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Regulation of Calbindin-D_{28k} Expression by Msx2 in the Dental Epithelium

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Summary

Amelogenesis involves the coordinated expression of a set of molecules that includes enamel matrix proteins and calcium-binding proteins. Msx2 is a member of the divergent homeobox gene family and is instrumental in dental morphogenesis and biomineralization. This study focused on an EF-hand calcium-binding protein, calbindin-D_{28k}, which is highly expressed in dental epithelium. In vivo data showed that calbindin-D_{28k} levels were higher in ameloblasts from $Msx2^{+/-}$ mice than $Msx2^{+/+}$ mice. Consistent with this finding, calbindin-D_{28k} distribution was affected in transgenic mice with ectopic expression in root epithelium in rests of Malassez in $Msx2^{+/-}$ and more clearly in $Msx2^{-/-}$ mice. In accordance with these in vivo data, calbindin-D_{28k} protein and mRNA levels were decreased in LS8 ameloblast-like cells by exogenous Msx2 overexpression. Furthermore, calbindin-D_{28k} promoter activity (nt-1075/+34) was specifically diminished in the presence of Msx2 overexpression, showing that Msx2 behave as a transcriptional repressor for calbindin-D_{28k} gene expression. In conclusion, Msx2 may control the spatiotemporally restricted frame of calbindin-D_{28k} production in the dental epithelium in relation to enamel mineralization, as previously shown for amelogenin. (J Histochem Cytochem 60:603–610, 2012)

Keywords

dental epithelium, calbindin-D_{28k}, Msx2, ameloblast differentiation, calcium

Enamel is unique in the vertebrate skeleton. This exceptionally acellular and highly mineralized tissue is formed by epithelium-derived ameloblasts. The functional differentiation of ameloblasts is driven by several developmental genes, including homeobox genes such as Tbx1 (Catón et al. 2009) and Msx2 (Satokata et al. 2000; Molla et al. 2010). Ameloblasts timely produce specific proteins whose self-assembly lends enamel its unique architecture (Hu et al. 2007). However, dental epithelium also comprises other cells such as the stratum intermedium, the stellate reticulum, the outer dental epithelium, or the root epithelium that are not directly involved in amelogenesis (Bosshardt and Nanci 2004; Tummers and Thesleff 2009). In adult physiology, dental epithelium forms rests of Malassez that also express a limited number of genes involved in enamel formation (e.g., calretinin but not calbindin-D_{28k}; Korkmaz et al. 2010).

In vivo, several pathways driving epithelial stem cell niches, ameloblast cell differentiation, and crown-root transition have so far been deciphered (Tummers and Thesleff 2009). They provide an interpretation of evolutionary changes that lead to tooth type and shape biodiversity. Ameloblast-related gene expression patterns are also under the spotlight in studies of various pathophysiological situations: When crown and root are formed, there is an ectopic and/or abnormally high expression of enamel

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proteins in non-ameloblastic cells. This pattern has been documented in root epithelial cells during repair (Nishio et al. 2010) and in $Msx2^{-/-}$ mice (Molla et al. 2010).

This study focused on calcium-binding proteins, as some of them are highly expressed in ameloblasts but not significantly expressed in the non-ameloblastic cells (Hotton et al. 1995; Hubbard 1995; Berdal et al. 1996; Korkmaz et al. 2010). Calbindin-D_{28k} is an intracellular EF-hand calcium-binding protein that is highly concentrated in vitamin D–dependent calcium-transporting epithelia, including those in the kidney, intestine, and enamel. Calbindin-D_{28k} represents 1% of total cytosolic proteins in dental epithelium (Berdal et al. 1996). Various studies have focused on transcriptional regulations of calbindin-D_{28k} expression (Gill and Christakos 1993, 1995) but it has yet to be explored in dental cells.

