ANIMAL MODELS

Distorted Patterns of Dentinogenesis and Eruption in *Msx2* Null Mutants

Involvement of *Sost*/Sclerostin

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The muscle segment homeogenes *Msx1* and *Msx2* play a major role in tooth and bone formation. Periodontal osteoclast impairment also occurs in *Msx2* null mutant mice, which is restored by overexpression of the receptor activator of NF-κB targeted in osteoclast lineage. Here, we investigated the role of *Msx2* in dentinogenesis. Experiments were performed on *Msx2*^{−/−} mice and the MDPC-23 odontoblastic cell line. After *Msx2* gene silencing, real-time quantitative RT-PCR data showed significant overexpression of *Runx2*, *Bglap*, *Dspg*, and *Alpl*. Of three inhibitors of Wnt/β-catenin signaling (*Dkk1*, *SostDc1*, and *Sost*/Sclerostin), only *Sost* was expressed in postnatal teeth and overexpressed in *Msx2*^{−/−} tooth samples. Initial crown dentin formation—primary dentinogenesis—occurred fairly normally in *Msx2*^{−/−} teeth, albeit with distorted cusp patterns. Later stages of tooth development were characterized by a deviation from secondary toward tertiary dentinogenesis with osteodentin formation and impaired dentin deposition leading to limited root elongation. In *Msx2*^{−/−}/receptor activator of NF-κB—transgenic double mutants, the dentin phenotype, notably in the roots, was rescued and sclerostin levels were normalized. These data suggest that *Msx2* may act indirectly on dentinogenesis by controlling osteoclast activity and the signaling network related to eruption, supporting and further extending the concept that *Msx2* controls formation of mineralized tissues by inhibition of the Wnt/β-catenin pathway; *Sost* in dentin and *Dkk1* in bone, as previously demonstrated. (*Am J Pathol* 2016, 186: 2577–2587; <http://dx.doi.org/10.1016/j.ajpath.2016.06.013>)

The muscle segment homeogenes *Msx1* and *Msx2* play a major role in morphogenesis.^{1–3} In addition, *Msx2* controls both physiological^{4,5} and pathologic tissue mineralization.⁶ In the growing craniofacial skeleton, *Msx2* expression is nearly ubiquitous, involving ameloblasts, odontoblasts, and bone-forming osteoblasts as well as periodontal osteoclasts.⁷ *Msx2* homeoprotein is a transcription factor that represses expression of genes important for mineralization: the osteocalcin,⁸ amelogenin^{9,10} and calbindin-D_{28k}.¹¹ *Msx2* is also engaged in cross talk with growth factors such as bone morphogenetic proteins in tooth morphogenesis¹² and with Wnt/β-catenin signaling in bone.¹³ *Msx2* may play a role in coupling bone remodeling and tooth growth. Indeed, *Msx2*

gene inactivation leads to regional osteopetrosis [secondary to reduced production of receptor activator of NF-κB (RANK) ligand] and impaired tooth eruption with affected tooth and root growth.⁷ Conversely, RANK-targeted overexpression in osteoclasts¹⁴ restores tooth eruption and also

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improves root elongation in *Msx2*^{-/-}/RANK-transgenic (Tg) double mutant mice.¹⁵

In *Msx2*^{-/-} mice, dentin abnormalities have received less attention to date. Our first aim was to explore *Msx2* function in this tissue. Dentin is the dental core tissue formed by odontoblasts, which are polarized secretory cells differentiated from mesenchymal progenitors at the dental pulp periphery.¹⁶ Throughout life, dentin formation occurs under different contexts, termed primary, secondary, and tertiary dentinogenesis. Primary dentinogenesis is the process of initial crown and root formation. When tooth eruption intervenes, the same odontoblasts now produce secondary dentin, which is characterized by a much lower rate of matrix deposition¹⁷ and a different gene expression profile¹⁸ compared with primary dentin. Primary and secondary orthodontins are characterized by long tubules running parallel through the dentin thickness.^{16,17} The tubules host odontoblast cell processes while the cell body moves retrogradely during matrix deposition. Thus, orthodontin-odontoblasts remain outside the matrix¹⁹ in contrast to what occurs in other mineralized tissues in which mature cells become entrapped, as exemplified by osteocytes in bone or cementocytes in cementum. In response to major external stresses such as excessive biomechanical loads, exposure to degradative chemicals, caries,^{20,21} or dental material filling,²² dentin-pulp healing proceeds. This is called tertiary dentinogenesis. Tertiary odontoblasts and the dentin they produce give rise to a spectrum of aberrant morphologies depending on the type, duration, and intensity of the inducing stress. Notably, after one single surgery with limited pulp exposure, the osteodentin that is laid down, in which odontoblasts are entrapped in the matrix, marks the transition from secondary to tertiary dentinogenesis.²² This is named the calciotraumatic line. Thereafter, orthodontin is produced by newly differentiated and polarized odontoblasts with cell processes elongating inside neo-tubules. Interestingly, in *Msx2*^{-/-} mutants, osteodentin is produced without any trauma.^{7,23} For comparison in mineralized tissues, osteocytes (cells entrapped in bone matrix) are involved in the response to bone mechanical stress, and they express sclerostin. Sclerostin, the product of the *Sost* gene, antagonizes β -catenin activation by Wnt growth factors^{24,25} and thus behaves as a negative regulator of bone formation. Here, we investigated the role of *Sost* expression on dentinogenesis in *Msx2* mutant mice and cells, other Wnt inhibitors, because *Dkk1* was previously shown to regulate *Msx2* effects on bone.¹³

