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Mesenchymal Stromal Cell-Derived Interleukin-6 Promotes Epithelial–Mesenchymal Transition and Acquisition of Epithelial Stem-Like Cell Properties in Ameloblastoma Epithelial Cells

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ABSTRACT

Epithelial–mesenchymal transition (EMT), a biological process associated with cancer stem-like or cancer-initiating cell formation, contributes to the invasiveness, metastasis, drug resistance, and recurrence of the malignant tumors; it remains to be determined whether similar processes contribute to the pathogenesis and progression of ameloblastoma (AM), a benign but locally invasive odontogenic neoplasm. Here, we demonstrated that EMT- and stem cell-related genes were expressed in the epithelial islands of the most common histologic variant subtype, the follicular AM. Our results revealed elevated interleukin (IL)-6 signals that were differentially expressed in the stromal compartment of the follicular AM. To explore the stromal effect on tumor pathogenesis, we isolated and characterized both mesenchymal stromal cells (AM-MSCs) and epithelial cells (AM-EpiCs) from follicular AM and demonstrated that, in in vitro culture, AM-MSCs secreted a significantly higher level of IL-6 as compared to the counterpart AM-EpiCs. Furthermore, both in vitro and in vivo studies revealed that exogenous and AM-MSC-derived IL-6 induced the expression of EMT- and stem cell-related genes in AM-EpiCs, whereas such effects were significantly abrogated either by a specific inhibitor of STAT3 or ERK1/2, or by knockdown of *Slug* gene expression. These findings suggest that AM-MSC-derived IL-6 promotes tumor-stem like cell formation by inducing EMT process in AM-EpiCs through STAT3 and ERK1/2-mediated signaling pathways, implying a role in the etiology and progression of the benign but locally invasive neoplasm. STEM CELLS *2017;35:2083–2094*

SIGNIFICANCE STATEMENT

Tumor initiating cells (TICs) play important role in tumorigenesis, metastasis, recurrence, and resistance to chemo-/radiation therapies in a variety of malignant tumors. However, much less work has been done to explore the potential role of TICs in the development of epithelial benign tumors. Here, we provide evidence that the solid/multicystic follicular ameloblastoma (AM), the most common and aggressive type of odontogenic benign tumors, harbor a subpopulation of epithelial cells (EpiCs) with epithelial–mesenchymal transition (EMT) phenotypes and express several epithelial stem cell-related genes. Both in vitro and in vivo studies show that mesenchymal stromal cell-derived IL-6 induces EMT and promotes acquisition of stem-like cell properties in AM EpiCs possibly via STAT3 and ERK1/2-mediated signaling pathways. This study demonstrates that stromal cell-derived IL-6 may play an important role in the pathogenesis of this aggressive benign tumor via driving EMT and tumor-like epithelial stem cell formation.

INTRODUCTION

Ameloblastoma (AM) is a benign but locally invasive tumor with a high recurrence rate, accounting for 1% of all oral tumors and about 11%–18% of odontogenic tumors [1]. In the third 2005 edition of World Health Organization (WHO) Classification of Head and Neck Tumors, AM was categorized into the solid/multicystic/conventional, extraosseous/peripheral, desmoplastic, and unicystic types [2]. Most recently, AM is reclassified into only three subtypes: AM, unicystic, and extraosseous/peripheral types in the fourth edition of WHO Classification of Head and Neck Tumors [3], among which the conventional AM is the

most common type, making up about 91% of all AM cases. Histologically, AM has a variety of histopathologic patterns such as follicular, plexiform, desmoplastic, basal cell, acanthomatous, and granular cells, among the follicular is the most common type [1]. Currently, the relatively high recurrence of AM remains a major clinical challenge for the management of this locally invasive tumor. It has been reported that the recurrence rate of AM could be as high as 75%–90% after conservative surgery and 15%–25% after radical treatment [4], whereas solid/multicystic or conventional AM has the highest recurrence rate [5]. Therefore, radical surgical treatment is usually recommended for AMs, but usually results in morbid defects involving bone, teeth, and orofacial soft tissues [5]. Although several hypotheses, including mutation of certain genes such as *BRAFV600E*, smoothened, and adenomatosis polyposis coli gene [6, 7], the exact etiology of AM remains unclear. Further delineating the cellular and molecular mechanisms underlying the pathophysiology of AM may lead to the development of novel, non-invasive, and targeted therapeutic modalities for this benign yet aggressive tumor.

Cellular heterogeneity is a hallmark of tumors. Cancer stem cells (CSCs), or tumor initiating cells, possess self-renewal, tumor initiation, and clonal long-term repopulation capabilities as well as the plastic property to reversibly transition between stem and nonstem cell states [8, 9]. Thus, CSCs have been proposed to play an important role in tumor growth, metastasis, and the development of treatment resistance and recurrence [10]. Several studies have demonstrated the existence of CSC-like cells in head and neck squamous cell carcinoma (HNSCC) [11, 12]; however, the origin of these CSC-like cells and their role in HNSCC development remains unknown, and there is still a lack of evidence whether tumor-like stem cells play a critical role in the development of epithelial benign tumors, particularly, the locally aggressive AM.

Epithelial–mesenchymal transition (EMT) is a fundamental biological process during development of multicellular organisms [13], which is governed by several signaling pathways interplayed by several core transcription factors such as SLUG, SNAIL, ZEB1, and TWIST [14]. EMT is a dynamic, reversible, and bidirectional (EMT vs. mesenchymal–epithelial transition) process which plays a critical role in tumor invasion and metastasis along with therapeutic resistance and recurrence [15], probably by facilitating the conversion or reprogramming of non-CSC tumor cell into CSC-like state [8, 16–19]. Interleukin (IL)-6, one of the key cytokines produced by stromal cells within the tumor microenvironment (TME), exerts versatile effects on tumor growth via multiple mechanisms, including facilitating the EMT process, CSC-like cell formation, and angiogenesis [20–23]. A recent study has shown that reciprocal cell–cell interaction between AM tumor cells and stromal fibroblasts via IL-1α-stimulated secretion of IL-6 and IL-8 may have contributed to the TME to support the progression of AM [24]; however, the underlying cellular and molecular mechanisms remain largely unknown. Furthermore, the histologic features of AM comprise of proliferating odontogenic epithelium among a fibrous stroma, raising the potential biological effect of stroma on tumor behaviors. In this study, we investigated the role of IL-6 in the regulation of EMT process and acquisition of stem-like cell properties in AM-derived epithelial cells (AM-EpiCs) as well as the underlying signaling mechanisms. We demonstrated that AM-derived mesenchymal stromal cell (AM-MSCs)-derived IL-6 significantly promoted the expression of EMT markers and several important stem cell-related genes in AM-EpiCs, while such effects were abrogated by blockade of STAT3 or MAPK activities, or by knockdown of the *Slug* gene expression. These findings suggest that AM-MSC-derived IL-6 facilitates tumor-stem like cell formation by inducing EMT process in AM-EpiCs through MAPK/STAT3/SLUG signaling pathways, thus possibly contributing to the outgrowth and invasiveness of this benign yet aggressive neoplasm.

Materials and Methods

Tumor Tissue Collection

The study was conducted in accordance with human subject research guidelines and a protocol approved by the institutional review board (IRB) at University of Pennsylvania (UPenn) (IRB#817407). This study focused on follicular AM, the most common histopathological variant of this benign tumor [1, 5]. Three fresh tumor samples were obtained post-surgical procedures following appropriate informed consents from patients who were diagnosed with primary and not recurrent follicular AM and underwent treatment at the Department of Oral and Maxillofacial Surgery, Penn Medicine Hospital of the UPenn. Five formalin-fixed paraffin-embedded samples were retrieved from the archives of the Department of Pathology, UPenn School of Dental Medicine. Diagnoses were made by two independent pathologists, including a board-certified oral and maxillofacial pathologist, based on the 2005 WHO Histologic Classification of Odontogenic Tumors [2].

