Crucial Roles of microRNA-16-5p and microRNA-27b-3p in Ameloblast Differentiation Through Regulation of Genes Associated With Amelogenesis Imperfecta

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Amelogenesis imperfecta is a congenital disorder within a heterogeneous group of conditions characterized by enamel hypoplasia. Patients suffer from early tooth loss, social embarrassment, eating difficulties, and pain due to an abnormally thin, soft, fragile, and discolored enamel with poor aesthetics and functionality. The etiology of amelogenesis imperfecta is complicated by genetic interactions. To identify mouse amelogenesis imperfecta-related genes (mAIGenes) and their respective phenotypes, we conducted a systematic literature review and database search and found and curated 70 mAIGenes across all of the databases. Our pathway enrichment analysis indicated that these genes were enriched in tooth development-associated pathways, forming four distinct groups. To explore how these genes are regulated and affect the phenotype, we predicted microRNA (miRNA)-gene interaction pairs using our bioinformatics pipeline. Our miRNA regulatory network analysis pinpointed that miR-16-5p, miR-27b-3p, and miR-23a/b-3p were hub miRNAs. The function of these hub miRNAs was evaluated through ameloblast differentiation assays with/without the candidate miRNA mimics using cultured mouse ameloblast cells. Our results revealed that overexpression of miR-16-5p and miR-27b-3p, but not miR-23a/b-3p, significantly inhibited ameloblast differentiation through regulation of mAIGenes. Thus, our study shows that miR-16-5p and miR-27b-3p are candidate pathogenic miRNAs for amelogenesis imperfecta.

Keywords: enamel, amelogenesis imperfecta, tooth defects, pathogenic gene, microRNA, ameloblast differentiation

INTRODUCTION

Enamel is composed of inorganic and organic matter and water. The inorganic component, called hydroxyapatite, mainly comprises calcium, phosphate, magnesium, potassium, fluoride, and sodium, whereas the organic component includes enamel matrix proteins and enzymes. FAM20C is a Golgi-localized serine/threonine-protein kinase that is activated by FAM20A
(Cui et al., 2015; Ohyama et al., 2016) and phosphorylates enamel matrix proteins, including Amelogenin (AMELX), Amelotin (AMTN), and Enamelin (ENAM), for mineralization (Ishikawa et al., 2012; Tagliabruni et al., 2012; Wang et al., 2013; Cui et al., 2015). The phosphorylated enamel matrixes provide a platform for further mineralization, during which they are cleaved and degraded by MMP20 and KLK4, and then removed from the hydroxyapatite crystals (Hu et al., 2007; Hu and Simmer, 2007). A failure in the degradation of the enamel matrixes leads to retention of enamel matrix residues between the hydroxyapatite crystals, abnormal crystal formation, and immature enamel formation (Simmer and Hu, 2002; Kwak et al., 2016; Yamazaki et al., 2019). Recent studies suggest that WDR72 may be important for the resorption of the enamel matrixes (especially for AMELX) from the extracellular matrix (ECM) through endocytosis of ameloblasts (Katsura et al., 2014; Wang et al., 2015).

Amelogenesis imperfecta (a.k.a. enamel hypoplasia) is a congenital disorder that affects the tooth surface and is characterized by abnormal enamel formation (Gadhia et al., 2012; Williams and Letra, 2018). The frequency of the condition varies among different populations worldwide, e.g., 1:700 in Sweden (Backman and Holm, 1986), 43:10,000 in Turkey (Altug-Atac and Erdem, 2007), and 1:14,000 in the United States (Crawford et al., 2007). The disorder may manifest by itself through a mutation in genes encoding enamel proteins or may accompany other morphological defects in tooth development (Aldred et al., 2003; Stephanopoulos et al., 2005; Smith et al., 2017). The affected enamel displays a wide range of severity of abnormalities, ranging from pits and grooves on the tooth’s surface to a complete loss of enamel, which results in easily brittle and worn teeth. These patients suffer from poor esthetic appearance due to tooth discoloration, abnormal tooth shape, open bite, and premature tooth loss, in addition to tooth pain, eating difficulties, and frequent and full-mouth dental maintenance and eating (Hashem et al., 2013).

Based on the distinct phenotype and mode of inheritance, amelogenesis imperfecta can be divided into four major categories: hypoplastic enamel, hypomaturation enamel, hypocalciﬁed enamel, and hypomaturation-hypoplastic enamel with taurodontism (Aldred and Crawford, 1995; Aldred et al., 2003). In hypoplastic enamel (type I), the enamel is thinner than usual but can retain its typical hardness and translucency. Due to the enamel matrix’s malfunction, the mature enamel layer often presents pits and grooves; other consequences of the thin enamel include lack of occlusion owing to small or absent cusps in the posterior molars. A distinct difference in density between dentin and the enamel layers can be seen in radiographs (Witkop, 1988; Wright, 2006). In the case of hypomaturation enamel (type II), the enamel is softer than normal due to a failure in protein removal during the maturation stage of amelogenesis. These enamel proteins that remain in the matrixes compromise the enamel matrix structure and crystal growth. While enamel thickness appears normal, its hardness is lower, resulting in pits on the surface and rapid wear. In radiographs, the enamel layer appears similar to dentin due to reduced density (Witkop, 1988; Wright, 2006). In hypocalciﬁed enamel (type III), the enamel is softer, rougher, and more prone to rapid wear than in type II cases due to abnormal mineralization (Witkop, 1988; Urzua et al., 2011). While the enamel appears to be of normal thickness, the abnormal mineralization leads to extremely brittle teeth without a smooth and translucent appearance. The dentin in these cases is more radiopaque than the enamel (Witkop, 1988; Wright, 2006). Lastly, in the hypomaturation/hypoplastic enamel with taurodontism (type IV), patients have thin, pitted enamel with enlarged pulp chambers in the molars (Witkop, 1988; Wright, 2006).

Clinically, patients often present a mixed phenotype. Treatment for amelogenesis imperfecta consists in the prevention of gradual occlusal wear, in which case early detection is beneficial. Full-mouth prosthetics can preserve the remaining enamel, prevent further tooth loss, and reduce pain caused by dentin exposure (Strauch and Hahnel, 2018).

While various genetic mutations have been reported in amelogenesis imperfecta, the regulatory network remains unknown. MicroRNAs (miRNAs), typically 21–22 nucleotide long, negatively regulate gene expression at the post-transcriptional stage and usually have multiple target genes and control their expression at the regulatory network level (Guo et al., 2010; Li et al., 2020). Recent studies suggest that miRNAs play crucial roles in tooth development (Fan et al., 2015; Farmer and Mcmanus, 2017; Jin et al., 2017); therefore, this study aimed to identify the regulatory network of genes and miRNAs associated with amelogenesis imperfecta. A better understanding of the mechanism of amelogenesis imperfecta can potentially lead to the development of novel preventive and therapeutic interventions.

**MATERIALS AND METHODS**

**Eligibility Criteria for the Systematic Review**

This systematic review followed the publishing guidelines and checklist established by PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analysis). Articles were included and excluded based on the following eligibility criteria: 1) Inclusion criteria: described genes causing or potentially associated with amelogenesis imperfecta and enamel hypoplasia in species other than humans; published as original articles (not as review articles, editorials, dissertations, conference proceedings, or comments); and published in the English language; 2) Exclusion criteria: gene mutations were not described in the original articles; enamel defects resulting from exposure to environmental risk factors; cell-based experiments, molecular and biochemical analyses, structural and component analyses, and evolutionary researches; and the articles failed to fit in any of the above criteria but did not include amelogenesis imperfecta candidate genes or related information.

