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ODAM promotes junctional epithelium-related gene expression via activation of WNT1 signaling pathway in an ameloblast-like cell line ALC

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Abstract

Objective: In this study, we investigated the potential and mechanism of odontogenic ameloblast-associated protein (ODAM) in the promoting junctional epithelium-related gene expression in an ameloblast-like cell line ALC.

Background: ODAM is expressed in ameloblasts and JE and acts as a component of the inner basal lamina (IBL) and intercellular matrix of JE. ODAM KO mice showed destruction of the integrity of the JE, which detaches from teeth. ODAM was confirmed to regulate the cytoskeleton through the ODAM-ARHGEF5-RhoA signaling pathway of the JE. Whether ODAM contributes to the regulation of ameloblast differentiation in JE remains unclear. After the formation of enamel, the ameloblast undergoes a series of morphological changes. Whether ODAM will affect the biological behavior of ameloblasts making them have the characteristics of JE is unclear.

Methods: A murine ameloblast-like cell line, ALC, was used to investigate the effects of ODAM on the JE-like changes of ALC cells in an epithelium-induced environment by generating ODAM overexpression and ODAM knockdown cells through a lentivirus transduction approach. The biomarkers of junctional epithelium CK19, SLPI, and ODAM and the potential regulatory gene WNT1 were investigated by real-time PCR, western blot, immunocytochemistry, immunostaining, luciferase reporter, and rescue assays.

Results: ODAM, CK19, and SLPI were significantly upregulated after epithelial induction. Overexpression of ODAM in ALC cells markedly increased CK19 and SLPI expression, while knockdown of ODAM in ALC cells clearly decreased CK19 and SLPI expression. A reporter luciferase assay showed that ODAM activated the WNT signaling pathway, especially through WNT1. Exogenous overexpression of ODAM upregulated WNT1 expression, while knockdown of ODAM reversed this effect. The WNT1 inhibition assay further confirmed the above results and showed that the WNT1 pathway was positively correlated with biomarkers of junctional epithelium CK19 and SLPI expression. Rescue studies showed that knocking down WNT1

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in the ODAM-overexpressing ALC cells decreased the expression of CK19 and SLPI. Immunocytochemistry showed that ODAM colocalized with CK19, SLPI, and WNT1 in the cells.

Conclusion: In conclusion, the research work showed that ODAM promotes junctional epithelium-related gene expression in ALC via the ODAM-WNT1 axis, which may provide new insight into the function of ODAM and JE formation.

KEYWORDS
ameloblast, junctional epithelium, ODAM, WNT1

1 | INTRODUCTION

ODAM is a member of the secretory calcium-binding phosphoprotein (SCPP) gene cluster,[1–3] and it is expressed in the nucleus and cytoplasm of ameloblasts and the extracellular matrix.[4] ODAM is highly expressed at the end of enamel maturation, continuously expressed in the reducing enamel organ and expressed in JE,[5] which indicates that it plays an important role in the end stage of amelogenesis and the early stage of JE generation.[4,6,7] Moreover, ODAM influences the integrity of JE, which detaches from healthy teeth and increases inflammatory infiltration in ODAM KO mice, especially in older mice.[8]

Ameloblasts undergo a series of morphological changes during development in which they form reduced enamel epithelium (REE)[9] and then become the primary JE after tooth eruption.[10] Moreover, previous studies have elucidated the functions of ODAM and the association between ODAM and JE since ODAM is expressed from the mature stage to the eruption of teeth. ODAM was found to regulate MMP20, which is controlled by RUNX2, in ameloblasts[3] and to affect the JE cell cytoskeleton by the fibronectin/laminin-integrin-ODAM-ARHGEF5-RhoA-axis.[9] Extensive evidence has indicated that ODAM is closely associated with junctional epithelium development.[11] ODAM has been proven to play a role in forming the JE and maintaining periodontal integrity.[5] However, the mechanism by which ODAM regulates the differentiation of ameloblasts into JE remains largely unclear.

JE expresses unique markers, including cytokeratin (CK)-7, -8, -14, -18, and -19,[11] under normal differentiation and pathological conditions.[12] The dynamic changes in CK19 expression may be related to changes in the function of ameloblasts.[12] Another biomarker for JE is the secretory leukocyte protease inhibitor (SLPI),[13] a member of the trappin gene family, which is related to immunity and protection against tissue damage[14] and is highly expressed in JE.[13,15]

The classical WNT/β-catenin signaling pathway is involved in various physiological and pathological processes, including embryonic development, apoptosis, differentiation, cell cycle arrest, and oxidative stress.[16–19] Previous reports have demonstrated that GSK3β, downstream of the WNT pathway, plays an important role in the differentiation of ameloblasts by combining with the TGF-β signaling pathway.[20] Our group also found that downregulation of WNT10A affects odontogenesis.[21] In the research of Yuan et al, they pointed out that WNT-responsive stem cells exist in the structure of the JE and they have the capacity to differentiate into function-specific daughter cells contributed to the integrity and function of JE.[22]

Mouse ameloblast cell lines include LS8[23] and ALC,[24] both of which are commonly used to investigate the expression of enamel-specific genes, including *Odam*.[25] Therefore, ALC cells are a suitable in vitro model to study maturation stage events of amelogenesis. In a study of ODAM on the mechanism of JE adhesion, ALC cells were also selected as *in vitro* cell model.[9]

In this study, we investigated the potential and mechanism of ODAM in the promoting junctional epithelium-related gene expression in ALC by generating stable cell lines with overexpression and knockdown of ODAM.

