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The Odontogenic Ameloblast-Associated Protein (ODAM) Cooperates With RUNX2 and Modulates Enamel Mineralization Via Regulation of MMP-20

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ABSTRACT

We have previously reported that the odontogenic ameloblast-associated protein (ODAM) plays important roles in enamel mineralization through the regulation of matrix metalloproteinase-20 (MMP-20). However, the precise function of ODAM in MMP-20 regulation remains largely unknown. The aim of the present study was to uncover the molecular mechanisms responsible for MMP-20 regulation. The subcellular localization of ODAM varies in a stage-specific fashion during ameloblast differentiation. During the secretory stage of amelogenesis ODAM was localized to both the nucleus and cytoplasm of ameloblasts. However, during the maturation stage of amelogenesis, ODAM was observed in the cytoplasm and at the interface between ameloblasts and the enamel layer, but not in the nucleus. Secreted ODAM was detected in the conditioned medium of ameloblast-lineage cell line (ALC) from days 14 to 21, which coincided with the maturation stage of amelogenesis. Interestingly, the expression of Runx2 and nuclear ODAM correlated with MMP-20 expression in ALC. We therefore examined whether ODAM cooperates with Runx2 to regulate MMP-20 and modulate enamel mineralization. Increased expression of ODAM and Runx2 augmented MMP-20 expression, and Runx2 expression enhanced expression of ODAM, although overexpression of ODAM did not influence Runx2 expression. Conversely, loss of Runx2 in ALC decreased ODAM expression, resulting in down-regulation of MMP-20 expression. Increased MMP-20 expression accelerated amelogenin processing during enamel mineralization. Our data suggest that Runx2 regulates the expression of ODAM and that nuclear ODAM serves an important regulatory function in the mineralization of enamel through the regulation of MMP-20 apart from a different, currently unidentified, function of extracellular ODAM. J. Cell. Biochem. 111: 755–767, 2010.

KEY WORDS: ENAMEL; ODAM; MMP-20; REGULATION; MINERALIZATION

Dental enamel formation is divided into secretory, transition, and maturation stages [Reith, 1970]. During the secretory stage, tall columnar ameloblasts secrete specialized proteins, including amelogenin [Hu et al., 2001], ameloblastin [Fukumoto et al., 2004], and enamelin [Hu et al., 2001] into the enamel matrix. Two novel molecules, odontogenic ameloblast-associated protein (ODAM) and amelotin (AMTN) have recently been described as members of the secretory calcium-binding phosphoprotein (SCPP) gene cluster [Kawasaki and Weiss, 2003; Sire et al., 2007; Kestler et al., 2008].

The cDNA transcript of ODAM (FLJ20512) was originally cloned from the human KATO III cell line [Sekiguchi et al., 1978] and has been detected in calcifying epithelial odontogenic tumor (CEOT)-associated amyloids designated as Apin [Solomon et al., 2003].

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ODAM has also been reported to be a gastric and breast cancer-specific gene based on the analysis of gene expression data [Aung et al., 2006]. Rat ODAM protein was identified from the secretome profile of rat enamel organ cells using the signal trap method [Moffatt et al., 2006]. ODAM contains a cleavable signal peptide and an abundance of glutamine and proline residues and is expressed in ameloblasts during the secretory and maturation stage of enamel development [Moffatt et al., 2006; Park et al., 2007]. The association of high ODAM expression with enamel maturation suggests a possible role for this protein in the final phases of enamel formation [Moffatt et al., 2008]. In addition to ameloblasts, ODAM is also expressed in odontoblasts, lactating mammary glands, nasal and salivary glands, tongue, and gingival tissue [Dey et al., 2001; Moffatt et al., 2008]. Taken together, these data suggest a broad physiological role for ODAM; however, the precise function of ODAM remains largely unknown.

Matrix metalloproteinase-20 (MMP-20, also known as enamelysin) and Kallikrein-4 (KLK4) have also been shown to function in enamel formation. MMP-20, which plays an important role in the degradation of amelogenin, is synthesized and secreted by ameloblasts. The amelogenin degradation induced by MMP-20 is believed to be essential for the axial growth of enamel crystals. During the maturation stage, low columnar ameloblasts synthesize and secrete less enamel matrix protein, but instead synthesize and secrete KLK-4, which degrades enamel proteins to promote enamel crystal thickening. During this stage, ameloblasts develop either a ruffle-end or a smooth-end, which plays an important role in the mineralization and maturation of enamel by removing water and enamel matrix degradation products, as well as transporting calcium [Hu et al., 2002; Lu et al., 2008]. Although there have been advances in our understanding of enamel formation, further studies are required to understand the precise mechanism underlying enamel mineralization.

In ameloblasts and other types of cells, the expression of MMP-20 correlates with ODAM expression. MMP-20 is primarily expressed in ameloblasts, although transient expression has been detected in odontoblasts [Hu et al., 2002]. MMP-20 expression has also been detected in pathological tissues, including the ghost cells of calcifying odontogenic cysts [Takata et al., 2000a], odontogenic tumors [Takata et al., 2000b], and human breast carcinomas [Hegedus et al., 2008].

Runx2, which is stimulated by BMP-2 or TGF-β, controls downstream factors that act on the development of the enamel organ epithelium [Golonzhka et al., 2009]. The importance of Runx2 in amelogenesis is evidenced by the lack of enamel in the incisor tooth germs of Runx2-deficient mice. Runx2 is also present in late secretory- and maturation-stage ameloblasts [D'Souza et al., 1999]. The promoter of the gene encoding ameloblastin, an extracellular matrix protein that may play a role in enamel crystal formation in the developing dentition, contains two Runx2-binding sites [Camilleri and McDonald, 2006]. The ODAM promoter also contains Runx2-binding sites [Harbron et al., 2009], suggesting that Runx2 may be involved in the early stages of enamel organ formation as well as tooth morphogenesis, and might also play a direct role in the formation of tooth enamel.

Recently, we reported that ODAM is primarily involved in mineralization of enamel that is mediated by up-regulating expression of MMP-20 [Park et al., 2007]. The aim of the present study was to determine whether Runx2 and ODAM co-operate to regulate the expression of MMP-20, thereby modulating enamel mineralization.

MATERIALS AND METHODS

TISSUE PREPARATION AND IMMUNOHISTOCHEMISTRY

All experiments involving animal were performed according to the Dental Research Institute guidelines of the Seoul National University. Mandibles and maxillae of 16-day-old mice were decalcified in 10% EDTA (pH 7.4) at 4°C and processed for immunohistochemistry. ODAM expression was detected using an ABC kit (Vector Lab) with rabbit anti-rat ODAM antibody (0.2 μg/ml) as the primary antibody and a biotin-labeled goat anti-rabbit IgG (1:200) as the secondary antibody. ODAM-specific antibodies were obtained by affinity purification of the ODAM antisera that had been produced by immunizing rabbits with a synthetic peptide (STSPKPDTGNF or QGGQAGQPDFSQQ; Peptron, Seoul, Korea), corresponding to the sequence of 241 through to 251 or 102 through to 114 of the 278-residue rat ODAM as previously described [Park et al., 2007].

CELL CULTURE

ALC is an ameloblastic cell line derived from tooth germs of newborn C57/Bl6J mouse lower molars [Nakata et al., 2003]. ALC was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat inactivated foetal bovine serum (FBS), 10 ng/ml of the recombinant human epithelial growth factor (EGF; Sigma–Aldrich), and antibiotic-antimycotic (Invitrogen) in a 5% CO_2 atmosphere at 37°C. ALC was kindly provided by Dr T. Sugiyama (Akita University School of Medicine, Akita, Japan). Odontoblast-like cells (MDPC-23) were provided by Dr. J.E. Nör (University of Michigan, MI, USA). C_2C_{12} (mouse myoblast cells), and MG-63 (human osteoblast-like cells) were obtained from ATCC. Cells were maintained in DMEM supplemented with 5% heat inactivated FBS.