Msx and Dlx transcription factors are expressed in the dental epithelium (Lézot et al. 2000; Ghoul-Mazgar et al. 2005) and have been shown to control amelogenin expression (Lézot et al. 2008; Venugopalan et al. 2011). More specifically, Msx2-null mutant mice exhibit defective amelogenesis (Satokata et al. 2000) due to the decrease in laminin-5α3, which maintains the organized structure of the ameloblast layer (Bei et al. 2004; Molla et al. 2010). In a syndromic case of amelogenesis imperfecta with cleft lip and palate and polycystic kidney disease, a T447C missense mutation was found that results in the conversion of methionine to threonine at position 129 (Suda et al. 2006). This further highlights the important role of Msx2 during amelogenesis. In vitro, Msx2 represses amelogenin expression by interacting with C/EBPα, which activates amelogenin transcription (Zhou et al. 2000; Xu et al. 2007). In vivo, haploinsufficiency in $Msx2^{+/-}$ mice leads to increased amelogenin expression, enamel thickness, and rod size, confirming the repressor role played by Msx2 in enamel morphogenesis (Molla et al. 2010).

Although calbindins have been shown to be vitamin D-dependent (Berdal et al. 1993), the regulatory effectors controlling calbindin-D_{28k} basal expression remain unknown in dental cells. Mouse calbindin-D_{28k} promoter has been characterized in terms of basal activity and response to 1,25-$(OH)_2$ vitamin D_3 (Gill and Christakos 1993), estrogen, and retinoic acid (Wang and Christakos 1995). Here, we investigated the role of Msx2 in the regulation of calbindin-D_{28k} gene expression in dental epithelial cells. This study was carried out in vivo in Msx2 knock-in mice and in vitro by measuring calbindin-D_{28k} promoter activity (nt-1075/+ 34), RNA, and protein levels in mouse ameloblast-like LS8 cells.

Materials and Methods

Collection of Biological Samples

Msx2 gene knock-in (KI) mutant mice were generated by inserting the bacterial lacZ gene into the Msx2 gene coding sequences leading to a dental phenotype (Aïoub et al. 2007). The same litters were used to analyze wild-type ($Msx2^{+/+}$), heterozygous, and homozygous ($Msx2^{-/-}$) mice. This study was performed in accordance with the French National Consultative Committee on Bioethics for Health and Life Sciences, following ethical guidelines for animal care. All experiments were performed by staff that had been trained to perform in vivo studies.

Cell Culture and Transfections

The LS8 cell line was previously established by immortalizing primary cultures of enamel organ epithelium with SV40 large T-antigen (gift from M. Snead, Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles). Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Sigma; La Verpillière, France). After reaching 40% confluence (24 hr after plating), LS8 cells were transfected according to the manufacturer's instructions (Qiagen; Courtaboeuf, France). At 48 hr posttransfection, cells were either collected for RNA and protein extractions or lysed for calbindin-D28k promoter activity assays.

Msx2 cDNA cloned into the pcDNA3 expression vector (Dr. Abate-Shen, Cancer Institute of New Jersey) was used for Msx2 overexpression studies. Mouse calbindin-D_{28k} promoter CAT construct (nt-1075/+34) was used for promoter activity investigations (Sylvia Christakos, University of Medicine and Dentistry of New Jersey, Newark), and pGL4.13 (Promega; Madison, WI) was used for transfection efficiency standardization. Final 25 nM Msx2 siRNAs (5-CAGUACCUGUCCAUAGCAG) or scrambled siRNAs used as controls (Eurogentec; Angers, France) were transfected using lipofectamine according to the manufacturer's procedure (Invitrogen; Saint Aubin, France) to directly and specifically repress Msx2 expression.

Immunohistochemistry

Fourteen-day-old $Msx2^{+/+}$, $Msx2^{+/-}$, and $Msx2^{-/-}$ mouse mandibles were collected and fixed overnight in 4% paraformaldehyde. The mandibles were then decalcified in ethylenediaminetetraacetate, dehydrated, and embedded in paraffin. Then, 8-µm sections were cut, deparaffinized, rehydrated, and stained for immunohistochemistry analysis. After a 1-hr incubation with rabbit anti–calbindin-D_{28k} (Swant, Bellinzona, Switzerland; 1/1000) or rabbit anti–β-galactosidase polyclonal antibodies (Abcam, Cambridge, MA; 1/500) (for Msx2 expression), slides were incubated with the goat anti-rabbit horseradish peroxidase (HRP)–conjugated polyclonal antibody (DAKO, Glostrup, Denmark; 1/ 200). All incubations were performed at room temperature.