2 | MATERIALS AND METHODS

2.1 | Tissue preparation and immunohistochemistry

C57BL/6J mice were sacrificed on days 5, 15, and 21 after birth in this study, which was approved by the Peking University Animal Welfare Committee. After anesthesia, the mouse mandible was dissected and immersed in 10% formalin overnight. After decalcification in 14% EDTA solution for 2 weeks, the specimens were dehydrated by gradient alcohol, embedded in paraffin, and cut into 5-μm thick slices. After antigen retrieval by the high-pressure method, the sections were treated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity, preincubated with goat blocking serum (ZSGB-Bio) for 20 min to block nonspecific binding, and incubated with rabbit anti-mouse ODAM antibody (Proteintech), anti-CK19 antibody (Proteintech), anti-SLPI antibody (Santa Cruz), and anti-WNT1 (Proteintech) antibody at 4°C overnight. After that, the sensitizer of the ZSGB-Bio System was applied for 30 min. Corresponding HRP-conjugated secondary anti-rabbit/anti-mouse IgG antibodies were added to the sections for 30 min at room temperature, followed by color development with diaminobenzidine (DAB) reagent for 3 min. After washing, the sections were counterstained with hematoxylin solution, dehydrated in ethanol (70%, 85%, 95%, and 100%, sequentially, for 3 min each), and cleared

with xylene. The staining of the sections was observed with a Nikon Eclipse Microscope (Nikon).

2.2 | Cell culture and epithelium induction

An immortalized ameloblast-lineage cell (ALC) line, originating from C57BL/6J mandible molar tooth germs, was a gift from Professor Gao Yuguang of Binzhou Medical University of China and was maintained in F-12/DMEM containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C, 5% CO_2, and 100% humidity.

The cells were seeded in a 6-well plate at a density of 5×10^5 cells/well with epithelial cell medium (EpiCM, ScienCell) mixed with F-12/DMEM complete medium (hereafter named differentiation medium, DM). The medium was changed every 2 days. Day 0 was defined as the day of epithelial medium supplementation to the cell culture. At the indicated time points, the JE biomarkers were detected by immunostaining, real-time PCR, and western blot assays. The cells cultured with F-12/DMEM complete medium in six-well plates served as a control.

2.3 | Real-time PCR

Total RNA of the cell lines was extracted by using TRIzol reagent (Invitrogen). Real-time PCR was performed in a Q3 real-time PCR system (Thermo Fisher) by using LightCycler® RNA Master SYBR Green I (Roche) according to the manufacturer's protocol. GAPDH was used as an internal control. Each reaction was performed at least in triplicate and repeated in at least three different samples. ODAM gene expression was determined by using the comparative CT ($2^{-\Delta\Delta Ct}$) method. All primers used for real-time PCR are listed in Table 1.

2.4 | Western blot

The total protein of the ALC and ALC induced by differentiation medium (DM) groups was extracted on day 7. Cells in the 6-well plate were harvested and lysed in RIPA buffer with protease inhibitors. The supernatant of whole-cell lysis was collected, and the protein concentration was measured by the BCA method.

Proteins (30 μg) from the cells were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked for 1 h with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% BSA for 1 h and then incubated overnight at 4°C with ODAM (Proteintech), CK19 (Proteintech) and SLPI (Santa Cruz) antibodies diluted in TBST buffer (1:1000). After the membranes were washed with TBST buffer, they were incubated for 1 h with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Proteintech). Immunoreactive proteins were visualized on a fusion imaging system (Odyssey LI-COR Biosciences). GAPDH (Proteintech) served as the internal control.

TABLE 1 The sequences of real-time PCR primers

Gene	Primer sequence	Species
Ck19	F:GGTGTCGACCTAGCCAAGAT	Mouse
	R:TCTGGATCTGCTCAGAGTGG	
Slpi	F:GGCCTTTTACCTTTCACGGTG	Mouse
	R:TACGGCATTGTGGCTTCTCAA	
Odam	F:GTCACATCCTCACCACAGCA	Mouse
	R:GAGTTTCTGGAGCTGTGCCT	
Wnt1	F:GGTTTCTACTACGTTGCTACTGG	Mouse
	R:GGAATCCGTCAACAGGTTCGT	
GAPDH	F:ACCACAGTCCATGCCATCAC	Mouse
	R:TCCACCACCCTGTTGCTGT	

2.5 | Immunocytochemistry (ICC)

DM-induced ALC and control cells were seeded on glass coverslips in 24-well plates and induced for 7 days. The cells were washed 3 times with PBS and then fixed with 4% paraformaldehyde for 15 min. The cell membrane was perforated with 0.1% Triton X-100 for 15 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. Other procedures were the same as the immunohistochemistry procedure. The primary antibodies were ODAM, CK19, SLPI, and WNT1 antibodies. Cells were observed under the microscope with a DP70 digital camera (Olympus).

2.6 | Generation of overexpression and knockdown of ODAM cell lines

The full-length *Odam* gene was chemically synthesized by GeneCopoeia and was introduced into the pEZ-Lv201 lentiviral vector (GeneCopoeia) in the unique EcoRI site to construct the lentivirus encoding the *Odam* gene, which was confirmed by nucleotide sequencing. The *Odam* knockdown lentivirus was constructed by cloning the complementary nucleotides of *Odam* into the GV493 lentiviral vector with EGFP (GeneChem, Inc.) between the AgeI and EcoRI sites.