RT–PCR AND REAL-TIME PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen), and 5 μg RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primers (New England Biolabs). RT products were amplified by PCR using the following primer pairs: Runx2 (280 bp), forward, 5′-tctggccttcctctctcagt-3′ and reverse, 5′-tatggagtgctgctggtctg-3′; ODAM (462 bp), forward, 5′-atgtcctatgtggttcctgt-3′ and reverse, 5′-ttatggttctcttaggctatc-3′; MMP-20 (458 bp), forward, 5′-agctgtgagcaactgatgactgga-3′ and reverse, 5′-acagctagagccaagaacacacct-3′; the amelogenin coding sequence (457 bp), forward, 5′-ccagagcatgataaggcagc-3′ and reverse, 5′-gaactggcatcattggttgc-3′; enamelin (465 bp), forward, 5′-gacctatgccatgatgcctg-3′ and reverse, 5′-cgctgataacggctgagtgt-3′; ameloblastin (473 bp), forward, 5′-aaaaggagaaggtccagaag-3′ and reverse, 5′-gcggaaggatagtaagtgt-3′; KLK4 (426 bp), forward, 5′-acaaacccttttataggagcc-3′ and reverse, 5′-aattaaaatttgggcctacc-3′;

and GAPDH (452 bp), forward, 5′-accacagtccatgccatcac-3′ and reverse, 5′-tccaccaccctgttgctgt-3′. The following PCR conditions were used: 94°C for 30 s; 55°C for 30 s; and 72°C for 1 min for 30 cycles. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. For real-time PCR, specific primers of ODAM, MMP20, and GAPDH were designed based on rat mRNA sequences. The primer sequences used were: ODAM, forward, 5′-aacactagagagctttgctgggct-3′ and reverse, 5′-agatggtgtctgctgctgtgagaa-3′; MMP-20, forward, 5′-tgtctaagctcaaggtgccctgtt-3′ and reverse, 5′-taagttgtccatgtgggtgctgga-3′; and GAPDH, forward, 5′-tccagaacatcatccctgcctcta-3′ and reverse, 5′-acaaagtggtcgttgagggcaatg-3′. PCR was carried out using SYBR® Premix Ex Taq™ II (Takara) according to the manufacturer's instructions. Expression quantity was analyzed by ABI PRISM® 7500 (Applied Biosystems). The PCR conditions were 94°C for 1 min followed by 95°C for 15 s and 62°C for 34 s for 40 cycles.

PLASMDS AND CLONING

All cDNAs were constructed using standard methods and verified by sequencing. Constructs encoding Flag (2×)- or HA-tagged Runx2, full-length ODAM or the ODAM signal peptide deletion mutant were inserted into pcDNA3 (Invitrogen). Based on the 19-nucleotide ODAM siRNA sequence (5′-AAGTGCCTCAAGATCAAAC-3′) selected using the siRNA Target Finder and design Tool (Ambion), plasmid expressing ODAM siRNA was prepared using the pSilencer 1.0-U6 siRNA expression vector (Ambion) according to the manufacturer's instructions. Commercially available mouse genomic DNA was used as a template to amplify a 1,500-bp DNA fragment upstream of the mouse MMP-20 start codon. The GenBank accession number AL603630 was used as the reference sequence of mouse MMP-20. Primers containing *Nhe*I and *Xho*I restriction enzyme sites were synthesized by Cosmo Genetech. The gel-purified PCR product was digested with *Nhe*I and *Xho*I, and subsequently subcloned into the *Nhe*I and *Xho*I sites of a promoter-less luciferase reporter pGL3-basic vector. The construct was confirmed by sequencing and was designated pGL3-mMMP20. The constructs used in experiments were as follows: a promoter-less pGL-3 basic vector, a SV40 promoter-driven luciferase reporter vector (pGL3-control), and pGL3-mMMP-20.

EXPRESSION AND PURIFICATION OF RAT RECOMBINANT ODAM PROTEIN (rODAM)

The coding region of ODAM was amplified by PCR using the following primers: 5′-caggctgctagcatgtcctatgtggttcc-3′ and 5′-gtaaactgcagcttatggttctcttaggctatc-3′. The PCR product was cloned into the Nhe I and Pst I sites of pRSET-A (Invitrogen) to generate pRSET-ODAM. The *Escherichia coli* strain, BL21 (DE3) pLysS, was transformed with pRSET-ODAM and cultured at 37°C in Luria-Bertani (LB) broth. The protein was extracted and purified from the cell lysates (Elpis-Biotech). Fractions were analyzed using SDS–PAGE and Western blotting.

FLUORESCENCE MICROSCOPY

Cells in Laboratory-Tek chambered cover glasses (Nunc) were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, and then permeabilized for 4 min in PBS containing 0.5% Triton X-100. After washing, the cells were incubated with anti-ODAM antibody (1:200 dilution) in blocking buffer (1% BSA in PBS) for 1 h and then with FITC-conjugated anti-rabbit IgG (1:200 dilution; Amersham Pharmacia Biotech). After washing, the cells were visualized using a fluorescence microscope (AX70; Olympus Optical Co). Chromosomal DNA in the nucleus was stained using propidium iodide.

PREPARATION OF CYTOPLASMIC AND NUCLEAR PROTEIN EXTRACTS

Plasmid expressing 2× Flag-tagged ODAM was transfected into ALC and C_2C_{12} cells using Lipofectamine Plus (Invitrogen). At the end of each experiment, the cells were collected by centrifugation at 3,000 rpm for 5 min at 4°C. Cell lysis was performed in ice cold hypotonic lysis buffer (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1% NP-40), supplemented with protease inhibitors (Roche) for 15 min. Nuclear and cytoplasmic fractions were separated by centrifugation at 3,000 rpm for 5 min at 4°C. The resulting supernatant (the cytoplasmic fraction) was stored at 4°C until further analysis. The membrane pellet was resuspended in ice-cold hypertonic lysis buffer (10 mM HEPES [pH 7.9], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol), supplemented with protease inhibitors and incubated for 15 min at 4°C. The soluble fraction was isolated by centrifugation at 3,000 rpm for 5 min at 4°C. The resulting supernatant (the nuclear fraction) was stored at 4°C until further analysis.

WESTERN BLOT ANALYSIS

Proteins were extracted from cell lysates after lysis in Nonidet P-40 (NP-40) lysis buffer (50 mM Tris–Cl [pH 7.4], 150 mM NaCl, 1% NP-40, 2 mM EDTA [pH 7.4]), supplemented with protease inhibitors. Conditioned medium (CM) was also collected and centrifuged at 23,700*g* for 5 min at 4°C to remove dead cell debris. The supernatants were precipitated for 1 h on ice with 10% trichloroacetic acid. The precipitated proteins were resuspended in lysis buffer as described above. Samples were separated on denaturing 10–12% Tris–HCl polyacrylamide gels and transferred to nitrocellulose membranes. Rabbit anti-Runx2 IgG (ARP 36678) was purchased from Aviva Systems Biology. Goat anti-MMP-20 IgG (sc-26926), rabbit anti-GAPDH IgG (sc-25778), goat anti-amelogenin IgG (sc-33109), goat anti-rabbit-IgG (sc-2004), and rabbit anti-goat-IgG (sc-2768) were purchased from Santa Cruz Biotechnology. Labeled protein bands were detected using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia).