Table 1. Quantitative PCR Primers

Gene Symbol		Primers	Annealing Temperature	Product Size (bp)
Calbindin-D_{28k}	Forward	5′-ACGGAAGTGGTTACCTGGAA-3′	60	552
	Reverse	5′-CACACATTTTGATTCCCTGG-3′		
GAPDH	Forward	5′-AACCATAGGAAGGATACGGCTG-3′	058	450
	Reverse	5′-GGGGTGTGGTAACATCAGCAC-3′		
Msx2	Forward	5′-CCTGAGGAAACACAAGACCA-3′	60	278
	Reverse	5′-AGTTGATAGGGAAGGGCAGA-3′		

Western Blot Analysis

Whole-cell lysates were prepared from transfected LS8 cells. After centrifugation at 3000 rpm for 15 min at 4C, protein concentrations of the supernatants were determined using a BCA protein assay kit (Pierce Thermo Scientific, Rockford, IL). Proteins were separated by electrophoresis and electroblotted onto PVDF membranes (Pierce Thermo Scientific). The membranes were first incubated with a primary antibody, that is, either anti–calbindin-D_{28k} 1/1000 (Swant) or anti-actin 1/5000 (Santa Cruz Biotechnology, Santa Cruz, CA; Sigma, St. Louis, MO) and finally with goat anti-rabbit peroxidase-conjugated secondary antibody (1/2000). The Immobilon enhanced chemiluminescent detection system (DAKO) was used to detect bound antibodies quantified in a LAS-4000 Imaging Analysis System (FUJIFILM Medical Systems, Asnières, France).

Real-Time PCR Analysis

Total RNAs from dental epithelial cells were isolated using TriReagent (Sigma) according to the manufacturer's instructions. Reverse transcription (RT) was performed on 1 µg of RNA template with SuperScript II RNase H Reverse Transcriptase (Invitrogen). All cDNA samples were diluted 100-fold, and quantitative PCR (qPCR) reactions were performed in triplicate using a Roche Light Cycler real-time PCR instrument (Roche Diagnostics, Neuilly-sur-Seine, France; Table 1). Mean, standard deviation (SD), and coefficient of variation (CV) of the triplicate qPCR reactions were calculated for each reaction plate.

Chloramphenicol Acetyl-Transferase and Luciferase Assays

Cells were lysed using a chloramphenicol acetyl-transferase (CAT)–ELISA kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Cell lysates were cleared by centrifugation, frozen, and stored at –80C until use. CAT activity was measured using the CAT-ELISA kit, and the resulting reaction was read at 450 nm on a FLUOstar Omega microplate reader (BMG LABTECH, Offenburg, Germany).

Luciferase activity was used as a transfection standard (1/10 of transfected plasmids) and was measured by mixing 25 µl of protein extract with 75 µl of Luciferase Assay Substrate (Promega).

Statistical Analysis

Unless otherwise stated, all data are presented as mean ± standard error of the mean (SEM). Statistical significance (*$p<0.05$ and **$p<0.01$) was determined by Student's *t*-test or analysis of variance (ANOVA).

Results

Calbindin-D_{28k} Expression in Msx2$^{+/-}$ and Msx2$^{-/-}$ Compared with Msx2$^{+/+}$ Mice

A strong immunoreactivity to calbindin-D_{28k} was localized in the cytoplasm of secretion stage ameloblasts from Msx2$^{+/+}$, Msx2$^{+/-}$, and Msx2$^{-/-}$ mice (Fig. 1A–C). Calbindin-D_{28k} protein was also present in hypertrophic epithelial rests of Malassez (ERM) (arrows) from Msx2$^{+/-}$ and Msx2$^{-/-}$ mice and appeared upregulated depending on Msx2 gene dosage (Fig. 1B, C; see Fig. 1G, H for greater magnification). It was not detected in ERM cells in Msx2$^{+/+}$ mice (Fig. 1A, F). The overall structure of the ameloblast layer was affected in Msx2$^{-/-}$ incisor as previously described (Molla et al. 2010).