**Information Sources and Search**

The search for articles was conducted through three central literature databases: Medline (Ovid), PubMed (National Library of Medicine), and Embase (Ovid). In addition, relevant articles were searched in Scopus (Elsevier) to retrieve any studies.
missed in the database searches. Concepts included in the search to identify studies were *amelogenesis imperfecta* and *genetics* (gene mutation). No specific species was included in the keywords since our review included all species. A combination of Medical Subject Headings (MeSH) terms and titles, abstracts, and keywords was developed to obtain the initial Medline search string, and then adapted to the searches of the other databases. The Mouse Genome Informatics (MGI) database was searched using keywords “amelogenesis imperfecta,” “enamel hypoplasia,” “tooth enamel,” “tooth mineralization,” and “enamel mineralization” in order to provide a means of comparison and validation for the systematic review and identify genes that were potentially missed in the database searches.

**Study Selection and Data Collection**

The citations searched were stored in Rayyan (https://rayyan.qcri.org/welcome), an online application for systematic reviews that stores the citations/results, automatically processes the removal of duplicates obtained through various database searches, and tracks the decisions made during the systematic review. The primary Excel workbook designed for the systematic review (http://libguides.sph.uth.tmc.edu/excel_SR_workbook) was also used for tracking search strategies and results. A Cohen’s kappa test was conducted by two screeners to check the reliability of study selection during title and abstract screening. After achieving a >90% score for the Cohen’s Kappa test, all the titles and abstracts found through the database search were full-text reviewed by the two screeners independently. All the screening results were recorded in the Primary Excel workbook, and a codebook for data collection from eligible articles was developed as previously described (Sangani et al., 2015).

**Bioinformatics Analysis**

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) was used for the gene set enrichment analysis. Gene Ontology (GO), including its Biological Process (BP), Molecular Function (MF), and Cellular Component (CC), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were used as reference gene sets (Sun H. et al., 2019). The top five most significant pathways or GO terms were selected for further analysis. k-means was used to cluster the gene functional enrichment results and the square error to extract the closest clusters. The highly-expressed mouse tooth miRNAs were retrieved from the publications (Cao et al., 2010). The miRNA-mAiGene regulations were integrated using the data from four databases: TargetScan (version 7.1) (Agarwal et al., 2015), miRanda (August 2010 Release) (John et al., 2004), miRTarBase (Release 7.0) (Huang et al., 2020), and PITA (version 6) (Kertesz et al., 2007). Considering the possibility of false results and multiple targets for each miRNA in these databases, the intersection of miRanda and PITA was merged with the intersection of TargetScan and miRTarBase to obtain reliable miRNA-mAiGene pairs. This conservative approach was demonstrated to effectively reduce the prediction of false-positive miRNA-mAiGene pairs (Jiang et al., 2016; Bonnet et al., 2020). Each gene set (GO term or KEGG pathway) containing at least two genes was used in the core miRNA family-based regulatory network. A Fisher’s exact test was applied to assess the enrichment significance of the miRNAs. All networks were visualized using Cytoscape (Shannon et al., 2003).

**Cell Culture**

The mHAT9d mouse dental epithelial cell line originated from the apical bud of the incisors was a gift from Dr. Hidemitsu Harada (Iwate Medical University, Iwate, Japan). mHAT9d cells were cultured in Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Thermo Fisher Scientific) supplemented with B-27 (Thermo Fisher Scientific), 25 ng/ml basic FGF (233-FB; R&D Systems), 20 ng/ml EGF (2028-EG; R&D Systems), and penicillin/streptomycin (Otsu et al., 2016). The LS8 cell line (Chen et al., 1992) was provided by Dr. Malcolm Snead (University of Southern California). Cells were plated at a density of 60,000 cells onto a 12-well cell culture plate and maintained until 80% confluence. The cells were treated with mimic for a negative control, miR-16-5p, miR-23a-3p, miR-23b-3p, miR-27b-3p, or miR-214-3p (mirVana miRNA mimic, Thermo Fisher Scientific) using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific), according to the manufacturer’s protocol (24 pmol of mimic and 3 µL of transfection reagent in 1 ml of medium per well). After 24 h of treatment, the cells at 100% confluence were cultured with differentiation medium [including 15 µg/ml retinoic acid (R2625, Sigma Aldrich) and 0.1 µM dexamethasone (D4902, Sigma Aldrich)] in order to induce ameloblast differentiation.

**Bromodeoxyuridine (BrdU) Incorporation Assay**

mHAT9d cells were plated onto ibiTreat 8-well µ-slides (ibidi GmbH, Munich district, Germany) at a density of 10,000/ chamber and cultured until 80% confluence. Cells were then treated with a mimic for miR-16-5p, miR-27b-3p, or control using Lipofectamine RNAiMAX transfection reagent (4.8 pmol of mimic with 0.48 µL of transfection reagent in 200 µL of proliferation medium). After 24 h of transfection, the cells were cultured under differentiation medium for 48 h. In addition, cells were treated with 100 µg/ml BrdU (Sigma Aldrich) for 1 h at day 2 of differentiation (n = 6 per group) and visualized with a rat monoclonal antibody against BrdU (ab6326; Abcam, 1:1,000), as previously described (Yoshioka et al., 2021a). BrdU-positive cells were quantified using images from six independent experiments.

**RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction**

Total RNAs were isolated from cells treated with mimics for the target miRNAs or negative control (n = 6 per group) using the QIAshredder and RNEasy mini extraction kit or the miRNeasy mini kit (QIAGEN), as previously described (Suzuki et al., 2019; Yan et al., 2020). In addition, total RNAs were isolated from ameloblasts at each stage of differentiation (pre-secretion, secretion, and maturation) in the lower incisors of 8-week old
males C57BL/6J mice (n = 3). Briefly, the lower incisors were extracted, and ameloblasts were manually dissected and separated into three parts [apical 1/3 (pre-secretion), middle 1/3 (secretion), and incisal 1/3 (maturation)] between the cervical loop and bony ridge of the incisor] under a dissection microscope. cDNA was reverse-transcribed with the iScript Reverse Transcription Super Mix (BioRad) and amplified with the iTaq Universal SYBER Green Super Mix (BioRad) using a CFX96 Touch Real-Time PCR Detection System (BioRad). The expression of genes was normalized with Gapdh. miRNA expression during ameloblast differentiation was detected with Taqman Fast Advanced Master Mix and Taqman Advanced miR cDNA Synthesis Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions. The PCR primers used are listed in Supplementary Table S1.

**Immunofluorescence Analysis**

The cells were plated onto ibiTreat 8-well μ-slides (ibidi GmbH, Munich district, Germany) at a density of 10,000/chamber and maintained until 80% confluency. The cells were then treated with mimics for miR-16-5p, miR-27b-3p, or a negative control, using Lipofectamine RNAiMAX transfection reagent (4.8 pmol of mimic with 0.48 µL of transfection reagent in 200 µL of differentiation medium) (n = 4 per group). After 24 h of treatment, the medium was replaced with differentiation medium for 2 days. AMELX expression was detected with anti-AMELX rabbit polyclonal antibody (ab153915, Abcam, 1:250), as previously described (Yoshioka et al., 2021b). Immunofluorescent images were captured with a confocal microscope (Ti-E, Nikon United States).