The cells were seeded in a 6-well plate at a density of 5×10^5 cells/well overnight and then infected with *Odam* overexpression lentivirus and its knockdown lentivirus when the ALC cells reached 50%~60% confluence. After puromycin (2.0 μg/ml) (Invitrogen) screening, the stable cell line was evaluated by observing EGFP expression by using a fluorescence microscope (Nikon) as well as ODAM expression by real-time PCR and western blots. The empty lentiviral vector with EGFP was used as a control. The four established cell lines are called EV (empty vector), ODAM-OE (ODAM-overexpressing cell line), shEV (short hairpin lentiviral empty vector), and ODAM-KD (ODAM knockdown cell line).

2.7 | Luciferase reporter assay

The WNT pathway reporter plasmid was constructed by a biotechnology company (GeneCopoeia). ODAM-OE and ODAM-KD cells and their controls were seeded in 24-well plates. When they grew to 50% confluence, the transfections were performed according to the manufacturer's instructions. At 24 h after transfection, the medium was replaced by DM. The luciferase assay was performed at 72 h after transfection as described by the manufacturer (GeneCopoeia). Each reaction was performed at least in triplicate. The values of GLUC and SEAP were read by MikroWin 2000, calculating the value of GLUC/SEAP.

2.8 | Rescue assay

For knockdown of endogenous WNT1 expression in ODAM-OE cells, small WNT1 interfering RNA (siWNT1) and negative control siRNA were constructed by a company (RiboBio). The ODAM-OE cells were seeded in a 6-well plate at a density of 5×10^5 cells/well. After cell adherence, it is advisable to change the DM. When the cell density was 50%–70%, according to the manufacturer's protocol, the siWNT1 and siRNA transfection complexes were prepared and added to 6-well plates. At 4–6 h after transfection, the medium was changed to DM. Western blotting was performed to determine the WNT1 knockdown efficiency. After culture with DM medium for 7 days, the transfected cells were collected for real-time PCR and western blot experiments to detect CK19 and SLPI expression.

2.9 | Statistical analysis

The data for real-time PCR and luciferase assay are presented as the mean ± SD, and Student's *t*-test was used for the comparison between the two groups. $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Detection of ODAM, CK19, and SLPI expression in the enamel organs of mice

IHC demonstrated that ODAM (Figure 1A–C) could be detected in the cytoplasm of ameloblasts at an early secretory stage of amelogenesis at 5 days, while there was no obvious labeling for CK19 (Figure 2A–C) and SLPI (Figure 3A–C) at the bell stage of the enamel organ. As the tooth erupts at day 15, ameloblasts and adjacent cell layers form the reduced enamel organ (EO), and thin connective tissue separates the enamel organ and oral epithelium. Expression of ODAM (Figure 1D–F) was detected in several cell layers of the EO and the overlying oral epithelium. CK19 (Figure 2D–F) and SLPI (Figure 3D–F) had similar spatial expression patterns. Finally, when the teeth were fully erupted at day 21, ODAM (Figure 1G–I), CK19 (Figure 2G–I), and SLPI (Figure 3G–I) in the JE were all strongly stained and highly expressed.

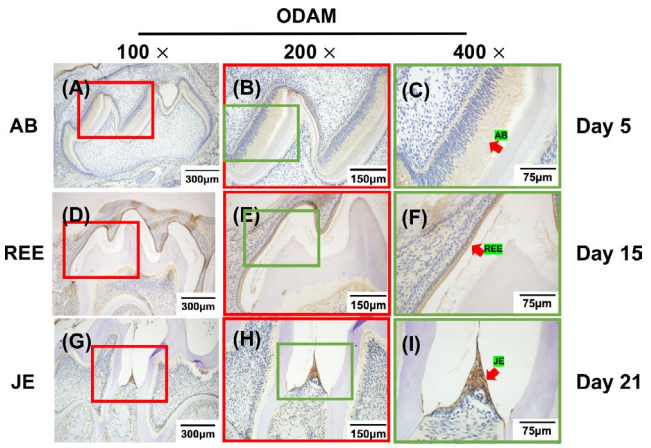

FIGURE 1 The spatiotemporal expression of ODAM in mouse during tooth eruption. IHC indicates that ODAM is expressed moderately in AB (A, B and C) and highly in REE (D, E and F) and JE (G, H and I). Red arrows indicate positive staining. Scale bars = 300, 150 and 75 μm. AB, ameloblast; JE, junctional epithelium; REE, reduced enamel epithelium

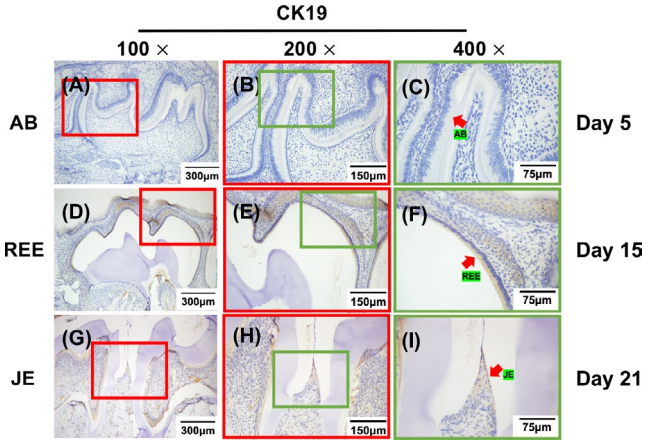

FIGURE 2 The spatiotemporal expression of CK19 in mice during tooth eruption. The staining of CK19 is strong in REE (D, E and F) and JE (G, H and I), whereas AB is negative (A, B and C) during the development. Red arrows indicate positive staining. Scale bars = 300 , 150 and 75μm. AB, ameloblast; JE, junctional epithelium; REE, reduced enamel epithelium

3.2 | The expression pattern of ODAM, CK19, and SLPI in ALC cells under epithelial induction condition