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

ALC or 293T cells were seeded in 12-well culture plates at a density of 1.5×10^5 cells per well. Cells were transiently transfected with the reporter constructs described above and a SV40-driven β-galactosidase expression vector as an internal control. Depending on the experimental conditions, expression vectors encoding ODAM, Runx2, an ODAM specific siRNA (U6-Apin siRNA-expressing plasmid) [Park et al., 2007], and/or a Runx2 specific siRNA (siGENOME SMARTpool M-064819-02-0005, Thermo scientific) were co-transfected. Following the addition of 50 μl

Luciferin to 50 μl cell lysate, luciferase activity was determined using an Analytical Luminescence Luminometer according to the manufacturer's instructions (Promega). β-Galactosidase activity was determined in 96-well plates that were read at 405 nm using an ELISA reader. The luciferase activity was normalized to β-galactosidase activity.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

ALC was transfected with Runx2 or ODAM expression vectors or siRNA specific for Runx2 or ODAM. Cells were treated with the cross linking reagent formaldehyde (1% final concentration) for 10 min at 37°C, rinsed twice with cold PBS, and swollen on ice in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl [pH 8.1]) for 10 min. Nuclei were collected and sonicated on ice. Supernatants were obtained by centrifugation for 10 min and were diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl [pH 8.1], 167 mM NaCl). The fragmented chromatin mixture was incubated with 2 ml anti-Runx2, anti-ODAM, anti-HA (Sigma), and anti-Flag (Sigma) antibodies on a rotator at 4°C for 4 h, then 20 μl protein A/G PLUS-agarose (Santa Cruz Biotechnology) was added and incubated for 1 h at 4°C with rotation to collect the antibody/chromatin complex. Cross-linked, precipitated chromatin complexes were recovered and reversed according to Upstate's protocol (Upstate). The final DNA pellets were recovered and analyzed by PCR using primers that encompass the ODAM promoter region (716 bp), forward, 5′-tccacctcatcttacctcaa-3′ and reverse, 5′-tgtagtggtcatagcactac-3′or the MMP-20 promoter region (446 bp), forward, 5′-accatgtaggtcctggggaatgaa-3′ and reverse, 5′-ctcctctcttgcttgatgat-3′.

ANALYSIS OF MMP-20 BY ZYMOGRAPHY

The activity of MMP-20 was assayed by casein zymography. Briefly, Conditioned medium was collected from cells following culture without serum for 24 h. Samples were mixed with loading buffer and electrophoresed on 12% SDS–polyacrylamide gels containing 2% casein (Invitrogen) at 140 and 110 V for 3 h. The gels were then washed twice in zymography washing buffer (2.5% Triton X-100 in double-distilled H_2O) at room temperature to remove SDS, followed by incubation at 37°C for 12–16 h in zymography reaction buffer (40 mM Tris–HCl [pH 8.0], 10 mM $CaCl_2$, 0.02% NaN_3). Gels were stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 0.1% amino black, 50% methanol, 10% acetic acid) for 1 h, then de-stained with de-staining solution (20% methanol, 10% acetic acid in 70% double-distilled H_2O). Non-staining bands representing the level of the latent form of MMP-20 were quantified by densitometry using a digital imaging analysis system.

ALIZARIN RED S STAINING

Cells were fixed with 70% ethanol for 20 min and stained with 1% alizarin red S (Sigma–Aldrich) in 0.1% NH_4OH at pH 4.2–4.4. Mineralization assays were performed by treatment of ALC with or without rODAM and staining with alizarin red S solution. The cells were evaluated at 0, 4, 7, 10, and 14 days.

STATISTICAL ANALYSIS

The data were analyzed for statistical significance using a nonparametric Mann–Whitney test.

RESULTS

EXPRESSION OF ODAM MRNA AND PROTEIN DURING AMELOGENESIS

In the first series of experiments, we determined the protein expression level of ODAM during different stages of ameloblast differentiation and the subcellular localization of ODAM protein during ameloblast differentiation by immunohistochemistry. Interestingly, the subcellular localization of ODAM varied in a stage-specific fashion during ameloblast differentiation (Fig. 1A). ODAM protein was not observed in presecretory phase that precedes the secretory and maturation stages. However, distinct expression was detected in secretory-stage ameloblasts (Fig. 1B). Strong staining was also observed in transition- and maturation-stage ameloblasts (Fig. 1C). In secretory-stage ameloblasts, ODAM staining was observed in the nucleus and cytoplasm, and the apex of ameloblasts stained strongly (Fig. 1D). However, in maturation-stage ameloblasts, ODAM was strongly detected in the supranuclear region (Golgi complexes) as well as the interface between ameloblasts and the enamel layer, but not in the nucleus (Fig. 1E).

Selective and time-dependent induction of enamel matrix proteins and enzymes was observed during ALC differentiation. The level of protein and mRNA expression was assessed using Western blots and RT-PCR respectively. Similar to a previous report [Park et al., 2007], expression of ODAM and KLK-4 gradually increased with time during culture (Fig. 2A,B). In contrast, transcription of amelogenin and enamelin mRNA gradually decreased with cell differentiation (Fig. 2B). Runx2 was steadi-lyexpressed during ALC differentiation (Fig. 2A,B). Expression of MMP-20 mRNA and protein increased slightly from the first day of culture until day 7 and decreased thereafter (Fig. 2A,B).

CELLULAR AND EXTRACELLULAR LOCALIZATION OF ODAM

The full-length rat ODAM cDNA encodes a 279-amino acid protein with 15-amino acid signal peptide at the N-terminus that includes a cleavage point for signal peptidase [Moffatt et al., 2006].

Exogenous ODAM protein was expressed in C_2C_{12} cells, which do not normally express ODAM, and was readily detected in intracellular compartments and the CM collected from serum-free cultures (Fig. 3A). To evaluate whether the signal peptide affected the localization of ODAM, we transfected ALC that expressed a reduced level of ODAM with an ODAM construct containing a mutant signal peptide. This mutant ODAM was expressed in the cytoplasm and nucleus, but not in the CM (Fig. 3B), indicating that the ODAM signal peptide plays an important role in ODAM protein secretion.

To examine the time-line of ODAM protein expression, we performed Western blot analysis of differentiating ALC. ODAM protein was detected in the nucleus from days 0 to 10 of ALC differentiation in vitro, but was observed in the CM after 10 days. The amount of ODAM protein in the cytoplasm increased after the