Materials and Methods

Animals for Experimentation

Wild-type (WT) CD1-Swiss mice and *Msx2* knockin (*Msx2*^{-/-}) mice were used in this study. *Msx2*^{-/-} mice were produced by insertion of the bacterial *lacZ* gene within the *Msx2* gene locus, replacing the *Msx2* coding sequence, as

previously described.²⁶ *Msx2*^{-/-} mice were obtained by mating heterozygous animals. Genotyping was performed by PCR with the following primers: *Msx2* forward, 5'-GTCTTCGCTTGAGAGTTGCCA-3', *Msx2* reverse, 5'-GTCCGACATGAGCGCCTC-3', and *LacZ*, 5'-GGCAAAGCGCCATTGCGCCATTCAGGC-3'.

WT and *Msx2*^{-/-} mice were studied. Mice were analyzed postnatally at 7, 14, 21, and 28 days and at 3 and 4 months, with at least five mice in each experimental group for a total of 54 mice.

RANK-Tg mice were generated by heterologous recombination of a cassette containing 1.5 kb of the human myeloid-related protein 8 (*Mrp8*) gene promoter²⁷ and the coding sequence of the mouse *RANK* gene. Thirty-two copies of the cassette were inserted in tandem in the RANK Tg line used in this study. Genotype was confirmed by PCR with the following two primers: RANK-Tg forward, 5'-ATGGACTACAAAGACGATGACGAC-3', and reverse, 5'-TGCCAGGATCCACCGCCACCA-3'.

A trained team authorized to conduct *in vivo* studies under Centre d'Exploration Fonctionnelle platform supervision (A75-06-12) performed all experiments. This conforms to European Union Council Directives (2010/63/UE) about the care and use of laboratory animals.

Sample Preparation for Immunohistochemistry and Dental Pulp Mesenchyme Microdissection

Five mice for each age group were used for immunohistochemistry analysis. Mice were anesthetized with an intraperitoneal injection of a blend containing 100 mg/mL ketamine hydrochloride (Imalgene 500; Merial, Toulouse, France) as tranquilizer and 20 mg/mL xylazine (Rompun 2%; Bayer Pharma, Berlin, Germany) for deeper sedation. After induction of general anesthesia, intracardiac perfusion was performed with 4% paraformaldehyde (Sigma-Aldrich, Darmstadt, Germany) in phosphate-buffered saline (PBS) 0.1 mol/L (Euro-medex, Souffelweyersheim, France) for 10 minutes. Whole maxillae were dissected out and immersed in the same fixative solution at 4°C overnight. Samples were decalcified with 4.13% disodium EDTA (Sigma-Aldrich) in a Biowave Pro microwave processor (Pelco, Clovis, CA) for 3 to 7 days, depending on mouse age. After washing in PBS, tissues were dehydrated in an ascending series of ethanol, placed in Clear-ene solvent, and embedded in paraffin surgipath paraplast-plus (Leica Microsystems, Milton Keynes, United Kingdom). Sagittal serial 7- μ m sections were cut with a microtome (Leica RM2145; Leica Microsystems, Wetzlar, Germany) and mounted on SuperFrostPlus slides (Menzel-Gläser, Braunschweig, Germany). Sections showing the complete crown and root of the first upper molars were subjected to histomorphologic analyses with hematoxylin and eosin and Masson's trichrome stainings, and immunochemistry.

