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# Long noncoding RNAs related to the odontogenic potential of dental mesenchymal cells in mice

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ABSTRACT

*Objectives:* The purpose of this study is to identify the lncRNAs that are associated with the odontogenic potential in mouse dental mesenchymal cells.
*Design:* The odontogenic potential of dental mesenchymal cells was found to be lost in the course of *in vitro* culture, so the lncRNA profiles were subsequently compared between freshly-isolated and cultured dental mesenchymal cells using RNA-sequencing. A co-expression analysis of differentially expressed lncRNAs and coding RNAs was performed to understand their potential functions. The expression of several selected lncRNAs was also examined in developing tooth germs.
*Results:* Compared with cultured dental mesenchymal cells, 108 lncRNAs were upregulated and 36 lncRNAs were downregulated in freshly-isolated dental mesenchymal cells. Coding genes correlated with the lncRNAs were mainly associated with DNA and protein metabolic processes and cytoskeletal anchorage. *Meg3*, *Malat1*, *Xist*, and *Dlx1as* were significantly downregulated in cultured dental mesenchymal cells but were upregulated in odontogenic dental mesenchymal tissues. Moreover, the levels of *Dlx1as* were negatively correlated with that of *Dlx1* in dental mesenchymal cells and dental mesenchymal tissues.
*Conclusions:* The lncRNA profiles of dental mesenchymal cells are significantly changed during culturing, and the dysregulation of lncRNAs is associated with the loss of odontogenic potential.


## 1. Introduction

Tooth formation, or odontogenesis, depends on self-regulatory epithelial-mesenchymal interactions, and is strictly regulated at the molecular level (Grobstein, 1967; Jernvall & Thesleff, 2000; Saxen & Thesleff, 1992; Thesleff, Vaahtokari, & Partanen, 1995). Odontogenic potential is transmitted back and forth between the dental epithelial and mesenchymal tissues (Kollar & Baird, 1970a, 1970b). After mouse embryonic day (E) 12.5, odontogenic potential shifts to the mesenchymal compartment, so the dental mesenchymal cells from the developing tooth germs of E14.5 mice are able to instruct tooth formation when recombined with dental or non-dental epithelium (Hu et al., 2014; Kollar & Baird, 1969, 1970a, 1970b; Mina & Kollar, 1987; Yamazaki, Tsuneto, Yoshino, Yamamura, & Hayashi, 2007). Dental mesenchymal cells are frequently used in the context of tooth development and tooth regeneration (Ikeda et al., 2009; Nakao et al., 2007).

Although multiple signaling pathways, including Wnt, Tgfβ/Bmp, Hh and Fgf signaling, and numerous transcription factors like Msx1 and Dlx1 play critical roles in the development of odontogenic potential, the regulation of odontogenesis by noncoding RNAs is poorly defined. MicroRNAs are thought to play a fine-tuning role during tooth development, and the differentiation of odontoblasts (Michon, Tummers, Kyyronen, Frilander, & Thesleff, 2010; Sun, Liu et al., 2015). An ectodermal organ oriented database containing the selected tooth enriched miRNAs was constructed (Michon et al., 2010). In contrast, although long noncoding RNAs (lncRNAs), tentatively defined as ncRNAs $\geq$ 200 nt in length, are known to play key roles in the pluripotency of stem cells (Bao et al., 2015; Chen et al., 2014) and the development of tissues and organs like the heart (Klattenhoff et al., 2013; Ramos et al., 2013), the lncRNAs associated with odontogenic potential remain to be identified.

Cultured dental mesenchymal cells showed impaired potential to instruct tooth formation (Keller, Kuchler-Bopp, Mendoza, Poliard, & Lesot, 2011), representing an accessible model to study

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the role of genes in odontogenesis. To investigate whether lncRNAs are associated with the odontogenic potential, lncRNA profiles were compared between freshly-isolated and cultured dental mesenchymal cells using RNA-sequencing. Our observations provide important insights into the molecular mechanisms that regulate odontogenic potential.

## 2. Materials and methods

### 2.1. Cell culture

Dental mesenchymal cells were isolated from the lower first molars of E14.5 ICR mice. The lower first molars were dissected from the tooth sockets of the mandible. The connective tissues around the tooth germs were dissected in order to avoid contamination from dental sac cells with the aid of a stereomicroscope (Leica MZ9.5; Leica Microsystèmes SA, Rueil-Malmaison, France). The isolated tooth germs were digested in 0.75% dispase to separate the dental epithelium from the dental mesenchyme. The dental mesenchyme tissues were then digested with 0.05% trypsin for 1 min and filtered through a cell sieve to obtain single cells for *in vitro* culture. The dental mesenchymal cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco), 100 U/ml penicillin, and 100 g/ml streptomycin. The culture media was changed every two days and cells were passaged with trypsin (Gibco) when confluency reached 80–100%. The first passage (P1) cells were harvested at about the 5th day and the second passage (P2) cells were harvested at about the 8th day. To investigate the relationship between *Dlx1* and *Dlx1as*, Fgf8 was used to treat the dental mesenchymal cells. Freshly-isolated dental mesenchymal cells were cultured in the medium with or without Fgf8. The cells were collected and the expression of *Dlx1* and *Dlx1as* was analyzed at 24 h.