In Vitro Culture of Primary MSCs and EpiCs from AM Specimens

One portion of fresh surgical AM samples were washed with sterile phosphate-buffered saline (PBS) and cut into 2–4 mm pieces followed by enzymatic digestion with 0.1% collagenase (Sigma-Aldrich; St. Louis, MO; https://www.sigmaaldrich.com/united-states.html) and 50 U/mL dispase (Life Technologies; Carlsbad, CA; http://www.lifetech.com) at 37°C for at least 2 hours in a shaking incubator. Single cell suspension was filtered through a 70-μm nylon mesh cell strainer and centrifuged at 1200 rpm for 10 minutes. For culture of AM-EpiCs, the cell pellet was resuspended at a concentration of 1×10^6 cells per milliliter in complete keratinocyte growth medium (KGM-2 Bullet kit; Lonza, Allendale, NJ; http://www.lonza.com/) and seeded onto 10-cm tissue culture dishes precoated with 0.1% gelatin and cultured in complete KGM-2 medium (2×10^5 cells per milliliter) in a humidified incubator at 37°C with 5% CO_2. Fresh media were changed twice a week and cell growth was observed and photographed under a microscope. Cells at 75%–100% confluence were subcultured following cell dissociation with 0.05% trypsin and 0.02% EDTA (Life Technologies). Early passages of primary cells were frozen in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide and stored in liquid nitrogen and cells less than six passages were used for further experiments. For culture of AM-derived MSCs (AM-MSCs), the resuspended single cell pellets derived from AM tissues were cultured with α-MEM containing 10% FBS and 1% antibiotics in a humidified 5% CO_2 atmosphere at 37°C.

Chemical Reagents and Antibodies

ERK1/2 inhibitor PD98059 and PI3K inhibitor LY294002 were purchased from EMD Millipore (Billerica, MA; http://www.emdmillipore.com/); STAT3 Inhibitor VI (S3I-201) was from Santa Cruz Biotechnology (Dallas, Texas; https://www.scbt.com); and IL-6R antagonist LMT-28 was from Sigma. For stem cell-related gene expression in immunohistochemical (IHC) staining, immunofluorescence (IF) staining, and flow cytometry, the following primary antibodies were used: aldehyde dehydrogenase 1 (ALDH1) (#12035S, Cell Signaling Technology, CST, Danvers, MA; https://www.cellsignal.com/), β-catenin (#8480P,CST), BMI-1 (sc-10745, Santa Cruz), SOX-2 (#3579S, CST), LIN28 (#3695, CST), E-cadherin (sc-21791, Santa Cruz), CD44 (103005, Biolegend), SSEA4 (330411, Biolegend, San Diego, CA; https://www.biolegend.com/), STRO-1 (340102, Biolegend), CD29 (555442, BD Pharmingen, San Diego, CA; http://www.bdbiosciences.com), CD73 (344016, Biolegend), CD90 (328109, Biolegend), CD105 (323202, Biolegend), CD146 (342002, Biolegend), and vimentin (sc-73258, Santa Cruz). For EMT-related marker expression in IF staining, Western blot and IHC staining, the following primary antibodies were used: SNAIL1 (#3879S, CST), SLUG (sc-166476, Santa Cruz), ZEB1 (sc-25388, Santa Cruz), and TWIST1 (GTX127310, Gene Tex, Irvine, CA; http://www.genetex.com/). For IL-6 detection in AM samples in IF staining, anti-IL-6 antibody (16-7069-81, eBioscience, San Diego, CA; http://www.eBioscience.com) and Collagen I (21787, ROCKLAND Immunochemicals Inc., Limerick, PA; http://www.rockland-inc.com/) were used. The following secondary antibodies are: anti mouse IgG-Fluorescein isothiocyanate (FITC) (sc-2010, Santa Cruz), anti-rabbit IgG-FITC (sc-2012, Santa Cruz), anti-mouse IgG-rhodamine (sc-2092, Santa Cruz), and anti-rabbit IgG-rhodamine (sc-2091, Santa Cruz). For IL-6 stimulation to AM EpiCs, human recombinant IL-6 (AF-200-06, PEPROTECH, Rocky Hill, NJ; https://www.peprotech.com/en-US) was used.

Histological, IHC, and IF Studies

The tumor tissue sample was fixed in 4% paraformaldehyde (Santa Cruz) and embedded in either paraffin or Optimal Cutting Temperature Compound. Paraffin-embedded (5 μm) and frozen (8 μm) sections were cut for H&E staining and IHC or IF studies, respectively.

For IHC staining, paraffin-embedded sections were deparaffinized, unmasked with 1 × IHC Antigen Retrieval Solution (E13399-110, eBioscience) and blocked with 2.5% goat serum in PBS for 1 hour at room temperature. Then, sections were immunostained overnight at 4°C with specific primary antibodies for human E-cadherin (EMD Millipore), vimentin (Santa Cruz), β-catenin, ALDH1, SOX2, LIN28, pERK1/2, pSTAT3, and pAKT1 (Cell Signaling Technology) (all in 1:200). After extensive washing with PBS, the the avidin-biotin complex (ABC) reagents (R.T.U. VECTASTAIN Kit, VECTOR, Laboratories, Burlingame, CA; https://vectorlabs.com/) were applied to the sections, followed by color development using Vector NovaRED substrate Kit (Vector NovaRED SUBSTRATE KIT, VECTOR Laboratories) and counterstained with hematoxylin. Images were observed and photographed under a microscope (Olympus, IX73, Center Valley, PA; http://www.olympusamerica.com/).

For dual-color IF studies, frozen sections were fixed with 4% PFA, permeabilized in 0.5% triton X-100 in PBS, and blocked with 2.5% goat serum in PBS for 1 hour at room temperature. Then, sections or cells were immunostained with a primary antibody (mouse IgG; 1:200) for human E-cadherin in combination with another antibody (Rabbit IgG; 1:200) for vimentin, ALDH1, BMI-1, SOX2, LIN28, β-catenin, SLUG, SNAIL1, and ZEB1 (Cell Signaling Technology), IL-6 (eBioscience), and type I collagen (Rockland Immunochemicals Inc.). After washing with PBS, cells were incubated with appropriate secondary antibodies at room temperature for 1 hour: goat anti-rabbit IgG–Alexa Fluor®488 (1:250; A11008, Life Technologies), goat anti-rabbit IgG–Alexa Fluor®568 (1:250; A11011, Life Technologies), goat anti-mouse IgG–Alexa Fluor®488 (1:250; A11029, Life Technologies), and goat anti-mouse IgG–Alexa Fluor®568 (1:250; A11004, Life Technologies). Isotype-matched control antibodies (BioLegend) were used as negative controls. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Life Technologies), and slides were observed under a fluorescence microscope (Olympus IX-73).

Immunocytochemical Studies

Cultured cells in eight-well chamber slides (Millicell® EZ SLIDES, EMD Millipore) were fixed with 4% PFA, blocked and permeabilized for 1 hour at room temperature in PBS + 2.5% goat serum + 0.5%Triton X-100, followed by incubation with the following primary antibodies (1:200) overnight at 4°C: E-cadherin, β-catenin, LIN28, ALDH1, BMI-1, SOX2, SLUG, SNAIL1, ZEB1, CD44, SSEA4, STRO-1, and ALDH1. After washing with PBS, cells were incubated with appropriate secondary antibodies (1:250) at room temperature for 1 hour: goat anti-rabbit IgG–Alexa Fluor®488, goat anti-rabbit IgG–Alexa Fluor®568, goat anti-mouse IgG–Alexa Fluor®488, and goat anti-mouse IgG–Alexa Fluor®568. Isotype-matched control antibodies (BioLegend) were used as negative controls. Nuclei were counterstained with DAPI. Images were captured using Olympus inverted fluorescence microscope (IX73). For semi-quantitative analysis, cells with positive signals in at least six random high-power fields were visualized, counted, and expressed as the percentage of total DAPI-positive cells [25].

Flow Cytometry

AM-MSCs were harvested and washed twice with PBS containing 2% heat-inactivated FBS and resuspended in cell staining buffer (BioLegend) at a concentration of 10^7 per milliliter, followed by incubation with FITC-conjugated antibodies (1:100) for human CD44, CD29, CD73, CD90, STRO-1, CD105, CD146, SSEA4, or an isotype-matched mouse IgG control at 4°C for 30 minutes. Then, cell samples were washed twice with PBS/2% FBS and submitted to flow cytometric analysis (BD LSRII).