Nine 3-month-old mice of each genotype (WT and *Msx2*^{-/-}) were used for microdissection of dental pulp mesenchyme. Pulp mesenchymal cells of the continuously

erupting incisor were microdissected out using a stereomicroscope MZIII (Leica Microsystems) as described.²⁸ The lower jaw tissues were isolated and quickly frozen in liquid nitrogen. For the pulp mesenchyme, all tissues around the tooth were excised, and the tooth was cleaned completely; the apical loop was removed to prevent epithelial contamination. Thereafter, the teeth were broken and the pulp mesenchyme was removed. Both, the WT and *Msx2*^{-/-} samples were derived from three pools that included three different mice per pool. Tissues were kept at -80°C until needed for RNA extraction.

Masson's Trichrome Staining

Deparaffinized and rehydrated sections were stained with Mayer's hematoxylin solution for 10 minutes and rinsed in tap water. After 10 seconds in acid alcohol and rinsing in water, samples were stained with Fuschin Ponceau solution for 7 minutes. Sections were immersed in phosphomolybdic acid solution for 10 to 15 minutes and stained by light Green solution for 3 minutes. After 4 minutes in acetic water and washing in tap water, the samples were dehydrated and mounted with resinous mounting medium (XAM; BDH Laboratory Supplies, London, United Kingdom).

Immunohistochemistry

Deparaffinized and rehydrated sections were incubated for 30 minutes in 3% H₂O₂/PBS to quench endogenous peroxidase activity and then were rinsed for 10 minutes in PBS.

DSP Immunolabeling

Nonspecific protein binding was blocked by incubation for 30 minutes in 5% goat serum (DakoCytomation, Glostrup, Denmark) in PBS. Specimens were incubated for 1 hour at room temperature in a humidified chamber with a polyclonal rabbit anti-bovine Dentin Sialophosphoprotein (DSP) antibody (kindly provided by Dr. Larry Fisher, NIH, Ref LF 153), with a 1:5000 dilution. Sections were washed three times in PBS at room temperature before treatment for 30 minutes at room temperature with secondary biotin-labeled goat anti-rabbit IgG antibody at a dilution of 1:500 (Streptavidin Multilink Kit; BioGenex, Kent, United Kingdom). Subsequently, sections were incubated for 30 minutes at room temperature with peroxidase linked to avidin (Vectastain ABC kit; Vector Laboratories, Burlingame, CA).

Sclerostin Immunolabeling

Before staining, sections were rinsed and permeabilized with 0.1% trypsin (Life Technologies, Glasgow, United Kingdom) for 20 minutes. Nonspecific background was reduced by treating the sections with 5% normal horse serum (Irvine Scientific, Irvine, CA) in PBS for 30 minutes

at room temperature. The sections were then incubated with goat anti-mouse polyclonal sclerostin antibody (R&D Systems, Wiesbaden, Germany) diluted 1:40 at 4°C overnight. Slides were washed with PBS three times for 5 minutes, after which time they were incubated with a horseradish peroxidase-conjugated rabbit anti-goat Signalway antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 in PBS for 30 minutes at room temperature.

A negative control involving omission of primary antibody was performed on mouse tooth sections for all experiments. Osteocytes and odontoblasts were considered as a positive control for sclerostin and DSP, respectively. After rinsing with three changes of PBS, the immunoreactivity was visualized by development for 2 to 5 minutes at room temperature with 0.1% 3,3'-diaminobenzidine and 0.02% H₂O₂ (DAB substrate kit; Vector Laboratories). Sections were examined briefly by light microscopy (Leica DMRB, Leica Microsystems, Nanterre, France) for a first visualization. Then, all slides were rinsed and counterstained with Mayer's hematoxylin and mounted with permanent mounting medium (XAM; BDH Laboratory Supplies) and examined by the same light microscopy. For advanced analysis, images were performed by a digital camera (Leica Microsystems).