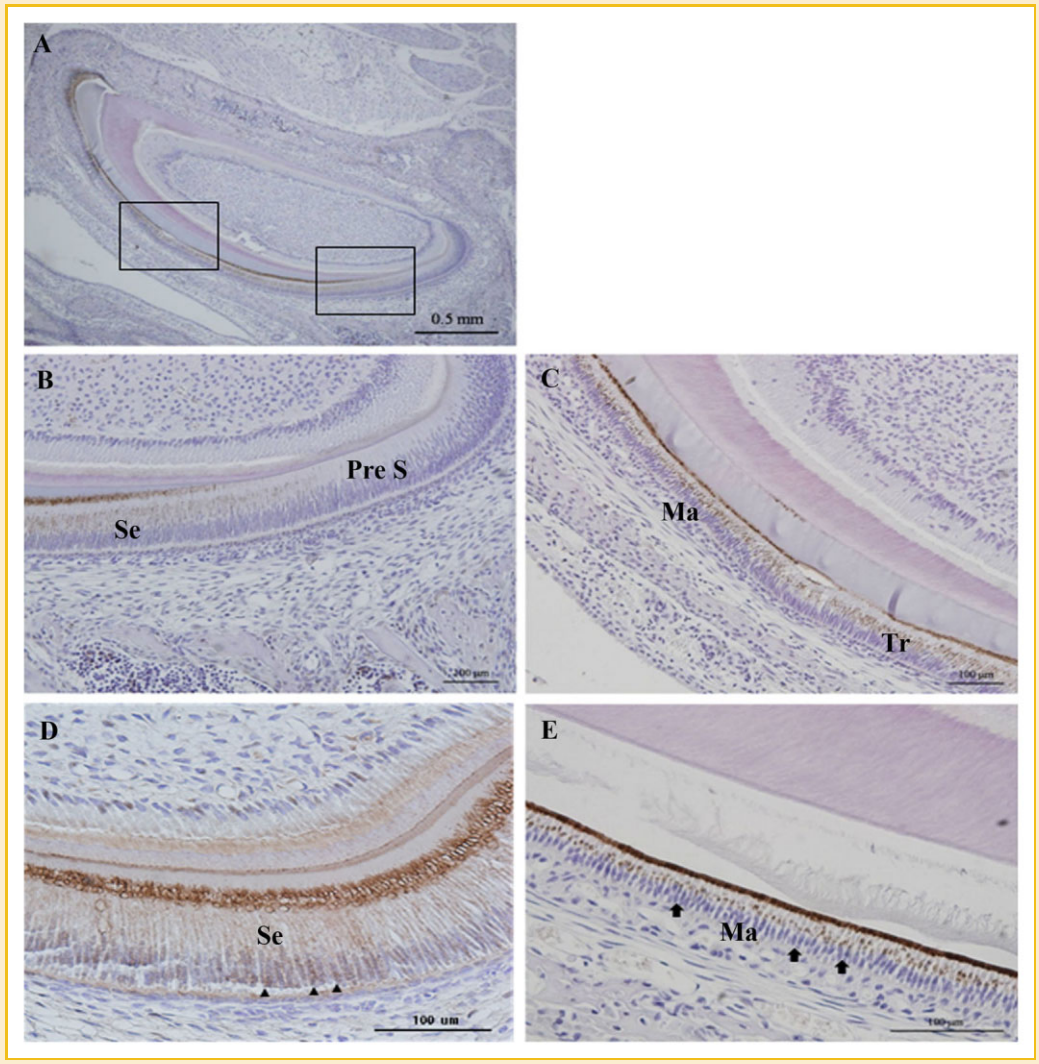

Fig. 1. Immunohistochemical analysis of ODAM expression during ameloblast differentiation in the mandibular incisor of a 16-day-old mouse. Presecretory ameloblasts lack ODAM protein expression (A,B). Transition-stage ameloblasts express ODAM protein in the supranuclear region of the cytoplasm (C). Secretory-stage ameloblasts express ODAM in their nucleus (arrowheads) and cytoplasm (D). Maturation-stage ameloblasts do not express ODAM protein in their nucleus (arrows, E). PreS, pre-secretory; Se, secretory; Tr, transition; Ma, maturation [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

initiation of ALC differentiation (Fig. 3C). These data suggest that the subcellular localization of ODAM varies in a stage-specific fashion during ameloblast differentiation.

To compare the subcellular localization of endogenous and exogenous ODAM, rODAM was treated into ALC cells. In control ALC, faint ODAM staining was seen in the nucleus and cytoplasm of ALC (Fig. 3D). After treatment with exogenous rODAM for 4 h, ODAM protein was clearly visible in the nucleus and cytoplasm. Under the same conditions, some cells showed a punctuate pattern of fluorescence throughout the cytoplasm, probably corresponding to rODAM internalized in endosomal compartments (Fig. 3D).

We also examined the expression of endogenous or exogenous ODAM protein (rODAM) in diverse cell lines. Immunofluorescence staining showed that endogenous or rODAM was localized in the nucleus and cytoplasm of ameloblasts (ALC) and odontoblasts (MDPC-23), but only in the cytoplasm of osteoblasts (MG-63) (Supplementary Fig. 1A,B).

EFFECT OF RUNX2 AND ODAM ON THE TRANSCRIPTIONAL ACTIVITY OF MMP-20

We next determined the effect of Runx2 and ODAM on MMP-20 expression. Overexpression of Runx2 or ODAM in ALC increased the expression of MMP-20 protein (Fig. 4A). In contrast, siRNA-mediated silencing of Runx2 or ODAM decreased MMP-20 expression (Fig. 4B). ODAM expression was higher in cells overexpressing Runx2 than in normal ALC (Fig. 4A). These studies suggest that Runx2 regulates ODAM expression, which in turn regulates MMP-20 expression. To confirm the function of Runx2 and ODAM in enamel formation, we examined the effect of overexpression or silencing of Runx2 or ODAM on the expression of mRNAs for enamel matrix proteins and enzymes by RT-PCR and real-time PCR. Consistent with the Western blot data, over-expression of Runx2 increased the mRNA level of ODAM and MMP-20, whereas their expression was decreased following Runx2 inactivation (Supplementary Fig. 2A,B). Overexpression of ODAM in

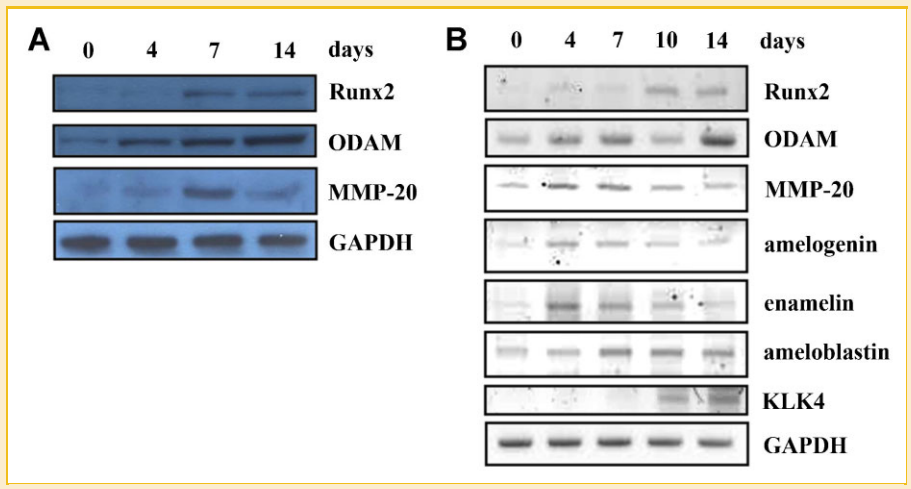

Fig. 2. ODAM and MMP-20 protein and mRNA expression during ameloblast differentiation in vitro. Western blot analysis of the expression of Runx2, ODAM, and MMP-20 during differentiation of ALC in vitro (A). ODAM expression was detected at the beginning of differentiation and increased at subsequent time-points. Expression of MMP-20 increased slightly from days 0 to 7 and decreased thereafter. RT-PCR analysis of mRNA expression of enamel matrix proteins and enzymes during differentiation of ALC in vitro (B). Expression of MMP-20 was strongest at 4–7 days (equivalent to the secretory stage). GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KLK-4, kallikrein-4; MMP-20, matrix metalloproteinase-20 [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

the ALC up-regulated the expression of MMP-20, whereas siRNA down-regulated MMP-20 (Supplementary Fig. 2A–C). However, expression of KLK4 remained unchanged when ODAM was either up- or down-regulated [Park et al., 2007].

The fact that TGF-β1 promotes MMP-20 expression in ameloblasts [Gao et al., 2009] suggests that TGF-β1 or BMP-2 influences Runx2 transcriptional regulation. To examine this, we treated ALC with TGF-β1 or BMP-2 and then determined the Runx2 expression level by Western blot. Both TGF-β1 and BMP-2 up-regulated Runx2 expression, although BMP-2 had a greater effect. In addition, increasing Ruxn2 expression enhanced the expression of ODAM and MMP-20 (Supplementary Fig. 3).

ODAM COOPERATES WITH RUNX2 TO REGULATE MMP-20

To correlate the role of Runx2 and ODAM in MMP-20 transcriptional activation with their function in vivo, ALC was transfected with a

