Molecular Pathogenesis of Genetic and Inherited Diseases

Enamel Protein Regulation and Dental and Periodontal Physiopathology in *Msx2* Mutant Mice

Muriel Molla,^{*†‡§¶} Vianney Descroix,^{*†‡§}
Muhanad Aïoub,^{*†‡§} Stéphane Simon,^{*†‡§}
Beatriz Castañeda,^{*†‡§||} Dominique Hotton,^{*†‡§}
Alba Bolaños,^{*†‡§} Yohann Simon,^{*†‡}
Frédéric Lezot,^{*†‡§} Gérard Goubin,^{*†‡§}
and Ariane Berdal^{*†‡§¶}

From the Centre de Recherche des Cordeliers,* University of Pierre and Marie Curie-Paris 6, UMRS 872, Paris, France; University of Paris-Descartes,[†] UMRS 872, Paris, France; INSERM U872,[‡] Paris, France; Laboratory of Molecular Oral Physiopathology,[§] University of Paris-Diderot, UFR of Odontology, Paris, France; Reference Center for Oral and Facial Malformations,[¶] Hôpital Hotel-Dieu, AP-HP, Paris, France; and the Department of Basic Sciences,^{||} University of Antioquia, Medellín, Colombia

Signaling pathways that underlie postnatal dental and periodontal physiopathology are less studied than those of early tooth development. Members of the muscle segment homeobox gene (*Msx*) family encode homeoproteins that show functional redundancy during development and are known to be involved in epithelial-mesenchymal interactions that lead to crown morphogenesis and ameloblast cell differentiation. This study analyzed the *MSX2* protein during mouse postnatal growth as well as in the adult. The analysis focused on enamel and periodontal defects and enamel proteins in *Msx2*-null mutant mice. In the epithelial lifecycle, the levels of *MSX2* expression and enamel protein secretion were inversely related. *Msx2*^{+/-} mice showed increased amelogenin expression, enamel thickness, and rod size. *Msx2*^{-/-} mice displayed compound phenotypic characteristics of enamel defects, related to both enamel-specific gene mutations (amelogenin and enamelin) in isolated amelogenesis imperfecta, and cell-cell junction elements (laminin 5 and cytokeratin 5) in other syndromes. These effects were also related to ameloblast disappearance, which differed between incisors and molars. In *Msx2*^{-/-} roots, Malassez cells formed giant islands that overexpressed amelogenin and ameloblastin that grew over months. Aberrant expression of enamel proteins is proposed to underlie the regional osteopetrosis and hyperproduction of cellu-

lar cementum. These enamel and periodontal phenotypes of *Msx2* mutants constitute the first case report of structural and signaling defects associated with enamel protein overexpression in a post-natal context. (Am J Pathol 2010, 177:2516–2526; DOI: 10.2353/ajpath.2010.091224)

Msx2 is a member of the Muscle segment homeobox gene (*Msx*) family, orthologous to the *Msh* gene of *Drosophila*.¹ The mammalian *Msx1* and *Msx2* genes share highly conserved sequences.² Their encoded homeoproteins show functional redundancy during development.^{2,3} They act as transcriptional repressors which bind specific DNA sequences.⁴ In several early developmental processes, *MSX2* signaling pathways have been elucidated. These ones include: cranial suture closing, epithelial-mesenchymal interactions leading to crown morphogenesis,⁵ and ameloblast cell differentiation.^{6,7} A gain-of-function mutation in the *Msx2* homeobox-gene sequence induces premature suture fusion (Boston-type 2 craniosynostosis; OMIM 604757).⁸ Conversely, a mutation leading to reduced *MSX2* DNA-binding is associated with impaired suture closing in the parietal foramina (OMIM 168500).⁹ Another mutation, in the homeodomain-coding region, has been described in a family with amelogenesis imperfecta and impaired tooth eruption.¹⁰ Different mouse models (null mutant mice,⁵ transgenic mice bearing overexpression constructs¹¹ and gene mutations^{12,13}) mimic the multiple craniofacial anomalies: precocious fusion of cranial bones, development of ectopic cranial bone,¹² or contrarily, persistent calvaria foramina and dental defects,⁵ and reduction of bone ossification¹³ and resorption.⁶ The present investigation focuses on the physiopathology of enamel and periodontal tissues in a knockin transgenic mouse model.⁶

MSX2 target-genes were first identified in osteoblasts.¹⁴ *In vitro*, the transcriptional repressor activity of

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Address reprint requests to Muriel Molla, D.D.S, MPhil, Ph.D., Laboratoire de Physiopathologie Orale Moléculaire, Centre de Recherche des Cordeliers, INSERM, UMRS 872, Université Paris-Diderot, Equipe 5, Esc. E-2ème étage, 15-21 rue de l'école de Médecine, F-75270 Paris Cedex 06, France. E-mail: murielmolla@gmail.com.

MSX2 was shown to be mediated through interactions with distinct transcriptional factors such as DLX homeoproteins for osteocalcin¹⁵ and the CCAAT/enhancer-binding protein α (C/EBP α) for amelogenin.¹⁶ In osteoblasts, MSX2 exerts either positive or negative effects on osteocalcin transcriptional activity depending on the stage of osteoblast differentiation and thus diverse MSX2 transcriptional partners.^{17–19} Therefore, the impact of MSX2 depends on the cellular circumstances. Recent *in vivo* studies addressed the role of MSX2 in late postnatal growth and adult homeostasis.^{3,6} These studies demonstrated additional roles for MSX2 in physiological regulation of postnatal mineralized tissue formation, modeling and homeostasis. In addition to enamel and dentin dysplasia and altered root formation, these mutant mice exhibit regional osteopetrosis resulting from site-specific down-regulation of RANKL expression in the alveolar bone⁶ which mechanisms are not understood.