Cell Culture and *Msx2* RNA Interference Transfection

The MDPC-23 cell line, a generous gift from Drs. Carl T. Hanks and Jacques E. Nör (School of Dental Medicine, University of Michigan, Ann Arbor, MI) was established from immortalized preodontoblastic cells originating from fetal mouse molar dental papilla.²⁹ MDPC-23 cells have the ability to differentiate into odontoblasts. They were cultured in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotics (penicillin/streptomycin, 50 IU/mL; Life Technologies), in a humidified atmosphere at 5% CO₂ in a 37°C incubator (Binder, Tuttlingen, Germany). At the onset of the experiments, MDPC-23 cells were seeded at a concentration of 10,000 cells/mL. The cells were grown in the culture medium noted above until reaching about 40% confluency for 1 day. Cells were transfected with 0.2 µg/µL *Msx2* expression vector¹¹ according to the Effecten transfection protocol (Qiagen, Courtaboeuf, France). Similar empty plasmid was used as a control for transfection of cells. After 6 hours, the cells were rinsed with PBS, and the culture medium was renewed with fresh Dulbecco's modified Eagle's medium, supplemented with 25 nmol/L siRNA targeting *Msx2* (Eurogentec, Patton, France) by using an RNAmix lipofectamin kit according to the manufacturer's protocol (Life Technologies). After 24 hours, the medium was removed again, and the cells were washed with PBS and resuspended in initial culture medium for 24 hours. On the third day, the cells were collected and subjected to total RNA extraction with the Trizol reagent kit (Life Technologies).

Reverse Transcription and Real-Time Quantitative PCR Analysis

Total RNA was extracted using a Trizol reagent kit (Life Technologies) according to the manufacturer’s protocol. After total RNA quantification, equal amounts of RNA (1 µg) were transcribed into cDNA using Super-Script II RNase H Reverse Transcriptase (Life Technologies) with oligo-dT and hexanucleotide random primers. Gene expression levels were determined by real-time PCR using specific primers (Table 1). Quantification of the gene expression was performed using CFX Manager software version 3.0 (Bio-Rad Laboratories, Hercules, CA). Optimal conditions were determined for the genes of interest, and *GADPH* was used as a housekeeping gene. To analyze the data, the comparative threshold cycle (CT) method was applied, and relative expression levels were normalized to *GADPH*.

Statistical Analysis

Statistical significance was assessed by using the nonparametric *U*-test on dental mesenchyme data. *P* < 0.05 was considered statistically significant.

Results

Msx2 Effects on Tooth Morphology and Dentinogenesis

Morphologic analysis showed that tooth morphology was strongly disrupted in *Msx2*^{−/−} null mutant animals. At early postnatal stages (7 days), *Msx2*^{−/−} animals presented fairly normal dental development, albeit with flat cusps (Figure 1, A and B). At later stages (14 and 21 days until adulthood) the process of root formation had already started before 14 days in WT mice, whereas it was delayed in *Msx2*^{−/−} mice (Figure 1, C and D). Dentin deposition was affected in *Msx2*^{−/−} teeth, and the pulp chamber seemed larger than in WT mice (Figure 1, E and F). The odontoblast row was disorganized in mutants, and this abnormal polarization ended with cellular inclusion within predentin and dentin matrix (Figure 1, G and H).
In *Msx2*^{−/−} crown dentin, morphologic differences were most noticeable at the end of primary dentinogenesis and/or

the start of secondary dentinogenesis (Figure 2). Different layers were visible in the dentin thickness of *Msx2*^{−/−} mice at 21 days.
An abnormal osteodentin layer was delimited by orthodentin; massive cellular inclusions and defective matrix secretion were observed within this osteodentin layer. Sparse inclusions were present in the thickness of orthodentin deposited after osteodentin (Figure 2, B and C). Consistent with this pathology, the odontoblastic layer was highly disorganized, with some cells partially embedded in the predentin. Within mineralized dentin, most sites of cell inclusion were empty of material, suggesting a lack of vitality of these embedded cells, by lack of nutritive supply in contrast to what is observed for osteocytes in bone. At later stages, the osteodentin layer was ground down due to rapid abrasion of coronal dentin (data not shown). This abnormal dentin is similar to the calciotraumatic line that delimits tertiary dentin after trauma and odontoblast cell death in WT mice.²²
To better understand this disorganized odontoblastic layer, we analyzed the expression of DSP, one of the most expressed proteins in dentin. Hence, DSP was expressed in dentin of *Msx2*^{−/−} and WT mice (Figure 2). Odontoblast nuclei were rarely present inside the lacunae observed in *Msx2*^{−/−} mice, presumably in relation with the lack of nutrients and oxygen in odontoblast cells which are normally not entrapped in dentin. DSP immunoreactivity was systematically strong (Figure 2, A–G), suggesting that embedded odontoblast had been actively producing matrix peptides. The immunoreactivity marking cell lacunae described above were evident throughout the secondary dentin thickness and appeared to be randomly localized. In contrast, WT mouse teeth showed orthodentin without cell inclusions throughout the dentin thickness (Figure 2, H–J), and DSP was evenly distributed (Figure 2, K–M).
We then hypothesized that the distorted dentin phenotype was caused by the inhibition in osteoclast activity secondary to RANK ligand decreased production in *Msx2*^{−/−} mouse jaw. Hence, to investigate the putative rescue of *Msx2*^{−/−} mice by overexpressing the RANK ligand receptors in osteoclasts, the morphology of the first upper molars in WT, *Msx2*^{−/−}, and double mutant *Msx2*^{−/−}/RANK-Tg mice was analyzed. Indeed, the used promoter *Mrp8* has been