Western Blot

Cell lysates were prepared by incubation with radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz) supplemented with a cocktail of protease inhibitors (Santa Cruz) and the total protein concentrations were determined using bicinchoninic acid (BCA) method (PIERCE Biotechnology, Rockford, IL; http://www.thermoscientific.com/pierce). Then proteins were subjected to SDS-polyacrylamide gel electrophoresis before being electroblotted onto a nitrocellulose membrane (BioRad, Hercules, CA; http://www.bio-rad.com/). Primary antibodies were used to detect the relevant protein, and β-actin was used as loading control. Bolts were developed with horseradish peroxidase-conjugated secondary antibodies, and the signals

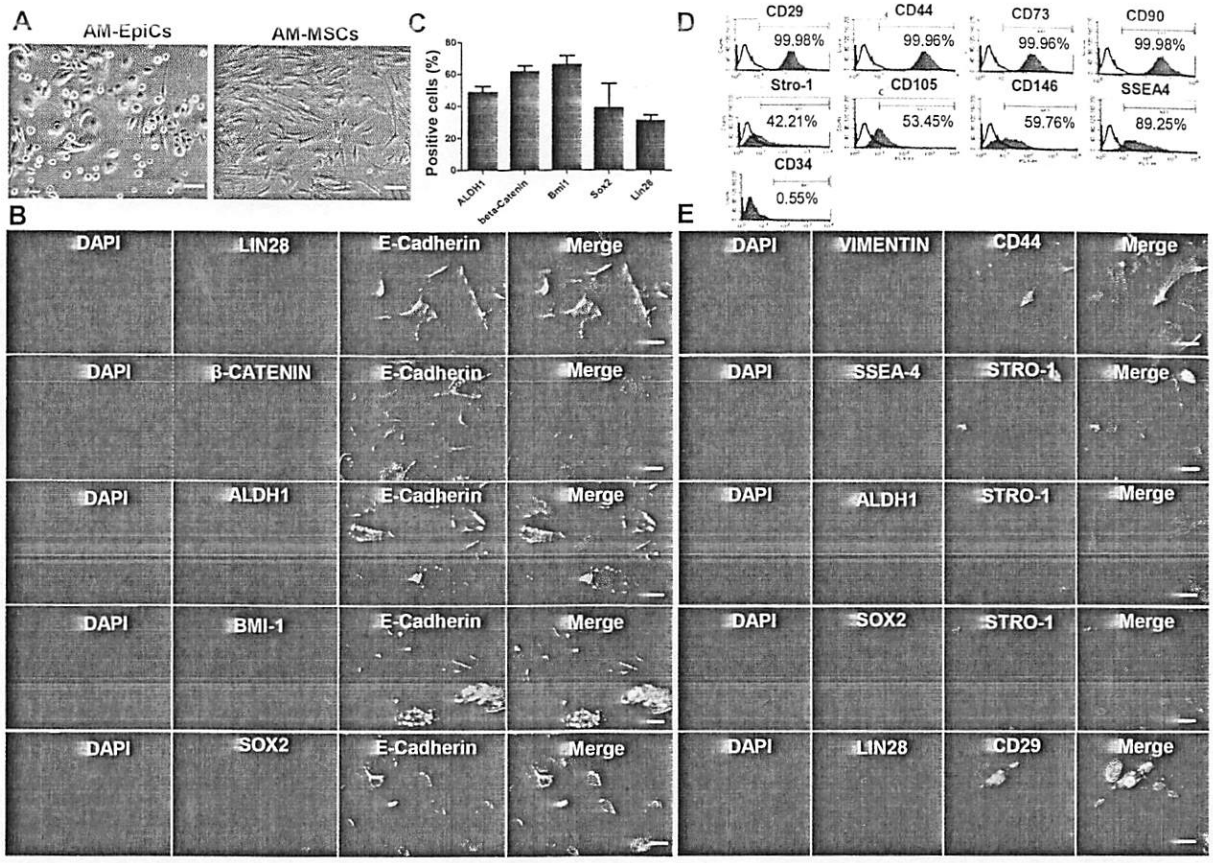

Figure 2. Isolation of epithelial stem-like cells and mesenchymal stromal cells (MSCs) from follicular ameloblastoma (AM). **(A):** Ex vivo-cultured AM-derived epithelial cells (AM-EpiCs) (left panel) and MSCs (AM-MSCs) (right panel). Scale bar = 100μm. **(B, C):** A proportion of AM-EpiCs expressed stem cell-related genes, LIN28, β-catenin, ALDH1, BMI-1, and SOX2, as determined by immunofluoresence studies. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Scale bar = 20 μm. **(D):** Flow cytometric analysis of the expression of a panel of MSC (MSC)-associated cell surface markers in AM-MSCs. **(E):** Immunofluoresence studies on the expression of MSC-related genes in AM-MSCs. An isotype control IgG was used as the control. The nuclei were counterstained with DAPI. Scale bar = 20 μm. Abbreviations: ALDH1, aldehyde dehydrogenase 1; AM, ameloblastoma; DAPI, 4′, 6-diamidino-2-phenylindole; EpiCs, epithelial cells; MSC, mesenchymal stromal cell.

Results

Follicular AM Harbor a Subpopulation of EpiSCs

Follicular AM is characterized by the presence of epithelial islands consisting of peripheral columnar cells reminiscent of ameloblasts with reverse polarization of the nuclei and central stellate-reticulum like cells, and a mature surrounding connective tissue stroma (Fig. 1A). IHC staining showed that several genes related to the self-renewal and pluripotency of stem cells, including ALDH1, β-catenin, BMI-1, SOX2, and LIN28, were positively expressed in follicular AM tissues (Fig. 1B). Dual-color IF staining further indicated that the positive signals for these stem cell-related genes were mainly localized within the epithelial islands or compartments of follicular AM (Fig. 1C).

Isolation of EpiSCs and MSCs from Follicular AM

To date, culture and maintenance of EpiCs from AM still remain challenging. Using our established conditions, we successfully isolated and cultured EpiCs derived from follicular AM (AM-EpiCs), which displayed round and polygonal morphological characteristic of EpiCs (Fig. 2A, the left panel) and expressed E-cadherin, a specific EpiC surface marker (Fig. 2B). Importantly, our results showed that >50% of these primary EpiCs expressed stem cell-related genes, ALDH1, β-catenin, and BMI-1, and 30%–40% of them expressed the SOX2 and LIN28 genes (Fig. 2B, 2C), which supported our in vivo findings that a subpopulation of epithelial stem-like cells (AM-EpiSCs) exist in the epithelial islands of follicular AM. Meanwhile, we also isolated and cultured a population of MSCs from follicular AM (AM-MSCs). These stromal cells exhibited typical spindle-like morphology (Fig. 2A, the right panel) and consistently expressed a panel of mesenchymal cell-associated surface markers and stemness-regulated genes as determined by both flow cytometry and IF staining (Fig. 2D, 2E). These primary epithelial and MSCs derived from human follicular AM would allow further investigation into the pathogenesis of this benign yet aggressive odontogenic tumor.

Follicular AM Harbors a Subpopulation of EpiCs Undergoing EMT Process

It has been recognized that the EMT process contributes to aggressive growth and invasion of a variety of tumors. We next explored whether EMT played a role in the pathogenesis of AM and determined the expression of EMT-related genes in follicular AM tissues using dual-color IF staining. Our results showed that a proportion of cells in the peripheral area of

