*, or double inhibitor [DI] embryos). The DI embryos were smaller than *PMIS-miR-200a* or *PMIS-miR-200c* E16.5 littermates (Supporting Information Figure S1B). Most of the DI embryos were embryonic lethal and only well-formed living E16.5 embryos were used in the following experiments.

Because the *PMIS* inhibitor is expressed as an RNA molecule, levels of the inhibitor transcript can be determined using RT-qPCR. Mandibular RNA (including the lower incisor) was isolated from WT and *PMIS-miR-200a/c* littermates and *PMIS* expression was measured (Supporting Information Figure S1C,D). High levels of PMIS expression were found in the DI embryos, and from the same samples, RT-qPCR demonstrated a reduction in the detectable levels of the entire *miR-200* family (Supporting Information Figure S1E-I). We detect residual levels of target miRs because the PMIS system binds the miRs with a high affinity and forms a stable PMIS-miR complex that is slowly degraded in cells. Thus, some miRs remain attached to the PMIS and are PCR amplified when the tissues/cells are lysed resulting in the detection of residual levels of miRs. Upon functional testing we have shown that the PMIS inhibits over 90% of miR activity.[10,11]

Previously, we reported that the function of the PMIS inhibitor is dependent on seed sequence homology, and that the substitution of a single base within the seed sequence prevents inhibition.[10,11] In order to confirm the specificity of the system in vivo, we identified miRs with three different types of relationships to the *miR-200* family: miRs containing an identical seed sequence, but with one base substitution, 1MM; miRs containing an identical seed sequence, but located in a different position on the miR, SS; and miRs with little homology to the *miR-200* family, MH (Supporting Information Figure S2A). Using RT-qPCR, the level of these miRs was then quantitated and compared between WT and *PMIS-miR-200a/c* samples. After examining 1 MM, 3 SS, and 3 MH miRs, we found that there was no significant difference in the levels of these miRs in *PMIS* inhibitor mice compared with WT controls and no effect on their functional targets (Supporting Information Figure S2B-D). The lack of inhibition of miRs selected for their similarity to the *miR-200* family demonstrates that the PMIS system specifically inhibits the *miR-200* family.

3.2 | The *miR-200* family compartmentalizes *Sox2* expression within the stem cell niche

Sox2 is required for dental stem cell self-renewal and has been validated as a direct target of *miR-200c*.[2,20,21] A depiction of early tooth development, with the location of the Sox2+ cells, the invaginating dental epithelia, formation of the lingual and labial cervical loops (LiCL and LaCL, respectively), the dental epithelial stem cells (DESCs) located in the LaCL and the adult murine mandible showing the molars and incisor are shown in the top panel of Figure 1. *Sox2* expression was determined in WT and *miR-200* inhibitor lower incisors at different developmental stages (Figure 1).[13,23] In E11.5 embryos, when a thickening in the oral epithelia forms a dental placode, *Sox2* was expressed in the posterior region of the placode in all genotypes. This expression pattern is consistent with previous reports of *Sox2* expression in E11.5 embryos (Figure 1A-D, arrow).[13]

At E13.5, the bud stage WT lower incisor expresses *Sox2* in the posterior region (Figure 1E), the *PMIS-miR-200a* incisor has reduced Sox2 expression and *PMIS-miR-200c* tooth buds have an increased Sox2 expression pattern (Figure 1F-G), although the tooth bud from *PMIS-miR-200c* lower incisors is smaller. Interestingly, in the *PMIS-miR-200a/c* or double inhibitor (DI) embryos, the entire tooth bud expresses Sox2 (Figure 1H), demonstrating that the *miR-200* family plays a role in compartmentalizing *Sox2* expression at this stage.

At E16.5, Sox2 expression is restricted to the posterior of the labial cervical loop stem cell niche in the lower incisor in WT and *PMIS-miR-200a* lower incisors (Figure 1I-J). Interestingly, when *miR-200c* is inhibited, *Sox2* expression is expanded within the stem cell niche, and cells exiting the loop in the transient amplifying cells (TAC) retain *Sox2* expression (Figure 1K, arrow). In the DI lower incisor, the LaCL stem cell niche is enlarged, and all the cells within the niche express Sox2 including the stellate reticulum (SR), outer enamel epithelium (OEE) and TAC (Figure 1L, magnified in Figure 2). Interestingly, Sox2 expression is not only expanded within the LaCL of the DI embryo, but is also activated in the lingual cervical loop (LiCL), where it is not expressed in WT embryos (Figures 1L and 2A,B). Cells throughout the lower incisor are *Sox2+* in the DI embryos (Figure 1L).