After ALC cells were induced by epithelial induction condition using DM medium, immunocytochemistry showed that the expression of ODAM, CK19, and SLPI was drastically enhanced (Figure 4A). Real-time PCR (Figure 4B) data indicated that the transcription of *Odam* ($p < .01$), *Ck19* ($p < .0001$), and *Slpi* ($p < .0001$) increased significantly

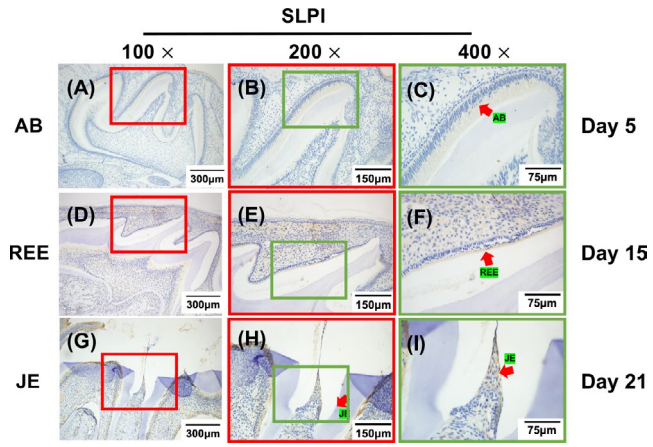

FIGURE 3 The spatiotemporal expression of SLPI in mice during tooth eruption. Cell staining strongly positive for SLPI are also seen in REE (D, E and F) and JE (G, H and I), whereas AB (A, B and C) exhibits a negative reaction in early stage. Red arrows indicate positive staining. Scale bars = 300 and 150 μm. AB, ameloblast; JE, junctional epithelium; REE, reduced enamel epithelium

in the DM-induced ALC cells compared to the noninduced cells, which was further confirmed by the western blot results (Figure 4C). These results suggested that the expression levels of CK19, SLPI, and ODAM were significantly increased after epithelial induction condition.

3.3 | Generation of ODAM overexpression and knockdown ameloblastic cell lines

To further study whether ODAM regulates CK19 and SLPI expression during DM induction, we generated ODAM-OE and ODAM-KD cell lines as described in the Materials and Methods. As the infected lentiviruses carried the EGFP fluorescence gene, the transfected cells were detected by EGFP fluorescence to monitor the transduction efficiency of the lentivirus particles. Under fluorescence microscopy, EGFP expression was observed at more than 90% confluence after puromycin screening (Figure 5A). Moreover, the *Odam* mRNA levels were significantly increased in the ODAM-OE group ($p < .001$) and decreased in the ODAM-KD group ($p < .05$) compared with their control groups after lentivirus infection (Figure 5B). Furthermore, the ODAM-OE group had stronger ICC staining than the EV group, while the ODAM-KD group had weaker staining than the shEV group (Figure 5C). A similar pattern was observed with ODAM protein expression assayed by western blots (Figure 5D). These results indicate that we successfully generated ODAM-OE cells as well as ODAM-KD cells, which provided tools for studying the subsequent mechanisms for ODAM-mediated junctional epithelium-related gene expression.

3.4 | ODAM regulates the expression of JE biomarkers

To investigate the effect of ODAM on the JE-like changes of ALC, we performed real-time PCR and western blotting after DM induction. As shown in the real-time PCR data, the biomarkers of junctional epithelium—*Ck19* ($p < .05$ and $p < .01$) and *Slpi* ($p < .0001$)—were increased in the ODAM-OE group compared to the EV group on days 7 and 14 (Figure 6A,B), respectively. However, *Ck19* ($p < .01$) and *Slpi* ($p < .0001$) were significantly reduced in the ODAM-KD cells (Figure 6D,E). Western blot analysis further confirmed the mRNA results (Figure 6C,F). These data suggested that under DM induction conditions in vitro, ODAM not only has a relationship with JE biomarkers but also may be involved in the regulation of their expression.

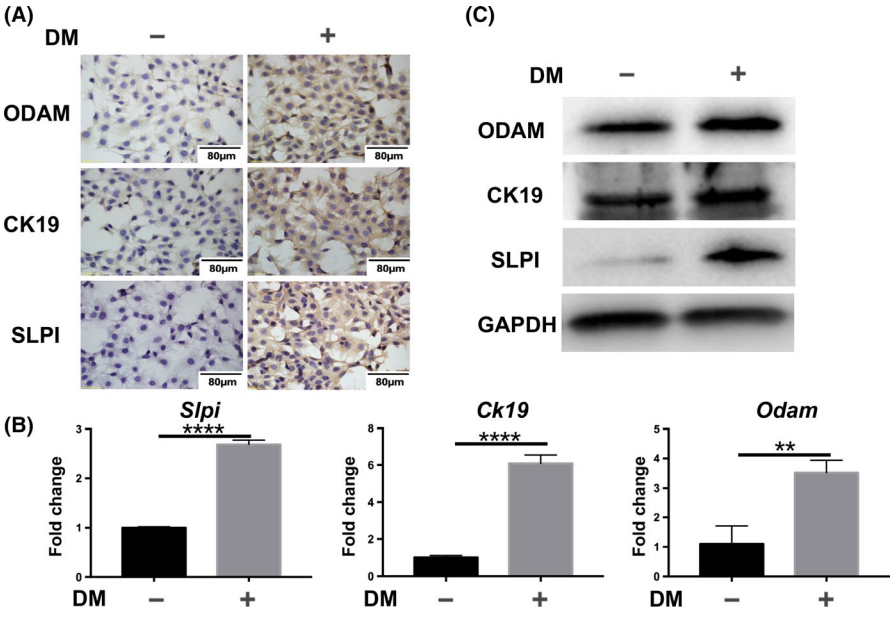

FIGURE 4 The morphological change of ALC after DM induction. A, ICC revealed that the staining of ODAM, CK19, and SLPI were deeper after DM induction. Scale bars = 80 μm. B, At mRNA level, *Odam*, *Ck19*, and *Slpi* were increased in ALC cells after DM induction. Uninduced ALC cells served as control. Data are mean ± SD of triplicate experiments. *$p < .05$ compared with the control. C, The protein of ODAM, CK19, and SLPI before and after induction was measured by western blot