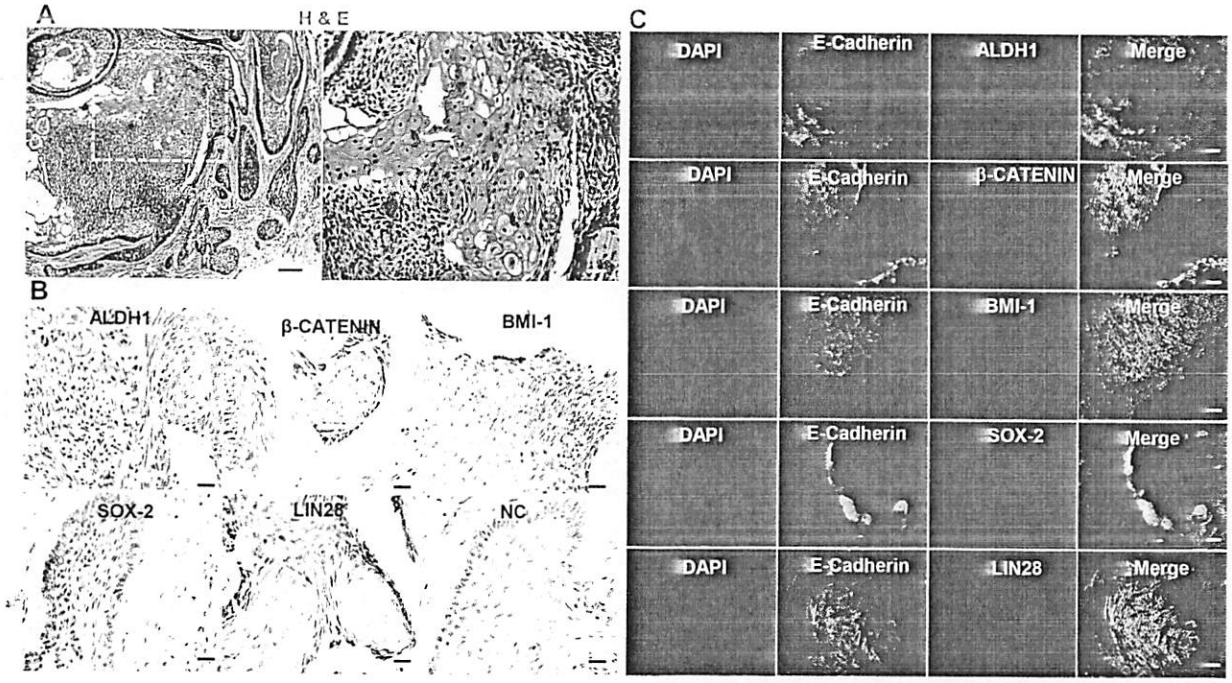

Figure 1. Expression of stem cell-related genes in epithelial compartment of follicular ameloblastoma (AM). **(A):** Representative H&E staining of follicular AM tissue samples. The left panel shows a lower magnification (Scale bar = 100 μm), while the yellow box highlights an area showing acanthomatous change; the right panel shows the magnified area of the yellow-boxed area in the adjacent panel (Scale bar = 20 μm). **(B):** Immunohistochemical studies on the protein expression of stem cell-related genes, aldehyde dehydrogenase 1 (ALDH1), β-catenin, BMI-1, SOX2, and LIN28, in follicular AM tissues. Scale bar = 100 μm. **(C):** Immunofluorescence studies on the protein expression of E-cadherin, ALDH1, β-catenin, BMI-1, SOX2, and LIN28, in follicular AM tissues, while an isotype control IgG was used as the control. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Scale bar = 100 μm. Abbreviations: ALDH1, aldehyde dehydrogenase 1; DAPI, 4′,6-diamidino-2-phenylindole; NC, negative control with an isotype control antibody.

were visualized using enhanced chemiluminescence detection (PIERCE), according to the manufacturer's protocol.

Cytokine Array

Tissue lysates were prepared from fresh AM or normal jaw bone samples in RIPA lysis buffer supplemented with a cocktail of protease inhibitors (Santa Cruz) and the total protein concentrations were determined using BCA method (PIERCE). Total protein (100 μg) was used per subarray from RayBio Human Cytokine Antibody Array III (RayBiotech, Inc., Norcross, GA; https://www.raybiotech.com/) and semiquantified following the manufacturer's instructions.

Enzyme-Linked Immunosorbent Assay

The concentration of IL-6 in supernatants of AM-EpiCs and AM-MSCs were detected using enzyme-linked immunosorbent assay (ELISA) kits (BioLegend).

In Vivo Tumorigenecity

Six- to eight-week-old female athymic NCr-nu/nu mice were purchased from Charles River Laboratories (Wilmington, MA; http://www.criver.com/) and maintained under standard conditions. All animal procedures were handled according to the guidelines of the Institutional Animal Care and Use Committee of the UPenn. Mice were group-housed in polycarbonate cages (five animals per cage) in the animal facilities with controlled temperature (23 ± 2°C), 40%–65% of humidity and a 12-hour light/dark cycle. Mice were acclimatized for at least 1 week before the study, fed with a standard laboratory diet and allowed ad libitum access to drinking water. For subcutaneous transplantation, 1×10^6 AM-EpiCs and 1×10^6 AM-MSCs were mixed with Matrigel and subcutaneously injected into nude mice. To further investigate the role of IL-6 in the EMT process and tumorigenic potential of AM-EpiCs, we mixed 2×10^6 AM-EpiCs with IL-6-loaded hydrogel and subcutaneously injected into the nude mice. Two weeks after transplantation, xenografted tumors were collected for histologic and IHC studies on the expression of EMT- and stem cell-related genes.

Short Interfering RNA Transient Transfection

AM-EpiCs (2×10^5 per well) were seeded into six-well plate precoated with 0.1% gelatin and incubated overnight, and then transfected with 60 nM Slug short interfering RNA (siRNA) (sc-38393; Santa Cruz) or a control siRNA (sc-36869, Santa Cruz). After transfection for 5 hours, the transfection medium was replaced with fresh complete KGM-2 medium. The next day, AM-EpiCs transfected with Slug-siRNA or the control siRNA were cocultured with 2×10^5 AM-MSCs in transwells for 3 days.

Statistical Analysis

All data are presented as mean ± SD and analyzed using the Student's paired or independent *t* tests. A value of $p < .05$ was considered to be statistically significant. Statistical analyses were performed using SPSS 18.1 statistical software program (SPSS Inc., Chicago, IL; http://www.spss.com/).

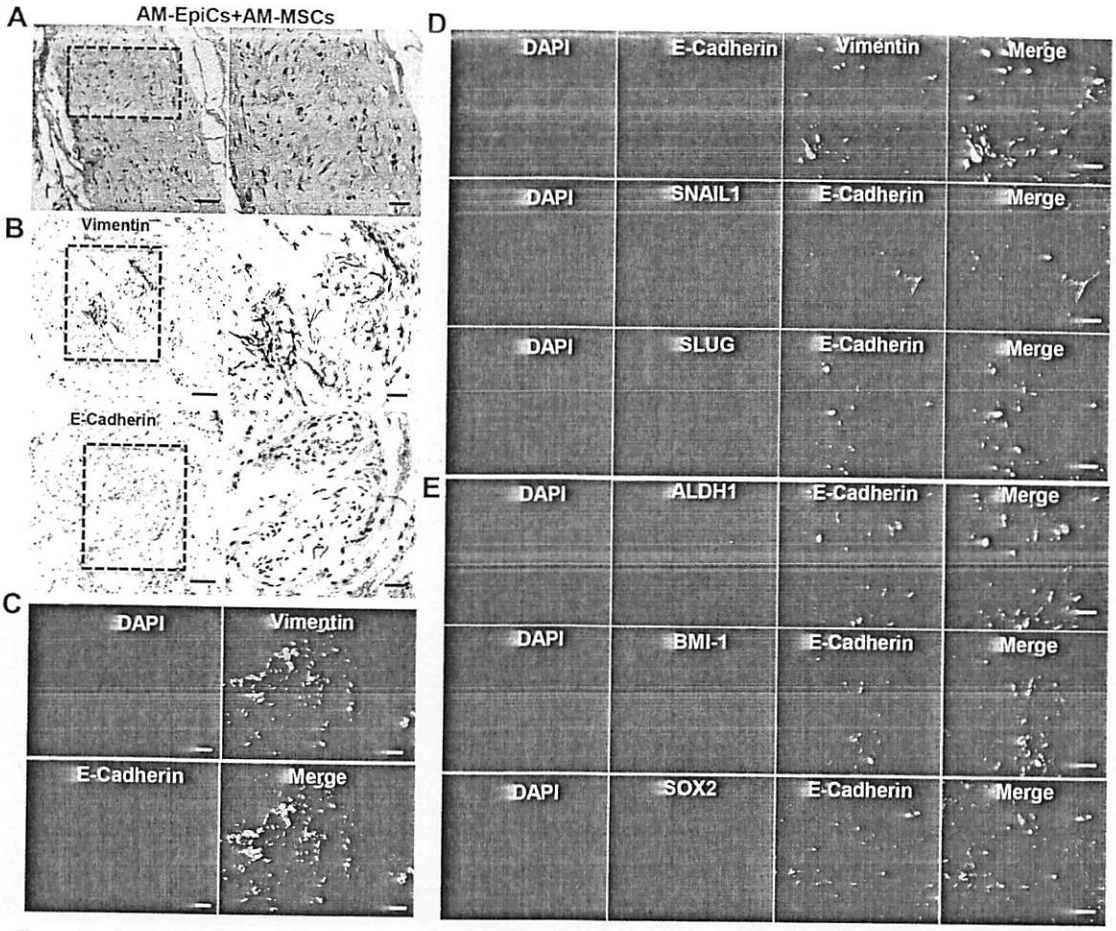

Figure 4. Tumorigenic potential of ameloblastoma (AM)-derived epithelial cells (AM-EpiCs) and mesenchymal stromal cells (MSC). **(A):** H&E staining of xenografted tumor tissues generated by cotransplantation of AM-EpiCs and MSCs (AM-MSCs). Scale bar = 100 μm (the left panel) and 20 μm (the right panel). **(B, C):** The expression of E-cadherin and vimentin in xenografted tumors was determined by immunohistochemical (B) and immunofluorescence staining (C). The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Scale bars = 100 μm (the left panel) and 20 μm (the right panel). **(D, E):** Expression of epithelial–mesenchymal transition- and stem cell-related genes in xenografted tumors as determined by dual color-immunofluorescence studies, while an isotype control IgG was used as the control. The nuclei were counterstained with DAPI. Scale bar = 100 μm. Abbreviations: ALDH1, aldehyde dehydrogenase 1; AM, ameloblastoma; DAPI, 4′, 6-diamidino-2-phenylindole; EpiCs, epithelial cells; MSC, mesenchymal stromal cell.