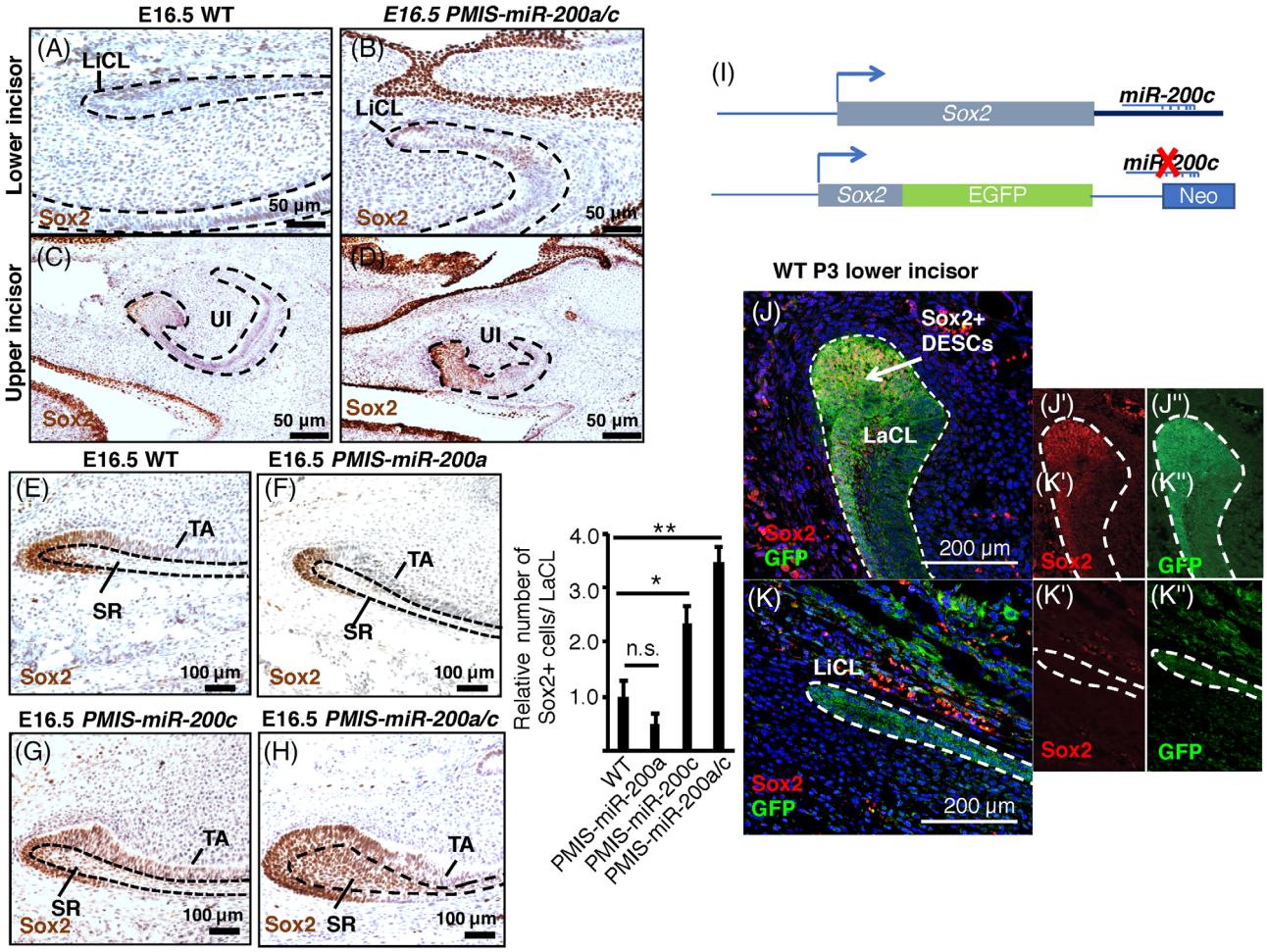


FIGURE 2 The *miR-200* family regulates *Sox2* expression in the lower incisor lingual and labial cervical loops and the upper incisor. A and B, IHC for *Sox2* protein reveals that while it is not expressed in the lingual cervical loop (LiCL) or incisor tip in WT E16.5 embryos, it is activated when both functional groups of the *miR-200* family are inhibited. C and D, Cells in the cervical loop of the WT upper incisor express Sox2 protein at E16.5. Upon inhibition of the *miR-200* family, Sox2 protein is more highly expressed and expanded in the upper incisor. E-H, Higher magnification of *Sox2* staining in the lower incisor labial cervical loop of the WT E16.5 embryo; the *PMIS-miR-200a* E16.5 embryo; the *PMIS-miR-200c* E16.5 embryo; and the *PMIS-miR-200a/c* E16.5 embryo. The relative number of Sox2+ cells in the LaCL were quantitated and values expressed from 3 or more biological replicates. The *Sox2* 3′UTR regulates its expression during incisor development. I, A schematic of the WT *Sox2* allele and the reporter line obtained from Jackson labs. The knock in of GFP eliminates the endogenous *miR-200c* binding site of the *Sox2* locus. J, J′, and J″, Staining for endogenous Sox2 protein (red) and GFP protein (green) in the LaCL region. Sox2 and GFP are coexpressed in the LaCL, but the GFP expression region is larger. J′ and J″ show single channels of J. K, K′, and K″, Staining for endogenous Sox2 protein (red) and GFP protein (green) in the LiCL region of the lower incisor and no endogenous Sox2 protein is detectable by IF, but GFP protein is present. K′ and K″ show single channels of K

Sox2 expression in the upper incisor was also increased in the *PMIS-miR-200* embryos. WT upper incisors (UI) at E16.5 express *Sox2* in the posterior cervical loop (Figure 2C); however, *Sox2* expression was increased and expanded outside of the stem cell niche in DI upper incisors (Figure 2D). Higher magnification of the E16.5 lower incisor LaCL shows the Sox2+ cells in WT (Figure 2E), in *PMIS-miR-200a* (Figure 2F), expanded into the SR and TA cells in *PMIS-miR-200c* (Figure 2G) and greatly expanded into these regions in the DI LaCL (Figure 2H). Quantitation of the number of Sox2+ cells in the LaCL is shown.

To better distinguish the type of cells in the expanded LaCL and TA regions we assayed for Pitx2 and Tbx1 expression by immunofluorescence and immunohistochemistry, respectively. We previously reported that both Pitx2 and Tbx1 mark both of these regions. Pitx2 expression is expanded in the TA (transient amplying) and SR (stellate reticulum) regions in the *PMIS-miR-200a/c* embryos (Supporting Information Figure S3). Tbx1 expression marks the stem cell region in the LaCL and TA cells and is also expanded in the *PMIS-miR-200a/c* embryos (Supporting Information Figure S3). *Pitx2* is directly regulated by *miR-200a*,[4] while *Tbx1* is not directly regulated by these miRs.

To confirm the regulation of *Sox2* expression by the *miR-200* family, we took advantage of a *Sox2* reporter mouse line (Jackson Labs 017592) where the GFP ORF has been knocked into the endogenous

Sox2 locus. An unintended consequence of generating the reporter line was the ablation of the *Sox2* endogenous 3′ UTR, which removes regulation by the *miR-200* family (Figure 2I). In order to examine the expression of the unregulated reporter compared with the endogenous *miR*-regulated Sox2 protein, WT P4 lower incisors were costained for EGFP and Sox2 protein. The LaCL and LiCL were imaged and the red (Sox2) and green (GFP) channels were examined separately (Figure 2J′-J″,K′-K″). In the LaCL, both Sox2 and GFP were detected, although the expression domain of GFP protein was greater than the endogenous Sox2 protein. In the LiCL, no native Sox2 was detectable (Figure 2K′); however, GFP expression was detected in this region (Figure 2K″). The merged images show endogenous Sox2 and GFP in the LaCL (Figure 2J) and only GFP in the LiCL (Figure 2K). These data indicate that the *Sox2* promoter drives expression in the LaCL and the LiCL, but endogenous Sox2 protein is only expressed in the *miR-200* negative LaCL due to *miR-200* targeting the 3′UTR and in the LiCL. These data suggest *miR-200* is required for Sox2 compartmentalization, as well as distinguishing the labial and lingual cervical loops of the incisor. Taken together, these data indicate that the *miR-200* family acts to compartmentalize the expression of the dental stem cell transcription factor Sox2 within the stem cell niche or LaCL during development.

3.3 | *miR-200* family inhibition regulates incisor tissue composition and length

The role of the *miR-200* family in tooth organogenesis was determined at different developmental stages. At E11.5, in wildtype embryos dental ectoderm development begins as a thickening of the oral epithelial tissue called the dental placode (Figure 3A, arrow). Individual *miR-200* family functional groups inhibited independently (Figure 3B,C, arrow) or together (DI embryos; Figure 3D, arrow) did not affect the formation of the placode, which was observed in all genotypes.