Table 1 Primer Sequences Used for RT-qPCR Analysis

Gene	Forward primer sequence	Reverse primer sequence
<i>GAPDH</i>	5'-ACCCCTTCATTGACCTCAACTAC-3'	5'-AGTTGTCATGGATGACCTTG-3'
<i>Msx2</i>	5'-CCTCGGTCAAGTCGGAATTC-3'	5'-CGTATAAGGATGCTGCTTGAG-3'
<i>Sost</i>	5'-CGTAGAACAACAGACCATG-3'	5'-ATCCGGGATGCAGCGGAAT-3'
<i>Dsp</i>	5'-TACTGGCCCTATCCAAGGTG-3'	5'-CCTCCTGTCTTGGTGTGGTT-3'
<i>Runx2</i>	5'-GGACGAGGCAAGAGTTTAC-3'	5'-TGCCTGCCTGGGATCTGTAA-3'
<i>Ocn</i>	5'-ACCTTATTGCCCTCCTGCTT-3'	5'-CTTGGTGCACACCTAGCAGA-3'
<i>Alpl</i>	5'-CCAGCAGGTTTCTCTCTTGG-3'	5'-CTGGGAGTCTCATCTGAGC-3'
<i>Col1a1</i>	5'-AAAAGGGTCATCGTGGCTTC-3'	5'-ACTCTGCGTCTTCCAGTCA-3'

RT-qPCR, real-time quantitative RT-PCR.

Figure 1 A–F: Histology of the first upper molar of WT (A, C, and E) and *Msx2*^{−/−} (B, D, and F) mice at 7 (A and B), 14 (C and D) and 21 (E and F) days postnatal; 7-μm thick sections of the first molar of WT and *Msx2*^{−/−} mice of different ages stained with hematoxylin and eosin. At 7 days, no pronounced differences in tooth morphology are evident, although the molars of *Msx2*^{−/−} mice have flat cusps (B, black dotted line) and reduced volume compared with WT (A). At 14 days, there are still no pronounced differences in morphology, but root growth seems to be initiated later in *Msx2*^{−/−} molar than WT (C and D, dotted arrows). At 21 days, the morphology of the first upper molar of *Msx2*^{−/−} mice is altered, exhibiting a shorter root and a 90° deviation of root growth orientation (E, arrows in F). G and H: The enlarged view of the Od predentin junction shows a disorganized row and cellular inclusions within matrix in *Msx2*^{−/−} molar compared with WT (G, arrows in H). Scale bars: 500 μm (A–F); 50 μm (G–H). Od, odontoblast; WT, wild-type.

evidenced as being efficient in the osteoclasts and their progenitors.^{14,15} At early stages, crowns of WT and *Msx2*^{−/−}/RANK-Tg mice shared a similar morphology, albeit with differences in their relative sizes (*Msx2*^{+/+} = *Msx2*^{−/−}/RANK-Tg > *Msx2*^{−/−}) (Figure 3, A–E). In adults, double Tg mice exhibited first upper molars with a relatively normal-length root and absence of deviation of the direction of mesial root growth, in contrast to *Msx2*^{−/−} mice (Figure 3, D and F). Finally, the osteodentin layer was absent in double Tg mice.

Modulation of Gene Expression Related to *Msx2* Gene Down-Regulation

Molecular analyses showed an increase of *DSPP* mRNA expression in *Msx2*^{−/−} mesenchymal cells. This held true also for *Runx2*, *Bglap*, and *Alpl*, whereas the level of collagen1 $\alpha 1$ mRNA was reduced (Figure 4A). *Runx2* and osteocalcin were significantly up-regulated, and collagen1 $\alpha 1$ was down-regulated after *Msx2* silencing *in vitro* (Figure 4B). *Sost*, as a Wnt inhibitor and a key factor in

bone-embedded osteocytes, was investigated. *Sost* was overexpressed compared with WT both in *Msx2*^{−/−} dental pulp mesenchyme (Figure 4C) and *in vitro* after silencing of *Msx2* in MDPC-23 cells (Figure 4D).