and the expression of EMT- and stem cell-related genes was determined by Western blot. Our results showed that exposure to IL-6 or coculture with AM-MSCs led to a decreased expression in E-cadherin, and a reciprocal increase in the expression of vimentin, SLUG, and ZEB1 in AM-EpiCs (Fig. 5E). Meanwhile, an increase in the expression of stem cell-related genes, ALDH1 and SOX2, was also observed in AM-EpiCs following IL-6 stimulation or coculture with AM-MSCs (Fig. 5E). Furthermore, we showed that treatment with a specific IL-6 receptor antagonist (LMT-28) [28] abrogated AM-MSC-induced downregulation of E-cadherin and upregulation of vimentin, SLUG, ZEB1, ALDH1, and SOX2 expressions in AM-EpiCs (Fig. 5F). Altogether, these findings suggest that AM-MSCs promote EMT process and stem cell-related gene expression in AM-EpiCs, possibly via secretion of IL-6.

Signaling Pathways Involved in IL-6-Mediated EMT Process and Acquisition of EpiSC Properties in AM

Next, we determined the downstream signaling pathways that are potentially involved in IL-6-mediated effects on EMT process and EpiSC formation in AM (AM-EpiSCs). IHC studies showed that phosphorylated ERK, AKT, and STAT3 were positively expressed in follicular AM (Fig. 6A). We next stimulated AM-EpiCs with IL-6 (10 ng/mL) at different time points (0–8 hours) and found that the activation of ERK1/2 and STAT3 peaked at 1–2 hours and then started to decline; while the AKT activity increased much earlier at 10 minutes, peaked at 1–2 hours, and maintained at the same level up to 8 hours following IL-6 stimulation (Fig. 6B). To identify which signaling pathway is essential in IL-6-mediated effects on the EMT process and EpiSC formation, AM-EpiCs were pretreated with specific inhibitors for STAT3 (STAT3 Inhibitor VI, S3I-201; 100 μM), ERK1/2 (PD98059, 50 μM), or PI-3K/AKT (LY294002, 50 μM) for 1 hour followed by stimulation with 10 ng/mL IL-6 for 72 hours. Our results showed that IL-6 stimulation induced spindle-like cell morphological change characteristic of the EMT process in AM-EpiCs, which was abrogated by pretreatment with a specific inhibitor of STAT3 or ERK1/2 but not AKT1 inhibitor (Fig. 6C). Furthermore, we showed that blocking the activity of STAT3 or ERK1/2 obviously abrogated IL-6 induced downregulation of E-cadherin and upregulation of vimentin, SLUG, ZEB1, ALDH1, and SOX2 expressions in AM-

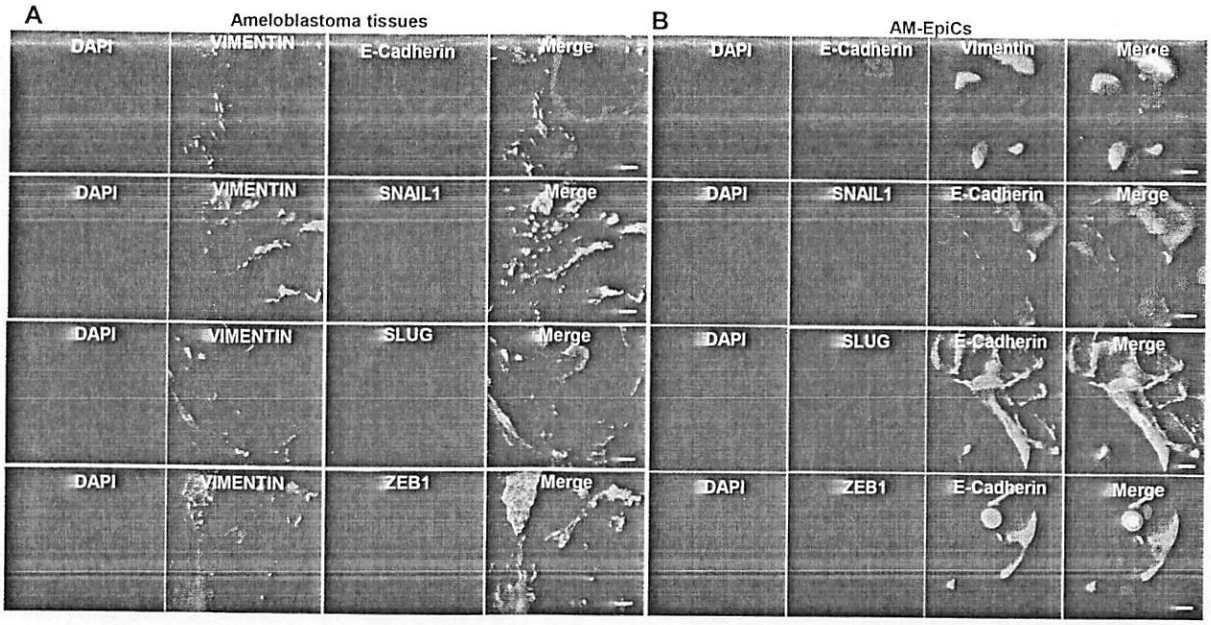

Figure 3. Follicular ameloblastoma (AM) harbor a subpopulation of epithelial cells (EpiCs) undergoing epithelial–mesenchymal transition (EMT) process both in vivo and in vitro. **(A):** Follicular AM tissues comprise E-cadherin$^+$/ vimentin$^+$-hybrid cells and express EMT-related genes, SNAIL1, SLUG, and ZEB1, as determined by dual-color immunofluorescence (IF) studies, while an isotype control IgG was used as the control. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Scale bar = 100 μm. **(B):** A proportion of isolated AM-EpiCs were double-positively stained with E-cadherin and vimentin and highly expressed EMT-related genes, SNAIL1, SLUG, and ZEB1, as determined by dual-color IF studies, while an isotype control IgG was used as the control. The nuclei were counterstained with DAPI. Scale bar = 20 μm. Abbreviation: DAPI, 4′, 6-diamidino-2-phenylindole.

epithelial islands were epithelial–mesenchymal hybrid cells, which were positively double-stained for both E-cadherin and vimentin (Fig. 3A). Meanwhile, an abundant expression of several EMT-related genes, such as SNAILl, SLUG, and ZEB1, was also observed in both epithelial and stromal compartments of follicular AM (Fig. 3A). These in vivo findings were further confirmed using in vitro cultured AM-EpiCs, showing that approximately 70% of cells were E-cadherin$^+$/vimentin $^+$ double-positive hybrid cells and expressed similar EMT-related genes (Fig. 3B).

Tumorigenic Potentials of AM-Derived EpiCs and MSCs

We next determined the in vivo self-renewal and tumorigenic potentials of cultured follicular AM-EpiCs and MSCs (AM-MSCs). To this end, 1×10^6 AM-EpiCs or 1×10^6 AM-MSCs were mixed with 100 μL of Matrigel either alone or in combination and then subcutaneously transplanted into NOD/SCID mice for 2 weeks. Our results indicated that transplantation of a combined mixture of AM-MSCs and AM-EpiCs, or AM-MSCs alone could form tumor-like structures (Fig. 4A), while transplantation of AM-EpiCs alone could not (data not shown). Histological analysis of the tumor xenografts showed a lack of characteristic epithelial islands (Fig. 4A), however, the vimentin-positive stromal cells and E-cadherin-positive EpiCs apparently reorganized into stromal and epithelial compartments (Fig. 4B, 4C). Similar to in vivo findings from AM tissues, tumor xenografts generated from transplantation of combined AM-EpiCs and AM-MSCs harbored a proportion of E-cadherin$^+$/vimentin$^+$ double positive hybrid cells (Fig. 4D; the top panel). The positive signals representing SLUG expression were overlapped with those of E-cadherin, but the highly expression signals for SNAILl were majorly localized outside E-cadherin positive EpiCs (Fig. 4D). Meanwhile, AM-EpiCs cotransplanted with AM-MSCs highly expressed several stem cell-related genes, including ALDH1, β-catenin, BMI-1, and SOX2 (Fig. 4E). These findings suggest that transplanted AM-EpiCs can survive and self-renew, which is supported by cotransplanted AM-MSCs.