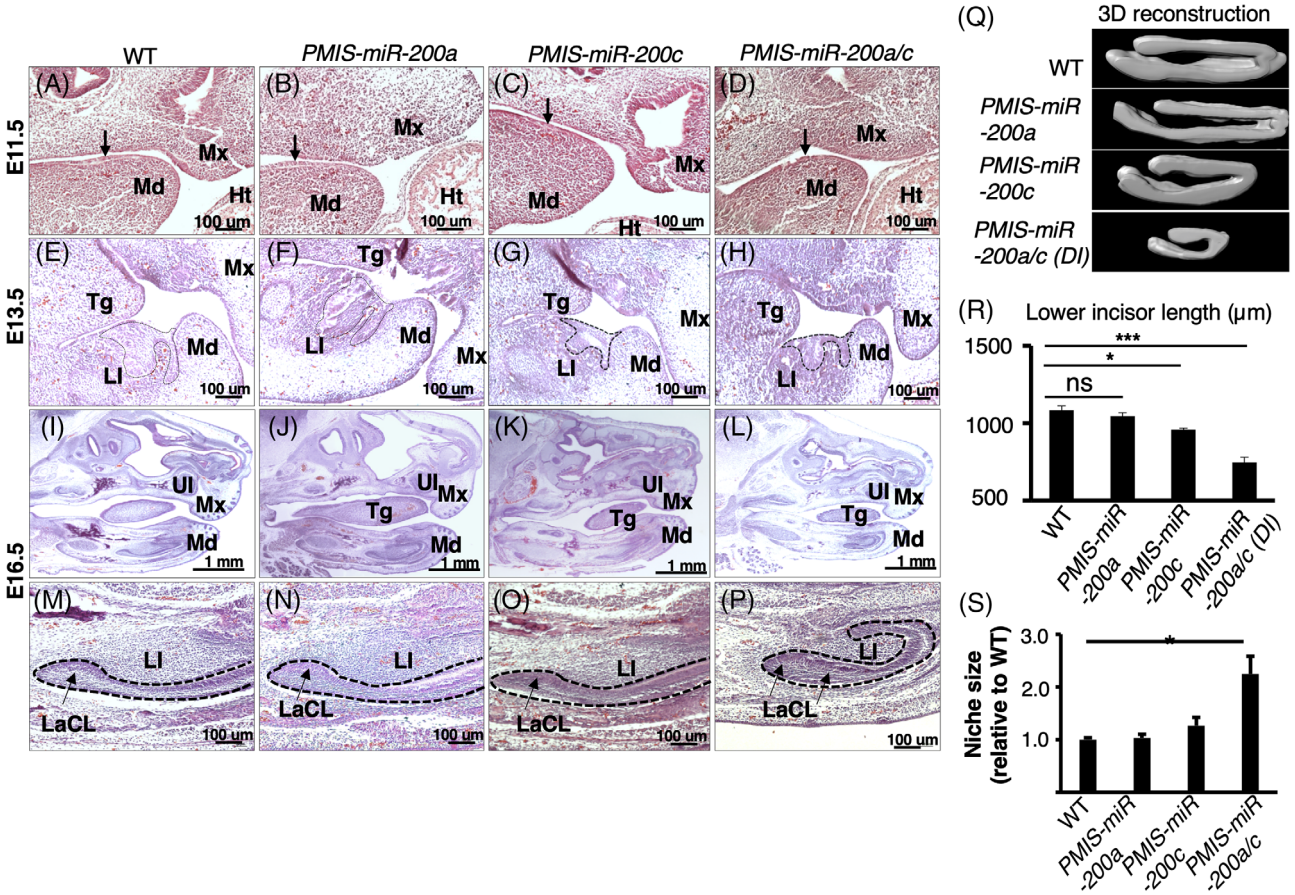


FIGURE 3 *miR-200* family inhibition regulates incisor tissue composition and length. A-D, Hematoxylin and Eosin (H&E) staining of WT, *PMIS-miR-200a*, *PMIS-miR-200c* and *PMIS-miR-200a/c* embryos at E11.5. E-H, H&E staining of *WT*, *PMIS-miR-200a*, *PMIS-miR-200c* and double inhibitor lower incisors at E13.5 revealed smaller tooth buds in *PMIS-miR-200c* and double inhibitor embryos. I-L, The heads of E16.5 inhibitor embryos were similar in size and grossly similar, containing upper and lower incisors, the tongue, palate and sinus cavity. M-P, Higher magnification of the *PMIS-miR-200a/c* lower incisor revealed an expanded labial cervical loop region compared with WT, *PMIS-miR-200a* and *PMIS-miR-200c* embryos. Q, The dental epithelial tissue from serial sections composing the entire lower incisor for each embryo was traced and Imaris imaging software was used to create 3-D models. R, Lower incisors from three different embryos of each genotype were measured and *PMIS-miR-200c* and *PMIS-miR-200a/c* lower incisors are shorter (N = 3, **P* < .05, ****P* < .01). S, Quantitation of the size of the LaCL stem cell niche relative to WT is shown. Md, Mandible; Mx, Maxilla; Ht, Heart; Tg, Tongue; LI, Lower incisor; UI, upper incisor; LaCL, Labial Cervical Loop

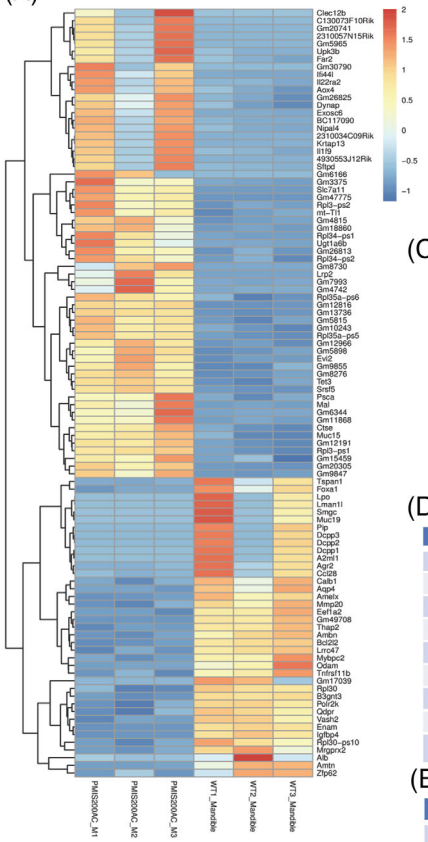
At E13.5, the oral epithelial tissue invaginates and mesenchyme condenses around the dental epithelium to form a tooth bud. In WT, *PMIS-miR-200a*, *PMIS-miR-200c*, and DI embryos, a lower incisor tooth bud was identified (Figure 3E-H). However, in *PMIS-miR-200c* and DI E13.5 embryos, there is a decrease in tooth bud size, associated with a decrease in the invaginating dental epithelial layer.