### 2.2. Tooth formation assays

Freshly-isolated and cultured dental mesenchymal cells were recombined with E14.5 dental epithelium. The recombinants were cultured for two days *in vitro* in DMEM supplemented with 10% FBS, L-glutamine (2 mM, Gibco), 100 U/ml penicillin, and 100 g/ml streptomycin. The recombinants were then implanted in the renal capsule of ICR mice and harvested after 3 weeks. The samples were then subjected to H&E staining.

### 2.3. RNA extraction and sequencing

RNA of dental mesenchymal cells was prepared using the Qiagen RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). RNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Ribosome depleted strand specific RNA libraries were prepared and sequenced using Illumina Hiseq$^{TM}$ 2000 (Illumina, San Diego, CA) in duplicate. Raw data of the performed RNA-Seq experiments was recorded in the GEO public database (accession number: GSE65164).

### 2.4. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from dental mesenchymal cells using TRIzol and reverse transcribed into complementary DNA (cDNA) according to the manufacturer's recommendations (Takara Bio, Otsu, Japan). Real-time PCR was performed using SYBR Premix Ex Taq (Takara Bio) on an ABI 7500 Fast Real-Time PCR machine. Relative changes in transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers for qRT-PCR were listed in Table 1.

### 2.5. Bioinformatics

Sequenced reads were mapped to the reference mouse genome (mm9) using Tophat (ver. 2.0.12) for Illumina (Trapnell, Pachter, & Salzberg, 2009). Cufflinks (ver.1.2.1; University of Washington, WA) was used to assemble transcript models and to estimate the expression of transcript (Trapnell et al., 2012). FPKM (fragments per kilo base of exon model per million mapped fragments) of genes were estimated (Mortazavi, Williams, McCue, Schaeffer, & Wold, 2008). Data was analyzed using hierarchical clustering and differential expression was performed using Cuffdiff (University of Washington, WA). The fold change cut-off was set at 2-fold and $p$-values $< 0.05$ were considered to be statistically significant. A coding-noncoding gene co-expression network was constructed based on Pearson correlation analysis of differentially expressed lncRNAs and mRNAs with a threshold of 0.99. The network was created using the Cytoscape program (ver. 3.2.0).

### 2.6. Immunofluorescence analysis

For the immunofluorescence analysis, dental mesenchymal cells were fixed with 4% paraformaldehyde, treated with 0.3% hydrogen peroxide and 0.05% Tween 20. Then the cells were blocked with phosphate-buffered saline (PBS) containing 1% goat serum and incubated with the primary antibodies.for mouse Pax9 (1:100; Cell Signaling, Danvers, MA) overnight at 4 °C The FITC-conjugated secondary antibody was applied for an hour before the cells were counterstained with DAPI (5 μg/mL) and sealed with mounting medium. To accomplish staining of endogenous F-actin, the Alexa Fluor 647-labeled phalloidin