AM-MSC-Derived IL-6 Promotes the Expression of EMT- and Stem Cell-Related Genes in AM-EpiCs

It has been reported that MSCs and the soluble factors secreted in the TME play an important role in tumor development and progression [26, 27]. Our results showed that cotransplantation of AM-MSCs could support the survival and self-renewal of AM-EpiCs in vivo, suggesting an important role of AM-MSCs in AM development. To further identify the AM-MSC-derived factors potentially involved in AM formation, we performed cytokine arrays using follicular AM tissue lysates. Our results showed an abundant level of IL-6, growth-regulated alpha protein, IL-8, and angiogenin, in follicular AM tissue lysates as compared to control normal jawbone ($p < .01$) (Fig. 5A, 5B). Dual-color IF staining further confirmed that the abundant IL-6 signals were mainly localized within vimentin and collagen I-expressing MSCs, while only a few were localized in E-cadherin-positive EpiCs (Fig. 5C), suggesting that IL-6 was likely secreted by MSCs within the follicular AM microenvironment. To substantiate our findings, we compared the secretion of IL-6 in the supernatants of in vitro cultured AM-EpiCs and AM-MSCs by ELISA. Our data indicated that AM-MSCs secreted a significantly robust level of IL-6 as compared to their epithelial counterparts (Fig. 5D).

We then determined the function of IL-6 secreted by AM-MSCs in regulating EMT and stem cell properties of AM-EpiCs. To this end, AM-EpiCs were stimulated with exogenous IL-6 (10 ng/mL) or cocultured with AM-MSCs (2:1) for 72 hours

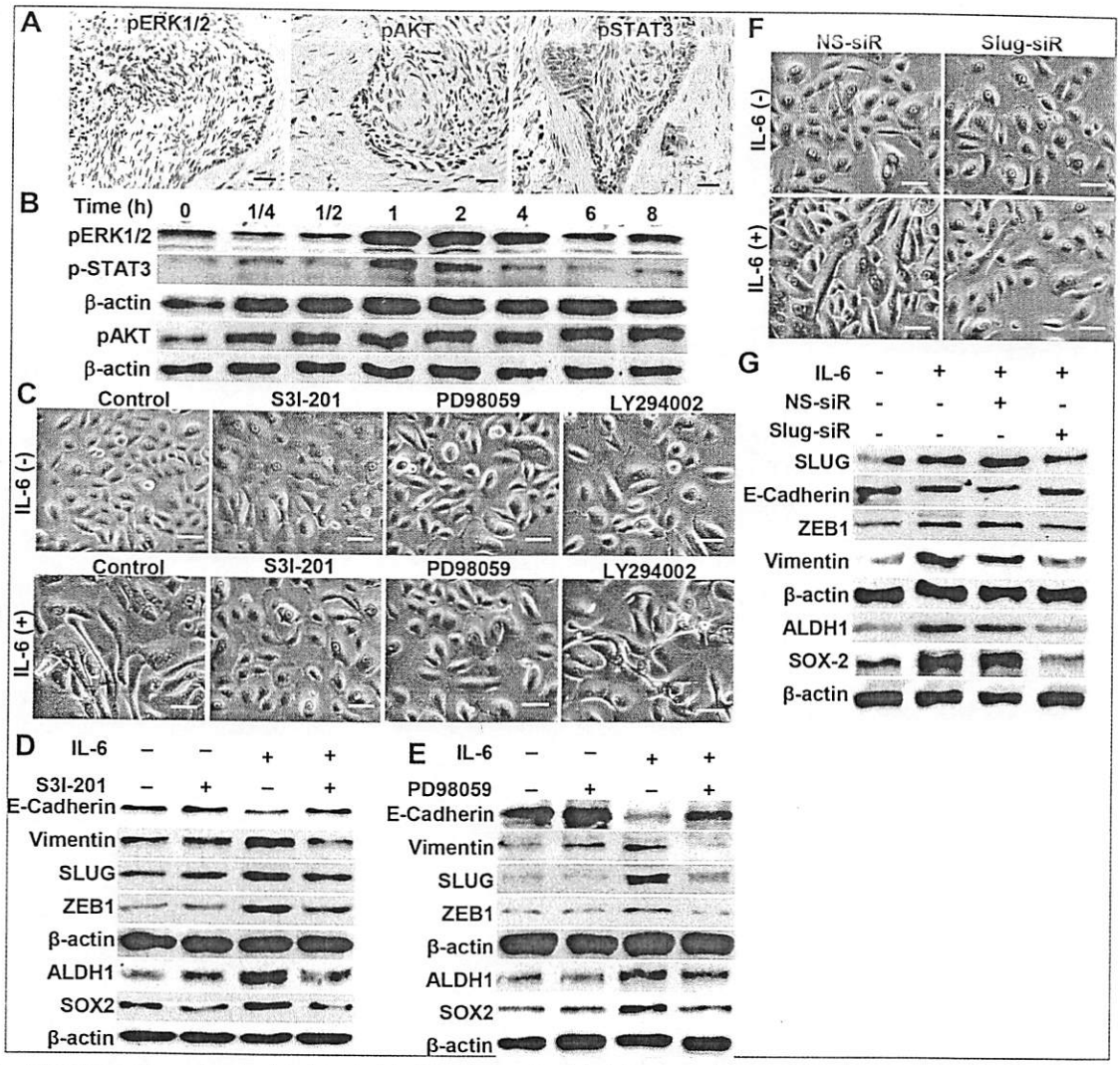

Figure 6. Signaling pathway involved in interleukin (IL)-6-induced epithelial–mesenchymal transition- and stem cell-related gene expressions in follicular ameloblastoma (AM) epithelial cells (EpiCs). **(A):** Immunohistochemical studies on the expression of pERK1/2, pAKT1, and pSTAT3 in follicular AM tissues. Scale bar = 50 μm. An isotype control IgG was used as the control. **(B):** Activation of ERK1/2, pAKT1, and pSTAT3 expression in AM-EpiCs in response to IL-6 stimulation (10 ng/mL) for different time periods. **(C)**: Morphological changes in AM-EpiCs stimulated by IL-6 in the presence or absence of specific inhibitors for STAT3 (S3I-201; 100 μmol/L), ERK1/2 (PD98059; 50 μmol/L), or PI3K/AKT (LY294002, 50 μmol/L). Scale bar = 100 μm. **(D, E):** Pretreatment with a specific inhibitors for STAT3 (S3I-201; 100 μmol/L) or ERK1/2 (PD98059, 50 μmol/L) abrogated IL-6-induced downregulation of E-cadherin expression and upregulation of vimentin, SLUG, ZEB1, aldehyde dehydrogenase 1 (ALDH1), and SOX2 expressions, in AM-EpiCs as determined by Western blot. **(F):** Knockdown of *SLUG* gene expression by short interfering RNA (siRNA) transfection (Slug-siR) reversed IL-6-induced spindle-like cell morphological changes in AM-EpiCs. Scale bar = 100 μm. **(G):** Knockdown of *SLUG* gene expression by siRNA transfection (Slug-siR) significantly abrogated IL-6-induced downregulation of E-cadherin expression and upregulation of vimentin, ZEB1, ALDH1, and SOX2 expressions, in AM-EpiCs as determined by Western blot. A nonspecific siRNA (NS-siR) was used as a control. Abbreviations: IL, interleukin; NS, nonspecific.

IL-6, exogenous or secreted by AM-MSCs, could promote AM-EpiCs to undergo EMT and acquire EpiSC properties in vivo.