Msx2 protein was located in the nuclei of dental cells of 14-day-old Msx2$^{+/-}$ mouse incisor sections (Fig. 1D). The control (without the primary antibody) was negative (Fig. 1E).

Msx2 Decreased Endogenous Calbindin-D_{28k} mRNA and Protein Levels

LS8 cells were transiently transfected with increasing amounts of Msx2 expression vector to study the effect of Msx2 on endogenous calbindin-D_{28k} expression.

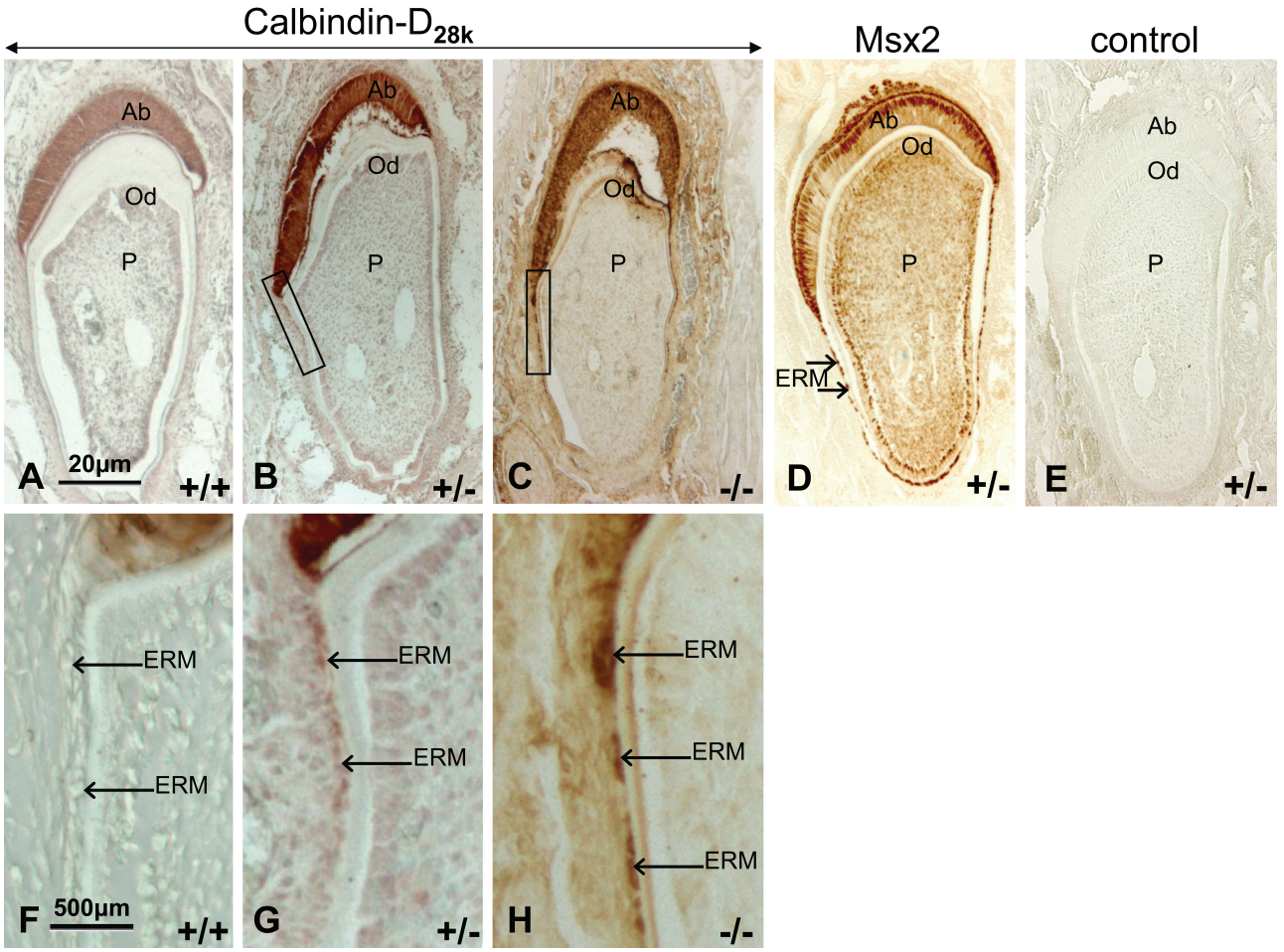

Figure 1. Calbindin-D_{28k} expression in mouse incisor. Calbindin-D_{28k} protein was comparatively immunodetected in the incisors of 14-day-old Msx2+/+ (A, F), $Msx2^{+/-}$ (B, G), and $Msx2^{-/-}$ (C, H) mice. Tissue sections (in ameloblast secretion stage) were fixed in 4% paraformaldehyde, decalcified in ethylenediaminetetraacetate, and embedded in paraffin. Then, 8-µm sections were analyzed by immunohistochemistry with rabbit anti–calbindin-D_{28k} or rabbit anti–β-galactosidase polyclonal antibodies (for Msx2) (D). Control sections (E) were incubated without primary antibody. Arrows indicate calbindin-D_{28k} increased staining in epithelial rests of Malassez cells from $Msx2^{+/+}$ to $Msx2^{-/-}$ incisors. Ab, ameloblasts; Od, odontoblasts; P, pulp; ERM, epithelial rests of Malassez. Top row, scale bar = 20 µm; bottom row, scale bar = 500 µm.