In addition to osteocalcin and amelogenin, other enamel matrix proteins constitute candidate MSX2 targets. Their aberrant expression is hypothesized here to underlie the broad dento-alveolar spectrum of alterations associated with *Msx2* mutation. Indeed, enamel proteins could play a dual role in mineralized tissues, acting both as a structural scaffold for amelogenesis and as signaling molecules for mineralized tissue formation and resorption. Amelogenin proteins self-assemble into nanospheres and coils which serve as an enamel scaffold.²⁰ These transient self-assemblies run throughout the enamel layer and guide apatite crystal formation and growth.^{21,22} Amelogenin null mice show a significant reduction of enamel thickness which is in addition devoid of prismatic pattern.²³ Two other enamel proteins, enamelin and ameloblastin, also cooperate in enamel morphogenesis, as shown by their extracellular distribution in forming enamel and the phenotype of their respective null mutants.^{24,25} On the other hand, dentin sialophosphoprotein (DSPP) is transiently expressed in presecretion-stage ameloblasts and initiates enamel biomineralization.²⁶ Therefore, these four enamel proteins (amelogenin, enamelin, ameloblastin, and DSPP) cooperate in the control of biomineralization events and are essential for enamel morphogenesis. Isolated amelogenesis imperfecta, which comprises a family of hereditary diseases affecting enamel structure, including hypoplastic, hypomaturational, and hypomineralization types, is associated with mutation of the corresponding genes in humans.²⁷

Besides their role as structural proteins, amelogenin and ameloblastin isoforms are proposed to play a role in cell signaling.²⁸ Amelogenin peptides were shown to induce bone-cell differentiation via Runx-2 pathway activation *in vivo* and *in vitro*.^{29,30} Amelogenin null mutants show regional hyperresorption mediated by activation of RANKL-RANK pathway.^{31,32} Interestingly, the amelogenin gene was one of the first identified MSX2 target-genes for which transcriptional repression was deciphered at the molecular level.³³ While this *in vitro* MSX2 action has been well documented, no *in vivo* studies have been performed regarding the relationship between MSX2 expression and enamel proteins.

Based on MSX2 transcriptional repression of the amelogenin gene established *in vitro* and the potential functions of enamel proteins, the purpose of this study was to scrutinize enamel proteins *in vivo* in *Msx2* mutants mice. Postnatal phenotypes were compared in *Msx2*^{+/+} (wild type), *Msx2*^{+/-} (heterozygous) and *Msx2*^{-/-} (homozygous) mice. MSX2 and enamel proteins were analyzed in the dental epithelium throughout its life cycle: enamel organ during amelogenesis, Hertwig's root sheath during root formation, and Malassez remnants in the adult periodontium. A inverse relationship was observed between *Msx2* gene dosage and expression levels of enamel-related genes in the dental epithelium. Our data revealed the existence of a beneficial impact of loss of a single *Msx2* allele on enamel formation in *Msx2*^{+/-} mice which was not previously described. In *Msx2*^{-/-} mice, the data suggest a parallel relationship between epithelial amelogenin and ameloblastin levels and alveolar bone and cementum homeostasis.

Materials and Methods

Expression Analysis in Knockin *Msx2/lacZ* Transgenic Mice

Knockin mice were produced by insertion of the bacterial *lacZ* gene within the *Msx2* gene, replacing the coding sequence.⁶ Heterozygous males and females were mated using a CD1 Swiss genetic background. Their litters were used to compare wild-type (*Msx2*^{+/+}), transgenic heterozygous (*Msx2*^{+/-}) and homozygous (*Msx2*^{-/-}) mice. All experiments were performed in accordance with the French National Consultative Bioethics Committee for Health and Life Science, following the ethical guidelines for animal care. All experiments were performed by staff trained to conduct *in vivo* studies.

RNA Extraction and RT-qPCR

Twenty-one 3-month-old mice were used for each group (*Msx2*^{+/+}, *Msx2*^{+/-}, and *Msx2*^{-/-}). Half mandibles were removed, quickly frozen in liquid nitrogen and ground up. Dental epithelial cells of the continuously erupting incisor and alveolar bone were microdissected out under a stereomicroscope. The cervical loops were excised to avoid mesenchymal contamination. Three pools including three different animals per pool were generated. Total RNA was extracted using a Trizol reagent kit (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's protocol. After total RNA quantification, equal amounts of RNA (1 μ g) were transcribed into cDNA using SuperScript II RNase H reverse transcriptase (Invitrogen) with oligo-dT and hexanucleotide random primers. Gene expression levels were determined by real-time PCR using specific primers (Table 1). Optimal conditions and cDNA dilutions were determined for each gene. q-PCR experiments were performed with a Roche LightCycler in triplicate using FastStart DNA Master SYBR Green I (Roche, Neuilly-sur-Seine, France).