**Immunoblotting**

The cells were plated onto 12-well plates at a density of 60,000 per well, maintained until 80% confluence, and treated with either miR-16-5p, miR-27b-3p, or a negative control mimic, for 24 h (n = 3 per group). The cells were then cultured in ameloblast differentiation medium for another 48 h. The treated cells were
| # | Gene Symbol | Gene Name | Location | Enamel Phenotype | Mouse Strain | PMID | Human Disease |
|---|---|---|---|---|---|---|---|
| 1 | Alpl | alkaline phosphatase, liver/bone/kidney | 4 D3 | hypoplastic | Alpl<sup>−/−</sup> | 10371245 | hypophosphatasia-enamel hypoplasia |
| 2 | Ambn | ameloblastin | 5 E1 | hypoplastic | Tg (under Amelx) | 12657627 | isolated AI |
| 3 | Amelx | amelogenin, X-linked | X F5 | hypoplastic | Amelx<sup>−/−</sup> | 11406633 | isolated AI |
| 4 | Amtn | amelotin | 5 E1 | hypomaturation and hypomineralized | Amtn<sup>−/−</sup> | 25715379 | isolated AI |
| 5 | Arhgap6 | Rho GTPase activating protein 6 | X F5 | hypoplastic | Arhgap6<sup>−/−</sup> | 16007484 | isolated AI |
| 6 | Ascl5 |achaete-scute family bHLH transcription factor 5 | 1 E4 | hypoplastic | Ascl5<sup>−/−</sup> | 30504223 | |
| 7 | Bcl11b | B cell leukemia/lymphoma 11B | 12 F1 | hypomineralized | Bcl11b<sup>−/−</sup> | 23727454 | |
| 8 | Bmp2 | bone morphogenetic protein 2 | 2 F2 | hypomineralized | Otsx-Cre;Bmp2<sup>F/F</sup> | 21597270; 25545831 | |
| 9 | Ctr | cystic fibrosis transmembrane conductance regulator | 6 A2 | hypomineralized | Ctr<sup>−/−</sup> | 9206347; 8708137; 12161463 | cystic fibrosis—AI |
| 10 | Cldn3 | claudin 3 | 5 G2 | hypomineralized | Cldn3<sup>−/−</sup> | 28596736 | familial hypercalciuria and hypomagnesemia with nephrocalcinosis (FHHNC) —AI |
| 11 | Cldn16 | claudin 16 | 16 B2 | hypoplastic and hypomineralized | Cldn16<sup>−/−</sup> | 2642691 | |
| 12 | Cnnm4 | cyclin M4 | 1 B | hypomineralized | Cnnm4<sup>−/−</sup> | 24339795 | Jalli syndrome—AI |
| 13 | Col17a1 | collagen, type XVII alpha 1 | 19 D1 | hypomaturation and hypomineralized | Col17a1<sup>−/−</sup> | 19036806 | Junctional epidermolysis bullosa—AI |
| 14 | Csfr1 | colony-stimulating factor 1 (macrophage) | 3 F2 | hypoplastic | OP/OP; OP/OP; Tg (csCSF-1) | 17126805 | |
| 15 | Ctnnb1 | catenin beta 1 | 9 F4 | hypomineralized | Amelx-Cre;Ctnnb1<sup>Δex3F/F</sup> | 30066216 | |
| 16 | Dlx3 | distal-less homeobox 3 | 11 D | hypomineralized | K14-Cre;Dlx3<sup>−/−</sup> | 27760456; 29745813 | trichodentosseous syndrome—AI |
| 17 | Dmp1 | dentin matrix protein 1 | 5 E5 | hypoplastic and hypomineralized | Dmp1<sup>−/−</sup> | 14966118; 14514755 | hypophosphatemia—AI |
| 18 | Dspp | dentin sialophosphoprotein | 5 E5 | hypoplastic | Tg (under Amelx) | 16014627 | dentinogenesis imperfecta type II—AI |

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| #  | Gene Symbol | Gene Name                        | Location | Enamel Phenotype                                           | Mouse Strain                  | PMID                     | Human Disease                                      |
|----|-------------|----------------------------------|----------|-----------------------------------------------------------|-------------------------------|--------------------------|---------------------------------------------------|
| 19 | Eda         | ectodysplasin-A                  | X C3     | hypoplastic (no enamel)                                   | Tg (under K14)                | 12812793                 | hypohidrotic ectodermal dysplasias not AI          |
| 20 | Enam        | enamelin                         | 5 E1     | hypomutation, hypoplastic, hypomineralized hypoplastic     | Enam^Rgapc521/Rgapc521 &     | 15649948; 20598351       | isolated AI                                       |
|     |             |                                  |          | hypoplastic or no enamel or hypoplastic                    | Enam^Rgapc514/Rgapc514 &     | 15649948                 |                                                   |
|     |             |                                  |          |                                                           | Enam^R176G/p.176X            | 15271968                 |                                                   |
|     |             |                                  |          |                                                           | Enam^P.S55I/p.55I            | 23034996                 |                                                   |
| 21 | Fam20a      | family with sequence similarity 20, member A | 11 E1 | hypoplastic and hypomineralized hypoplastic hypoplastic    | Fam20a<sup>-/-</sup>          | 22373258                 | enamel-renal-gingival syndrome—AI                 |
|     |             |                                  |          | hypoplastic (no enamel)                                   | K14-Cre;Fam20a<sup>FF</sup>  | 27280136                 |                                                   |
|     |             |                                  |          |                                                           | Sox2-Cre;Fam20a<sup>FF</sup> | 31067961                 |                                                   |
| 22 | Fam20c      | family with sequence similarity 20, member C | 5 G2 | hypoplastic (no enamel)                                   | Fam20c<sup>-/-</sup>          | 22373258                 | Raine syndrome—Al                                  |
|     |             |                                  |          | hypoplastic and hypomineralized hypoplastic hypomineralized| K14-Cre;Fam20c<sup>FF</sup>  | 24026952                 |                                                   |
|     |             |                                  |          | hypoplastic and hypomineralized                            | Sox2-Cre;Fam20c<sup>FF</sup> | 22936805                 |                                                   |
| 23 | Fam83h      | family with sequence similarity 83, member H | 15 D3 | hypoplastic hypoplastic                                   | Fam83h<sup>-/-</sup>          | 30774028                 | isolated Al                                       |
|     |             |                                  |          | Tg (truncated protein 1–206)                              | K14-Cre;Fam83h<sup>FF</sup>  | 31067961                 |                                                   |
| 24 | Fgfr1       | fibroblast growth factor receptor 1 | 8 A2     | hypoplastic                                               | K14-Cre;Fgfr1<sup>FF</sup>   | 18296907                 | Pfeiffer syndrome—not AI Jackson-Weiss syndrome—not AI |
| 25 | Foxo1       | forkhead box O1                   | 3 C      | hypomutation                                              | Rx-Cre;Foxo1<sup>FF</sup> & K14-Cre;Foxo1<sup>FF</sup> | 22291914                 |                                                   |
| 26 | Gdnf        | glial cell line derived neurotropic factor | 15 A1 | hypoplastic                                               | Gdnf<sup>-/-</sup>            | 11878293                 | Hirschsprung disease type 3—not Al                |
| 27 | Gja1        | gap junction protein, alpha 1     | 10 B4    | hypoplastic hypoplastic                                   | PGK-Cre;Cx43G138R<sup>FF</sup> | 18003637                 | oculodentodigital dysplasia - Al                  |
|     | (a.k.a. Cx43)|                                  |          | Gja1<sup>G60S</sup>/Gja1<sup>jrt</sup>/a.k.a. Gja1<sup>FF</sup> | 16155213; 20127707          | 16155213; 20127707       |                                                   |
| 28 | Hmgr2       | high mobility group nucleosomal binding domain 2 | 4 D3 | hypoplastic                                               | Tg (under K14)                | 23975681                 |                                                   |
| 29 | Hras        | Harvey rat sarcoma virus oncogene | 7 F5     | hypominalized                                             | Caggs-Cre;Hras<sup>G12V</sup> | 24057668; 19416908       | Costello syndrome—enamel defect                   |
| 30 | Ifit6       | interferon regulatory factor 6    | 1 H6     | hypoplastic                                               | Pb2-Cre;Ifit6<sup>FF</sup>   | 27369889                 | van der Woude syndrome—not Al popliteal pterygium syndrome—not Al |
| 31 | Itgb1       | integrin beta 1                   | 8 E2     | hypoplastic                                               | K14-Cre;Itgb1<sup>FF</sup>   | 25830530                 |                                                   |
| 32 | Itgb6       | integrin beta 6                   | 2 C1.2   | hypominalized                                             | Itgb6<sup>-/-</sup>           | 23264742                 | isolated Al                                       |
| 33 | Kld4        | kallkrein-related peptidase 4 (prostate, enamel matrix, prostate) | 7 B3 | hypominalized                                             | Kld4<sup>lacZ2;lacZ2</sup>   | 19578120                 | isolated Al                                       |
| 34 | Lama3       | laminin, alpha 3                  | 18 A1    | hypoplastic                                               | Lama3<sup>-/-</sup>           | 10366601                 | junctional epidermolysis bullosa—Al               |
| 35 | Lamb3       | laminin, beta 3                   | 1 H6     | unknown                                                   | Lamb3<sup>−</sup>a<sup>lacZ2</sup>| 27626380                 | junctional epidermolysis bullosa—Al               |
| 36 | Lamc2       | laminin gamma 3                   | 1 G3     | pitted enamel hypominalized                               | Spontaneous (Lamc2<sup>FF</sup>) Tg (TetO-Lamc2<sup>FF</sup>;K14-rTa;TetO-HumLAMC2) | 20336083; 26956061; 23029085 | cortical malformation, occipital—not Al            |