**Table 1**
Primers for quantitative real-time PCR.

| | Forward | Reverse |
|---|---|---|
| Actin | 5′-GGC TGT ATT CCC CTC CAT CG-3′ | 5′-CCA GTT GGT AAC AAT GCC ATG T-3′ |
| Dlx1as | 5′- GCA GAC AGA ATT GGG TCG TT-3′ | 5′- CTC AAC TAC CGC CTG CAA A-3′ |
| Meg3 | 5′- GAA CAG AAG CAT TCT AGG CTG G-3′ | 5′- TTC TAA GTG AAT TAC GGT GGG TG-3′ |
| H19 | 5′- TCC TCA CCT CCA ATT TCC CCT −3′ | 5′- GAG CGA GAG CCG TTC GAT G-3′ |
| Neat1 | 5′- GCT CTG GGA CCT TCG TGA CTC T-3′ | 5′- CTG CCT TGG CTT GGA AAT GTA A-3′ |
| Xist | 5′- ACC GAG GAG CAC AGC GGA CT −3′ | 5′- CGG CTA GCA CAA CCC CGC AA-3′ |
| Malat1 | 5′- GAA AGA AGA ATG GTA GAT GGC AAG T-3′ | 5′- AGA CAG ACC TAA GGG GAA AAG AAC-3′ |
| Luc7l | 5′- TCG GGA CGG AGA TGA AAC CA −3′ | 5′- GAA GAG CCA AGT CGT GGA TTT −3′ |
| Timeless | 5′- ATG AAC TGT GAA CTT CTA GCC AC −3′ | 5′- CCT CAG GTA TCG GAT CAA ATC CT −3′ |
| Ezh2 | 5′- AGT GAC TTG GAT TTT CCA GCA C −3′ | 5′- AAT TCT GTT GTA AGG GCG ACC −3′ |
| Rbp1 | 5′- CTG AGC AAT GAG AAT TTC GAG GA −3′ | 5′- GCG GTC GTC TAT GCC TGT C −3′ |
| Ttpal | 5′- TCC GAC CAG ACA GAT GGA TAC −3′ | 5′- GCA AGG ATA ACA ATC CCG TTC A −3′ |
| Cntn4 | 5′- GGA CAT TGT GTT TAC GTG GAC A −3′ | 5′- CAG TTG GAT GTT TCG GAT CAT CA −3′ |

(1:500; Molecular Probes) was applied to the dental mesenchymal cells instead of the primary and second antibodies.

### 2.7. Statistical analysis

Statistical analyses were performed using the SPSS for Windows software package (ver. 18; SPSS Inc., Chicago, IL). RNA-sequencing analyses were performed in duplicate, and all experiments involving cell culture were performed at least three times. All data are expressed as the means ± standard deviations (SD). Differences among groups were analyzed using one-way ANOVA and a two-tailed $p$-value $<0.05$ was considered statistically significant.