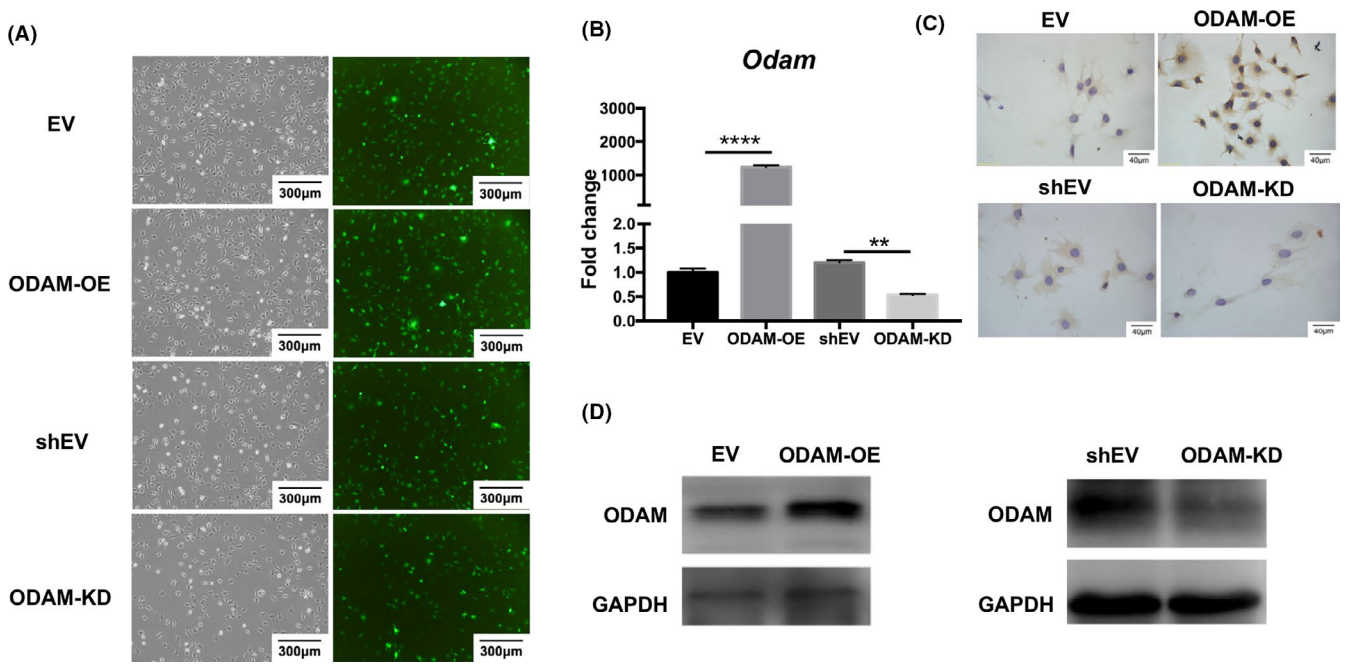

FIGURE 5 Generation of ODAM-OE and ODAM-KD cell lines. A, Lentiviruses infection efficiency representative images of ALC after puromycin selection 2 weeks. Left panel is a bright field image of ALC after infection, and the right image is in fluorescence field. Scale bars = 300 μm. B, ODAM mRNA in ODAM-OE and ODAM-KD was measured by real-time PCR. EV and shEV serves as control. Data are mean ± SD of triplicate experiments. *$p < .05$ compared with the control. C, The expression of ODAM protein in ODAM-OE and ODAM-KD was detected by ICC. EV and shEV serves as control. Scale bars = 40 μm. D, ODAM protein expression in ALC cells before and after infection was detected by western blot