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| #  | Gene Symbol | Gene Name                               | Location | Enamel Phenotype       | Mouse Strain                  | PMID                      | Human Disease                                      |
|----|-------------|-----------------------------------------|----------|-----------------------|-------------------------------|---------------------------|---------------------------------------------------|
| 37 | Ltbp3       | latent transforming growth factor-beta binding protein 3 | 19 A     | hypoplastic            | Ltbp3<sup>−/−</sup>          | 25699657; 28084688        | dental anomalies and short stature (DASS)—AI       |
| 38 | Map3k7      | mitogen-activated protein kinase kinase 7 | 4 A5     | hypomineralized        | CaMap3k7 (under Amelx)       | 29024853                  | cardiospondylocarpofacial syndrome—not AI         |
| 39 | Med1        | mediator complex subunit 1              | 11 D     | hypomineralized        | K14-Cre;Med1<sup>F/F</sup>   | 24949995; 28673966        | potent inhibition of transcription factors         |
| 40 | Mmp20       | matrix metallopeptidase 20 (enamelysin) | 9 A1     | hypoplastic            | Mmp20<sup>−/−</sup>          | 12393861; 15557396; 24466234; 29481294 | isolated Al                                       |
| 41 | Mtx2        | msh homeobox 2                          | 13 B1    | hypoplastic            | Mtx2<sup>LacZ/LacZ</sup>     | 20934968; 17878071        | isolated Al enlarged parietal foramina—Al         |
| 42 | Nectin1     | nectin cell adhesion molecule 1         | 9 A5     | hypomineralized        | Nectin1<sup>−/−</sup>        | 18703497; 21038445        | clef lip and palate/ectodermal dysplasia 1—not Al |
| 43 | Nectin3     | nectin cell adhesion molecule 3         | 16 B5    | unknown                | Nectin3<sup>−/−</sup>        | 21038445                  |                                                   |
| 44 | Pax9        | paired box 9                            | 12 C1    | hypoplastic            | Pax9<sup>neo/neo</sup>       | 16236760                  | tooth agenesis, selective, 3—not Al               |
| 45 | Plau        | plasminogen activator, urokinase        | 14 A3    | unknown-chalky white  | Tg (under K5)                | 9927592; 15161862         |                                                   |
| 46 | Ptb2        | paired-like homeodomain transcription factor 2 | 3 G3 | unknown                | Ptb2<sup>−/−</sup>           | 27826380                  | Axenfeld-Rieger syndrome—not Al                   |
| 47 | Postn       | periostin, osteoblast-specific factor    | 3 C      | unknown-chalky white  | Postn<sup>−/−</sup>          | 16314533                  |                                                   |
| 48 | Rac1        | Rac family small GTPase 1               | 5 G2     | hypoplastic and hypomineralized | K14-Cre;Rac1<sup>F/F</sup> | 22243243                  | mental retardation, autosomal dominant, 48—not Al |
| 49 | Ref         | RELT tumor necrosis factor receptor     | 7 E2     | hypomineralized        | Ref<sup>−/−</sup>; P530<sup>−/−</sup>; P50<sup>−/−</sup> | 30506946                  | isolated Al                                       |
| 50 | Rhoa        | ras homolog family member A             | 9 F1-F2  | hypoplastic            | Tg (dominant-negative, under Amelx) | 21576911; 23941790       | Braddock-Carey syndrome (BCS)—Al                  |
| 51 | Runx1       | runt-related transcription factor 1     | 16 C4    | hypoplastic            | K14-Cre;Runx1<sup>F/F</sup> | 30026553                  | metaphyseal dysplasia with maxillary hypoplasia and brachydactyly—Al |
| 52 | Runx2       | runt-related transcription factor 2     | 17 B3    | hypomineralized        | K14-Cre;Runx2<sup>F/F</sup> | 29941908                  | cleidocranial dysplasia—not Al                    |
| 53 | Slc4a4      | solute carrier family 4 (anion exchanger), member 4 | 5 E1 | hypoplastic and hypomineralized | Slc4a4<sup>−/−</sup>          | 20520845; 25012520        | proximal renal tubular acidosis—Al                 |
| 54 | Slc10a7     | solute carrier family 10 (sodium/bile acid cotransporter family), member 7 | 8 C1 | hypoplastic            | Slc10a7<sup>−/−</sup> Slc10a7<sup>−/−</sup> | 30082715; 30082715        | skeletal dysplasia—Al                              |
| 55 | Slc12a2     | solute carrier family 12, member 2      | 18 D3    | hypomineralized        | Slc12a2<sup>−/−</sup>         | 29209227                  |                                                   |
lysed with RIPA buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail (Roche) and centrifuged at 21,130 × g for 20 min at 4°C. The protein concentration of the supernatants was measured with the BCA protein kit (Pierce). Protein samples (30 μg) were applied to Mini-PROTEAN TGX Gels (Bio-Rad) and transferred to a polyvinylidene fluoride (PVDF) membrane. Anti-AMELX rabbit polyclonal antibody (ab153915, Abcam, 1:1,000), anti-KLK4 rabbit polyclonal antibody (PA5-109888, Thermo Fisher Scientific, 1:750), anti-MMP20 rabbit polyclonal antibody (55467-1-AP, Proteintech, 1:750), and anti-GAPDH mouse monoclonal antibody (MAB374, Millipore, 1:6,000) were used for immunoblotting. Peroxidase-conjugated anti-rabbit IgG (7074, Cell Signaling Technology, 1:100,000) and anti-mouse IgG (7076, Cell Signaling Technology, 1:100,000) were used as secondary antibodies. All immunoblotting experiments were performed three times to validate the results.

### Rescue Experiment

Cells were plated on 12-well cell culture plates at a density of 60,000 cells per well, or on ibiTreat 8-well μ-slides (ibidi GmbH, Munich district, Germany), at a density of 10,000 cells per well and maintained until 80% confluence. The cells were treated with mimics for a negative control, miR-16-5p, or miR-27b-3p (4.8 pmol for 12-well plates and 1.2 pmol for ibiTreat 8-well μ-slides) with a combination of overexpression vectors [100 ng (12-well plates) or 25 ng (ibiTreat 8-well μ-slides)] using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific), according to the protocol provided by the manufacturer. The cells were harvested after 24 hours of transfection and processed for immunoblotting as described above.