Table 1. Primer Sequences and PCR Conditions Used for RT-qPCR Analysis

Gene	Oligonucleotides	Size (bp)	Tm (°C)
<i>Amelogenin</i>			
P1	5'-GGATCAAGCATCCCTGAGTTTCAAAC-3'	370	60
P2	5'-ATCATTTGGTTGCTGGGGGATCAC-3'		
<i>Ameloblastin</i>			
P1	5'-CACTTACTATCCTTCCGCAG-3'	181	58
P2	5'-GTGCTGACACTTAGACTTGC-3'		
<i>Enamelin</i>			
P1	5'-TCGGAGGGATGTTCTGAAAC-3'	232	58
P2	5'-AGGACTTTCAGTGGGTGTGG-3'		
<i>MSX2</i>			
P1	5'-CCTGAGGAAACACAAGACCA-3'	278	60
P2	5'-AGTTGATAGGGAAGGCAGA-3'		
<i>Laminin5α3</i>			
P1	5'-GGGTGTGACCAAAAAGTGCT-3'	206	58
P2	5'-CATCTTCCAGGGTGACCAGT-3'		
<i>Cytokeratin 5</i>			
P1	5'-TCAAGAAGCAGTGTGCCAAC-3'	217	58
P2	5'-TCCAGCAGCTTCCTGTAGGT-3'		
<i>DSPP</i>			
P1	5'-ACAAGAGTGGGACCCTGTTT-3'	399	58
P2	5'-TACAGTGTGGGCGTTACAAC-3'		

Scanning Electron Microscopy Analysis of Mandible Incisor and Molar Enamel

Six mandibles from 3-month-old *Msx2*^{+/+}, *Msx2*^{+/-} and *Msx2*^{-/-} mice were fixed for 24 hours in 4% paraformaldehyde in PBS, pH 7.4. Samples were then cut along the frontal axis into two fragments before the first molar by using a rotating diamond wheel. Previous microscanner analysis was used to validate the cutting axis (perpendicular to incisor axis). Dehydration was performed using increasing concentrations of ethanol for 24 to 48 hours each at 4°C. Each fragment was embedded in calibrated cylinders with clear polyester resin. The first fragment, corresponding to the buccal part of the incisor, was ground through bone emergence using optically controlled monitoring. The second fragment was ground until the mesial surface of the first molar appeared. Sample surfaces were polished with sandpaper of successively decreasing grits. Conditioning of the enamel surface was achieved by etching with 37% phosphoric acid for 10 seconds. Each sample was coated with palladium-gold in a vacuum evaporator and observed with a scanning electron microscope (JSM-6400, JEOL, Tokyo) at 10 kV. The morphology of incisor was used as a criteria to calibrate the section planes based on the established μ CT landmarks.

Sample Preparation for Histomorphology, in Situ Hybridization, and Immunolabeling

Msx2^{+/+}, *Msx2*^{+/-}, *Msx2*^{-/-} mouse mandibles (four animals for each group, at ages 1, 2, 3, 6, 12, and 24 weeks) were dissected out, fixed by immersion in 4% paraformaldehyde for 24 hours at 4°C, and rinsed in PBS. Half of the samples were decalcified at 4°C with EDTA (4%) for 4 to 64 weeks, depending on mouse age. Right half-mandibles were rinsed, dehydrated and embedded in paraffin for sectioning. Sections of 7 μ m were deparaf-

finized and rehydrated before being stained with hematoxylin and eosin for histological analysis or subjected to immunohistology. For the left half-mandibles, 10- μ m cryostat sections without decalcification were made with a Leica CM 3050S Microtome (Leica, Rueil-Malmaison, France) and deposited onto poly-L-lysine (Sigma)-coated slides, dehydrated in a graded series of ethanol, and stored at -20°C.

In Situ Hybridization

Amelogenin sense and antisense RNA probes were prepared from full-length cDNA (covering exons 2-3-5-6-7; see Bonass et al³⁴), subcloned into a Bluescript plasmid and linearized with XhoI or PstI endonucleases.

For radioactive *in situ* hybridization, [³⁵S]UTP-labeled single-stranded antisense and sense probes were synthesized using T7 and T3 RNA polymerase, respectively (Boehringer, Meylan, France).³⁴ For nonradioactive *in situ* hybridization, the same probes were labeled with UTP-digoxigenin (Roche, France).

Radioactive *in situ* hybridization was performed as previously described by Hotton et al³⁵ on non-decalcified samples. Briefly, cryostat sections were pretreated with proteinase K (Sigma), hybridized with 20 μ l of labeled probes containing 60,000 cpm/ μ l in a humid chamber overnight at 50°C, and washed under high-stringency conditions. The slides were dipped into NTB2 autoradiographic emulsion (Kodak, Paris, France) and exposed from 2 to 4 weeks at 4°C. After developing the film, the sections were stained with hematoxylin, dehydrated, and mounted under a coverslip. The sections were examined and photographed using a DRMB photomicroscope (Leica, France) under bright- and dark-field illumination.

For nonradioactive *in situ* hybridization, sections were deparaffinized, dehydrated, and hybridized overnight with 50 μ l of labeled probe (1 ng/ μ l) in a dark, humid chamber. The reaction was revealed by immu-

nocytochemistry using an anti-digoxigenin alkaline phosphatase-conjugated polyclonal antibody (Vector NovaRED; Abcsyss, Paris, France). The sections were then stained with Mayer's hematoxylin and dehydrated before being mounted under a coverslip and photographed.