Wnt Inhibitors during Postnatal Tooth Development

Three Wnt inhibitors (*Dkk1*, *SostDc1*, *Sost*) expressed in early tooth development were explored here at the postnatal stage. Because we demonstrated that the *Sost* expression was increased in the absence of *Msx2* *in vitro*, we investigated sclerostin expression in postnatal teeth (Figure 5). This glycoprotein was present in odontoblasts and pulp mesenchyme of WT and at higher levels in *Msx2*^{−/−} mice during the first 7 postnatal days (Figure 5, A–D). Double mutants did not show this intense labeling (Figure 5, E and F). After eruption, sclerostin expression was not detectable in the mesenchyme of 3-month-old WT mice (Figure 6, A and B), whereas it remained expressed in odontoblasts of *Msx2*^{−/−} mice (Figure 6, A–D). In *Msx2*^{−/−} mice, sclerostin was continuously expressed postnatally. Maximal expression was

Figure 2 Dentin structure of a first upper molar from 21 days postnatal *Msx2*^{-/-} and WT mice showing different areas of the crown. **A:** *Msx2*^{-/-} mouse molars contained primary and secondary dentins, which were differentially affected (see enlargements in **B–D**). In *Msx2*^{-/-} molar, primary dentinogenesis (I in **B**) appears to be normal. Secondary dentinogenesis (II in **B**) is disturbed and appears like a combination of orthodontin and reparative dentin (osteodontin). The CL (**B**) delimits the primary normal dentin from the secondary. Cellular inclusions in the dentin or presence of vacuoles in the mineralized tissue thickness are noticeable (**B** and **C**, black arrow). A second wave of abnormal structure is visible in secondary dentin (**B** and **C**, red arrows), and the odontoblast layer is highly disorganized with cells partially embedded (**B** and **C**, blue arrows). Root growth direction is deviated and a 90° change in the mesial root orientation is noticeable (**A**, black arrow). DSP immunoreactivity is clearly visible in the embedded material in the dentin thickness (**D**, black arrows in **E**, **F** and **G**). In WT molar, dentinogenesis occurs normally with tubular orthodontin (**H–J**), and DSP immunoreactivity is clearly noticeable (**K–M**). Masson's trichrome staining (**A–C**, and **H–J**). DSP immunohistochemical analysis counterstained with hematoxylin (**D–G**, **K**, **L**, and **M**). Scale bars: 500 μm (**A**, **D**, **H**, and **K**); 100 μm (**B**, **C**, **E–G**, **I**, and **J**); and 80 μm (**L** and **M**). CL, calcitragic line; DSP, dentin sialophosphoprotein; WT, wild-type.

detected on odontoblasts of the extreme coronal and radicular positions. RANK overexpression in osteoclasts of *Msx2*^{-/-} was correlated with the disappearance of sclerostin labeling in adult molars of double mutant mice (Figure 5, E and F; Figure 6, E and F). Normalization of both eruption and tooth morphology of *Msx2*^{-/-}/RANK-Tg mice was associated with this sclerostin down-regulation.

Discussion

Our data support and extend to odontoblasts the known impact of *Msx2* on dental cells.^{10,12} *In vitro* and *in vivo*, we observed up-regulation of *Runx2*, *Bglap*, and *Dsp* in odontoblasts as a consequence of reduced *Msx2* expression levels. This is consistent with the known repressive

transcriptional activity of *Msx2* on mineralizing genes such as *Bglap* in bone⁸ or *Amelx* and *Calb1* in enamel.⁴ Thus, dentinogenesis regulation by *Msx2* might be secondary to particular cell-autonomous events targeting at least *Dspp*, *Bglap*, and *Runx2*.

As previously demonstrated for amelogenesis under *Msx2* inactivation,^{10,12} initial dentinogenesis was not seriously affected, although there was some distortion of cusp patterns.^{4,12} In contrast, at postnatal days 7 to 14, a pathologic osteodontin layer was deposited, characterized by entrapped odontoblasts able to secrete extracellular DSP but then disappearing, resulting in empty lacunae. After this transient event that simultaneously involved the entire odontoblast layer, the dentin exhibited hybrid features combining normal ordinary orthodontin morphology and sparse odontoblast inclusions reminiscent of tertiary osteodontinogenesis.