### Table 1 (Continued)

| # | Gene Symbol | Gene Name | Location | Enamel Phenotype | Mouse Strain | PMID | Human Disease |
|---|-------------|-----------|----------|------------------|--------------|------|----------------|
| 56 | Slc13a5 | solute carrier family 13 (sodium-dependent citrate transporter), member 5 | 11 B4 | hypoplastic | Slc13a5−/− | 28406943 | Kohlschütter-Tönz syndrome (KTS)—AI early infantile epileptic encephalopathy 25 (EIEE25)-tooth hypoplasia and hypodontia—not AI |
| 57 | Slc24a4 | solute carrier family 24 (sodium/potassium/calcium exchanger), member 4 | 12 E | hypomineralized | Slc24a4−/− | 23375655 | isolated AI |
| 58 | Smad3 | SMAD family member 3 | 9 C | hypomineralized | Smad3−/− | 12763048 | Loeys-Dietz syndrome—not AI |
| 59 | Sp3 | trans-acting transcription factor 3 | 2 C3 | hypoplastic (no enamel) | Sp3−/− | 10675334 | |
| 60 | Sp6 | trans-acting transcription factor 6 | 11 D | hypoplastic | Sp6−/− | 30504223; 18156176; 18297738 | |
| 61 | Sp7 (a.k.a. Osx) | trans-acting transcription factor 7 (osterix) | 15 F3 | unknown (die at birth) | Sp7−/− | 29405385 | osteogenesis imperfecta type XII - not AI |
| 62 | Stim1 | stromal interaction molecule 1 | 7 E2-E3 | hypomineralized | K14-Cre;Stim1F/F Amelx-Cre;Stim1F/F | 28732182; 31329049 | AI tubular aggregate myopathy—not AI Stormorken syndrome—not AI |
| 63 | Tdx1 | T-box 1 | 16 A3 | hypoplastic (no enamel) | Tdx1−/− | 19233155 | 22q-11.2 deletion syndrome (DiGeorge syndrome)—AI |
| 64 | Torg1 (a.k.a. ATPase, H+ transporting, lysosomal V0 protein A3) | T cell, immune regulator 1, | 19 A | hypomineralized | spontaneous | 23174213 | autosomal recessive osteopetrosis—not AI |
| 65 | Tgfb1 | transforming growth factor, beta 1 | 7 A3 | hypoplastic | Tg (under Dspp) Tgfb1 Tgfb3/Tgfb3 K14-Cre;Tgfb1F/F | 16674659; 11116156; 24056969; 30243146 | Camurati-Engelmann disease—not AI |
| 66 | Tgfr2 | transforming growth factor, beta receptor II | 9 F3 | hypoplastic and hypomineralized | Amelx-Cre;Tgfr2−/− | 24278477 | Loeys-Dietz syndrome—not AI familial thoracic aortic aneurysm and dissection - not AI |
| 67 | Tmbim6 | transmembrane BAX inhibitor motif containing 6 | 15 F1 | hypomineralized | Tmbim6−/− | 30965569 | |
| 68 | Wdr72 | WD repeat domain 72 | 9 D | hypomaturation and hypomineralized | Wdr72LacZ/LacZ | 25008349; 26247047 | isolated AI |

AI: amelogenesis imperfecta; OP: osteopetrotic; Tg: transgenic.
to the manufacturer’s protocol, which was followed by treatment with *Eda* (Antibodies-online Inc., ABIN3291185), *Relt* (Antibodies-online Inc., ABIN4054001), or *Smad3* (Antibodies-online Inc., ABIN3809504) for the miR-16-5p mimic, or *Bmp2* (Antibodies-online Inc., ABIN4045152), *Pax9* (Antibodies-online Inc., ABIN4216431), or *Slc24a4* (Addgene, 75208) for the miR-27b-3p mimic (n = 6 per group). After 24 h of transfection, the medium was switched to differentiation medium for 2 days.

**Statistical Analysis**

Statistical comparisons between two groups were performed with a two-tailed Student’s *t*-test. Multiple comparisons were conducted with one-way analysis of variance with the Tukey–Kramer post hoc test. A *p*-value of less than 0.05 was considered as statistically significant. For all groups, data were represented as mean ± SD.

**RESULTS**

**Literature and Database Search**

A total of 4,846 articles were extracted from a database compilation of multiple sources through a search conducted using Rayyan (Ouzzani et al., 2016). After resolving duplicates with RefWorks, 2,306 articles were selected for further screening. A total of 2,207 articles were excluded because there was no underlying genetic mechanism dictating the gene findings or the articles did not mention any relevant study or research conducted

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**TABLE 2 | Compound mutant mouse models with enamel defects.**

| # | Gene Symbol | Gene Name | Location | Enamel Phenotype | Mouse Strain | PMID |
|---|---|---|---|---|---|---|
| 1 | Ambn and Enam | ameloblastin and enamelin | 5 E1 and 5 E1 | hypoplastic | Ambn<sup>−/−</sup>; Enam<sup>−/−</sup> | 31478359 |
| 2 | Bmp2 and Bmp4 | bone morphogenetic protein 2 & bone morphogenetic protein 4 | 2 F2 and 14 C4 | hypomineralized | K14-Cre;Bmp2<sup>−/−</sup>; Bmp4<sup>+/−</sup> | 27146352 |
| 3 | Klk4 and Mmp20 | kallikrein related-peptidase 4 and matrix metalloprotease 20 | 7 B3 and 9 A1 | hypoplastic and hypomineralized | Klk4<sup>−/−</sup>;Mmp20<sup>−/−</sup> | 27066511 |
| 4 | Stim1 and Stim2 | stromal interaction molecule 1 and stromal interaction molecule 2 | 7 E2-E3 and 5 C1 | hypomineralized | K14-Cre;Stim1<sup>−/−</sup>; Stim2<sup>−/−</sup> | 28732182 |

**TABLE 3 | Classification of enamel defects.**

| Phenotype | Gene Symbols |
|---|---|
| hypoplastic/no enamel/chalky-white | *Amtn, Col17a1, Enam, Foxi1, Slc10a7, Wdr72* |
| hypomaturation | *Amtn, Col17a1, Enam, Foxi1, Slc10a7, Wdr72* |
| hypomineralized/hypocalciﬁed | *Amelx, Amtn, Bcl11b, Bmp2, Cfr, Col17a1, Ctnnb1, Dlx3, Dmp1, Fam20a, Fam20c, Fgf1, Gdnf, Gja1, Hmgcr2, Itgb1, Itgb6, Lama3, Ltbp3, Mmp20, Mx2, Pax9, Plau, Postn, Rac1, Rhoa, Run1, Slc4a4, Slc13a5, Sp3, Sp6, Tbx1, Tgif1, Tgif2, Ambn and Enam, Klk4 & Mmp20* |
| unknown | *Lamb3, Nectin3, Pitx2, Sp7* |