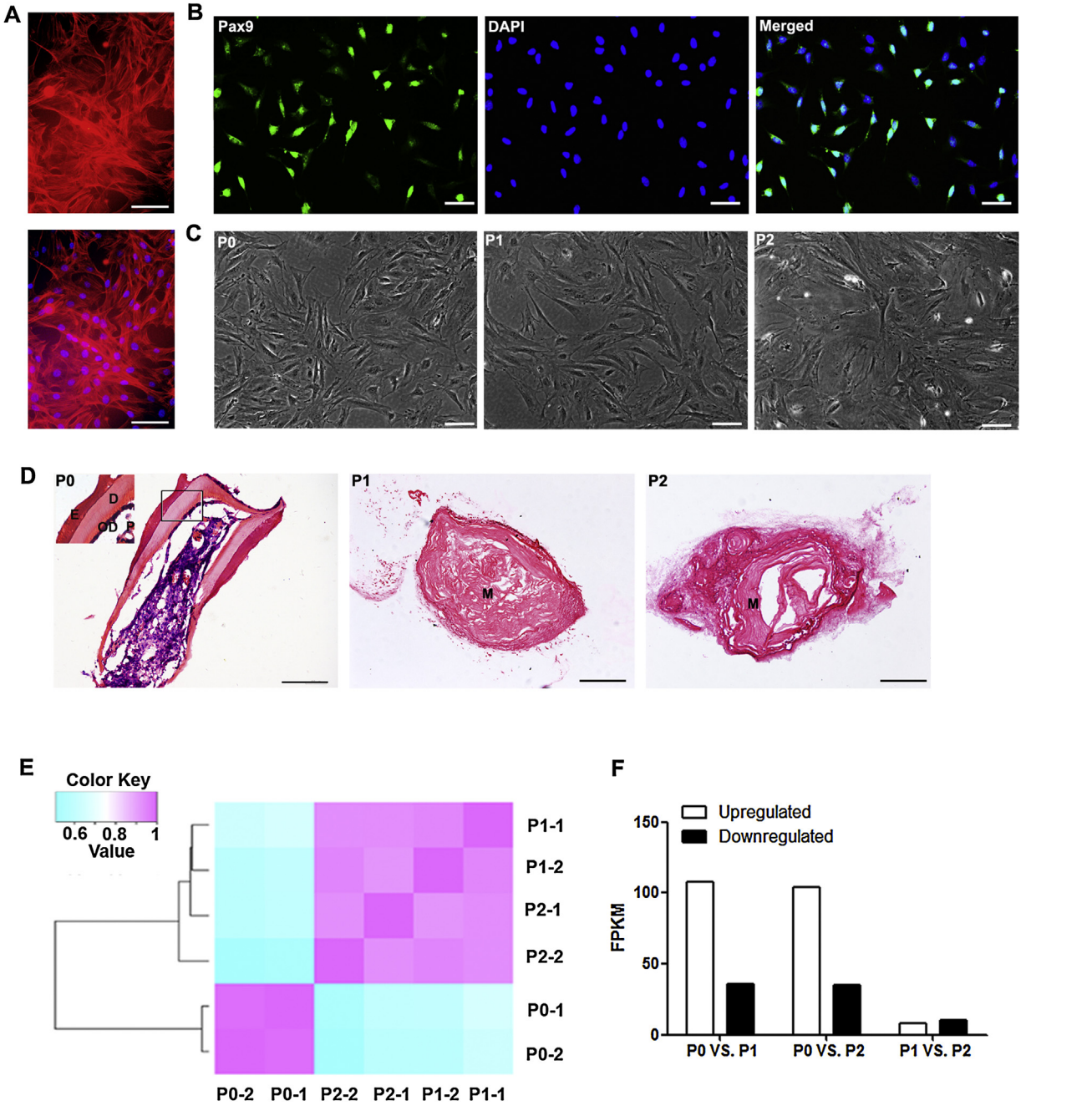


**Fig. 1.** A shift in the profiles of lncRNAs associated with the loss of odontogenic potential in cultured dental mesenchymal cells. (A) Phalloidin staining is shown. The longitudinal bundles of F-actin were visible. Scale bar: 50 μm. (B) Immunofluorescence staining of freshly-isoalted dental mesenchymal cells with anti-Pax9 antibody. Scale bar: 50 μm. (C) Images of the freshly-isolated dental mesenchymal cells (P0), the first passage (P1), and the second passage (P2) dental mesenchymal cells. Scale bar: 50 μm (D) Freshly-isolated dental mesenchymal cells developed into well-structured tooth, whereas the cultured dental mesenchymal cells failed to support tooth formation. E, enamel; D, dentin; P, dental pulp; OD, odontoblast; M, matrix. Scale bar: 100 μm. (E) Hierarchical cluster analysis of lncRNAs. The cultured dental mesenchymal cells (P1-1, P1-2, P2-1, and P2-2) clustered away from the freshly-isolated cells (P0-1 and P0-2). (F) The number of differentially expressed genes in dental mesenchymal cells. In total, 144 genes were differentially expressed between the P0 and P1 cells, 139 genes were differentially expressed between the P0 and P2 cells, but only 18 genes were differentially expressed between the P1 and P2 cells. P, passage.

## 3. Results

### 3.1. Odontogenic potential loss is reflected by the change in lncRNA profiles in the cultured dental mesenchymal cells

Dental mesenchymal cells from the molars of E14.5 mice showed fibroblast-like cell morphology with longitudinal bundles of F-actin (Fig. 1A). The cells positively expressed Pax9, a specific marker for dental mesenchymal cells (Fig. 1B). During *in vitro* culturing, the P1 and P2 cells showed a higher cytoplasmic to nuclear ratio and a flatter morphology compared with the freshly-isolated dental mesenchymal cells (Fig. 1C). To examine the odontogenic potential, the dental mesenchymal cells were recombined with E14.5 dental epithelium. The freshly-isolated dental mesenchymal cells gave rise to well-structured teeth, whereas the cultured dental mesenchymal cells failed to regenerate any tooth (Fig. 1D).

To identify the lncRNAs that participate in odontogenesis, the expression profiles of freshly-isolated and cultured dental mesenchymal cells were compared. Hierarchical clustering analysis of lncRNAs showed that the replicates clustered with each other, whereas cultured cells clustered away from the freshly-isolated dental mesenchymal cells (Fig. 1E). Compared with the freshly-isolated dental mesenchymal cells, 108 genes were downregulated in the P1 cells and 104 genes were downregulated in the P2 cells, while 36 genes were upregulated in the P1 cells and 35 genes were upregulated in the P2 cells. Compared with the P2 cells, 8 genes were upregulated and 10 genes were downregulated in the P1 cells (Fig. 1F). Thus, the loss of odontogenic potential in dental mesenchymal cells is accompanied by a shift in lncRNA profile, demonstrating the potential role of lncRNAs in tooth formation.

### 3.2. Validation of RNA-seq data

Based on the differentially expression analysis and putative function, the expression of six lncRNAs (*H19*, *Meg3*, *Malat1*, *Neat1*, *Dlx1as*, and *Xist*) that participate in organ development was examined using qRT-PCR. The qRT-PCR and RNA-seq results were consistent (Fig. 2). Although the expression of *H19* and *Malat1* was comparable between freshly-isolated and cultured dental mesenchymal cells, the expression of *Dlx1as* and *Meg3* was significantly decreased and the expression of *Neat1* was significantly increased in cultured dental mesenchymal cells.