At E16.5, *miR-200* family inhibition did not grossly alter craniofacial organization (Figure 3I-L). The WT bell stage lower incisor contains a labial cervical loop (LaCL) as well as polarized ameloblasts and odontoblasts (Figure 3M). Loss of the *miR-200a* functional group did not cause obvious morphological defects at E16.5 (Figure 3N). Interestingly, *miR-200c* functional group inhibition lead to a 20% decrease in incisor length compared with WT (Figure 3O,R). Upon inhibition of both functional groups in DI embryos, lower incisors were more severely decreased in length (by about 45%) and had other apparent defects in epithelial cell structure (Figure 3P,R). In contrast, the *PMIS-miR-200c* and DI embryos have an expanded stem cell niche compartment (Figure 3O,P, respectively). Quantitation of the stem cell niche size is shown (Figure 3S). 3-D reconstructions of the lower incisors from these embryos confirmed the differences observed in individual sections (Figure 3Q).

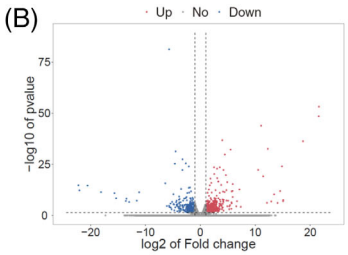
3.4 | *miR-200* inhibition decreased gene expression associated with enamel formation

The observed defects in incisor length suggested that the gene expression program was altered upon inhibition of the *miR-200* family. In order to understand these changes, triplicate samples of WT and *PMIS-miR-200a/c* RNA isolated from mandibles were used to perform RNA sequencing (Figure 4). In *PMIS-miR-200a/c* mandibles, 583 genes were upregulated, and 392 genes were downregulated compared with the control, and a heatmap of the most significantly differentially expressed genes was constructed (Figure 4A). A Volcano plot of differentially expressed genes demonstrates that most of the changes in gene expression were contained within ±5 of the Log2 of fold change

(A)



(B)



(C)

GO #	GO term	*P* value
GO:0097186	amelogenesis	2.97791723608473E-07
GO:0031214	Biomineral tissue development	0.0000863240198158
GO:0007267	cell-cell signaling	0.0002197395533062
GO:0030057	desmosome	0.0011564844107996
GO:0070175	positive regulation of enamel mineralization	0.0034437255184897
GO:0042475	odontogenesis of dentin-containing tooth	0.003933782519004
GO:0002934	desmosome organization	0.0109637045317256

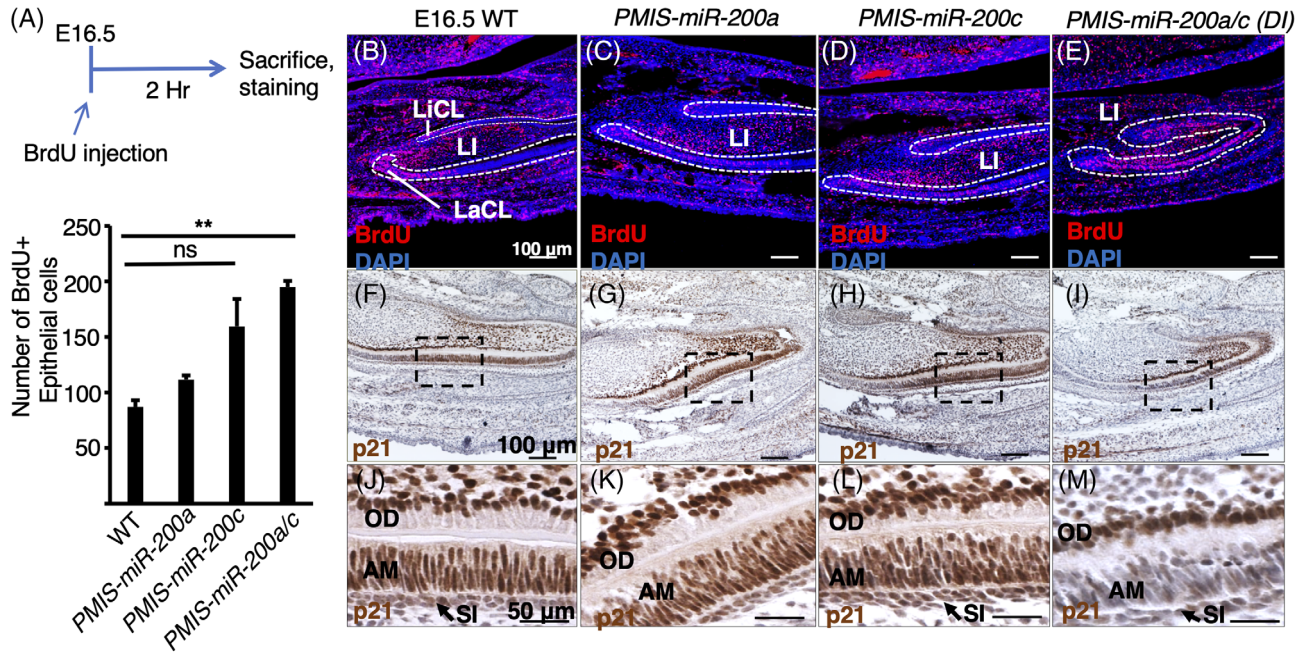
(D) **Genes regulating the dental epithelial stem cell niche (N = 3)**

Gene	Fold change	*P* value
Tbx1	1.15	0.44
Pitx2	1.25	0.270473058
Klf4	1.79	1.05812E-05
Lef-1	1.36	0.099586697
Lgr5	1.37	0.0914652258375379
ABCG2	1.07	0.60785567089843
Yap	1.11	0.423320866967171
Gli1	1.09	0.550195143487359
ITGA6	1.02	0.861591249447506

(E) **Signaling pathways involved in dental epithelial cell regulation (N = 3)**

Gene	Fold change	*P* value
Noggin	1.17	0.498993832
Bmp4	1.51	0.235622921
Shh	0.35	7.75222E-05
Notch2	1.25	0.089519148

FIGURE 4 *miR-200* family inhibition regulates several gene and cell fate pathways. A, Heatmap comparing statistically significantly altered genes in triplicate samples of WT and *PMIS-miR-200a/c* mandibular RNA. B, A volcano plot showing the differences in gene expression. Blue points represent genes that were significantly reduced with a fold change >2, and red represents significantly upregulated genes with a fold change >2. C, A subset of some of the most significant GO terms are displayed. D, Altered stem cell type genes in triplicate samples of WT and *PMIS-miR-200a/c* mandibular RNA identified by RNA-sequencing. Green letters in fold change indicate an increase in expression and red letter are decreases in gene expression. E, Signaling pathways affected in the *PMIS-miR-200a/c* embryos. (N = 3)