Discussion

EMT, a critical process associated with tumor growth and cancer metastasis, is commonly reported in tumor samples from head and neck cancer patients [30–32]. A growing body of evidence has suggested a dynamic interaction between cancer cells and the TME which orchestrates the EMT process and cancer-stem like cell generation, and thus contributing to the increased capabilities of tumor cells to survive, proliferate, metastasize, and resist chemo- and radiation therapies [26, 33–35]. However, it still remains largely unknown whether TME-regulated EMT process and tumor-like stem cell formation contribute to the pathogenesis of the epithelial benign yet aggressive neoplasm. Here, we chose to study a common odontogenic neoplasm, follicular AM, which although its benign nature, has been recognized for its locally aggressive growth and high recurrence [36]. This study has provided the first line of evidence that the epithelial islands or compartments of follicular AM, the most common histopathological variant type, harbor a subpopulation of epithelial-mesenchymal hybrid cells which expressed both EMT-related markers and several important stem cell-related genes, thus

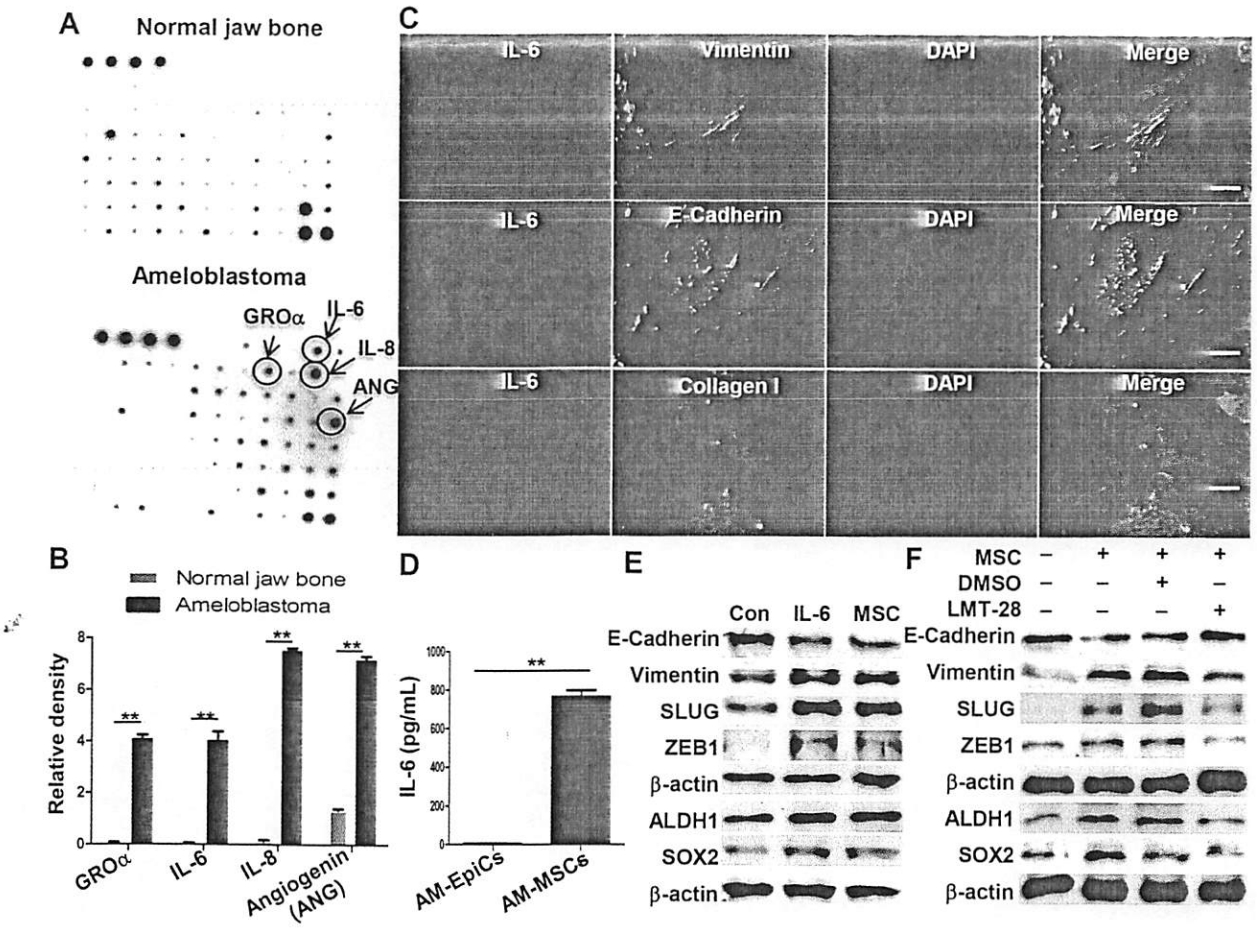

Figure 5. Ameloblastoma (AM) mesenchymal stromal cell (MSC)-derived interleukin (IL)-6 promotes epithelial–mesenchymal transition (EMT)- and stem cell-related gene expression in AM epithelial cells (EpiCs). **(A):** Cytokine arrays on the expression profile of cytokines and growth factors in follicular AM and normal jaw bone tissues. **(B):** Semi-quantification of the expression of growth-regulated protein alpha, IL-6, IL-8, and angiogenin from cytokine arrays (A). **, $p < .01$. **(C):** Expression of IL-6 in the stromal compartment of follicular AM as determined by dual color-immunofluorescence studies, while an isotype control IgG was used as the control. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole. Scale bar = 100 μm. **(D):** The secretion of IL-6 in the supernatant of cultured AM-EpiCs and MSCs (AM-MSCs) was determined by enzyme-linked immunosorbent assay. **, $p < .01$. **(E):** Stimulation with human recombinant IL-6 or coculture with AM-MSCs downregulated the expression of E-cadherin, while upregulated the expression of EMT-regulatory transcriptional factors (SLUG and ZEB1) and stem cell-related genes (aldehyde dehydrogenase 1 [ALDH1] and SOX2) in AM-EpiCs as determined by Western blot. **(F):** Specific IL-6 receptor antagonist abrogated AM-MSC coculture-induced downregulation of E-cadherin expression as well as the upregulation of SLUG, ZEB1, ALDH1, and SOX2 expression in AM-EpiCs as determined by Western blot. Abbreviations: ALDH1, aldehyde dehydrogenase 1; ANG, angiogenin; Con, control; DAPI, 4′, 6-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; EpiCs, epithelial cells; GROα, growth-regulated alpha protein; IL, interleukin.

EpiCs (Fig. 6D, 6E). Altogether, these findings suggest that activation of STAT3 and ERK1/2 signaling pathways possibly contributed in IL-6-mediated EMT process and acquisition of EpiSC properties in AM-EpiCs.

Since our results indicated that stimulation of AM-EpiCs with IL-6 significantly increased the expression of SLUG and ZEB1, two key transcriptional factors involved in regulating EMT process, next we asked which transcriptional factor was directly involved in the IL-6-mediated EMT process and stem cell-related gene expression in AM-EpiCs. Our results showed that knockdown of *SLUG* by transfection of specific siRNAs reversed the spindle-like morphological changes in AM-EpiCs in response to IL-6 stimulation (Fig. 6F). In addition, knockdown of *SLUG* expression markedly abrogated IL-6-induced downregulation of E-cadherin and upregulation of vimentin, ZEB1, ALDH1, and SOX2 in AM-EpiCs (Fig. 6G). These results suggest that SLUG plays a crucial role in orchestrating IL-6-induced EMT process and stem-like cell formation in AM-EpiCs.

IL-6 Promoted EMT and Tumorigenic Properties in AM-EpiCs In Vivo

We next determined the in vivo effects of IL-6 on EMT process and EpiSC formation of AM-EpiCs using the tumor xenograft model. To this end, AM-EpiCs (2×10^6) and IL-6 (10 ng/mL) were mixed with polyethylene glycol hydrogel, a scaffold material widely used for a controlled release of biomolecules [29], and then subcutaneously transplanted into nude mice. Our results showed that transplantation of AM-EpiCs mixed with hydrogel in the presence of IL-6 promoted formation of tumor-like structures, whereas no obvious tumor-like structure was generated in the control group without IL-6 (Fig. 7A). IF study revealed a highly expression of EMT-related genes, such as vimentin, SNAIL1, SLUG, and ZEB1, as well as stem cell-related genes, such as ALDH1, BMI-1, and SOX2 in tumor xenografts formed by transplanted AM-EpiCs mixed with IL-6 (Fig. 7B, 7C). These findings have provided further evidence that

in AM-EpiCs; however, only specifically blocking ERK1/2 or STAT3 signaling significantly abrogated IL-6-mediated EMT- and stem cell-related gene expression in these cells. Of note, blocking ERK1/2 activity alone by PD98059 treatment induced the expression of certain EMT-related markers in AM-EpiCs, which was consistent with a previous study by Kanda et al., reporting that exposure to PD98059 increased cell motility by promoting EMT in human renal carcinoma cells [45]. Since ERK and STAT3 were involved in a variety of tumor cell responses, inhibition of these signaling pathways was considered a potential strategy in the treatment of advanced cancer [23, 44, 46]. Thus, a number of synthetic small molecule inhibitors have been screened for tumor cell responses in vitro and tumor progression in vivo. Most recently, a study reported that oral administration of SC144, a specific gp130 inhibitor, delayed tumor growth in a mouse xenograft model of human ovarian cancer without significant toxicity to normal tissues [46]. Taken together, these studies suggest that in-depth delineating of the IL-6 mediated cell context-dependent biological effects and the corresponding signaling pathways can provide a new platform for the development of novel targeted therapeutic approaches for the treatment of both malignant and benign tumors, particularly, AM [27].