Immunohistochemistry

Deparaffined and rehydrated sections were incubated for 30 minutes in 3% H_2O_2 /PBS to quench endogenous peroxidase activity, and then rinsed for 10 minutes in PBS. Nonspecific protein binding was blocked by incubation for 30 minutes in 10% normal goat serum and 1% bovine serum albumin in PBS. Specimens were incubated for 1 hour at room temperature in a humidified chamber with a polyclonal rabbit anti-mouse β -galactosidase antibody (Genetex), anti-amelogenin (generous gift of Pr. S. Sasaki, Tokyo Medical and Dental University, Department of Biochemistry, diluted at 1:1000) and anti-ameloblastin (generous gift of Pr. Tilmann Wurtz, Centre de Recherche des Cordeliers, Paris, France; diluted at 1:1400). Sections were then washed extensively three times in PBS at room temperature before treatment for 30 minutes at room temperature with the secondary biotin-labeled goat anti-rabbit IgG antibody (StrAvidin Multilink kit, Biogenex, UK) for β -galactosidase, and with horseradish peroxidase goat anti-rabbit IgG antibody (DAKO) for

Figure 2. Immunolocalization of β -galactosidase protein in *Msx2* knock-in heterozygous mouse root. Epithelial life cycle is followed in an incisor (A) and first molar (B-E). Different cells are recognized according to their morphology and position. At postnatal week 2, *MSX2* expression is evident in odontoblasts, pulp cells, ERM (arrows), and Hertwig epithelial root sheath (between the vertical black lines). HERS, Hertwig epithelial root sheath. Black rectangles in B are shown enlarged in D and E.

amelogenin and ameloblastin. Subsequently, sections were incubated for 30 minutes at room temperature with peroxidase linked to avidin (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for β -galactosidase, and

Figure 1. Immunolocalization of β -galactosidase protein in *Msx2* knock-in heterozygous mutant mouse molar crown. *MSX2* expression is shown labeled in brown at 1 (A, D, H) and 2 (B, C, I) postnatal weeks. A represents a sagittal view of a molar, with the cervical area on the left and the occlusal part on the right. Black rectangles are enlarged in E-H. Ameloblast stage was determined according to their morphology and location in the molar crown. At 1 week, most ameloblasts are at the secretion stage while at 2 weeks, most of the ameloblasts are at the maturation stage. Ab PS, prescretion stage ameloblasts; Ab S, secretion stage ameloblasts in 3 areas: Ab S1 corresponds to the cervical part of the crown where enamel thickness is reduced, Ab S2 corresponds to the middle part of the crown where enamel thickness is greatest, and Ab S3 corresponds to the cusp area where enamel thickness decreases. Ab EF, ameloblast facing the enamel-free area; Ab M, maturation stage ameloblasts; IEE, inner enamel epithelium; SR, stellate reticulum; SI, stratum intermedium. Strong labeling is noted in prescretion stage ameloblasts (D), maturation stage ameloblasts (I) and in ameloblasts facing the enamel-free area in the occlusal crown (H). In secretion stage ameloblasts, labeling decreases with enamel thickness: at the middle region of the crown, corresponding to the thickest enamel layer, brown labeling is less intense (Ab S2, F); at the cervical and occlusal parts of the crown where the enamel layer is thinner, labeling is stronger (Ab S1 and Ab S3, E-G). Experiments without primary antibody are controls (B and C).

with a NovaRED kit (Vector Laboratories) for amelogenin and ameloblastin. After rinsing in PBS, the immunoreactivity was visualized by development for 2 to 10 minutes with 0.1% 3,3-diaminobenzidine and 0.02% H₂O₂ (DAB substrate kit, Vector Laboratories). For β -galactosidase analysis, sections were counterstained with Mayer's hematoxylin, mounted with permanent mounting medium (XAM, BDH Laboratory, England) and examined by light microscopy. Amelogenin and ameloblastin labeling samples were not counterstained but mounted with aqueous medium (Labonord, Lille). A positive control was performed on untreated mouse teeth and a negative control on mouse oral mucosa. A further negative control experiment that omitted primary antibody was also performed on mouse tooth sections.

Results

MSX2 Expression in Odontogenic Cells

MSX2 expression was analyzed during the first 4 postnatal months in the mandibular first molar of *Msx2*^{+/-} mice by using β -galactosidase immunostaining. Developmental stages of ameloblasts have been identified based on their cell morphology. Presecretion stage ameloblasts are short, and are adjoining differentiated mesenchymal cells forming predentin and dentin with no enamel deposition. Secretion stage ameloblast are polygonal and elongated with a large basal nucleus and distal Tomes processes. Enamel deposition is visible. Maturation stage ameloblasts do not possess Tomes process, become abruptly much shorter and reduced in size. The enamel thickness is stable.