(N) **Genes promoting G1/S cell cycle progression**

Gene symbol	Fold change	*P* value
Camk2a	2.93	0.0015623093082440
Camk2b	0.46	0.0018930015138194
Spdya	3.43	0.0029846440388276
Pik3	2.51	0.0009485662223296

FIGURE 5 The *miR-200* family regulates DESC proliferation. A, A timeline for the experiment. Pregnant mice staged to E16.5 were injected with BrdU 2 hours prior to sacrifice. Embryos were harvested and staining for BrdU was used to identify proliferating cells. B-E, Immunostaining for BrdU+ cells. Inhibiting either *miR-200* functional group resulted in an increase in the domain of BrdU+ cells and in DI embryos the proliferating cells were throughout the tooth germ. Quantitation of the number of BrdU+ epithelial cells is shown. F-I, p21 staining of the lower incisor. p21 is expressed in the differentiated ameloblasts and odontoblasts in WT and single inhibitor mice. p21 staining was reduced in DI embryo ameloblasts. J-M, enlargements of the boxed regions in F-I. N, Genes identified as upregulated by RNA-seq that promote cell cycle progression beyond the G_1/S checkpoint

(Figure 4B). Interestingly, GO term analysis revealed differences in groups of genes associated with amelogenesis, biomineral tissue development and odontogenesis (Figure 4C). Several stemness genes were upregulated indicating that other than *Sox2*, other stem cell factors were increased by the inhibition of the *miR-200* family (Figure 4D). Interestingly, *Shh* was significantly down regulated in the DI embryos (Figure 4E). Addition of Shh ligands to cell cultures specifically inhibits *miR-200* expression and Shh is highly expressed in the LaCL stem cell niche (data not shown). Thus, Shh acts to inhibit *miR-200* in the LaCL stem cell niche to maintain stemness. Other GO terms suggested cell signaling and cellular adhesion are affected in the *PMIS-miR-200a/c* embryos.

The RNA-seq. Results corroborate the histology data in which many transcripts required for epithelial cell differentiation, as well as the production of enamel, were significantly downregulated in *PMIS-miR-200a/c* embryos. These results support the hypothesis that the *miR-200* family promotes the differentiation of DESCs.

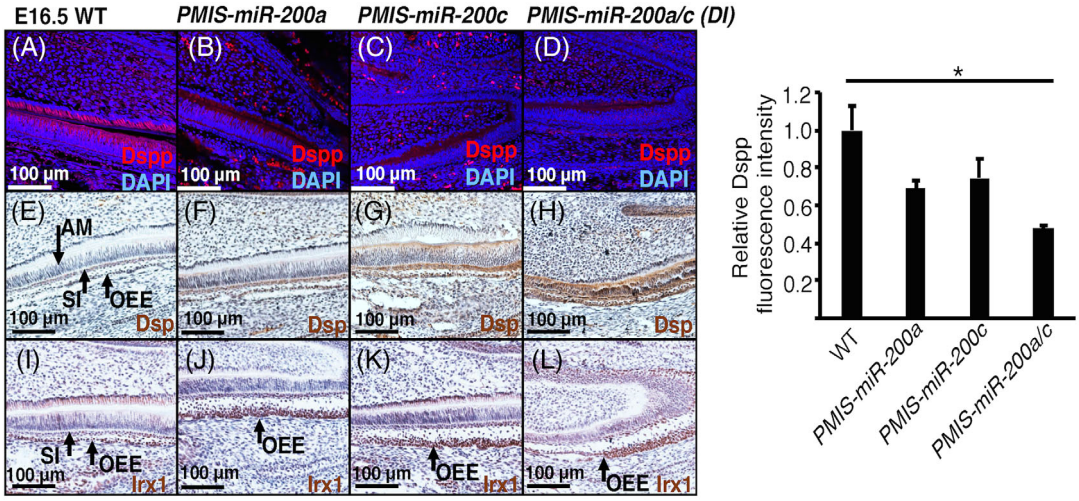
3.5 | The *miR-200* family compartmentalizes proliferating epithelial cells to the LaCL and TAC regions

The small lower incisor when the *miR-200* family was inhibited suggested a possible defect in epithelial cell proliferation. To determine if cell proliferation was affected, we injected pregnant E16.5 mice with BrdU, and sacrificed after 2 hours to label proliferating cells (Figure 5A). In WT E16.5 lower incisors, BrdU+ cells were compartmentalized in the labial and lingual (LiCL) cervical loops as has been reported previously (Figure 5B). Surprisingly, upon inhibition of either *miR-200a* or *miR-200c*, there was an increase in the BrdU positive domain in the dental epithelium (Figure 5C,D), compared with WT. Epithelial cell proliferation was increased throughout the lower incisor in DI lower incisors, and the majority of dental epithelial cells were BrdU positive (Figure 5E). Quantitation of the number of BrdU+ cells is shown.

Typically, differentiating ameloblasts derived from proliferating DESCs express the CDK inhibitor p21, promoting cell cycle exit.[24] To test if p21 expression was defective in the hyper-proliferative DI lower incisors, we performed p21 immunohistochemical staining in WT, *PMIS-miR-200a*, *PMIS-miR-200c* and DI lower incisors (Figure 5F-I). At this stage, p21 is strongly expressed in WT and single inhibitor lower incisors, and a higher magnification shows p21 is expressed in the odontoblasts (OD), ameloblasts (AM), and some stratum intermedium (SI) cells (Figure 5J). This expression pattern is maintained in *PMIS-miR-200a* and *PMIS-miR-200c* lower incisors. However, in *PMIS-miR-200a/c* DI lower incisors, p21 expression is reduced in the dental epithelium including ameloblasts and SI cells (Figure 5I), although some epithelial cells at the distal tip of the incisor retain p21 expression. The expression of p21 was not altered in the mesenchymal-derived odontoblast cells, consistent with the observation that these cells were not labeled by BrdU and therefore, not proliferating in any of the examined genotypes. Several genes associated with overcoming the G_1/S cell cycle checkpoint were found to be upregulated by RNA-seq in DI lower incisors (Figure 5N), suggesting a possible mechanism.

3.6 | The differentiation of epithelial cells to ameloblasts requires *miR-200* expression

The defects observed in cell polarity and lower incisor stratification prompted us to examine if other aspects of cellular differentiation were affected in the PMIS inhibitor mice. In order to do so, we used immunostaining to label proteins expressed in ameloblasts, SI cells, and OEE cells. To label ameloblasts we stained for dentin sialoprotein (Dspp) in WT, single and double inhibitor embryos (Figure 6A-D). Interestingly, while Dspp was highly expressed in the presecretory ameloblasts and odontoblasts in the WT E16.5 lower incisor (Figure 6A), Dspp was decreased in embryos expressing *PMIS-miR-200a* and *PMIS-miR-200c* (Figure 6B,C). DSP expression was almost completely absent in the DI lower incisor (Figure 6D). Quantitation of Dspp fluorescence intensity is shown for panels A-D.