Conclusion

In conclusion, we have shown for the first time that a subpopulation of cells with EMT phenotypes and EpiSC properties exist in the epithelial islands/compartments of follicular AM. Both in vitro and in vivo studies have demonstrated that AM-MSC-derived IL-6 plays an important role in orchestrating the EMT process and EpiSC formation in AM-EpiCs via IL-6R/ERK1/2/STAT3/SLUG signaling pathways. However, it is noteworthy that this study only focused on the primary and non-recurrent follicular AM, whereas there are a variety of subtypes of AM with different clinical and histopathological features, distinct activating mutations in certain signaling pathways, varied recurrence rates and prognoses [1, 3]. Therefore, further studies are warranted to elucidate whether current findings are also applicable to other subtypes of AM, particularly the recurrent tumors, thus providing substantial evidence to support the notion that TME factors may play an important role in the pathogenesis, progression and recurrence of AM, and eventually leading to novel targeted therapies of this benign but aggressive odontogenic tumor.

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Author Contributions

C.J.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Q.Z., R.M.S., L.C., and F.A.: conception and design, data analysis and interpretation, manuscript writing; S.S. and T.-H.C.: collection and/or assembly of data; A.D.L.: conception and design, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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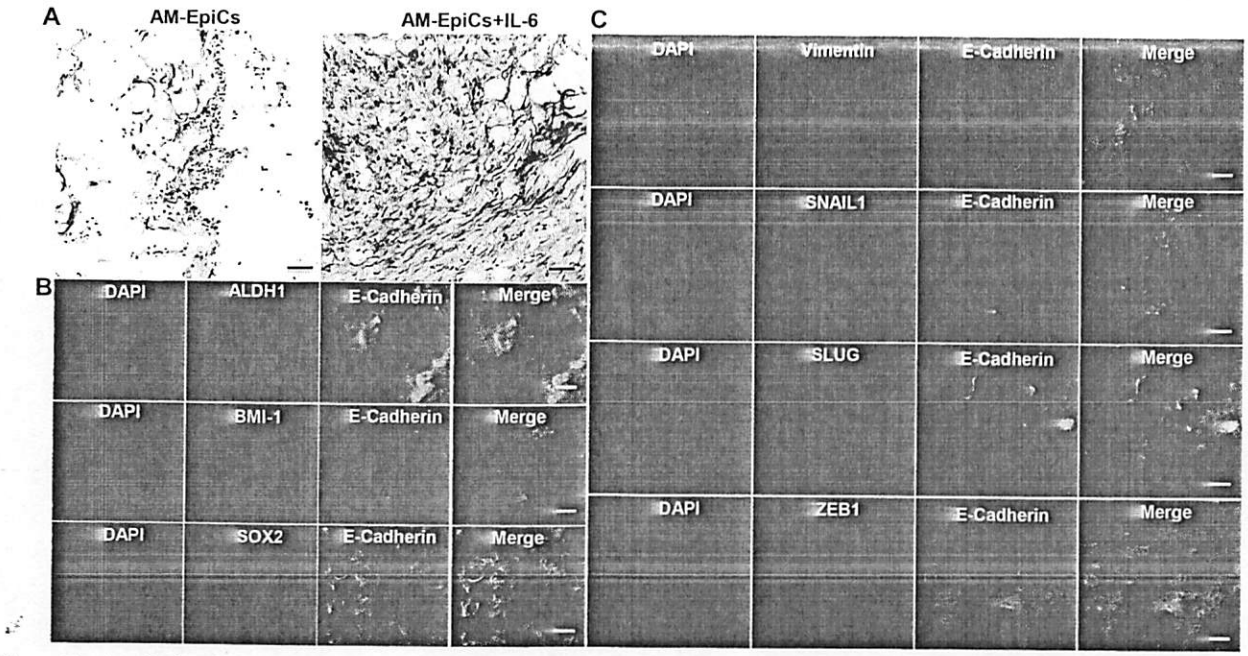

Figure 7. Interleukin (IL)-6 promoted in vivo tumorigenic potential of follicular ameloblastoma (AM) epithelial cells (EpiCs). AM-derived EpiCs (AM-EpiCs) (2×10^5) mixed with 100 μL of hydrogel in the absence or presence of IL-6 (10 ng/mL) were subcutaneously injected into nude mice and 2 weeks later tumor-like tissues were harvested. **(A):** H&E staining indicated that IL-6 promoted tumor-like structure formation by transplanted AM-EpiCs (the right panel). Scale bar = 100 μm. **(B):** Transplanted AM-EpiCs in the presence of IL-6 in xenografted tumors highly expressed stem cell-related genes, ALDH1, BMI-1, and SOX2, as determined by dual color-immunofluorescence (IF) studies, while an isotype control IgG was used as the control. The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Scale bar = 100 μm. **(C):** Transplanted AM-EpiCs in the presence of IL-6 in xenografted tumors highly expressed epithelial–mesenchymal transition-regulatory transcription factors, SNAIL1, SLUG, and ZEB1, as determined by dual color-IF studies, while an isotype control IgG was used as the control. The nuclei were counterstained with DAPI. Scale bar = 100 μm. Abbreviations: ALDH1, aldehyde dehydrogenase 1; AM, ameloblastoma; DAPI, 4′, 6-diamidino-2-phenylindole; EpiCs, epithelial cells; IL, interleukin.

supporting the notion that an active/dynamic EMT process and a subpopulation of EpiSCs co-exist in follicular AM tissues.

The TME is usually dominated by tumor-associated stromal cells which are known to secrete an array of protumorigenic factors, including IL-6, IL-8, stromal-derived factor-1 alpha, and vascular endothelial growth factor, among others [26]. Of these protumorigenic factors, an abundant amount of IL-6 and IL-8 is usually produced by tumor associated stromal cells, macrophages, and other types of immune cells [37], possibly linking chronic inflammation and tumor growth [38]. Accumulating evidences have shown that stromal cell-derived IL-6 promotes EMT process, the generation and self-renewal/expansion of various types of CSCs [27, 37–41] and potentially serves as key mediator of a positive feedback loop among tumor associated stromal cells, cancer cells and CSCs [37, 42]. Therefore, further deciphering the role of IL-6 in EMT, malignant transformation, and CSC properties may lead to a comprehensive understanding of its indispensable role in tumor development, progression, and recurrence.

Most recently, a study reported that IL-6 secreted by AM-derived fibroblasts cultured in vitro may play a role in the reciprocal cell-to-cell interaction between AM tumor cells and stromal fibroblasts [24]; however, there remains a lack of direct evidence whether and how this cytokine contributes to the pathogenesis of AM. In this study, we have provided first line of evidence, both in vitro and in vivo, that: (a) AM-MSCs produced an abundant amount of IL-6; (b) AM-EpiCs, in response to IL-6 stimulation or coculture with their stromal counterparts, underwent EMT characterized by altered expression of E-cadherin, vimentin, SLUG, and TWIST, and showed increased expression of several stem cell-related functional genes. Mechanistically, we showed that the knockdown of *Slug* gene expression, one of the key transcription factors involved in EMT regulation, partially reversed IL-6 induced EMT phenotype and stem cell-related gene expression in AM-EpiCs. Using tumor xenograft model in nude mice, we further demonstrated the critical role of AM-MSCs and IL-6 in the formation of tumor-like structures by transplanted a combined mixture of AM-EpiCs and AM-MSCs; these in vivo tumor xenografts harbored a subpopulation of epithelial–mesenchymal hybrid cells which expressed both EMT- and stem cell-related genes, and recapitulated to some extent the histological and biological properties of human follicular AM. These compelling findings support the notion that AM-MSC-derived IL-6 plays an important role in the pathogenesis of follicular AM, possibly by facilitating AM-EpiCs to undergo EMT process and acquire stem-like cell properties. However, further studies are warranted to explore whether our findings also apply to other subtypes of AM or odontogenic epithelial benign tumors.

It has been characterized that IL-6 exerts its biological functions by binding to IL-6R/gp130 complex and activating the downstream STAT3, MAPK, or AKT signaling pathways. Previous studies have shown that IL-6 promotes EMT and self-renewal of cancer stem cells via the IL-6 receptor/GP130 complex-mediated activation of STAT3 signaling pathway [43, 44]. In this study, we demonstrated that stimulation with IL-6 led to the activation of STAT3, AKT, ERK1/2 signaling pathways

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