During amelogenesis (Figure 1), MSX2 expression was stronger in presecretion-stage ameloblasts (Ab PS) (Figure 1D) and in mature, postsecretion-stage ameloblasts (Ab M) (Figure 1I) than in secretion-stage ameloblasts (Ab S) (Figure 1, A, D–H). Figure 1, B and C, show controls without primary antibody. Ab S1, S2, and S3 represent secretion-stage ameloblasts adjoining enamel area with different thicknesses, in the cervical part, the middle to upper part, and the occlusal part of the molar crown, respectively. In secretion-stage ameloblasts, MSX2 expression appeared to be less intense in areas of greater enamel thickness (Ab S2 versus Ab S1 and Ab S3). Strong MSX2 expression was observed in zones of mouse molar corresponding to enamel-free areas (Ab EF). MSX2 expression was also detected in forming and adult periodontium. During root elongation, MSX2 was expressed in Hertwig epithelial root sheath and, after its disruption, in epithelial rests of Malassez (ERM) (Figure 2, A–E). Epithelial rests of Malassez cells continued to express significant levels of *Msx2* in adults. Quantitative analysis of *Msx2* transcripts in ameloblasts (Figure 3) validated the absence of *Msx2* transcripts in *Msx2*^{-/-} mice and a half reduction in *Msx2*^{+/-} heterozygous mice as compared to *Msx2*^{+/+} control mice. These results indicate the occurrence of a haploinsufficiency phenomenon in heterozygous mice.

Figure 3. Quantitative RT-PCR analysis of *Msx2* (A) and enamel mRNA isolated from microdissected incisor epithelial cells in *Msx2*^{+/+}, *Msx2*^{+/-}, and *Msx2*^{-/-} 12-week-old mice (B). Each level of mRNA expression is normalized with stable ribosomal protein RS15 mRNA. The histograms show the mean $\pm$ SEM of data obtained from nine mandibles for each group (in triplicate). Significance was evaluated using the Mann-Whitney test (**P* < 0.05, ***P* < 0.005).

Msx2 Dosage Regulates Enamel Proteins

To estimate the variation in the levels of the mRNAs encoding enamel proteins in relationship to MSX2 expression levels, dental epithelium was microdissected from mouse mandible incisor and total mRNA was isolated. To analyze mRNA expression in a homogenous population of ameloblasts, the cervical loop was carefully removed. Levels of mRNA encoding amelogenin, ameloblastin, amelogenin, laminin 5 α 3, cytokeratin 5, and *Dspp* were measured by RT-qPCR in *Msx2*^{-/-}, *Msx2*^{+/-} and *Msx2*^{+/+} mice. The amelogenin primers were chosen to amplify the M180 major transcript. As expected, sequencing revealed that the amplified amelogenin cDNA contained exons 2, 3, 5, and 6. In *Msx2*^{+/-} heterozygous mice, only the modulation of amelogenin expression was found to be statistically significant among all enamel protein-encoding genes. As shown in Figure 3, amelogenin expression increased twofold in heterozygous mice as compared to wild type. In contrast, *Dspp* transcript expression was significantly reduced in heterozygous mice. In *Msx2*^{-/-} null mice, the amounts of amelogenin, enamel, laminin 5 α 3, cytokeratin 5, and *Dspp* mRNA were all significantly decreased, whereas no significant variation of ameloblastin mRNA was observed.

To localize amelogenin mRNA expression in secretion-stage ameloblasts, *in situ* hybridization was performed on incisor sections of 21-day-old mouse mandible (Figure 4, A and B). mRNA labeling was stronger in *Msx2*^{+/-} (Figure 4, C and D) than in *Msx2*^{+/+} mice (Figure 4, A and B). Scattered ectopic mRNA was observed in stratum intermedium cells. In *Msx2* null mice, no significant hybridization signal was detected in incisor (Figure 4E) as expected because of the complete disappearance of ameloblasts. Detailed analysis in molar showed some areas with few ameloblasts corresponding to irregular

Figure 4. Radioactive *in situ* hybridization of amelogenin mRNA in *Msx2*^{+/+}, *Msx2*^{+/-}, and *Msx2*^{-/-} mouse incisors at 21 postnatal days. In *Msx2*^{+/+} mice, amelogenin expression is located in the cell body of secretion-stage ameloblasts (**A** and **B**). In *Msx2*^{+/-} mutant mice, labeling is stronger (**C** and **D**). In *Msx2*^{-/-} mutant mice, amelogenin labeling was absent, as expected (**E** and **F**). Ameloblasts are not polarized or rounded (**F**). Ab, ameloblast; E, enamel; D, dentin; EO, enamel organ.

amelogenin labeling (Figure 4F). Indeed, some polarized ameloblasts were present in the cervical area, whereas in the most occlusal part, epithelial cells in contact with dentin showed no evidence of ameloblast polygonal structure and organization, but rather a shorter and round morphology (Figure 4F).

Histological studies of *Msx2*^{-/-} mouse mandibular molars showed anomalies of root-tissue development inducing root dysmorphology, with a gradient of severity from the third to the first molar. In the first molar the root dentin was formed and no obvious anomaly was observed until day 10 (data not shown). The second molar exhibited in some areas a total absence of root dentin formation and a massive cellular cementum-like structure (hypercementosis). Some ectopic mineralized tissue was seen in the pulp chamber. The third molar showed a complete root shape and structural disorganization. Moreover in all molars, hypertrophy of epithelial cells was observed at several sites. The first major site was localized adjacent to the crown-root junction and the second was more diffuse. These cells comprised clusters of only a few cells termed the ERM in wild-type mice (Figure 5, G and H). This analysis revealed three different sizes of cluster in *Msx2* null mutant mice: small ones composed of two or three cells (as in wild type), medium ones composed of five or six cells, and giant ones composed of more than 10 cells. The bigger these islands, the more extracellular matrix was present and the more the cells were enlarged. Histomorphometric analysis was performed