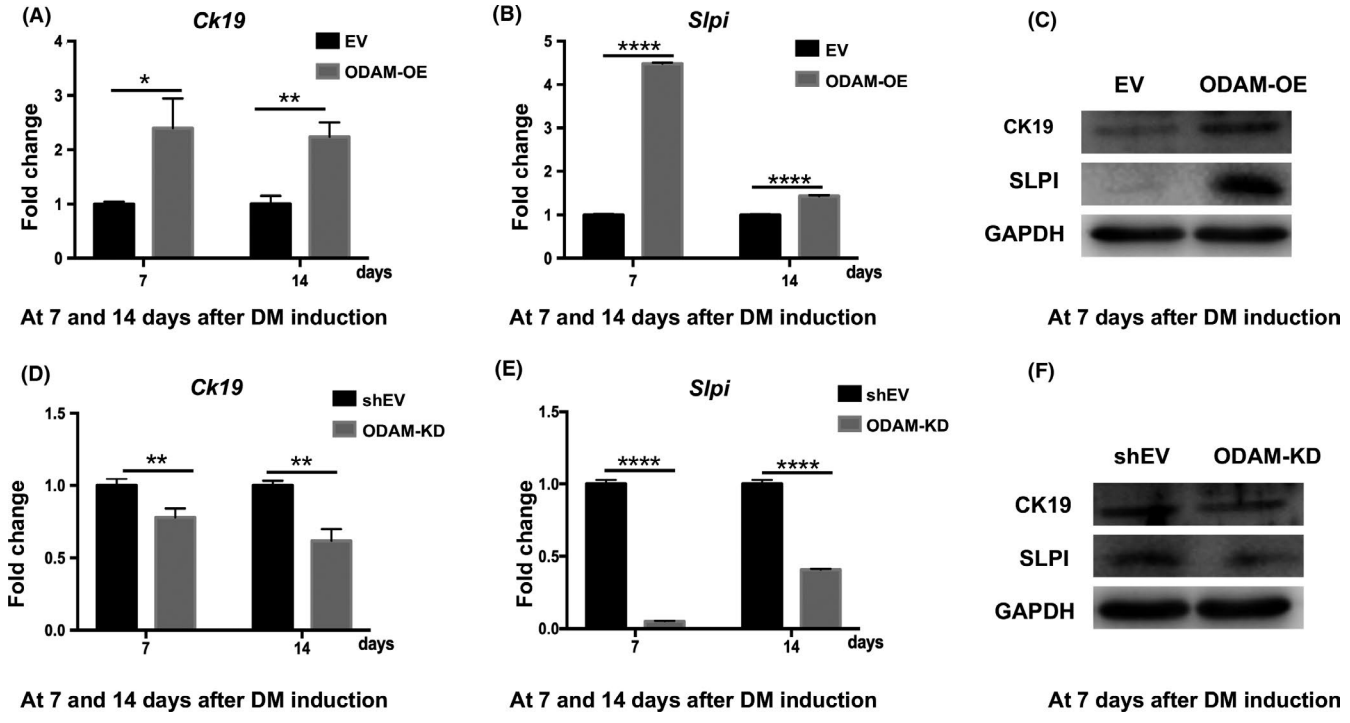

FIGURE 6 The influence of ODAM to the JE bio-makers of CK19 and SLPI. A and B, The effect of ODAM overexpression on the mRNA expression level of *Ck19* and *Slpi* was detected by real-time PCR after induction for 7 and 14 days. The effect of knockdown is shown in D and E. EV and shEV served as control. Data are mean ± SD of triplicate experiments. *$p < .05$ compared with the control. C and F, CK19 and SLPI protein expression were evaluated in ODAM-OE cells and ODAM-KD after DM induction for 7 days by western blot

3.5 | The WNT pathway is involved in the regulation of ODAM in JE-like changes of ALC

Since the expression of ODAM is positively correlated with the expression of JE biomarkers, the regulatory mechanism of this change was investigated through luciferase experiments. After the WNT reporter plasmid was transfected into DM-induced ODAM-OE and ODAM-KD cells, a luciferase assay showed that when ODAM was overexpressed, the WNT promoter was activated ($p < .001$, Figure 7A), while knockdown of ODAM inhibited the WNT pathway ($p < .01$, Figure 7A).

To further determine which member of the WNT family is involved in this process, we performed real-time PCR. The results of real-time PCR demonstrated the pattern of *Wnt1* expression corresponding to the luciferase assay experiments (Figure 7B). Increased expression and decreased expression of *Wnt1* were observed in the ODAM-OE ($p < .05$) and ODAM-KD ($p < .05$) groups, respectively, compared to their controls.

To confirm that WNT1 indeed participated in the process of JE-like changes of ALC, we transfected siWNT1 into the ODAM-OE cell line according to the above experimental procedures. The expression levels of JE biomarkers were detected when the WNT1 pathway was inhibited. The knockdown efficiency of siWNT was detected after transfection in the ODAM-OE cells by western blots (Figure 7C). The results showed that the protein levels of WNT1 in the siWNT1-001 and siWNT-002 groups were lower than those of the control group transfected with siNC. When the WNT1 pathway was inhibited, the expression of the JE biomarkers *Ck19* ($p < .05$) and *Slpi* ($p < .001$) was significantly reduced at the mRNA and protein levels (Figure 7D-F). The IHC experiment showed that WNT1 was moderately expressed in ameloblasts (Figure 8A,B) during the early development of the enamel organs. When REE (Figure 8C,D) and JE (Figure 8E,F) were formed, WNT1 showed strong positive staining.

These data showed that inhibition of WNT1 in the ODAM-OE cells suppresses the expression of JE markers expression, indicating ODAM promotes JE-related gene expression and has an impact on the JE-like changes of ALC.

4 | DISCUSSION

The development of teeth begins with the formation of dental lamina. Odonto-derived epithelial precursor cells differentiate into many types of cells through the spatiotemporal interaction of the