RT-qPCR analysis showed that calbindin-D_{28k} transcript expression was 2.4-fold and 6.0-fold lower in the presence of 0.3 and 0.5 µg Msx2 vector, respectively (both $p<0.05$) (Fig. 2A).

The polyclonal antibody used in Western blotting specifically recognises the 28-kDa protein. Calbindin-D_{28k} protein levels appeared lower in LS8 cells overexpressing Msx2 compared with controls (Fig. 2B). Quantification analysis showed that transfecting 0.3 µg and 0.5 µg of Msx2 expression vector decreased calbindin-D_{28k} protein expression by 59.1% and 40.9%, respectively, compared with control cells transfected with the same amounts of the empty vector pcDNA3 (Fig. 2B).

The presence of Msx2 and its ability to inhibit endogenous calbindin-D_{28k} expression in the ameloblast-like LS8 cells clearly indicated that Msx2 is a repressor of calbindin-D_{28k} expression.

Msx2 Dose-Dependent and Specific Repression of Mouse Calbindin-D_{28k} Promoter Activity

To test whether Msx2 can function as a transcriptional modulator of the mouse calbindin promoter, LS8 cells were transiently co-transfected with calbindin-D_{28k} promoter (nt-1075/+34) CAT (Gill and Christakos 1993), with either pcDNA3 (empty vector used as negative control) or increasing amounts of Msx2 expression vector. Reporter CAT expression was significantly lower in the presence of