**TABLE 4 | Functional category of amelogenesis imperfecta-related genes.**

| Category Name | Gene Symbols |
|---|---|
| Extracellular matrix | *Ambn, Amelx, Amtn, Col17a1, Col1, Dmp1, Dsp, Eda, Enam, Fam20a, Fam20c, Fam83h, Fgf1, Gdnf, Gja1, Hmgcr2, Itgb1, Itgb6, Relt, Tgfb2* |
| Enzyme | *Alpl, Fgf20, Fam20c, Hras, Irf6, Kif4, Map3k7, Mmp20, Plau, Rac1, Rhoa, Tgfb1, Tgfb2* |
| Receptor | *Cfr, Cnnm4, Slc4a4, Slc10a7, Slc12a2, Slc13a5, Slc24a4* |
| Receptor binding molecule | *Itgb3* |
| Ion exchanger or transporter | *Cfr, Cnnm4, Slc4a4, Slc10a7, Slc12a2, Slc13a5, Slc24a4* |
| Calcium sensor or regulator | *Stim1, Stim2, Tmbim6* |
| Cell-cell or cell-ECM adhesion molecule | *Cldn3, Cldn16, Ctnnb1, Ctnnb3, Ctnn1, Ctnn3* |
| Growth factor | *Bmp2, Bmp4, Gdnf, Tgfb1* |
| Transcriptional factor | *Ascl5, Bcl11b, Ctnnb1, Dlx3, Foxo1, Irf6, Mx2, Pax9, Pitx2, Runx1, Runx2, Smad3, Slc4a4, Slc10a7, Slc12a2, Slc24a4, Stim1, Tgfb1, Tgfb2, Tmbim6, Wdr72, Bmp2 & Bmp4, Klk4 & Mmp20, Stim1 & Stim2* |
| Transcriptional regulator | *Fam83h, Wdr72* |
| Signal mediator | *Fam83h, Wdr72* |

A total of 2,207 articles were excluded because there was no underlying genetic mechanism dictating the gene findings or the articles did not mention any relevant study or research conducted using Rayyan (Ouzzani et al., 2016). After resolving duplicates with RefWorks, 2,306 articles were selected for further screening. A total of 2,207 articles were excluded because there was no underlying genetic mechanism dictating the gene findings or the articles did not mention any relevant study or research conducted
in humans. A total of 99 articles were further reviewed and qualified through a full-text review (Figure 1A), referring to 89 studies in mice, seven in rats, two in dogs, and one in cattle. A total of 44 genes [42 genes in mice with single gene mutations and two additional genes (Bmp4 and Stim2) in compound mutant models] were identified in mice as genes associated with amelogenesis imperfecta through the systematic review (Supplementary Table S2). A search of the Mouse Genome Informatics (MGI) database identified a total of 59 mouse lines after the removal of duplicates. Upon validation of the enamel phenotype through review of the extracted articles, we identified 35 genes primarily associated with amelogenesis imperfecta (Supplementary Table S3). Among these 35 genes, 15 were uniquely found in the MGI search, and 19 were common in the systematic review and MGI search. Through a manual literature search, we identified additional 11 genes associated with amelogenesis imperfecta (Supplementary Table S4). As a result, a total of 70 genes were identified and curated [68 genes in single-gene mutant mice (Table 1) and two additional genes (after exclusion of overlapping genes in Table 1) in compound mutant mice (Table 2)] as genes associated with amelogenesis imperfecta (a.k.a. enamel hypoplasia) in mice (Figure 1B), hereafter referred as mouse amelogenesis imperfecta-related genes (mAIGenes). In addition, we found that three genes in rats, three genes in dogs, and one gene in cattle were reported in amelogenesis imperfecta (Supplementary Table S5). Among the 70 genes, mutations in 33 genes were reported in humans with amelogenesis imperfecta in isolated or syndromic cases.

These mAIGenes were further categorized into three classes of amelogenesis imperfecta based on gross anatomical observation, histological analysis, microCT, and component analyses, which all are established in human cases: hypoplastic/enamel hypoplasia/no enamel (40 genes), hypomaturation (6 genes), hypomineralized/hypocalcified (39 genes), and unknown detailed classification (4 genes) (Table 3). Some genes exhibited a combined phenotype, as seen in humans. It should be noted that different mutational strategies for deletion, overexpression, or knock-in of the same gene sometimes

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**FIGURE 2 |** Functional enrichment analysis of mouse amelogenesis imperfecta-related genes (mAIGenes). (A) Flowchart of the functional enrichment analysis. Significant Gene Ontology (GO) terms and KEGG pathways were determined by a false discovery rate (FDR) < 0.01. The significant gene sets were then clustered into functional modules using a k-means clustering algorithm. (B) Top 20 GO terms or KEGG pathways. Gene sets related to tooth development and cell proliferation were among the top enriched sets. (C) The gene set network showed four groups, which were ordered by the number of gene sets (the smallest number of gene sets was in Group 1). These gene sets were related to protein banding, enamel mineralization, cancer, and bone development, respectively.
TABLE 5 | Top functional enrichment clusters.

| Pathway                                           | Cluster # |
|---------------------------------------------------|-----------|
| positive regulation of cell migration             | 1         |
| transforming growth factor-beta receptor signaling pathway | 1         |
| growth factor activity                            | 1         |
| transforming growth factor-beta receptor binding  | 1         |
| TGF-beta signaling pathway                        | 1         |
| apical junction complex                           | 2         |
| cell adhesion molecule binding                    | 2         |
| adherens junction                                 | 2         |
| colorectal cancer                                 | 2         |
| basement membrane                                 | 2         |
| laminin-5 complex                                 | 3         |
| pathways in cancer                                | 3         |
| focal adhesion                                    | 3         |
| enamel mineralization                             | 4         |
| biomineral tissue development                      | 4         |
| odontogenesis of dentin-containing tooth          | 4         |
| structural constituent of tooth enamel            | 4         |
| proteoanaceous extracellular matrix               | 5         |
| extracellular region                              | 5         |
| protein binding                                   | 6         |

resulted in different tooth phenotypes. This suggests that subtle changes in the expression or deletion of non-coding genomic sequences may affect the expression and function of genes that are crucial for enamel formation.

Among the mAIGenes, 12 genes (Amnb, Amelx, Amtn, Col17a1, Csf1, Dmp1, Dpp, Enam, Lama3, Lamb3, Lamc2, and Postn) were grouped in the extracellular matrix (ECM) pathway, 11 genes (Alpl, Fam20a, Fam20c, Hras, Klk4, Map3k7, Mmp20, Plau, Rac1, Rhoa, and Tcrg1) in the enzyme pathway, and seven genes (Cftr, Cnmm4, Slc4a4, Slc10a7, Slc12a2, Slc13a5, and Slc24a4) in the ion exchanger/transporter pathway. Moreover, three genes (Stim1, Stim2, and Tmbim6) were related to a calcium ion sensor or regulator, and six genes (Cldn3, Cldn16, Ctnnb1, Gja1, Nectin1, and Nectin3) were involved in cell-cell or cell-ECM adhesions. Since ameloblasts secrete enamel proteins, mutations in genes related to ECM and enamel proteins support their causal roles in amelogenesis imperfecta. In addition, a substantial number of genes were involved in growth factor signaling cascades: four were growth factors (Bmp2, Bmp4, Gdnf, and Tgfb1), five receptors (Fgfr1, Itgb1, Itgb6, Retl, and Tgfr2), 15 transcription factors (Ascl5, Bcl11b, Ctnnb1, Dlx3, Foxo1, If6, Mnx2, Pax9, Pitx2, Runx1, Runx2, Sp3, Sp6, Sp7, and Tbx1), two transcriptional regulators (Hmgn2 and Med1), and one a signal mediator (Smad3). Since these factors are involved in various developmental processes, the mutations would be related to syndromic cases with various developmental defects beyond amelogenesis imperfecta (Table 4).