Figure 3 A–F: First upper molar in WT (A and D), *Msx2*^{−/−} (B and E), and *Msx2*^{−/−}/RANK-Tg (C and F) at 7 days (A, B and C), and 3 months (B, D and F) postnatal. No pronounced differences in morphology are evident at 7 days among WT, *Msx2*^{−/−}, and *Msx2*^{−/−}/RANK-Tg crowns, although *Msx2*^{−/−} molars exhibit flat cusps and reduced volume (A, C, and E). At 3 months, differences can be observed in *Msx2*^{−/−} molar, displaying short roots (D, red arrow) and deviation of the mesial root growth orientation (D, black arrows). A more developed (ie, WT-like) root elongation is noticeable in double Tg mice compared with *Msx2*^{−/−} mice (F, red arrow) with only the latter showing deviation of the mesial root (F, black arrows). Masson's trichrome staining. Scale bar: 500 μm. RANK, receptor activator of NF-κB; Tg, transgenic; WT, wild-type.

Furthermore, root dentin deposition was drastically reduced. This phenotype suggests that after initial primary dentin deposition, *Msx2*^{−/−} odontoblasts bypass secondary dentinogenesis and switch to a tertiary dentinogenesis pattern.

This distinct behavior of odontoblasts illustrates once more that, in fact, odontoblast cells can demonstrate a very heterogeneous phenotype, depending on their stage and location.^{18–30}

Figure 4 Molecular effects of deletion of *Msx2* in dental mesenchyme of 3-month-old mice (A and C) and silencing of *Msx2* in MDPC-23 cells (B and D). **A:** Quantitative RT-PCR analysis of different genes based on mRNA isolated from incisor pulp mesenchymal cells of 3-month-old WT and *Msx2*^{−/−} mice shows that increased expression of Runx2, Dspp, Ocn, and Alpl correlates with deletion of *Msx2*, together with a reduction of Col1a1 expression. **C:** An antagonistic effect on expression was revealed between *Msx2* and *Sost* according to the markers of differentiation in mesenchyme pulp cells. **B:** *In vitro* modeling using MDPC-23 cells odontoblast-like demonstrated an overall increase in cell differentiation markers after silencing of *Msx2* mRNA. **D:** After 3 days of RNAi transfection, *Msx2* mRNA drops by half, whereas *Sost* expression increases considerably compared with controls. **P* < 0.05. Alpl, alkaline phosphatase; Col1a1, collagen1α1; Ocn, osteocalcin; RNAi, RNA interference; WT, wild-type.

Figure 5 A–F: Immunohistochemical staining for sclerostin expression in the first upper molar from WT (A and B), *Msx2*^{−/−} (C and D), and *Msx2*^{−/−}/RANK-Tg (E and F) mice at 7 days postnatally counterstained with Mayer's hematoxylin. Sclerostin is expressed in the odontoblast layer of WT and *Msx2*^{−/−} molars but not in *Msx2*^{−/−}/RANK-Tg molars (B and D). The protein is localized in the odontoblast process and predentin, and there is also detectable expression in the dental pulp mesenchyme of *Msx2*^{−/−} mice. Sclerostin is expressed in osteocytes of the three genotypes (red arrows). Sagittal sections (7-µm thick) were counterstained with Mayer's hematoxylin. Scale bars: 500 µm (A, C, and E); 100 µm (B, D, and F). Od, odontoblast; RANK, receptor activator of NF-κB; Tg, transgenic; WT, wild-type.

In *Msx2*^{−/−} mice, we hypothesized that the odontoblast pathophysiology could be secondary to cellular stress during tooth eruption and subsequent crown abrasions in *Msx2*^{−/−} molars in the maxilla.⁷ Indeed, the deposition of osteodentin layer in *Msx2*^{−/−} mice during a specific developmental window to produce a calciotraumatic line in the crown suggests the occurrence of one simultaneous transient deposition event at the tooth organ level. Owing to its shared temporal window with the osteoclast activity peak during tooth eruption, we hypothesize that osteoclast inhibition in *Msx2*^{−/−} mice might be causative in this qualitatively and quantitatively distorted dentinogenesis pattern. To test this hypothesis, RANK overexpression was driven in the osteoclasts by crossing the *Msx2*^{−/−} mouse strain with the RANK-Tg strain. RANK overexpression has been previously shown to increase the number and efficiency of osteoclasts in the double mutant mice and rescue regional osteopetrosis as well as root elongation.¹⁴ In double mutant *Msx2*^{−/−}/RANK-Tg mice, we here observed that the aberrant osteodentin layer was not generated.