(M) **Differentiation genes decreased in DI embryos**

Gene symbol	Fold change	*P* value
Shh	0.35	0.00007
Odam	0.10	6.15198E-23
Phex	0.69	0.03096691089188
Amtn	0.01	1.97500647E-16
Enam	0.02	5.8296525E-82
Ambn	0.11	3.5435962E-28
Amelx	0.04	4.95150117E-32
Dspp	0.27	1.3198295E-06

(N) **SI and OEE specific genes increased in DI embryos**

Gene symbol	Fold change	*P* value
Dsp	1.98	0.000114158546080
PERP	2.30	0.000042034234299
DSC1	4.26	.0002687502167471
Irx1	1.51	0.12541471705727
Notch2	1.25	0.089519147857705
Notch1	1.10	0.430035474416539
Gli2	1.57	0.00245530850753432
Gli3	1.52	0.003071233632756

FIGURE 6 The *miR-200* family promotes cell fate decisions of the SI/OEE layers. A-D, Dentin sialoprotein (Dspp), a transient marker of presecretory ameloblasts at E16.5, is highly expressed in the WT lower incisor at E16.5. The inhibition of the *miR-200* family decreased Dspp expression in most of the cells, with only a few *Dspp+* ameloblasts present at the distal tip of the DI lower incisor. The relative Dspp expression in the sections is shown. E-H, *Desmoplakin* (*Dsp*) expression a component of desmosomes, was increased in the ameloblast region upon inhibition of the *miR-200* family. I-L, The outer enamel epithelium, which expresses *Irx1*, is thicker when the *miR-200* family is inhibited. M, A list of differentially expressed markers of ameloblasts/odontoblasts identified by the RNA sequencing experiment were down regulated in DI mandibles compared with WT. N, A list of genes expressed in the SI/OEE layer are upregulated in DI mandible samples

SI cells contact ameloblasts, presumably functioning to anchor them to the other tooth structures through desmosomes.[25] To assay for defects in this layer, we stained for the marker desmoplakin (Dsp) (Figure 6E-H). In WT and *PMIS-miR-200a* lower incisors, we found that Dsp expression was specifically expressed by SI cells. Interestingly, when *miR-200c* was inhibited, Dsp expression was increased in the SI, and observed in the ameloblast region as well. In the DI embryos, Dsp expression was greatly upregulated, and ubiquitously expressed throughout the different layers of the lower incisor. To examine the outer enamel epithelium (OEE) and SI, we stained for Irx1, which was specifically expressed in both WT cell layers (Figure 6I). In *PMIS-miR-200a*, and *PMIS-miR-200c* lower incisors, the Irx1+ cell layers are expanded (Figure 6J,K). The combined layer of apparent SI and OEE cells in the DI embryos show an increase in Irx1 expression (Figure 6L).

The immunostaining experiments suggested that ameloblast differentiation had been compromised while the SI and OEE layers were expanded. To further confirm these results, the expression of markers for these different regions were examined in the RNA-seq data set (Figure 6M,N). Markers specific for ameloblasts, were downregulated in *PMIS-miR-200a/c* lower incisors compared with WT. Conversely, the expression of markers associated with the SI and OEE layers were increased. From these data, we conclude that the differentiation of ameloblasts requires the *miR-200* family and that the inhibition of *miR-200* results in the loss of ameloblasts and the expansion of the OEE layer or a combined SI and OEE layer. These data indicate that the *miR-200* family plays an essential role in dental epithelial cell organization and differentiation of ameloblasts.

3.7 | *miR-200c* regulates autophagy mechanisms of the dental epithelium promoting cell survival

Although epithelial cell proliferation increases when the *miR-200* family is inhibited, the overall length of the lower incisor is decreased (Figure 3Q,R), suggesting a loss of cells through a programmed cell death pathway. Probing the lower incisors from E16.5 embryos using terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining to identify dying cells revealed that WT E16.5 lower incisor epithelia did not contain dying cells in the enamel organ, and inhibiting the *miR-200a* functional group did not result in cell death (Supporting Information Figure S4A,B). However, inhibiting the *miR-200c* functional group resulted in epithelial cell death, and inhibiting both functional groups resulted in an increase in the detectable number of dying cells (Supporting Information Figure S4C,D). By quantifying the number of TUNEL+ cells in each group, we found on average 9.66 dying cells/incisor when only the *miR-200c* functional group was inhibited, and 22 dying cells/incisor when both functional groups are inhibited (Supporting Information Figure S4O, N = 3).

To further understand the mechanism by which the epithelial cells were dying in the DI lower incisor, IHC staining for cleaved caspase 3 (CC3) was performed. CC3 is a marker for cells undergoing apoptosis through a caspase dependent cell death pathway.[26] Surprisingly, although TUNEL+ and CC3+ cells were identifiable in the vestibular lamina of WT and DI lower incisors at this stage (Supporting Information Figure S4M,N,M′-N′, arrows), no CC3+ cells were found in WT and *PMIS-miR-200a* lower incisor enamel organs, or in the TUNEL+ region observed in the *PMIS-miR-200c* and DI lower incisors (Supporting Information Figure S4I-L), suggesting that the cells are not undergoing caspase-dependent programmed cell death.

Previous work has demonstrated a link between dental epithelial cell death and autophagy,[27] and it has been demonstrated that *miR-200c* negatively regulates autophagy through indirectly inhibiting levels of LC3 protein.[28,29] To test the lower incisors for indications of autophagic cell death, we stained for LC3, a marker of autophagy. Interestingly, in WT and *PMIS-miR-200a* lower incisors, only a few LC3+ cells were observed in the transient amplifying zone of the lower incisor (Supporting Information Figure S4E,F), which is consistent with previous reports. However, in *PMIS-miR-200c* and double inhibitor lower incisors, LC3 expression is expanded, indicating an increase in autophagy (Supporting Information Figure S4G,H). Thus, we conclude that *PMIS-miR-200c* and DI lower incisors are smaller due to an increase in autophagy-dependent programmed cell death, and that the *miR-200c* functional group regulates autophagy in the dental epithelium.

To better understand the mechanisms of autophagy in the *PMIS-miR-200* embryos and dental epithelial cells, *PMIS-miR-200a/c* embryos were analyzed using the LC3AB antibody, which recognizes two forms of LC3 protein (LC3I and II), including one (LC3II) that is processed under autophagy conditions. As shown in the previous figure (Supporting Information Figure S4) we detect increased LC3 protein in the *PMIS-miR-200a/c* embryos (Supporting Information Figure S5A). LS-8 oral epithelial cells and cells transfected with PMIS empty vector were starved to induce autophagy and treated with chloroquine to accumulate autophagosomes, demonstrated an increase in LC3 expression, while untreated cells did not show expression of the LC3 autophagy marker (Supporting Information Figure S5B). However, *PMIS-miR-200a*, *PMIS-miR-200c* and *PMIS-miR-200a/c* transfected cells all showed LC3 expression and starvation and treatment with chloroquine did not significantly increase LC3 expression (Supporting Information Figure S5B). Interestingly, we assayed for PRAS and P-PRAS expression (part of mTORC1 pathway) in LS-8 cells transfected with *PMIS-miR-200* constructs and found no significant differences in the levels of these proteins (data not shown). These data demonstrate that the *miR-200* family acts independently of mTORC1 to inhibit autophagy.