miRNA-mAIGene Regulatory Network and Identification of Critical miRNAs

For the miRNA-mAIGene regulatory network analysis, we performed miRNA-mAIGene enrichment analysis and miRNA regulatory network analysis (Figure 3A). We identified 35 Highly Expressed MiRNAs (HEMs) in mouse incisors and 32 HEMs in molars with a frequency >1%; 26 mouse tooth HEMs were then curated by taking the intersection of the incisor and molar HEMs (Supplementary Table S6) [26]. A total of 21 of these HEMs did not have a confident -3p or -5p; therefore, we considered that these had both -3p and -5p and identified 47 HEMs, all with a certain -3p or -5p. Based on these 47 HEMs, we predicted that 32 HEMs might target the 42 mAIGenes by using our pipeline and the four miRNA-target gene databases: TargetScan, miRanda, mirTarBase, and PITA. By performing the miRNA-mAIGene regulatory relationship enrichment analysis with a cutoff adjusted p-value < 0.05, we identified 27 notable miRNAs, 41 genes, and 161 miRNA-mAIGene pairs. A total of 17 miRNAs or mRNA groups, 41 genes, and 103 miRNA-mAIGene pairs were extracted after merging the miRNAs or mRNA groups that shared the same targets (such as miR-23a/b-3p and miR-125a/b-5p) (Table 6). Three miRNAs (miR-16-5p, miR-27b-3p, and miR-
FIGURE 3 | miRNA-mAIGene regulatory network and features. (A) Flowchart of the miRNA regulatory network analysis. The miRNA-mAIGene pairs were first identified using four miR-target databases with adjusted p-value < 0.05. Next, the miRNA regulatory network analysis was performed. (B) The miRNA regulatory network, which included 17 miRNAs, 41 mAIGenes, and 103 miRNA-mAIGene pairs. Three miRNAs (i.e., miR-16-5p, miR-27b-3p, and miR-23a/b-3p) were the hub miRNAs in the network. (C) Degree distribution of the miRNAs in the miRNA-mAIGene regulatory network in B, with miR-16-5p, miR-27b-3p, and miR-23a/b-3p having the highest degrees. (D) The sub-network of miR-16-5p, miR-27b-3p, and miR-23a/b-3p. (E) The sub-network of genes regulating more than two miRNAs in Figure 3D.
23a/b-3p) were considered to be hubs in the miRNA regulatory network (Figure 3B) because they had the highest degrees (Figure 3C, Supplementary Table S7), which are defined as the number of partners that immediately interact with a node of interest in the network (Sun et al., 2012), and the lowest adjusted p-values (Table 6). The sub-network of miR-16-5p, miR-27b-3p, and miR-23a/b-3p showed that Smad3 was regulated by all the three hub miRNAs. Stim2, Csf1, Slc10a7, Bcl11b, Slc12a2, Slc12a2, Slc4a4, and Pax9 were regulated by two of these three hub miRNAs or miRNA group, whereas the other genes were regulated by one miRNA or miRNA group (Figures 3D,E). As above, miR-16-5p, miR-27b-3p, and miR-23a/b-3p were considered to be promising miRNA candidates for amelogenesis imperfecta in mice.

### Experimental Validation

To evaluate the function of the miRNAs predicted by the bioinformatic analyses, we conducted ameloblast differentiation assays using mHAT9d cells, a mouse dental epithelial cell line. Although the mouse ameloblast-like cells LS8 (Chen et al., 1992) have been widely used for ameloblast studies, they are limited in their ability to differentiate. We analyzed both LS8 and mHAT9d cells under differentiation conditions and found that mHAT9d cells reacted better to the induction of differentiation (Figure 4A). For instance, the expression of the ameloblast differentiation maker genes was induced more strongly in mHAT9d cells compared to LS8 cells (Supplementary Figures S2, S3). Therefore, mHAT9d cells were used in this study. We found that expression of ameloblast differentiation marker genes (i.e., Ambn, Amelx, Enam, Klk4, and Mmp20) was induced with ameloblast differentiation medium (Figure 4B, Supplementary Figure S3). In addition, we tested whether other genes associated with amelogenesis imperfecta were induced. Among the 27 genes regulated by miR-16-5p, miR-23a-3p, miR-23b-3p, miR-27b-3p, and miR-214-3p, we found 14 genes that were upregulated under differentiation conditions (Supplementary Figure S4). miR-16-5p and miR-27b-3p were induced at relatively high expression levels in mHAT9d cells, and their expression did not change under differentiation conditions (Supplementary Figure S5A). In addition, we found that miR-16-5p and miR-27b-3p were expressed at the pre-secretion, secretion, and maturation stages of ameloblast differentiation in mouse lower incisors (Supplementary Figure S5B). Overexpression of either miR-16-5p or miR-27b-3p significantly anti-correlated with downregulation of expression of Amelx and Enam, but not Ambn, Klk4, and Mmp20, in mHAT9d cells (Figure 4B). We confirmed that the expression levels of AMELX, but not KLK4 and MMP20, were decreased by overexpression of miR-16-5p and miR-27b-3p with immunoblotting (Figure 4C). The expression of AMELX was further confirmed by immunocytochemical analysis (Figure 4D). By contrast, mimics for miR-23a-3p, miR-23b-3p, and miR-214-3p did not affect the gene expression of the ameloblast differentiation makers (Figure 4B). These results indicate that miR-16-5p and miR-27b-3p may play a critical role in ameloblast differentiation through the regulation of genes that are crucial for ameloblast differentiation.

Next, to identify the miRNA-mAIGene regulatory mechanism(s), we conducted quantitative RT-PCR (RT-PCR) analyses for the predicted target genes for each miRNA (Bcl11b, Csf1, Edf, Fgf1, Med1, Relt, Slc4a4, Slc10a7, Slc12a2, Smad3, Stim1, and Stim2 for miR-16-5p; Bmp2, Csf1, Foxo1, Pax9, Runx1, Slc10a7, Slc24a4, Smad3, Sp6, Sp7, Stim2, Tmbim6 for miR-214-3p; Csf1, Ctnnb1, Fgf1, Irf6, Sp7, Stim2 for miR-27b-3p; Csf1, Ctnnb1, Fgf1, Irf6, Sp7, Stim2 for miR-23a-3p). We found that treatment of inhibitor for miR-16-5p mimic (Figure 5A, Supplementary Figure S6). Similarly, the expression of Bmp2, Pax9, and Slc24a4 was significantly downregulated in mHAT9d cells treated with miR-16-5p mimic (Figure 5A, Supplementary Figure S6). Furthermore, we confirmed that treatment of inhibitor for either miR-16-5p or miR-27b-3p had no effect on expression of Amelx and Enam, while the expression of the target genes of
Each miRNA was upregulated (Supplementary Figure S7). Indeed, the predicted target genes contained miRNA recognition sites for their correlated miRNAs on the 3′-UTR (Supplementary Figure S8). By contrast, there was no potential recognition site for miR-16-5p on Amelx and Enam, and for miR-27b-3p on Amelx, while there was a potential recognition site for miR-27b-3p on Enam, and treatment with either mimic or inhibitor for miR-16-5p and miR-27b-3p failed to alter the expression of Amelx and Enam, suggesting that these genes are indirectly regulated by miR-16-5p and miR-27b-3p in mHAT9d cells.

Finally, to examine the functional relevance of genes that were significantly downregulated under treatment with either miR-16-5p or miR-27b-3p mimic, we conducted rescue experiments by...
overexpressing the target genes (Figure 6A). We found that overexpression of \( Eda \), \( Relt \), and \( Smad3 \) under conditions of overexpression of miR-16-5p partially restored mRNA and protein expression of \( Amelx \) and \( Enam \) (Figures 6B, C). Similarly, overexpression of \( Bmp2 \), \( Pax9 \), and \( Slc24a4 \) partially restored mRNA and protein expression of \( Amelx \) and \( Enam \) when miR-27b-3p was overexpressed (Figures 6B, C). Taken together, our results show that overexpression of miR-16-5p and miR-27b-3p inhibits ameloblast differentiation through the regulation of \( mAI Genes \).