### 3.3. Co-expression of lncRNAs and coding RNAs

The co-expression of lncRNAs and protein-coding genes was then investigated based on their correlation coefficients to predict their putative functional relatedness and infer their potential functions. An lncRNA-mRNA co-expression network was

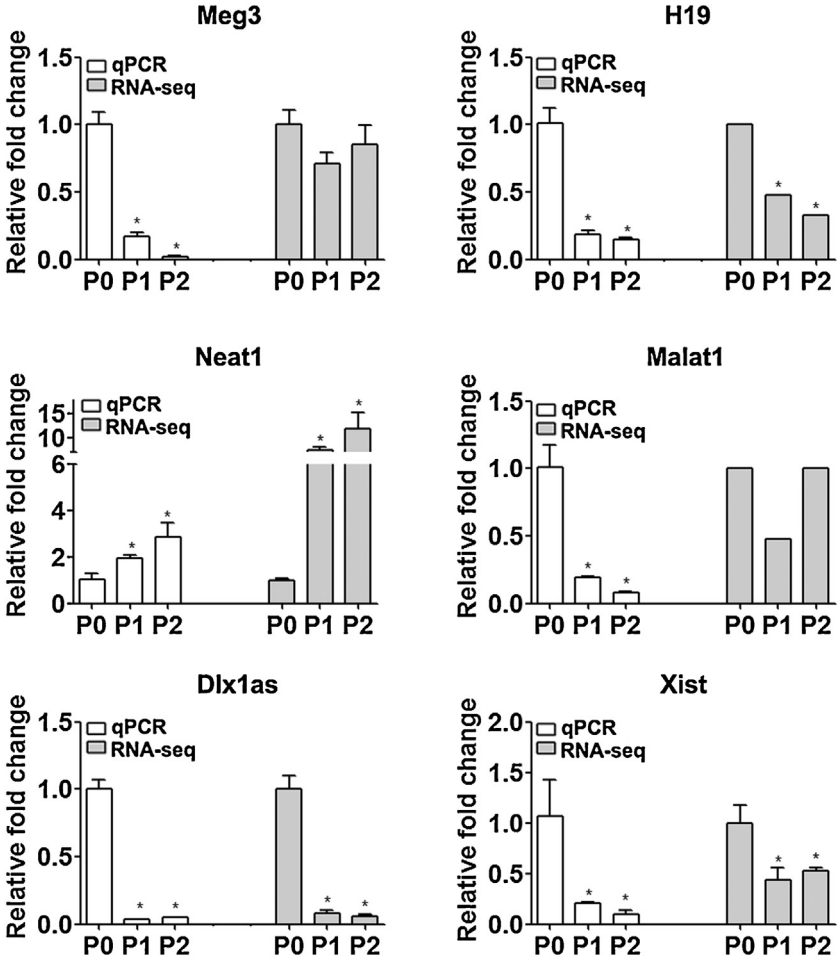


**Fig. 2.** Validation of RNA-seq data. The expression of six lncRNAs was validated using qRT-PCR and the results of qRT-PCR and RNA-seq were consistent. P, passage. The white bars represent the results of qRT-PCR, and the gray bars represent the results of RNA-seq. $^{*}p < 0.05$ compared with the P0 cells.