with image analysis software to calculate the area occupied by and the number of epithelial cluster cells along the roots (Figure 5I). In *Msx2*^{-/-} mice, these clusters appeared less numerous but much larger than in wild-type mice and their size appeared to increase with age (4.6-fold at 2 postnatal weeks, Figure 5, A–C; 14-fold at 4 postnatal weeks; 30-fold at 8 postnatal weeks, Figure 5, D–F). At 2 to 6 months most of these clusters were entrapped in the cellular cementum (Figure 5, D–F). In addition, *in situ* hybridization and immunolabeling showed that they overexpressed both amelogenin and ameloblastin (Figure 6, B–E) compared to wild type (Figure 6A).

SEM Analysis of Msx2^{+/+}, *Msx2*^{+/-} and *Msx2*^{-/-} Mouse Enamel

For comparison, the corresponding areas of mature enamel in *Msx2*^{+/+}, *Msx2*^{+/-} and *Msx2*^{-/-} mouse mandible incisors are shown in Figure 7. The thickness of the enamel layer differed between the three mouse genotypes. The enamel layer of the *Msx2*^{-/-} null mouse was markedly reduced (2.6-fold) (Figure 7E), whereas the thickness of the enamel layer in the heterozygous mouse was increased 1.3-fold (Figure 7C). In addition to this thickness modification, the enamel had a different structural pattern in the different genotypes. In the *Msx2* null mouse, the enamel was reduced to a thin layer, homogeneous in structure with cracks inside and lacking its usual prismatic appearance (Figure 7F). Heterozygous mice showed a regular prismatic structure similar to normal enamel with rods cut in alternating sagittal and transversal orientations (Figure 7C). Yet, some differences were noticed. The external prismatic enamel layer, in which all prisms were cut transversally, was much larger in the heterozygous mouse tooth than in the wild-type mouse tooth (increasing twofold) (Figure 7, A–D). Most of the enamel body consists of rod and inter-rod enamel. Rods present a classical keyhole shape when they are transversally cut. Each rod is separated with inter-rod enamel connecting to the rod on one side. *Msx2*^{+/-} heterozygous mice exhibited much larger prisms (2.7 ± 0.2 μm in width in *Msx2*^{+/-} mice versus 1.9 ± 0.2 μm in *Msx2*^{+/+} mice) (Figure 7, G and H).

Discussion

Disruption of one (*Msx2*^{+/-}) or two (*Msx2*^{-/-}) copies of the *Msx2* gene led to specific abnormalities in teeth. In the *Msx2*^{+/-} mouse, both enamel thickness and amelogenin expression in ameloblasts were increased. In *Msx2*^{-/-} mice, epithelial cells showed divergent alterations in the crown and root: reduced expression of enamel matrix proteins in the crown was associated with the disappearance of ameloblasts, whereas in the root, epithelial Malassez cells accumulated progressively throughout postnatal growth and in adult periodontium (the calculated area of the ERM was increased 4.6-fold at 2 postnatal weeks and 30-fold at 8 postnatal weeks). The latter cells formed hypertrophic islands (showing increased width and numbers of

Figure 5. Analysis of epithelial rest of Malassez in *Msx2*^{+/+} and *Msx2*^{-/-} mouse molars. Pictures represent epithelial cells in the periodontal ligament along root, in contact to cementum. Epithelial clusters were analyzed at 2 weeks (**A–C**), 4 weeks (not shown), and 8 weeks (**D–F**) in *Msx2*^{+/+} mice versus controls (2 weeks, **G**; 8 weeks, **H**). Surface areas were measured on four different first mandibular molars from *Msx2*^{+/+} and *Msx2*^{-/-} mice along the root length. These measurements were performed at 2, 4, and 8 postnatal weeks. In *Msx2* null-mutant mice, three types of clusters could be observed: **A**, a small cluster with only a few cells, similar to the clusters in wild-type mice; **B**, medium-sized with 5–6 cells; and **C**, a large cluster including more than 10 cells. At 8 postnatal weeks, most of the cell clusters are included in cementum (**D–F**). In wild-type mice, epithelial rest of Malassez are present all along the root and composed of one or very few cells at 2 weeks (**G**, arrows), 4 weeks (not shown), and 8 weeks (**H**, arrow). Image analysis software (Image J) was used to measure epithelial cell areas (**I**). ERM areas increased from 2 weeks (ratio *Msx2*^{-/-} versus *Msx2*^{+/+} = 4) to 4 weeks (ratio = 14) to 8 weeks (ratio = 30).

cells) that overexpressed enamel proteins at both the mRNA and protein levels.