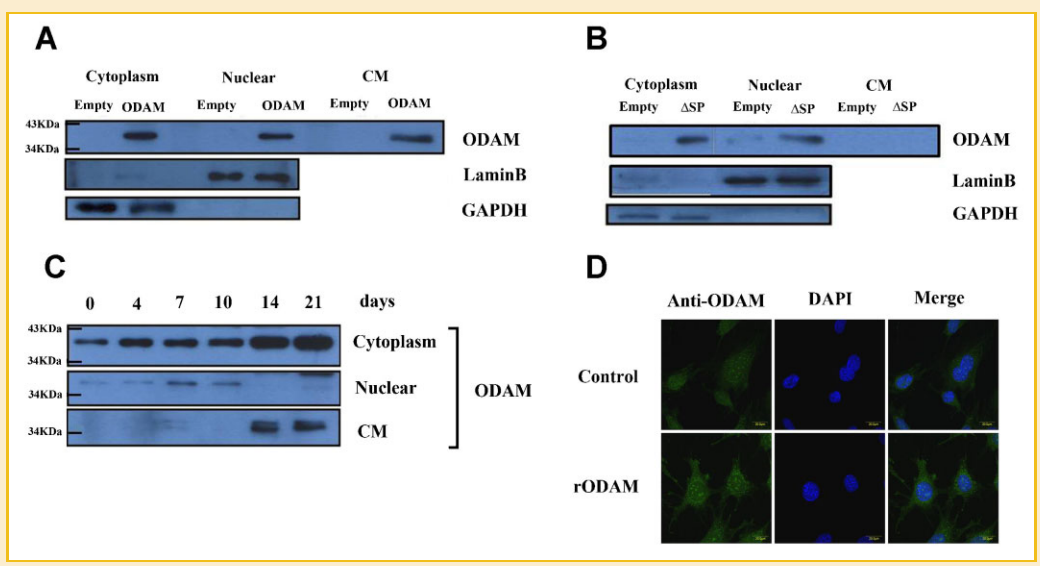

Fig. 3. Subcellular and extracellular localization of ODAM. C_2C_{12} cells were transfected with an ODAM-expression plasmid and the cell lysates and conditioned medium (CM) were analyzed for the presence of ODAM protein by Western blot (A). A strong ODAM signal was observed in both the cell lysates and CM. ALC stably expressing low levels of ODAM protein were transfected with an expression construct encoding an ODAM signal peptide mutant and analyzed by Western blot (B). The cell lysates exhibited a strong ODAM signal, whereas the CM did not show a strong signal for the ODAM protein. Western blot analysis of the expression pattern of ODAM in different cellular compartments during in vitro differentiation of ALC (C). Note the gradual increase in expression of ODAM in the cytoplasm throughout differentiation. ODAM was detected in the nucleus from days 0 to 10. The presence of ODAM in the CM was strongly detected after 10 days. Subconfluent ALC was cultured in the absence or presence of 10 μg rODAM (D). ODAM localization was detected by immunostaining. Bars: 20 μm [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

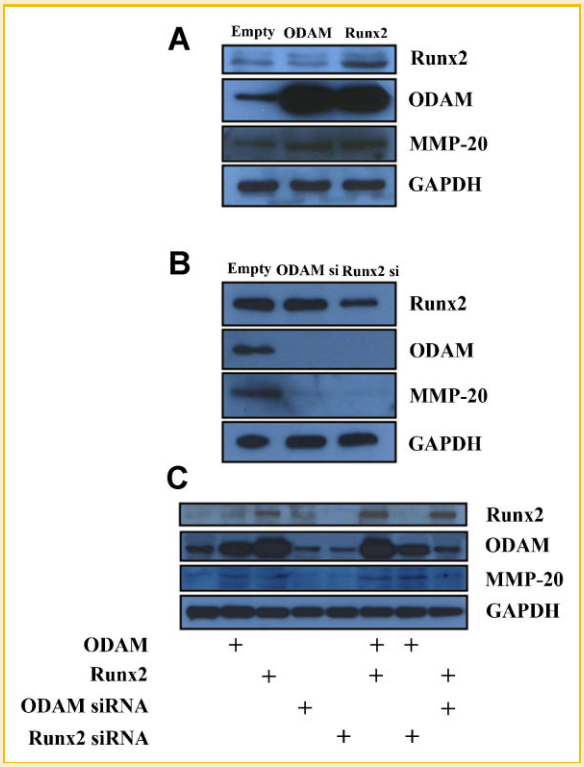

Fig. 4. Regulation of MMP-20 by Runx2 and ODAM. ALC was transfected with Runx2- or ODAM-expression plasmids and cell lysates were analyzed by Western blot with antibodies against Runx2, ODAM, MMP-20, or GAPDH (as a control, A). Overexpression of Runx2 or ODAM induced an increase in the MMP-20 expression level. ALC was transfected with either a control siRNA (100 pM) or siRNA specific for ODAM or Runx2 (100 pM, B). After 2 days, the expression level of Runx2, ODAM, and MMP-20 was determined by Western blot with the indicated antibodies. MMP-20 expression was decreased following the expression of each siRNA. GAPDH was used as a loading control. ALC was transfected with constructs expressing Runx2, ODAM, Runx2 specific siRNA, ODAM specific siRNA, or control siRNA, alone and in various combinations (C). Protein levels were analyzed by Western blotting with antibodies against Runx2, ODAM, MMP-20, and GAPDH. The data show that Runx2 regulated ODAM expression and ODAM induced MMP-20 expression [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

HA-tagged Runx2 expression construct, a Flag-tagged ODAM expression construct, and/or specific siRNA constructs. Runx2 specifically induced ODAM and MMP-20 protein expression, and overexpression of ODAM augmented MMP-20 expression. However, ODAM expression did not affect the expression of Runx2 (Fig. 4C). In agreement with the Western blot data, overexpression of Runx2 further increased the mRNA level of ODAM-induced MMP-20 (Supplementary Fig. 2D).

RUNX2 ATTENUATES ODAM-MEDIATED MMP-20 TRANSCRIPTIONAL ACTIVATION

To investigate the functional consequences of Runx2- or ODAM-induced MMP-20 expression, we determined the effect of Runx2 on ODAM-mediated transcriptional activation. Increasing concentrations of ODAM or Runx2 significantly increased the expression of a luciferase reporter gene under the control of the mouse MMP-20 promoter (Fig. 5A). As expected, depletion of ODAM or Runx2 using specific siRNA suppressed the promoter activity of the MMP-20 reporter construct (Fig. 5B). Next, we investigated whether Runx2, acting as an upstream regulator of ODAM, is required for ODAM-mediated MMP-20 transcriptional regulation using the mouse MMP-20-luciferase construct in ALC, which expressed a quantifiable level of ODAM. As expected, overexpression of Runx2 or ODAM significantly induced MMP-20 transcriptional activity in ALC cells. Overexpression of Runx2 and ODAM showed a synergistic effect on MMP-20 transcriptional activity (Fig. 5C). On the other hand, when endogenous Runx2 or ODAM was suppressed using siRNA, the positive effect of Runx2 or ODAM on the activity of the MMP-20 promoter was disrupted (Fig. 5C). Moreover, ODAM increased the activity of the MMP-20 promoter 2.5-fold in Runx2-deficient cells, whereas following the knockdown of ODAM expression in ALC cells, Runx2 only weakly induced the activity of the MMP-20 promoter (Fig. 5C). These results confirm the data presented in Figure 4 with respect to the role of the Runx2-ODAM cascade in promoter activity.

RECRUITMENT OF ODAM TO THE MMP-20 PROMOTER

To test whether ODAM-mediated activation of the MMP-20 promoter occurs through recruitment of ODAM to the endogenous MMP-20 promoter and Runx2 has an influence on the ODAM promoter, which has 10 expected Runx2 binding sites, but not the MMP-20 promoter, we performed ChIP assay. Chromatin DNA fragments were precipitated with the indicated antibodies, and the DNA was amplified using primers selective for the ODAM-response element in the MMP-20 promoter or Runx2-response element in the ODAM promoter. As shown in Figure 6A, the MMP-20 promoter could be precipitated using an ODAM-specific antibody but not with the negative control antibody (pre-immune serum) or a Runx2-specific antibody. In addition, the result, that the ODAM promoter could be precipitated using a Runx2-specific antibody, showed that Runx2 was recruited to the ODAM promoter, but not the MMP-20 promoter (Fig. 6A).

We confirmed the interaction of Runx2 or ODAM with DNA of ODAM or MMP-20 promoters by ChIP assay. ALC was transfected with the Flag-tagged ODAM or HA-tagged Runx2 expression construct. ChIP assays using a primer set for ODAM or MMP-20 indicated that Runx2 was not recruited to the MMP-20 promoter, but ODAM promoter. Furthermore, Flag-tagged ODAM was also recruited to the MMP-20 promoter following expression of ODAM (Fig. 6B). The interaction of Runx2 or ODAM protein and their specific antibody was not likely to interact non-specific binding.