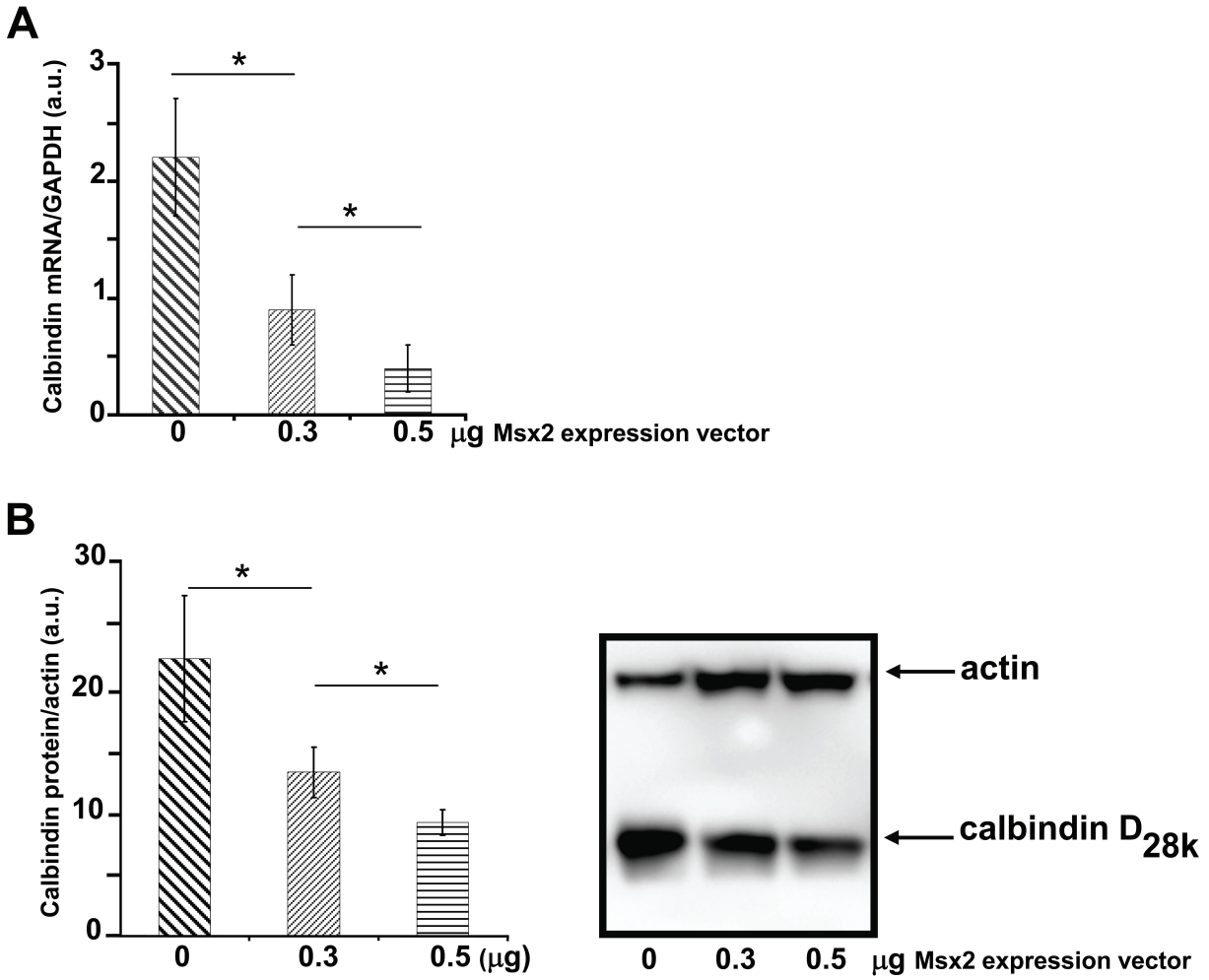

Figure 2. Msx2-mediated dose-dependent inhibition of calbindin-D_{28k} expression. (A) Calbindin-D_{28k} transcript levels were quantified by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). RNAs were isolated from LS8 cells after transient transfection with the Msx2 expression vector. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level was used for normalization. Differences were considered significant at $p<0.05$ (*). Mean ± SEM from a representative set of three independent experiments are shown. (a.u. for arbitrary units). (B) Whole-cell lysates were prepared from LS8 cells transiently transfected with Msx2 vector. Comparable amounts of cell lysate proteins were loaded into each lane and immunoblotted with a goat anti-actin antibody (top) and a rabbit anti–calbindin-D_{28k} antibody (bottom). The numbers indicate the molecular masses of marker proteins (in kilodaltons). The left panel shows the graphical representation of calbindin signal quantification (related to actin) of three independent experiments.