MSX2—A Regulator of Enamel Architecture via Transcriptional Control of Amelogenin Levels

The amelogenin promoter harbors five response elements containing canonical TAATT-binding motifs for

both MSX and DLX homeoproteins.³⁶ Four DLX genes are expressed in ameloblasts.^{36,37} *In vitro*, both positive and negative DLX effects on amelogenin promoter activity were shown to depend on the identified homeodomain-binding sites. In contrast, MSX2 behaves as a repressor of the amelogenin promoter,^{33,38} in part by antagonizing C/EBP α .¹⁶ These *in vitro* findings motivated the present investigation. Here, the observed increased level of amelogenin expression in *Msx2*^{+/+} ameloblasts is interpreted as resulting from reduced transcriptional repressor activity of MSX2. Indeed, *Msx2* RNA levels mirrored *Msx2* gene copy number (that is [*Msx2*^{+/+}] > [*Msx2*^{+/+}] > [*Msx2*^{-/-}] = 0; this study and Berdal et al³). Amelogenin mRNA was increased twofold in *Msx2*^{+/+} mice compared to wild-type mice. For other molecular species, such as matrix (ameloblastin, enamelin), cytoskeletal (cytokeratin 5) and cell adhesion (laminin 5 α 3) proteins, no significant differences were found between the heterozygous and wild-type mice.

On the other hand, we found that the cells with the highest levels of MSX2 expression were not actively forming enamel matrix or producing significant amounts of amelogenin. These cells include presecretion- and maturation-stage ameloblasts,³⁹ enamel-free zone ameloblasts⁴⁰ and, even more strikingly, the cells of the Hertwig root sheath⁴¹ and ERM.⁴² This suggests that MSX2 down-regulation is a prerequisite for significant enamel deposition, exclusively realized by secretion-stage ameloblasts. Indeed, the present data establish a link between MSX2-controlled amelogenin levels and enamel morphogenesis (regional enamel thickness and rod/inter-rod proportion). An increase of approximately 1.3-fold in

Figure 6. Amelogenin and ameloblastin expression in *Msx2*^{+/+} (**A**) and *Msx2*^{-/-} (**B–E**) mice at 3 postnatal weeks. **A–C**: Nonradioactive *in situ* hybridization to amelogenin mRNA reveals a significant increase in giant ERM in *Msx2* null mutants (arrows). **D** confirms increased amelogenin protein expression by immunoperoxidase staining (arrows). **E** shows significant ameloblastin protein levels by immunoperoxidase staining in the same ERM (arrows). Such intensity of amelogenin and ameloblastin immunolabeling is not observed in wild-type mice (data not shown); D, dentin; Od, odontoblast; Period., periodontium.

Figure 7. Scanning electron microscopy of transverse sections of mature enamel incisor (sagittal view) from *Msx2*^{+/+}, *Msx2*^{+/-}, and *Msx2*^{-/-} mice at 3 postnatal months. The accuracy of each section location was established by the size and shape of incisor enamel and dentin, based on a preliminary study using microCT-3D reconstruction (data not shown). Compared to the situation in wild type (A), enamel thickness is higher ($\times 1.3$) in *Msx2*^{+/-} mice (C) and lower in *Msx2*^{-/-} mice ($\times 0.38$) (E). The most significant difference between *Msx2*^{+/+} (B) and *Msx2*^{+/-} (D) enamel is observed for the external prismatic enamel (between the dashed lines). *Msx2*-null mutants show a hypoplastic enamel dysplasia with absence of rods and many internal cracks (F). Compared to wild type (G), in *Msx2*^{+/-} mutant mice the rods are modified (H). Prism width was $1.9 \pm 0.2 \mu\text{m}$ in wild-type mice, and $2.7 \pm 0.2 \mu\text{m}$ in heterozygous mice, as established by analysis of 20 different areas of the inner prismatic enamel. The external prismatic enamel layer (EPE) is much larger in *Msx2*^{+/-} than in *Msx2*^{+/+} mice. E, enamel; D, dentin; R, resin; OAE, outer aprismatic enamel; IPE, inner prismatic enamel; IRo, inter-rod rod enamel.

enamel thickness and shifting of the rod/inter-rod proportion in favor of the rods was documented in *Msx2*^{+/-} mice (rods were enlarged to $2.7 \mu\text{m}$ in the heterozygous genotype versus $1.9 \mu\text{m}$ in wild type). Divergent phenotypes *in vivo* also suggest that amelogenin levels control enamel thickness and rods. In amelogenin ^{-/-} mice, enamel is considerably reduced in thickness and loses its rod structure.²³ This phenotype is also observed to a lesser extent in vitamin D-deficient rats, in which lower amelogenin levels led to a decrease in both the rod/inter-rod ratio and enamel thickness.³⁹ Consistent with this, in secretion-stage ameloblasts, *MSX2* (as shown previously for *DLX2*⁴³) was variably down-regulated, depending on the enamel thickness. The *MSX2* (and *DLX2*³⁶) expression gradient mirrors the amelogenin and enamel thickness patterns.

The present study revealed a key role for *MSX2* in amelogenin repression, and suggests two functions for *MSX2 in vivo*: i) up-regulation of *MSX2* would limit amelogenin production and block enamel deposition in other cell stages and types than secretion stage ameloblasts; and ii) during the enamel matrix deposition, *MSX2* controls enamel thickness and rod morphogenesis. In conclusion, *MSX2* regulates enamel architecture, as previously documented for developmental genes in mineralizing tissues⁴⁴ in our group.