We then performed ChIP assays to examine whether over-expressed or silenced Runx2 or ODAM influenced ODAM or MMP-20 promoters using ALC transfected with the HA-tagged Runx2, Flag-tagged ODAM, ODAM siRNA expression construct, or Runx2 siRNA oligo. Before the ChIP assay, we performed immunoprecipitation of Runx2 and ODAM to determine whether Runx2 was recruited with ODAM to the MMP-20 promoter. Runx2 did not interact with ODAM in ALC (data not shown). ChIP assays using a primer set for MMP-20 indicated that inducing ODAM was recruited to the MMP-20 promoter following expression of either ODAM or Runx2, which increases expression of ODAM. In addition, increasing

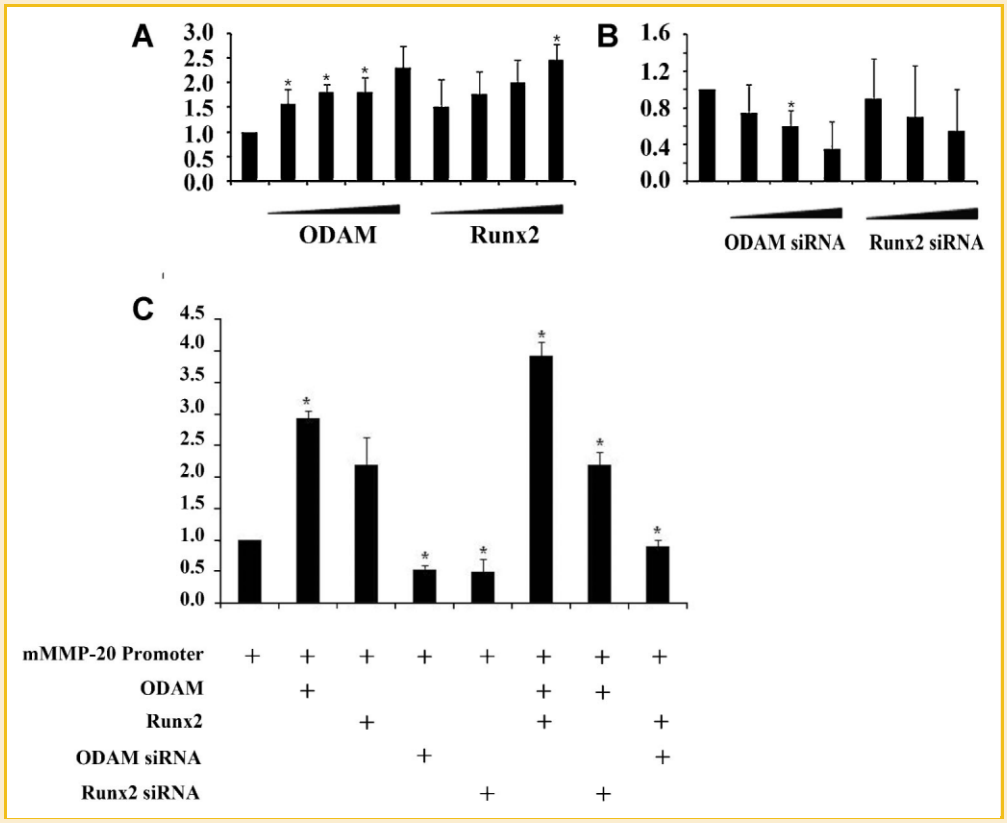

Fig. 5. MMP-20 promoter activity is induced by the cooperation of Runx2 and ODAM. The transcriptional activity of the MMP-20 promoter was altered by expression of Runx2 and ODAM in ALC (A). ALC was transfected with increasing amounts of plasmid expressing Runx2 or ODAM (0.1, 0.5, 1.0, or 2.0 μg). The MMP-20 promoter activity increased in a dose-dependent manner in response to increased concentration of Runx2 or ODAM. Runx2 or ODAM knockdown abolished MMP-20 transcriptional activity (B). ALC was transfected with 40, 80, or 160 pmol of Runx2 specific siRNA or 0.1, 0.5, or 1.0 μg of ODAM specific siRNA expression plasmid. The MMP-20 promoter activity decreased in a dose-dependent manner with reduced levels of Runx2 or ODAM. ALC cells were transfected with luciferase reporter under control of the MMP-20 promoter (0.1 μg), and with various combinations of Runx2 or ODAM DNA (1 μg DNA) or Runx2 or ODAM specific siRNA (100 pmol, C). Cells extracts from the transfected cells were analyzed by luciferase assay. The data are presented as the mean ± standard deviation for triplicate experiments. An asterisk denotes values significantly different from the control ($P < 0.05$).

Runx2 bound the ODAM promoter but not MMP-20 promoter (Fig. 6C). Together, these results suggest that Runx2 interacted with the ODAM promoter in vivo, and ODAM was specifically recruited to the MMP-20 promoter, where it induced MMP-20 transcription.

ROLE OF ODAM DURING AMELOGENESIS IN VITRO

Amelogenin is digested by MMP-20 during amelogenesis [Caterina et al., 2002; Bourd-Boittin et al., 2005]. To directly demonstrate that MMP-20 cleaves amelogenin in vivo, amelogenins were extracted from ALC that had been differentiated for 4 days and the digestion was analyzed by Western blot with amelogenin specific antibodies. MMP-20 cleaved intact amelogenin, generating a lower molecular weight fragment of approximately 17 kDa. Markedly different patterns of amelogenin degradation were observed in ALC in which Runx2 or ODAM were overexpressed or silenced. One amelogenin band less than 24 kDa in size was not present in the enamel from cells in which ODAM was silenced, whereas the cleavage product in the controls and cells overexpressing ODAM or Runx2 had a lower molecular mass (Fig. 7A). Therefore, in vivo MMP-20 activity resulted in different amelogenin isoforms that are present in naturally maturing dental enamel. The pattern of amelogenin cleavage products generated through in vitro digestion was similar to that observed in Western blot analyses of amelogenin cleavage products in porcine secretory stage enamel extracts [Bourd-Boittin et al., 2005].

To confirm the expression and function of MMP-20, we performed Western blot analysis and casein zymography. Secretion of MMP-20 into the extracellular matrix was induced by ODAM (Fig. 7B). Since the crude protein extract containing the MMP-20 enamel enzyme was used in the zymography assay, alterations in enzyme activity would probably go unnoticed. Therefore, to observe whether alterations in the activity of MMP-20 were present, we prepared zymograms containing casein as the substrate. The zymograms revealed no band in the negative control; however, as seen in Figure 7B, in addition to the expected MMP-20 fragment, a fragment at 78 kDa could be detected with expression of ODAM or Runx2. MMP-20, induced by Runx2 or ODAM, was identified in the crude extract used in this study. The main lysis band was not observed with extracts from cells treated with Runx2- or ODAM-specific siRNAs (Fig. 7B).

Finally, we determined the effect of altered ODAM expression on enamel mineralization. In normal ALC mineralized nodules,