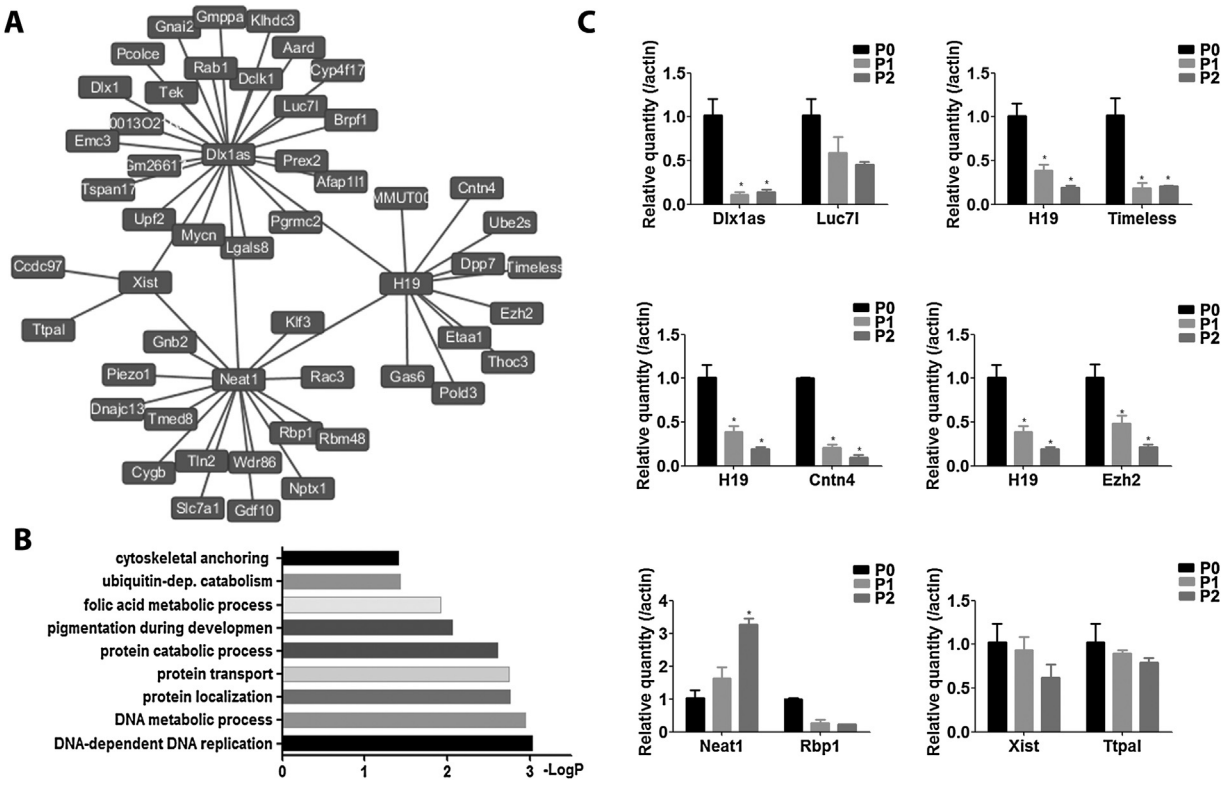


**Fig. 3.** Co-expression of lncRNAs and mRNAs. (A) An lncRNA-mRNA co-expression network was constructed based on the Pearson's correlation coefficients. (B) The Gene Ontology analysis of the mRNAs that are co-expressed with differentially expressed lncRNAs. (C) The co-expression of some lncRNAs and mRNAs was validated using qRT-PCR. $^{*}p < 0.05$ compared with the P0 cells.

constructed centering around *Xist*, *H19*, *Dlx1as*, and *Neat1* (Fig. 3A). In total, 54 pairs of highly inter-connected genes were identified, with 44.4% and 55.6% negatively and positively correlated pairs, respectively. Genes correlated with the lncRNAs were mainly associated with DNA and protein metabolic processes and cytoskeletal anchorage (Fig. 3B). The co-expression of genes was also validated by qRT-PCR, which revealed a consistent expression pattern between lncRNAs and the co-expressed mRNAs (Fig. 3C).

### 3.4. The expression of lncRNAs during tooth development

To assess the potential role of *H19*, *Meg3*, *Malat1*, *Xist*, *Dlx1as* and *Neat1* in tooth formation, their expression in developing tooth germs was examined. The odontogenic instructive signaling shifts from the dental epithelium to the dental mesenchyme at E12.5 (Mina & Kollar, 1987). E14.5 and E18.5 dental mesenchyme elicit the formation of tooth when recombined with dental or non-dental epithelium, while the E10.5 dental mesenchyme can not. The expression of *Meg3*, *Malat1*, *Neat1*, and *Xist* in E14.5 and E18.5 dental mesenchyme was significantly increased compared with E10.5 dental mesenchymal. *Dlx1as* was significantly increased at E18.5, and the level of *H19* was comparable between E10.5, E14.5, and E18.5 dental mesenchyme. (Fig. 4A).

Since Dlx family homeobox genes play a pivotal role in early tooth development (Thomas et al., 1997), the relationship between *Dlx1* and *Dlx1as* was investigated. The expression of *Dlx1* was decreased, while *Dlx1as* was increased, in E18.5 tooth germ compared to E14.5 tooth germ (Fig. 4B). Furthermore, Fgf8 that was shown to induce *Dlx1* expression (Bei & Maas, 1998) was used to induce the expression of *Dlx1*. Fgf8 induced the expression of *Dlx1*, but downregulated the expression of *Dlx1as* in dental mesenchymal cells (Fig. 4C). These results suggest a negative correlation between the levels of *Dlx1* and *Dlx1as*.