**DISCUSSION**

This study aimed to identify regulatory networks for the genes and miRNAs involved in amelogenesis imperfecta in mouse models. Through a literature and MGI searches, we identified 70 genes associated with ameloblast imperfecta and predicted 27 miRNAs to be involved in the development of amelogenesis imperfecta in mice. We found that overexpression of miR-16-5p and miR-27b-3p in mHAT9d cells suppresses \( Amelx \) and \( Enam \) under ameloblast differentiation conditions, respectively.

In this study, we found that overexpression of miR-16-5p inhibited expression of \( Eda \), \( Relt \), \( Slc24a4 \), and \( Smad3 \). miR-16-5p has been detected in osteosarcoma, osteoarthritis, and bone fracture healing. Its overexpression induces suppression of \( SMAD3 \), resulting in inhibition of cell proliferation, migration, and invasion in osteosarcoma cells (Gu et al., 2020), and in downregulation of \( COL2A1 \) and \( Aggrecan \) and upregulation of \( ADAMTS \) in chondrocytes, which may be involved in the
FIGURE 6 | Overexpression of target genes following overexpression of miR-16-5p and miR-27b-3p. (A) Schematic of the experiment. (B) Gene expression of Amelx and Enam following overexpression of Eda, Relt, and Smad3 under overexpression of control and miR-16-5p mimic, or of Bmp2, Pax9, and Scl24a4 under overexpression of control and miR-27b-3p mimic, in mHAT9d cells \((n=6)\). *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\). (C) ICC for AMELX in mHAT9d cells under the indicated conditions. Scale bar, 50 μm. Graph shows the quantification of images from four independent experiments. **\(p < 0.01\); ***\(p < 0.001\).
development of osteoarthritis (Li et al., 2015). In addition, overexpression of miR-16-5p suppresses BACH2 in gingival epithelial cells and Bcl2 and Ccdn1 in MC3T3-E1 cells, resulting in apoptosis and G1/S cell cycle arrest (Sun Y. et al., 2019; Liu et al., 2020).

RELT, a TNF receptor superfamily, is cleaved at the extracellular domain by ADAM10, a metalloprotease that is expressed at the apical loop during the transition stage of ameloblasts (Ikeda et al., 2019). ADAM10 also cleaves type XVII collagen, a component of the basement membrane (Franzke et al., 2009). Mice deficient for either Relt or Col17a1 display a hypomineralized enamel defect (Asaka et al., 2009; Kim et al., 2019). Currently, no mutations in ADAM10 have been reported in amelogenesis imperfecta in humans and mice; therefore, the role of ADAM10 in amelogenesis imperfecta is unclear.

EDA is a TNF family transmembrane protein that binds to its receptor EDAR and initiates NF-κB signaling. Overexpression of Eda in mice results in hypoplastic amelogenesis imperfecta (Mustonen et al., 2003); in humans, mutations in either EDAR or EDAR have been found in hypohidrotic ectodermal dysplasia and isolated tooth agenesis, but not in amelogenesis imperfecta (Shen et al., 2019; Wright et al., 2019; Yu et al., 2019; Andreoni et al., 2021).

SLC4A4, a sodium bicarbonate co-transporter (NBCe1), is involved in the regulation of bicarbonate transportation and intracellular pH homeostasis (Bernardo et al., 2006; Urzua et al., 2011). Mice deficient for Slc4a4 exhibit hypomineralized amelogenesis imperfecta; therefore, NBCe1 is responsible for a change in extracellular pH during enamel maturation (Lacruz et al., 2010; Jalali et al., 2014).

SMAD3 transduces canonical TGF-β signals together with SMAD2 and SMAD4 in the regulation of downstream genes under developmental and pathological conditions. Smad3 knockout mice exhibit hypomineralized amelogenesis imperfecta through downregulation of genes involved in biominerization (e.g., Ambn, Amel, Enam, Mmp20, Klk4, and Gja1) (Yokozeki et al., 2003; Poche et al., 2012).

In addition, we found that overexpression of miR-27b-3p inhibits expression of Bmp2, Pax9, and Slc24a4. Previous studies suggest that overexpression of miR-27b-3p in stem cells in the bone marrow or the maxillary sinus membrane suppresses osteogenic differentiation via suppression of KDM4B or Sp7, respectively (Peng et al., 2017; Zhang et al., 2020). Moreover, miR-27b-3p is downregulated in cartilage in patients with rheumatoid arthritis compared to healthy individuals. In chondrocytes, overexpression of miR-27b-3p suppresses Caspase-3 and upregulates BCL-2, resulting in apoptosis inhibition (Zhou et al., 2019).

BMP2 is a TGF-β superfamily growth factor involved in the development and homeostasis of mineral tissues (Chen et al., 2004; Halloran et al., 2020). Mice with a deletion of Bmp2 in osteogenic and odontogenic cells (Osx-Cre:Bmp2fl/f cKO) exhibit hypomineralized amelogenesis imperfecta and incisal malocclusion through downregulation of Enam, Amelx, Mmp20, and Klk4 (Feng et al., 2011; Guo et al., 2015). Moreover, mice with an odontoblast-specific deletion of Bmp2 (Dmp1-Cre;Bmp2 and Wnt1-Cre;Bmp2 cKO) show dentinogenesis imperfecta without enamel formation defects (Jani et al., 2018; Malik et al., 2018).

PAX9, a transcription factor, plays a role in craniofacial and skeletal development, including the development of tooth, bone, cartilage, and muscle (Monsoro-Burq, 2015; Farley-Barnes et al., 2020). Several single nucleotide polymorphisms (SNPs) in PAX9 are reported to be associated with tooth size and shape as well as tooth agenesis (Lee et al., 2012; Wong et al., 2018; Safari et al., 2020; Alkhatib et al., 2021). While Pax9 null mice exhibit cleft palate and tooth developmental arrest at the bud stage (Zhou et al., 2011), hypomorphic Pax9 mutant mice exhibit hypoplastic amelogenesis imperfecta in the lower incisors and tooth agenesis of the third molars (Kist et al., 2005).

SLC24A4, a potassium-dependent sodium/calcium exchanger (NCKX4), is expressed in ameloblasts at the maturation stage and plays an important role in calcium ion transport by exchanging intracellular Ca²⁺ and K⁺ with extracellular Na⁺ for Ca²⁺ supply into the developing enamel crystals (Hu et al., 2012; Bronckers et al., 2015). A deficiency of Slc24a4 causes hypomineralized amelogenesis imperfecta in mice (Parry et al., 2013), and mutations in SLC24A4 are associated with isolated amelogenesis imperfecta (either hypomineralized or hypomaturation types) in humans (Parry et al., 2013; Seymen et al., 2014; Herzog et al., 2015; Khan et al., 2020).

Our results from the rescue experiments suggest that miR-16-5p and miR-27b-3p are involved in amelogenesis imperfecta through dysregulation of mAIGenes. In summary, our systematic search for mAIGenes provides an overview of the genes involved in this condition. Our bioinformatics pipeline identified three potential miRNAs that may actively interact with mAIGenes, and two of these miRNAs were experimentally validated in mouse cell lines. These results will expand our knowledge of the genetics of amelogenesis imperfecta in animal models, which can be translated into human studies and help develop clinical approaches for diagnosis and treatment. We will need to further evaluate the functional significance of these miRNA-gene regulatory networks in vivo. Both the negative and positive feedback loops between the miRNAs and target genes should also be further evaluated in various cell lines and in vivo since miRNAs may regulate the expression of multiple genes and multiple miRNAs may regulate the expression of a single gene. In addition, transcription factors may be involved in these miRNA-gene regulatory networks; for example, a direct regulation between miRNA and mAIGenes may be bypassed through other transcription factors.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.
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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2022.788259/full#supplementary-material

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