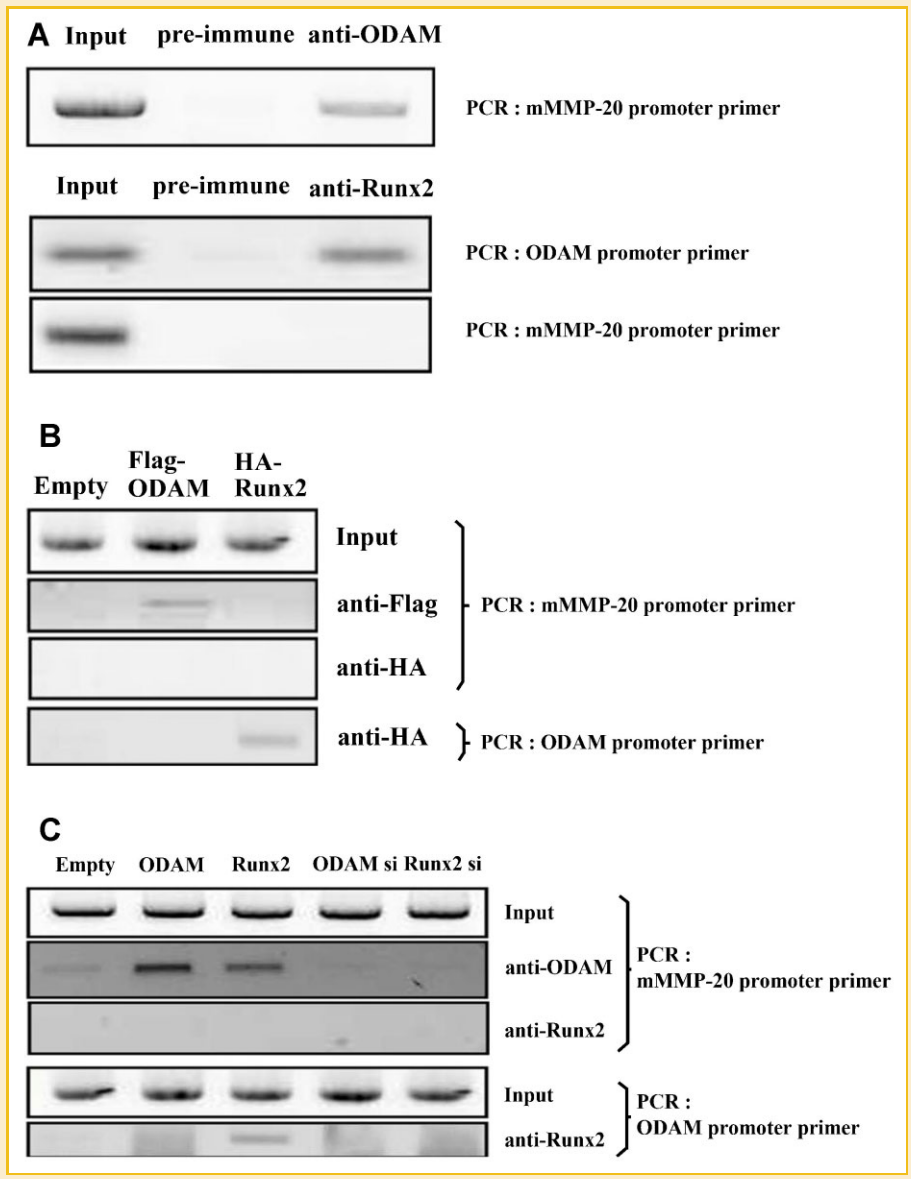

Fig. 6. Recruitment of ODAM to chromatin. Cross-linked chromatin was prepared and immunoprecipitated with pre-immune serum or ODAM specific antibody (A). The precipitated DNA was analyzed by PCR with primer pairs spanning the mouse MMP-20 promoter. The control represents PCR product obtained before precipitation. ALC was transfected with expression vectors for Flag-ODAM or HA-Runx2 (B). ChIP was performed as in (A). Over-expressed ODAM was recruited to the MMP-20 promoter and over-expressed Runx2 was recruited to the ODAM promoter. ALC was transfected with expression vectors for Flag-ODAM or HA-Runx2 or with ODAM- or Runx2-specific siRNAs (C). PCR was performed with primer pairs spanning the mouse ODAM or MMP-20 promoter.

visualized by staining with alizarin red S, appeared after 14 days of culture (Fig. 7C-a). In ALC that overexpressed ODAM or were treated with rODAM mineralized nodule formation was initially observed on day 7 (Fig. 7C-b,C-c). Inactivation of ODAM resulted in cells that failed to mineralize even after prolonged culture (Fig. 7C-d).

DISCUSSION

The secretory stage precedes the maturation stage during amelogenesis [Reith, 1970]. If ODAM is primarily expressed during the maturation stage of enamel development, it could not positively regulate MMP-20 expression because MMP-20 primarily detected during the secretory stage. The data supporting the expression of ODAM in secretory ameloblasts are controversial. Although our laboratory has previously shown that ODAM is expressed in secretory ameloblasts [Park et al., 2007], other reports have indicated that ODAM is not expressed during the secretory stage of amelogenesis [Moffatt et al., 2006, 2008]. Therefore, one aim of the present study was to clarify the expression pattern of ODAM during early amelogenesis. In the present study, ODAM protein was localized to the nucleus and cytoplasm of the secretory ameloblasts in vivo. In addition, when ALC was cultured in differentiation media, expression of nuclear ODAM was induced immediately and

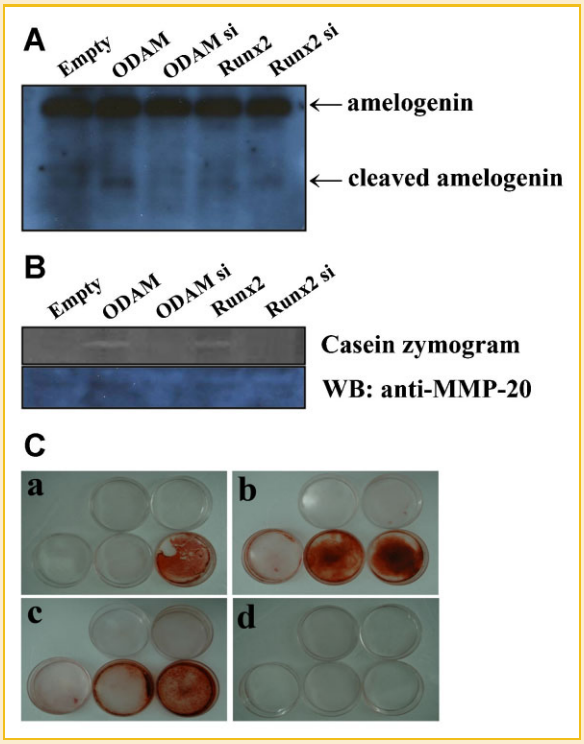

Fig. 7. Increased expression of ODAM enhances mineralization in ALC. ALC that were exposed to differentiation media for 4 days and then transfected with the appropriate constructs were used to examine amelogenin cleavage by endogenous MMP-20 by Western blot analysis (A). Proteins from ALC were extracted in the presence of EDTA-free protease inhibitors cocktail. Increasing MMP-20 levels induced increased cleavage of amelogenin. The kinetics of MMP-20 expression of in ALC (B). Casein zymogram showing the relative proteolytic activity in equal volumes of transfected ALC samples; 10 ml of sample solution prepared from serum-free media was applied to each well (Top). Western blot analysis of 60 μg protein of extract using an anti-MMP-20 antibody (Bottom). Induction of MMP-20 resulted in increased protease activity. Alizarin red S staining over a 14-day time course in differentiation and mineralization media for normal ALC (a), ALC treated with rODAM (3 μg/ml, b), ODAM overexpressing cells (c), and ODAM specific siRNA-expressing cells (d). The cells were evaluated at 0, 4, 7, 10, and 14 days (C) [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

continued until day 10, which coincided with the secretory stage of amelogenesis. Interestingly, secreted ODAM was detected in the CM of ALC from days 14 to 21, which coincided with the maturation stage of amelogenesis. We also showed that MMP-20 was expressed during the same stage of amelogenesis as the expression of nuclear ODAM. These results suggest that nuclear ODAM may influence MMP-20 expression during the secretory stage of amelogenesis.

To elucidate stage-specific role of ODAM during amelogenesis, we used the ALC line as a study model. ALC is an ameloblastic cell line derived from neonate molar tooth organs [Nakata et al., 2003]. ALC maintained the expression of several ameloblast specific genes (amelogenin, enamelin, MMP-20, and KLK4) and also formed calcified nodules in long-term culture. It has been reported that ALC seems to maintain its original property as secretory ameloblasts [Takahashi et al., 2007]. In the present study, the increase of KLK4 with time during the culture also implies the maturation of ALC. Collectively, these findings suggest that ALC expressing a typical ameloblast phenotype might be used for studying the stage-specific localization of ODAM and further the mechanisms of MMP-20 regulation mediated by ODAM.

MMP-20 cleaves amelogenin to produce fragments commonly observed in vivo, and is thought to regulate enamel mineralization [Lu et al., 2008]. Using the broad-spectrum MMP inhibitor marimastat, inhibition of mineralization was found to be associated with the inhibition of MMP-20 activation during amelogenesis [Bourd-Boittin et al., 2005]. Enamel formation in MMP-20-deficient mice is severely defective, with enamel mineral content reduced by 50% and hardness decreased by 37% [Caterina et al., 2002]. In the present study, stable cell lines in which ODAM had been knocked down failed to initiate mineralized nodule formation. Notably, treatment of ALC that stably overexpress ODAM or rODAM caused formation of an increased number of mineralized nodules and induced nodule formation earlier than in normal cells. These results suggest that increasing level of ODAM, as the result of overexpression or treatment with rODAM, enhanced the onset of mineralization whereas ODAM inactivation suppressed mineralization by inhibiting MMP-20 activation.