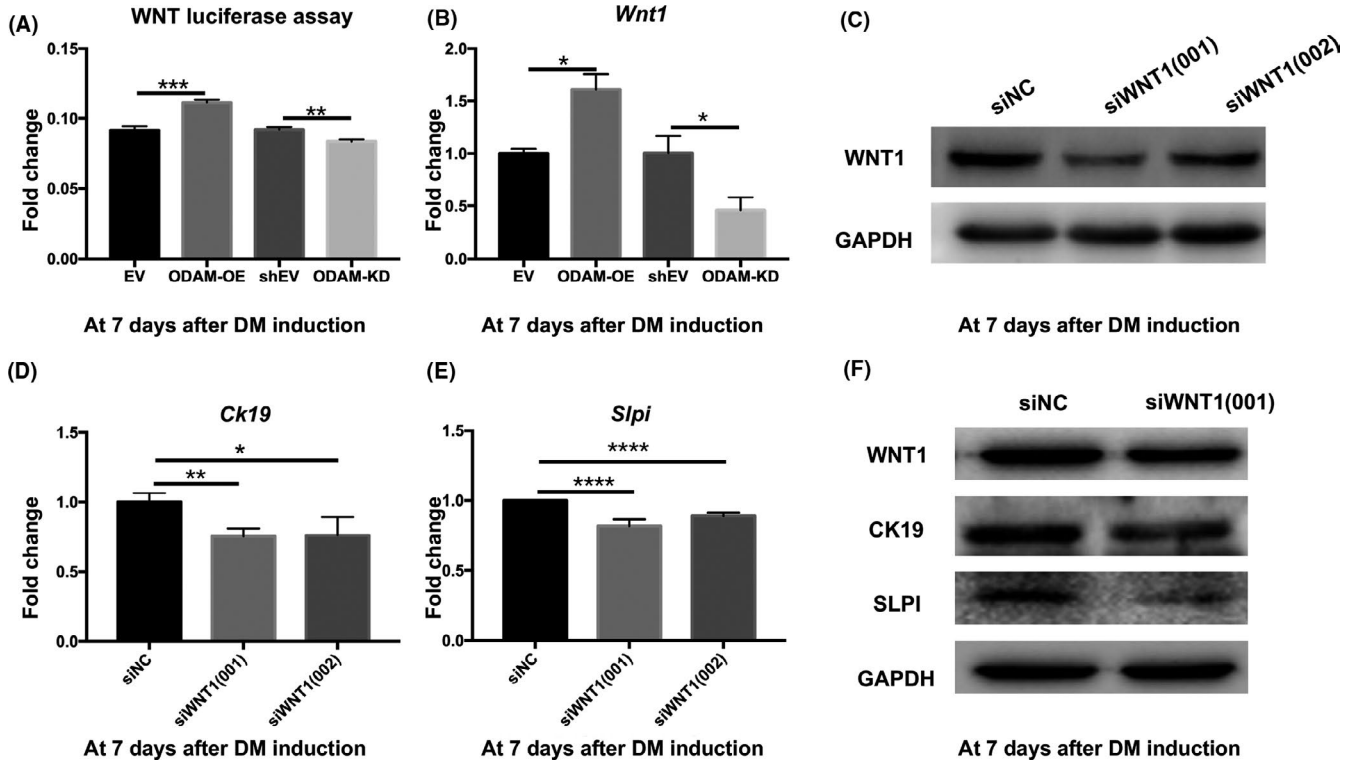

FIGURE 7 WNT1 is involved in ODAM-induced JE differentiation, inactivation of WNT1 by siRNA reduced ODAM-induced JE differentiation. A, The activation and inhibition pathways of ODAM-OE and ODAM-KD cell line were detected by luciferase assay, and the test was performed after induction for 7 days. Data are mean ± SD of triplicate experiments. *$p < .05$ compared with the control. B, Confirming it is the WNT1 that participate the regulation is by the method of real-time PCR. The test was performed after induction for 7 days. Data are mean ± SD of triplicate experiments. *$p < .05$ compared with the control. C, Knockdown efficiency of siWNT1 in ODAM-OE cell line was detected by western blot. D and E, The changes of *Ck19* and *Slpi* mRNA levels after WNT1 Knockdown in ODAM-OE cell line were detected by real-time PCR. siNC severs as control. Data are mean ± SD of triplicate experiments. *$p < .05$ compared with the control. F, The changes of CK19 and SLPI protein levels after WNT1 Knockdown in ODAM-OE cell line were detected by western blot

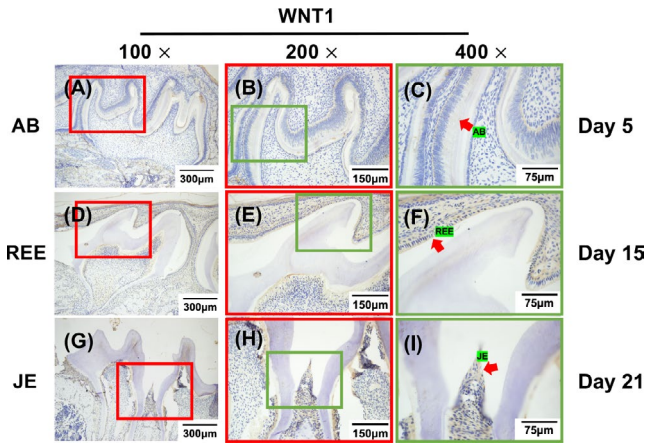

FIGURE 8 The spatiotemporal expression of WNT1 in mice during tooth eruption. IHC indicates that WNT1 was expressed weakly in AB (A, B and C) and highly in REE (D, E and F) and JE (G, H and I). Red arrows indicate positive staining. Scale bars = 300 and 150 μm. AB, ameloblast; JE, junctional epithelium; REE, reduced enamel epithelium

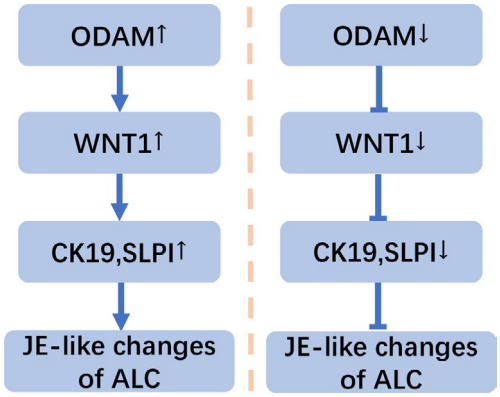

FIGURE 9 The scheme shows the mechanism of the JE-like changes of ameloblast

epithelium and mesenchyme.[26] In the process of tooth development, inner enamel epithelial cells undergo a series of morphological changes and gradually differentiate into secretory, transitional, mature, and reduced ameloblasts.[27] Harada et al[28] reported that the stratum intermedium adjacent to the inner enamel epithelium is also derived from the ameloblast lineage and eventually becomes the papillary layer.[29] During tooth eruption, JE is formed by the reduced enamel organ, including the reduced ameloblast and the papillary cells at the side of connective tissue.[13] However, it is still unclear whether this primary JE will eventually be replaced.