0.3 to 0.5 μg Msx2 ($p<0.03$) (Fig. 3A). This inhibitory effect of Msx2 was dose-dependent and specific to the wild-type (WT) promoter, as the Msx2 inhibitory effect was not observed in the deleted promoter without the Msx2 responsive element (nt-1003/+34) (Fig. 3C). The Msx2-mediated inhibition of calbindin-D_{28k} promoter activity was specific, as demonstrated by concomitant transfection of Msx2 siRNAs, which reduced Msx2 expression by 62.5%. In the presence of 25 nM Msx2 siRNAs, the Msx2 effect was abolished, whereas it remained intact in the presence of scrambled siRNAs (Fig. 3B).

Discussion

This study focused on the regulation of calbindin-D_{28k} gene expression in ameloblasts and dental epithelium by the Msx2 transcription factor. The Msx2 homeoprotein is required for numerous cellular processes as well as for the development of several tissues and organs. Msx2 is a potent transcriptional repressor of many genes involved in mineralized bone and tooth tissues (Marijanović et al. 2009; Molla et al. 2010), including osteocalcin (Hassan et al. 2004) and amelogenin (Zhou et al. 2000; Xu et al. 2007). Msx2 can interact directly

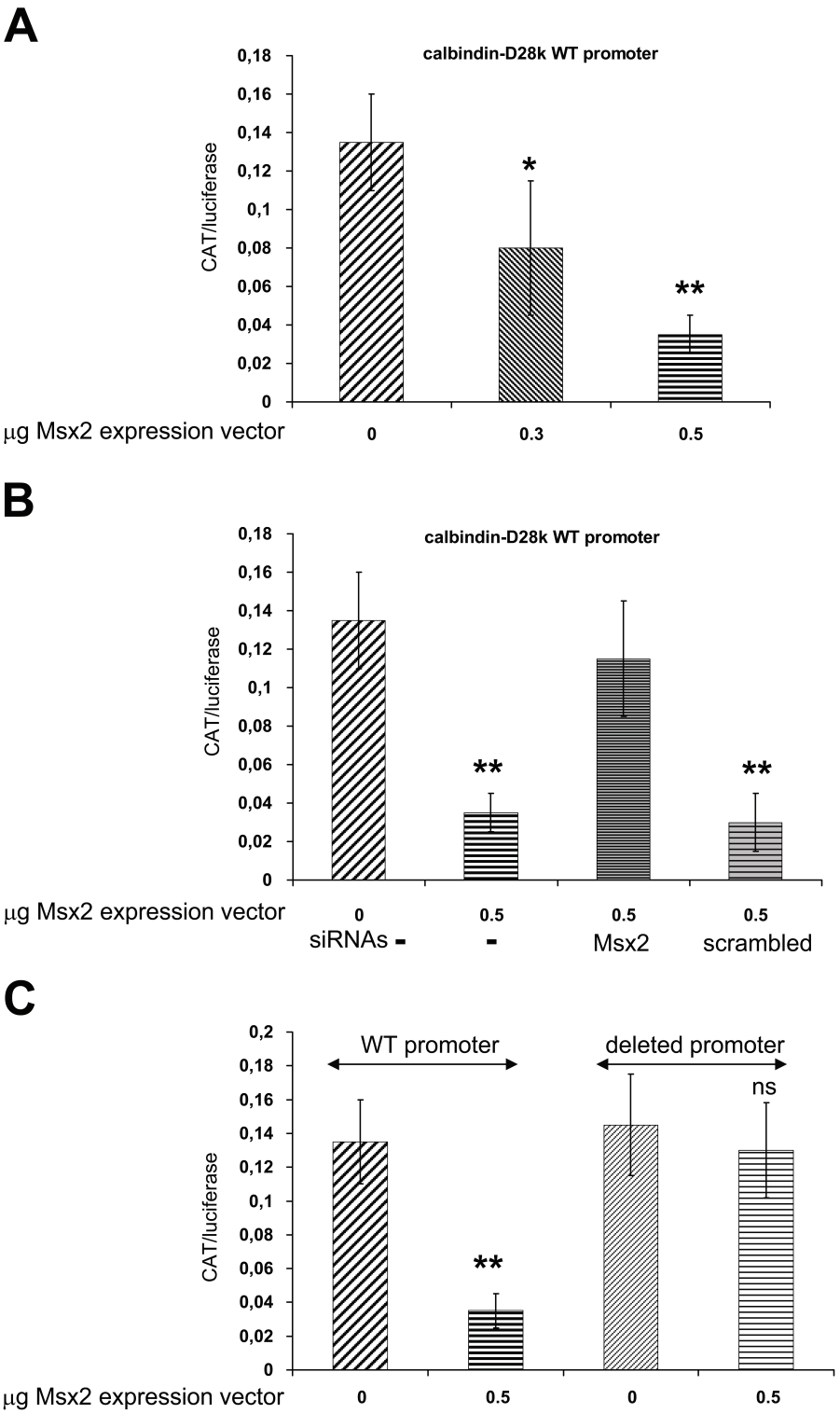

Figure 3. Calbindin-D_{28k} promoter activity. Varying amounts of Msx2 expression plasmid were used (A) with or without appropriate siRNAs (B) and were transiently co-transfected into LS8 cells with 0.5 μg reporter construct. The involvement of Msx2 binding element was shown with the loss of inhibition of the deleted promoter activity (C). Each bar represents the mean ± SEM of three independent experiments. $^{**}p<0.01$, $^{*}p<0.05$. CAT, chloramphenicol acetyl-transferase; WT, wild-type.

with its DNA target sequence as the rat osteoclacin promoter (–84 to –92 nt), thus decreasing its activity (Towler et al. 1994). Msx2 could also act indirectly on target genes by binding other transcription factors such as C/EBPα for amelogenin (Xu et al. 2007) or Runx2 for osteocalcin (Hassan et al. 2004; Sierra et al. 2004). Otherwise, Msx2 and Dlx homeoproteins can recognize the same response elements (AC/TAATTGG) and compete for common target-gene promoters (Bendall and Abate-Shen 2000; Diamond et al. 2006, Lézot et al. 2008). Here, Msx2-induced repression was demonstrated at both protein and RNA