Several secretory proteins that contain the signal sequence for targeting to the endoplasmic reticulum (ER), for example angiotensin converting enzyme, have been reported to localize to the nucleus [Arnoys and Wang, 2007; Lucero et al., 2010]. Interestingly, ODAM contains the newly identified nuclear localization signal (NLS) motif that responds to extracellular stimuli and requires phosphorylation for transfer to the nucleus [Chuderland et al., 2008], in addition to several potential consensus nuclear export sequence (NES) motifs [Hutten and Kehlenbach, 2007]. This is similar to the secreted form of phospholipid transfer protein (PLTP), which contains an abnormal NLS and a consensus NES [Vuletic et al., 2009]. Therefore, nuclear import of the ODAM fragment could be accomplished by a piggyback mechanism, involving binding to a NLS-containing partner [Steidl et al., 2004]. The predicted NES could play a role in the exit of intact ODAM or ODAM fragments from the nucleus. ODAM showed strong positive nuclear and cytoplasmic expression in human breast tissue [Siddiqui et al., 2009]. Because ODAM is expressed in different cellular locations during the various stages of differentiation, it is likely to perform different functions intracellularly and extracelluarly. However, the functionality of these sequence motifs remains to be established.

Although the functional roles of ODAM in the extracellular matrix are not currently known, there are several possibilities. It is interestingly to note that the FDC-SP locus, which is adjacent to ODAM in the SCPP cluster, is expressed in late dental development, as well as in neoplasms of the breast. Furthermore, it has been included as a gene signature screening component indicative of breast cancer invasiveness [Zucchi et al., 2004; Nakamura et al., 2005]. It is also noteworthy that ODAM expression is increased approximately sixfold in the early stages of breast cancer, compared with the normal mucosa [Kita et al., 2006]. Furthermore, we posit that ODAM is up-regulated by the Shh pathway, which is involved in carcinogenesis. This would explain the concomitant expression of the zinc finger transcription factor Gli-1 (a Shh pathway member) in CEOT and other odontogenic neoplasms [Zhang et al., 2006], as well

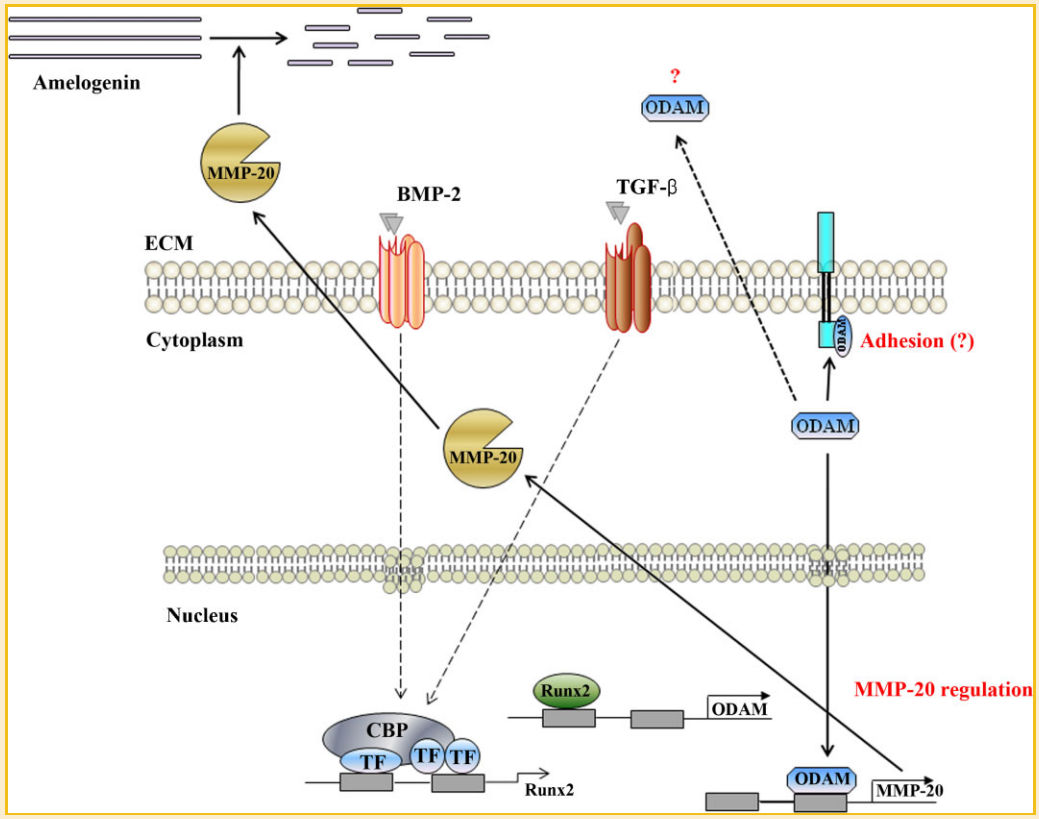

Fig. 8. Proposed model for transcriptional activation of MMP-20 through the co-operation of Runx2 and nuclear ODAM. Runx2 is associated with the ODAM promoter and ODAM is recruited to the MMP-20 promoter in the nucleus. Runx2 regulates the expression of ODAM and ODAM has an effect on MMP-20 activation [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com].

as gastric [Ma et al., 2005], breast [Mukherjee et al., 2006], and lung cancers [Lemjabbar-Alaoui et al., 2006]. The discovery, that ODAM may be implicated in neoplastic transformation, in addition to its role in the development of teeth and other body tissues, has potential diagnostic and therapeutic relevance. In addition, tight junction (TJ) proteins have been shown to play a role in malignant transformation, tumor invasion, and metastasis [Naik et al., 2008; Martin and Jiang, 2009]. Certain TJ proteins such as ZO-1, ZO-2, and huASH1 are co-expressed in the nucleus and the extracellular matrix with some TJ proteins serving prominent roles in signal transduction [Nakamura et al., 2000]. TJ proteins, such as VMP-1, JAM-A, Claudin-4, and ZO-1, have been shown to play a role specifically in breast tumor cell invasion [Naik et al., 2008]. ODAM contains six PDZ (postsynaptic density 95/Dlg/zona occludens 1) motifs suggesting typical of some TJ proteins [Hung and Sheng, 2002; Siddiqui et al., 2009]. In the presence of recombinant ODAM, binding of cultured human breast tumor cells to the extracellular matrix is increased while tumor cell invasion is inhibited.

If ODAM proves to be a junction protein or is otherwise associated with cell signaling and/or barrier functions, then its ability to inhibit in vitro tumor cell invasion could reflect an ability to decrease the rate of invasion or metastasis. In this regard, we have tried to investigate the role of ODAM in cancer cells. ODAM expressed highly in normal tissues and early-stage cancer cell lines. In addition, protein microarray showed that ODAM interacted with other metastasis suppressor genes. Furthermore, we found that ODAM was a new metastasis suppressor for cancer cell lines, including MDA-MB231 (data not shown), suggesting that the role of ODAM in invasiveness regulation is closely related to the cell survival by metastasis suppressor in vivo.

As summarized in Figure 8, our data show that Runx2 is associated with the ODAM promoter and ODAM is recruited to the MMP-20 promoter in the nucleus. Our results demonstrate that Runx2 regulates expression of ODAM protein level, which in turn regulates MMP-20 promoter activity, thus suggesting that ODAM plays a key role in efficient amelogenesis in higher eukaryotic cells. The presence of ODAM at cell–enamel interface suggested involvement in the adhesion of junctional epithelial cells to the tooth surface [Moffatt et al., 2008]. However, although we speculate that the role of extranuclear ODAM may involve cell adhesion and attachment, the function of cytoplasmic or secreted ODAM in maturation stage ameloblasts and various cancer cells remains to be determined.

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