Physiopathological Triad of Hypoplastic Enamel Dysplasia in *Msx2*^{-/-} Mice

In *Msx2*^{-/-} mice, the enamel, when present, presented a reduced thickness ($40 \pm 10 \mu\text{m}$ versus $104 \pm 10 \mu\text{m}$ in wild type) and displayed an irregular surface. Enamel was confined to an amorphous layer without

rod architecture. Some cracks were observed in this thin enamel layer. Various genetic forms of amelogenesis imperfecta⁴⁵ are secondary to mutations in human genes (enamelin,⁴⁶ kallikrein 4,⁴⁷ and enamelysin⁴⁸) and transgenic mouse lines.^{23,25,49–52} Among these clinical disorders and experimental models, the enamels of *Msx2*^{-/-} and amelogenin ^{-/-} mice harbor the most significant similarities, both corresponding to hypoplastic type of enamel defects. Similarly, enamelin null mutants show structural alterations in heterozygous mice and the absence of enamel in homozygous mutant mice.⁵³ Consistent with such phenotypes, the present RT-qPCR analysis revealed reduced expression of both amelogenin and enamelin in *Msx2*^{-/-} mouse dental epithelium, whereas ameloblastin mRNA expression was unaffected.

In addition to enamel matrix defects, ameloblast differentiation was impaired in *Msx2*^{-/-} mice. Ameloblasts lost their intercellular junctions, became round, and detached. Reduced amounts of mRNA encoding laminin 5 α 3 (as previously observed *in situ*⁷) and cytokeratin 5 were observed by RT-qPCR. In humans, mutation of the laminin 5 α 3 gene leads to epidermolysis bullosa, which is associated with enamel dysplasia and impaired tooth eruption as observed in *Msx2* null-mutant mice.⁶ Laminin 5 is implicated in cell-cell junctions during ameloblast differentiation. Ameloblast anchorage to secreted matrix, membrane and mineral is crucial for rod orientation, structure, and mineralization.⁵⁴ Cytokeratin 5 is a component of intermediate filaments of epithelial tissues. Cytokeratin 5 binds to the amelogenin trityrosyl motif peptide (ATMP) sequence of amelogenin and is suspected to play a chaperone role for nascent amelogenin polypeptides.⁵⁵ Thus, the reduction of cytokeratin 5 expression

would additionally contribute to the enamel defects observed in *Msx2* null mice.

Therefore, it may be concluded that the enamel dysplasia results from a physiopathological triad in *Msx2*^{-/-} mice: i) a progressive deficit of differentiated ameloblasts, which was shown here to vary between incisors and molars; ii) altered expression levels of cellular proteins involved in cell-cell junctions (laminin 5 α 3) and the amelogenin biosynthetic pathway (cytokeratin 5); iii) decreased levels of specific enamel-related species (amelogenin, enamelin, and DSPP, but not ameloblastin) in the extracellular matrix compartment.

*Overexpressed Enamel Proteins – Signals in *Msx2*^{-/-} Mouse Periodontal Anomalies*

Amelogenin function seems not to be restricted to a structural role. The first evidence of amelogenin signal function via Runx 2 activation was supplied by Arthur Veis Dental School of the Aristotle University of Thessaloniki, Thessaloniki, Greece.²⁹ A number of studies further supported this first experiment. Amelogenins are osteo-inductive in both fibroblasts²⁹ and embryonic stem cells.³⁰ In vitro studies assigned a function to amelogenins in cementum and alveolar bone remodeling.^{56–58} Immortalized cementoblasts exposed to amelogenin overexpress bone sialoprotein (BSP) in a dose-dependent manner.⁵⁹ In bone cells, amelogenin severely inhibits osteoclastogenesis through down-regulation of RANKL, M-CSF, and fibronectin production by osteoblasts.⁶⁰ Conversely, *in vivo*, amelogenin^{-/-} mice display cementum resorption and increased RANKL expression.^{23,31}

In the periodontium of *Msx2* null-mutant mice, regional osteopetrosis and delayed tooth eruption were previously reported.⁶ In the present study, transformation of ERMs into hypertrophic epithelial islands and neighboring hypercementosis were documented. This ended in young adults with the growth of cementum-bone-like tissue structures which entrapped giant epithelial islands within the roots. These epithelial cells appeared enlarged and produced amelogenin and ameloblastin even though enamel protein detection is technically difficult in postnatal periodontium at the basal level^{41,61}. Evident enamel protein detection in epithelial root Malassez cells is related to physiopathological situations such as inflammation,⁶² cementum repair and, here, periodontal defects in *Msx2* null mice. Alterations to dental hard tissues may have consequences in mastication function and could contribute to these periodontal defects.

Therefore, the MSX2 decreased and the overexpression of amelogenin and ameloblastin in continuously growing, giant epithelial islands and cells observed here play a role in the pluri-tissular alterations of *Msx2*^{-/-} mouse periodontium: increased cementum, alveolar bone deposition (via increased Runx2 levels specifically in the alveolar bone, RT-qPCR data non shown), and decreased alveolar bone resorption (via decreased RANKL-ligand levels).⁶

To conclude, *Msx2*^{-/-} mice encompass phenotypic characteristics of both isolated enamel dysplasia, because of the severe reduced expression in enamel struc-

ture genes (amelogenin, enamelin), and syndromic enamel dysplasia, through alterations in cell-cell junctions (laminin 5 α 3, cytokeratin 5). In addition, the enamel and periodontal phenotypes demonstrate a potential dual impact: both structural (enamel morphogenesis in *Msx2*^{+/-} and *Msx2*^{-/-} mutant mice) and signaling (periodontal positive balance in *Msx2*^{-/-} mice) roles of enamel proteins in a physiopathological context.

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