4 | DISCUSSION

4.1 | The *miR-200* functional groups regulate different developmental aspects of an ectodermal appendage

We examined the role of the *miR-200* family in regulating an ectodermal stem cell niche. This is the first in vivo report demonstrating the

functional role of the *miR-200* family in compartmentalizing and maintaining a stem cell niche. Furthermore, *miR-200* expression is required for progenitor cells exiting the niche to differentiate. By inhibiting miR activity based on seed sequence homology, we found distinct roles and functions of each *miR-200* family functional group. For example, inhibiting *miR-200c*, but not *miR-200a*, resulted in increased autophagy and cell death. *PMIS-miR-200c* and DI E16.5 embryos have reduced size incisors, defects in cellular differentiation due to a lack of stem cell compartmentalization and epithelial cell differentiation (Figure 7). Interestingly, the phenotype of DI embryos was more exaggerated than the phenotype of embryos expressing either individual inhibitor, suggesting a genetic interaction between the distinct targets of the *miR-200* family functional groups.

4.2 | The *miR-200* family regulates DESC differentiation and the compartmentalization of the epithelial stem cell niche

We demonstrate that DESC differentiation can be specifically attributed to the *miR-200* family. Indeed, inhibiting the *miR-200* family resulted in the complete loss of typical ameloblast cells, which failed to polarize and did not express typical differentiation markers, such as *Dspp* and *p21* in early development. Instead, these cells were positive for *Desmoplakin*, a marker typically only expressed by the SI. The SI and OEE layers were also physically disorganized in DI incisors, and indiscriminately expressed *Desmoplakin* and *Irx1*, which are specific SI and OEE markers in WT lower incisors.[30] Markers of the SI and OEE layers were increased in *PMIS-miR-200a* and *PMIS-miR-200c* lower incisors, suggesting the *miR-200* family promotes the ameloblast cell fate and inhibits the SI/OEE fates. However, the SI layer appears to be disrupted in the *PMIS-miR-200a/c* lower incisors. Interestingly, the differentiation of odontoblasts was also decreased by the inhibition of the *miR-200* family. This would be predicted as a result of the loss of ameloblast differentiation, as previous reports have identified that odontoblast differentiation requires signaling from preameloblast cells.[31]

4.3 | The *miR-200* family regulates multiple stem cell factors

The inability of the dental epithelium to properly organize in DI embryos is not surprising given that many of these cells failed to exit the cell cycle. While inhibiting either *miR-200* family functional group resulted in the expansion of the proliferative zone of the lower incisor, the inhibition of both functional groups simultaneously resulted in the complete loss of the compartmentalization of progenitor cells undergoing proliferation. The maintenance of the lower incisor DESC population depends on proliferation of these cells within the LaCL, and our

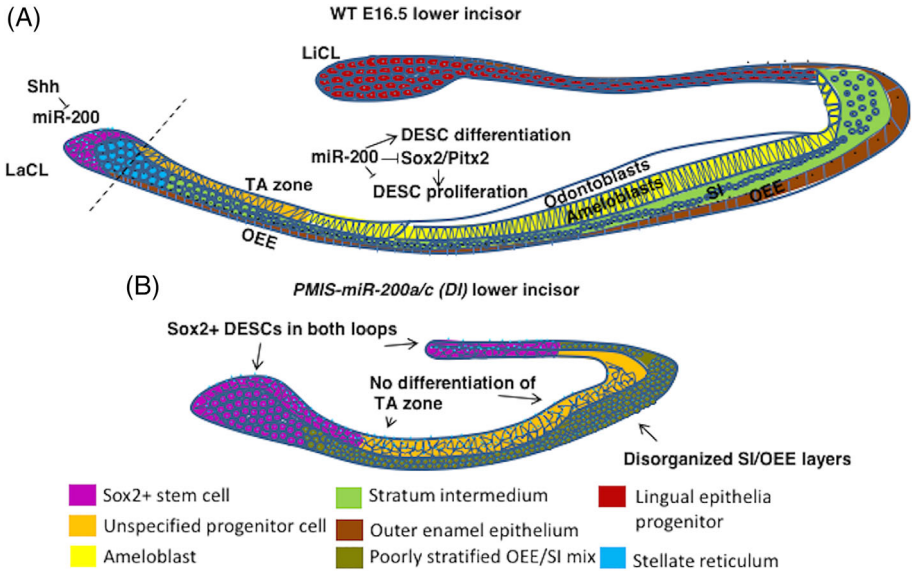


FIGURE 7 A model for the *miR-200* family in promoting the differentiation of DESCs in the lower incisor. A, In WT lower incisors, the labial cervical loop does not express the *miR-200* family due to Shh signaling. This allows for the expression of different transcription factors, including *Sox2*, *Pitx2*, and *Tbx1*, among others, to be strongly expressed and promote DESC maintenance. When these cells migrate distally away from the LaCL, the *miR-200* family becomes activated and shuts down the expression of these transcription factors, promoting the differentiation of the DESCs into the three major stratified layers of the lower incisor (ameloblasts, stratum intermedium, outer enamel epithelium). B, When the *miR-200* family is inhibited, the transcription factors that maintain the stem and progenitor status of the dental epithelium are no longer repressed. They become decompartmentalized, and the dental epithelial tissue becomes poorly differentiated and disorganized. The DESC program, which under normal conditions is only present in the labial cervical loop, becomes active in the lingual cervical loop as well. Markers of the ameloblast cell lineage are downregulated and ameloblast differentiation is impaired, while markers of the SI and OEE layers are upregulated and these regions are expanded

group and others have demonstrated that this proliferative capacity depends on the expression of Sox2.[13] In the *PMIS-miR-200a/c* inhibitor embryos, Sox2 expression was expanded outside of the LaCL, contributing to the hyperproliferative phenotype observed in DI lower incisors. Other DESC markers were also increased in the expanded LaCL including *Tbx1*, *Klf4*, *Lef-1*, *Lgr5*, *ABCG2*, *Yap*, *Gli1*, *ITGA6*, and *E-cadherin*. These factors are expressed in other types of stem cells and regulate the stem cell niche.[15,32-35] The RNA-seq. Data provides compelling evidence that the *miR-200* family controls the expression of multiple stem cell factors either directly or indirectly. Several animal models have shown that *Pitx2*, *Bmi-1*, *Gli-1*, *Ptch-1*, *Lef-1*, and miRs can all modulate the stem cell niche.[2,3,13,15,33,36] Interestingly, the *miR-200* family directly regulates *Pitx2*, *Sox2*, *Klf4*, *Bmi-1*, and *Lef-1*.[3,4,20] Other factors are indirectly regulated by the increased expression of selected transcription factors or signaling pathways.