## 4. Discussion

Recently, the expression of lncRNAs has been analyzed in several tissues and cell types, and their expression is generally more cell type-specific than the expression of coding genes (Mercer, Dinger, Sunkin, Mehler, & Mattick, 2008). For example, region-specific ncRNA expression was identified in particular subregions of the brain and olfactory bulb (Mercer et al., 2008). An understanding of the transcriptional regulatory circuitry that is responsible for odontogenesis is fundamental to understanding the molecular mechanism of tooth development, providing insight into tooth regeneration. Although the role of mRNAs has been widely investigated, the role of lncRNAs in odontogenesis is poorly understood. Thus, the expression of lncRNAs in dental mesenchymal cells with and without odontogenic potential was examined, especially the lncRNAs that play roles in cell differentiation and the development of other organs. Specifically, *H19* is abundantly expressed maternally in embryonic tissues but is strongly repressed after birth, and significant transcription persists in skeletal muscle (Dey, Pfeifer, & Dutta, 2014). *H19* has been found to promote myogenesis and osteogenesis (Dey et al., 2014; Huang, Zheng, Jia, & Li, 2015). *Neat1* is an integral component of nuclear paraspeckles (Clemson et al., 2009), and is important for the development of mammary (Standaert et al., 2014). *Xist* is involved in silencing the inactive X chromosome (Avner & Heard, 2001), and it is first expressed at the 4-cell stage, coincident with the onset of embryonic genome activation (Briggs, Dominguez, Chavez, & Reijo Pera, 2015). *Meg3* is a maternally expressed imprinted gene and mouse embryonic stem cells with highly expressed *Meg3* displayed

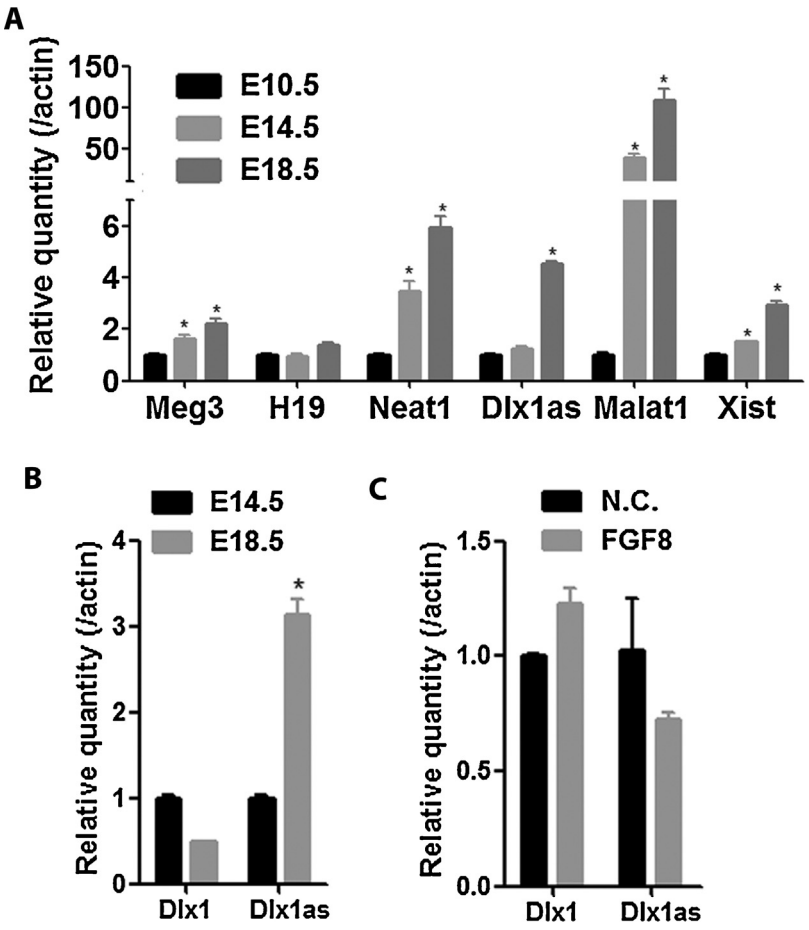


**Fig. 4.** The expression of *Meg3*,*Neat1*, *H19*, *Malat1*, *Xist*, and *Dlx1as* during tooth development. (A) The expression of *H19*, *Meg3*, *Dlx1as*, *Malat1*, *Xist*, and *Neat1* in the developing tooth germs. $^{*}p < 0.05$ compared with the E10.5 dental mesenchyme. (B) The levels of *Dlx1as* and *Dlx1* were negatively correlated in the developing tooth germ. (C) FGF8 increased the expression of *Dlx1* while decreasing the expression of *Dlx1as*. $^{*}p < 0.05$ compared with the E14.5 dental mesenchyme in B or the negative control (N.C.) in C.

increased capacity for neural lineage differentiation (Mo et al., 2015). *Dlx1as* is the antisense of *Dlx1* and is involved in the skeletal and neural system development (Kraus et al., 2013). *Malat1* is a highly conserved long non-coding RNA and plays a critical role in maintaining the proliferation potential of early-stage hematopoietic cells (Ma et al., 2015).