levels. Msx2 was shown to specifically repress calbindin-D_{28k} promoter activity as demonstrated by co-transfection of Msx2 siRNAs. The involvement of the putative Msx2 responsive element (–1068 to –1061 nt) on the mouse calbindin-D_{28k} promoter was evident by the loss of Msx2 inhibition on the deleted promoter. These data raise the question of putative Msx2 functions in other systems, such as Purkinje cells of the cerebellar cortex. These cells, where calbindin-D_{28k} is also selectively concentrated in the basal situation, are altered in $Msx2^{-/-}$ mice (Satokata et al. 2000).

The findings reported here could explain at least in part the physiological expression pattern of calbindin-D_{28k} in teeth. Indeed, during amelogenesis, the expression patterns of Msx2 (Molla et al. 2010) and Dlx (Lézot et al. 2008) mirror the stage-specific regulation of enamel protein and certain ion-handling molecules. Although in $Msx2^{+/-}$ ameloblasts, calbindin-D_{28k} level increased compared with $Msx2^{+/+}$, this was not observed in $Msx2^{-/-}$ incisors. This may be related to reduced laminin-5α3 and cytokeratin 5 expression levels that leads to the loss of ameloblast intercellular attachment and finally to their disorganization and disappearance (Bei et al. 2004; Aïoub et al. 2007; Molla et al. 2010). In parallel, calbindin-D_{28k} was upregulated in the rests of Malassez of $Msx2^{+/-}$ mice and even more in $Msx2^{-/-}$. This pattern is not physiological (Korkmaz et al. 2010), as confirmed here in Msx $2^{+/+}$ control mice. The same observation was also repeated for amelogenin in the same pathophysiological context. Indeed, amelogenin gene transcription, which is also repressed by Msx2 (Zhou et al. 2000; Xu et al. 2007), was overexpressed in rests of Malassez in $Msx2^{-/-}$ mice (Molla et al. 2010). These convergent in vivo results on amelogenin and calbindin-D_{28k} and the present findings led us to propose a new concept, where in addition to its developmental function, Msx2 may also limit the expression of its target genes in differentiated cells of dental epithelium within a defined frame related to amelogenesis.

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Declaration of Conflicting Interests

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