4.4 | Several signaling pathways are modulated by the *miR-200* family

Several signaling pathways are enhanced in the dental stem cell niche. For most stem cell types the simultaneous activation of several pathways are required for continuous stem cell self-renewal. In neural stem cells (NSC) the fibroblast growth factor (FGF), brain-derived neurotrophic factor (BDNF), and sonic hedgehog (Shh) signaling pathways are required for mammalian NSC self-renewal in vivo.[8] Therefore, a combination of signals are needed to control stem cell self-renewal, and many appear to function as short-range signals. Therefore, stem cells must stay inside the niche in order to maintain long-term self-renewal.[37] Furthermore, adhesion molecules such as E-cadherin are required to anchor stem cells in their niche. In the DI expanded DESC niche we find that *E-cadherin* was increased (1.15-fold, $P = .3$), consistent with E-cadherin as a stem cell marker. Interestingly, *Shh* is downregulated while *Bmp4* is increased in the DI embryos, it has been shown that Shh is required for the maintenance of the epithelial stem cell niche and the generation of ameloblast cells from Sox2+ cells.[7,33,35,38] BMP expressed in mesenchymal cells may inhibit epithelial Shh function to allow for maintenance of the dental epithelial stem cells and inhibit differentiation. Shh inhibits *miR-200* family expression (data not shown) but also that a lack of *miR-200* decreases Shh expression.

Shh is required for the generation of ameloblasts, but not stratum intermedium cells from the epithelial stem cells.[33] Shh is highly expressed in the DESCs and inner enamel epithelial progenitor cells that give rise to the ameloblasts. We show that both the pool of outer enamel epithelium and stratum intermedium cells are increased in the *PMIS-miR-200* embryos, although they are poorly differentiated. Notch signaling controls the maintenance and differentiation of many different stem cell types.[39-41] *Notch2* is upregulated in the *PMIS-miR-200* embryos and *Notch2* may play a role in progenitor specification of the OEE and SI cell layers.[42] This adds another level of cell fate and signaling pathway regulation by the *miR-200* family.

4.5 | Dental epithelial cell survival requires the modulation of autophagy levels by *miR-200c*

In addition to promoting the differentiation of DESCs, *miR-200c* inhibited autophagy in the developing incisor. The inhibition of *miR-200c* resulted in an unchecked increase in autophagy leading to epithelial cell death, which was exacerbated in DI lower incisors as demonstrated by the greater number of TUNEL+ cells. These cells did not activate the canonical apoptotic program, suggesting their demise can be attributed directly to increased levels of cellular autophagy.[43] The autophagy program is a check to control stimulated stem cell proliferation without appropriate progenitor cell differentiation.

4.6 | The *miR-200* family promotes ameloblast fate choice

The epithelium of the lower incisor was classically described as arising from a single population of *Sox2+* cells that then gave rise to all cell lineages through a transient *Srfp5+* cell population.[15] A more recent work revised this classical model by examining incisor epithelial cell differentiation using scRNA-seq, but also concluded that the distinct epithelial cell layers of the lower incisor are derived from a common pool of cycling progenitors.[44] However, the cell autonomous and nonautonomous mechanisms that influence cell fate decisions are unclear. In this work, we show that the *miR-200* family is required for unspecified epithelial cells to differentiate into ameloblasts, and that inhibiting the *miR-200* family results in the expansion and disorganization of the SI and OEE layers. Our results suggest that the SI/OEE fate is the default of differentiating epithelial cells and that the *miR-200* family is required to alter this fate decision.

4.7 | Stem cell niche organization depends on the expression of miRs

Overall, we demonstrate that the functional groups of the *miR-200* family have independent as well as overlapping roles during development that promote the organization of the lower incisor through balancing opposing processes including cell differentiation and proliferation. These miRs function through regulating the spatial expression of target genes. This work demonstrates a previously unrecognized strategy by which tissues can compartmentalize different cell types, including stem cells. This strategy might be unique to ectodermal organs such as teeth, but it is far more likely that it is a conserved mechanism utilized by other organs to promote tissue homeostasis as well. Small RNAs are rapidly advancing toward the clinic and understanding how they regulate stem cell niche behavior in different biological contexts will create powerful tools in the fields of regenerative medicine.

5 | CONCLUSIONS

We developed the Plasmid based miR Inhibitor System (PMIS) to study each functional group by inhibiting miRs through seed sequence complementation. The PMIS is efficient, specific, shows no off-target effects and is non-toxic. The PMIS inhibitor system is one of the most efficient technologies for studying the in vivo function of miR families and individual miRs within a cluster. This new approach has identified several new scientific advancements in stem cell niche biology.

*miR-200 family members are required for in vivo epithelial stem cell differentiation and stem cell niche maintenance.

*The miR-200 family regulates genes required for generating the different dental epithelial cell lineages.

*miR-200 expression may be a common mechanism for defining the boundries of adult epithelial stem cell niches.

*By inhibiting the miRs that become active when a progenitor cell becomes fated to differentiate, the in vivo cell differentiation programs are determined assuming their terminal fates.

*Dental epithelial stem cells are regulated by members of the miR-200 family to yield the differentiated progeny that become stratum intermedium, outer enamel epithelium, inner enamel epithelium, stellate reticulum and ameloblasts.

The PMIS is different than chemically modified anti-miR oligonucleotides (AMOs) and locked-nucleic acids (LNAs) that bind miRs transiently and inefficiently, do not remain in dividing cells and require repeated large doses of oligos in cells to be effective and they have severe off-target effects. Furthermore, these oligos cannot distinguish between a one nucleotide difference in miR families such as miR-141 and miR-200c however the PMIS can effectively and efficiently inhibit one without affecting the function of the other closely related miR. This technology demonstrates the differential effects of the miR-200 family in the regulation of the epithelial stem cell niche.

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CONFLICT OF INTEREST

B.A.A. is the owner and CSO of NaturemiRI, LLC, which produces the PMIS microRNA inhibitors. The other authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

M.S.: collected and analyzed data, prepared the manuscript, contributed to the design of the study; Y.S., W.Y., D.S., R.J.L., S.L.E.: performed experiments, analyzed data, contributed to the manuscript; B.A.A.: contributed to the design of the study, data interpretation and acquisition, prepared the manuscript.

DATA AVAILABILITY STATEMENT

RNA sequencing datasets will be deposited in the appropriate repository and be fully accessible upon publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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