The research of Sara Yajima-Himuro shows that the JE originates from odontogenic epithelium by reconstitution and transplantation of a bioengineered tooth germ combined with GFP-transgenic mouse-derived odontogenic organ and normal mouse-derived mesenchymal cells. In particular, odontogenic epithelial-derived JE cells were maintained for 3 months and were not eventually replaced by oral gingival epithelial cells.[30] JE, a nonkeratinized epithelium, is a specialized structure that seals off the tooth from the oral cavity, providing a frontier of defense against oral bacterial endotoxins. The integrity of JE is essential for maintaining periodontal health. Although the structure and function of the JE have been well studied, the exact mechanisms of its formation remain unknown.[31]

IHC analysis confirmed that ODAM is restricted to the bell stage of amelogenesis and the REE and gingival epithelium above the enamel surface as well as the JE surrounding the tooth. Its expression showed a transition from low-level expression to high-level expression. In addition, CK19 and SLPI both showed a trend from low to high expression, similar to ODAM.

ODAM is encoded by a gene originally called EO-009,[32] and the function of the ODAM sequence has revealed its relevance to the development of odontogenic tissues and tumors.[32] Studies have shown that ODAM is not only an important component of the inner basal lamina,[10,33] which is a special structure promoting the adherence of JE to the tooth surface, but also plays an important role in regulating the formation and regeneration of JE.[5] Animal experiments showed that after gingivectomy, the expression of ODAM can be detected in the migrating cell cluster at the leading edge of the gingival wound.[5] ODAM knockout mice do not show an obvious defect phenotype of enamel hypoplasia, but the JE cannot adhere to healthy teeth, and the structure of the JE is also changed, manifesting apparent parakeratinization.[8] After the end of enamel development, ameloblasts gradually transform to REE, covering the tooth surface to form a primary JE when teeth erupt. During this process, ameloblasts undergo a series of morphological changes. Related studies have shown that there is continuous expression of ODAM in JE formation.[9]

ODAM is a key regulator in the maturation stage of ameloblasts[3] and the junctional epithelium in which it has continuous expression of the ODAM protein.[5,33,34] In 2013, Nishio found that ODAM was also clearly expressed in JE.[35] However, the formation process of JE is still not understood. Therefore, in this experiment, we selected the ameloblast cell line ALC to investigate the mechanism of JE formation.

The expression data of ODAM, CK19,[36-38] and SLPI[15] obtained *in vitro* from real-time PCR are consistent with the temporal and spatial expression *in vivo*. Another interesting observation is that ODAM has a regulatory effect on the JE markers CK19 and SLPI in vitro under epithelial induction conditions.

Domingues MG et al. reported that cytokeratins are tissue-specific and related to the differentiation state of the tissue.[36] CK19 is an important biomarker of odontogenic epithelium[30] that is upregulated during the conversion of IEE to ameloblasts[36] and is continuously expressed in the basal layer of the JE[38] and occasionally expressed in the basal layer of gingival epithelium.[11,39] Therefore, this phenomenon that CK19 locates in ameloblast and the basal layer of JE provides a new perspective: is the primary JE formed by the REE actually replaced by gingival epithelium or is it ultimately converted to the basal cells of the JE, maintaining the ability to continually update? It is unknown at this time whether JE stem cells are

derived from REE and there is no direct evidence has been shown for its origin. This hypothesis requires further experiments for verification, such as the lineage tracing. Another biomarker, SLPI, was detected in all layers of the junctional epithelium. Sara Yajima-Himuro[30] showed that SLPI is characteristically expressed in JE. Moreover, it is associated with inflammatory responses and maintaining tissue integrity. The reduced expression of SLPI may cause periodontal tissue disease and destruction.[15]

Our data from the luciferase assay indicated that the WNT promoter was triggered in ODAM-OE cells and suppressed in ODAM-KD cells. WNT production is closely related to the development of odontogenic epithelium.[40] Moreover, the data from the tissue sections and immunohistochemistry staining revealed the spatiotemporal expression of WNT1. Notably, we identified colocalization of WNT1, ODAM, CK19, and SLPI in REE and JE. Since the junctional epithelium is composed of several layers of cells during tooth eruption, the *in vitro* study of a single cell has limitations. Some scholars noted that the four types of cells that make up the enamel organ show multipotential. Enamel organ serves as several stem cell reservoirs, whose functions include the formation of enamel, transportation of ions and promotion of epithelial sealing after tooth eruption.[29] Regarding whether the JE of odontogenic origin is eventually replaced by the oral epithelium, some scholars note that the cells directly attached to the tooth surface have no direct evidence of degeneration and migration between gingival basal cells.[41] Of course, this process may also need to be determined by cell labeling technology and tracing technology to trace the source of mature JE.

In summary, we conclude that ODAM promotes JE-related biomarkers via WNT1 under epithelial induction in ALC (Figure 9) which may act as a potential cell model for investigation of JE formation. Our preliminary results also shed light on the understanding of JE biology.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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