In this study, the expression of lncRNAs was found to differ significantly between dental mesenchymal cells with and without odontogenic potential, indicating that lncRNAs potentially play a role in odontogenesis apart from the coding RNAs. An lncRNA-mRNA co-expression network was constructed to predict their putative role in the regulation of odontogenic potential. The co-expressed genes mainly clustered in the DNA and protein metabolic processes and cytoskeletal anchorage function. In agreement with these function, *H19* has been found to promote cancer cell proliferation and migration (Sun, Wang et al., 2015; Yan et al., 2015), while *Neat1* is an integral component of nuclear paraspeckles (Clemson et al., 2009). The co-expression of some lncRNAs and mRNAs was also validated in the present study. Actually, some connections in the network were validated by others. For example, *H19* is associated with enhancer of zeste homolog 2 (EZH2), contributing to the pathogenesis of bladder cancer (Luo et al., 2013). Thus, the highly connected lncRNAs and mRNAs may represent interesting candidates for further studies.

In the present study, the expression of *Meg3*, *Malat1*, and *Xist* was decreased in cultured dental mesenchymal cells compared to freshly-isolated dental mesenchymal cells, and increased in dental mesenchyme tissues with vigorous odontogenic potential compared to the dental mesenchyme with no odontogenic potential. According to the microarray data, the level of *Meg3* and *Xist* in E13 dental mesenchyme is also higher than the level in E10 dental mesenchyme (O'Connell et al., 2012). This suggests that these genes may be involved in the regulation of odontogenic potential in dental mesenchymal cells, and the downregulation of these lncRNAs may indicate the compromised odontogenic potential. The expression of *H19* was decreased in cultured dental mesenchymal cells, but its expression was almost comparable between the dental mesenchyme with and without odontogenic potential, which is also consistent with the microarray data (O'Connell et al., 2012). This indicated that the downregulation of *H19* does not contribute to the loss of odontogenic potential in cultured dental mesenchymal cells. *Neat1* was increased in cultured dental mesenchymal cells and the dental mesenchymal tissues with odontogenic potential. Therefore further studies are needed to identify the correlation between the expression of *Neat1* and odontogenic potential. However, the upregulated expression of *Neat1* may indicate the loss of multipotency of dental mesenchymal cells considering that its expression is induced upon embryonic stem cells differentiation (Chen & Carmichael, 2009).

The antisense of *Dlx1* was also decreased in cultured dental mesenchymal cells, and may be involved in odontogenesis. Antisense transcripts are transcribed from the strand opposite to that of the originally annotated sense transcript of either protein-coding or non-protein-coding genes (Pelechano & Steinmetz, 2013). In the present study, the increased expression of

*Dlx1as* in E18.5 dental mesenchyme was accompanied by decreased expression of *Dlx1*. Moreover, FGF8, one of the growth factors upstream of *Dlx1* (Park et al., 2004; Shigetani, Nobusada, & Kuratani, 2000), induced the downregulation of *Dlx1as* and upregulation of *Dlx1* in dental mesenchymal cells. This indicates a reverse correlation between the expression of *Dlx1as* and *Dlx1*. Consistenly, the function of *Dlx1as* seems to be opposite to that of Dlx1 and mice devoid of *Dlx1as* display a mild phenotype reminiscent of a *Dlx1* gain-of function phenotype (Kraus et al., 2013). Similarly, the antisense of *Msx1*, *Msx1as*, was previously demonstrated to inhibit tooth morphogenesis by limiting *Msx1* expression during particular stages (Babajko et al., 2009; Coudert et al., 2005; Petit et al., 2009). Therefore, a similar mechanism may exist for the reciprocal regulation of the expression of *Dlx1* and *Dlx1as*.

In conclusion, we identified a group of differently expressed lncRNAs in dental mesenchymal cells with odontogenic potential compared with those without odontogenic potential., The results suggest that the downregulation of *Meg3*, *Malat1*, *Xist* was associated with the loss of odontogenic potential. Moreover, a negative correlation between *Dlx1as* and *Dlx1* was indicated. This study provides new insights into the regulation of odontogenic potential and tooth formation.

## Funding

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## Ethical approval

All animal experiments were performed under the guidelines of the Committee of Animal Care and Use Committee of Guangzhou Institutes of Health and the Peking University in China (permit Number: CMU-B20100106).

## Conflicts of interest

There is no conflict of interest of any authors.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2016.03.001.

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