Thus, impaired osteoclast activity may play a role in *Msx2*^{−/−} odontoblast phenotype. In bone, it is known that osteoclasts control neighboring osteoblasts by releasing factors stored in the mineralized matrix such as transforming growth factor β or insulin-like growth factor 1.³¹ These growth factors are important in odontoblast differentiation.³² Furthermore, osteoclasts secrete clastokines controlling osteoblast cell fate and activity³³; the putative role on dental cells is however unknown. Osteoclasts being distant

from the odontoblasts, the interposed tissues and cells—dental pulp, epithelial root sheath, and follicular sac—might mediate intermediary signals.

However, a bony crypt size reduction secondary to osteoclast inactivation might modify biomechanical constraints on the tooth germ cells. The contribution of Wnt inhibitors known as being the major mechanical transducers in bone was explored here in bone-dentin cross talks. Indeed, i) harmonious Wnt–β-catenin signaling is crucial for root elongation and eruption; both Wnt activation and repression result in abnormal dentinogenesis (root elongation impairment, osteodentin generation, and eruption anomalies) as evidenced in rootless β-catenin mutants^{13,34} and in mice overexpressing either β-catenin³⁵ or the Wnt inhibitor Dkk1.³⁶ ii) *Msx2* controls osteoblast differentiation via regulation of the Wnt–β-catenin pathway by repressing Dkk1 expression.³⁶ iii) In early tooth development, Wnt pathway plays a major role. iv) Among Wnt inhibitors previously studied in tooth development (sclerostin,³⁷ Dkk1,³⁸ and ectodin³⁹), preliminary results (data not shown) indicated that only sclerostin is expressed in post-natal stages. In addition, we demonstrated the sclerostin expression in dental mesenchyme, transiently in WT, and continuously in *Msx2*^{−/−}. Sclerostin expression was physiologically down-regulated at the transition from primary to secondary dentinogenesis and/or during the crown/root developmental transition in control mice. *Sost*/sclerostin expression was found to be increased in the dental mesenchyme of *Msx2*^{−/−} mutants compared with controls.

Figure 6 A–F: Immunohistochemical staining for sclerostin in the first upper molar from WT (A and B), *Msx2*^{-/-} (C and D), and *Msx2*^{-/-}/RANK-Tg (E and F) 3-month-old mice. Sclerostin is not expressed in odontoblasts of WT mice (A and B) but is expressed and localized in the odontoblast layer in *Msx2*^{-/-} mice (C and D). RANK overexpression in *Msx2*^{-/-} mice (E and F) abrogates sclerostin expression in odontoblasts. Scale bars: 500 µm (A, C, and E); 100 µm (B, D, and F). RANK, receptor activator of NF-κB; Tg, transgenic; WT, wild-type.

Moreover, RANK overexpression in *Msx2*^{-/-}/RANK-Tg osteoclasts led to normalized sclerostin expression. Thus, our findings indicate that osteoclast impairment due to *Msx2* inactivation is a key factor for dentinogenesis control, in part by increasing sclerostin levels in dental mesenchyme.

Wnt inhibitor repression may end when the Wnt signal cascade proceeds in WT mouse root, whereas continuing *Sost* in *Msx2*^{-/-} mutants might lock the process. Within the bone tissue, *Sost*/sclerostin expression is modulated under biomechanical constraints,⁴⁰ its production by osteocytes being increased in the unloaded bone. By binding the lipoprotein receptor–related protein 5 and 6,^{41,42} sclerostin inhibits canonical Wnt/β-catenin signaling⁴³ and Wnt/β-catenin–dependent recruitment as well as differentiation of osteoblast progenitors proceeds. This study shows that in bone, sclerostin is produced by mature cells which retro-control their proper progenitors.⁴⁴ This is not the case for mature odontoblasts in secondary orthodentin. In all mineralized tissues, sclerostin is secreted by cells embedded within the mineralized matrix (chondrocytes in cartilage, cementocytes in cementum, osteocytes in bone⁴⁴). We did not find it to be the case in dentin. In fact, sclerostin expression in the odontoblasts might mediate bone and tooth cell interplays, which regulate harmonious tooth allometric growth and bone crypt enlargement. Biomechanical stress transmitted from

bone to teeth when eruption proceeds may elicit down-regulation of sclerostin levels, thus enabling Wnt/β-catenin activation in the mesenchyme. In addition, this may further trigger dentinogenesis through the recruitment and differentiation of odontoprogenitors.^{45,46} However, it may not be excluded that additional signals produced by osteoclast activity (growth factors released from bone and clastokines)³³ intervene.

Conclusion

Our data delineate a putative integrative regulatory path in the complex formed by tooth and bone: sclerostin down-regulation in odontoblasts induced by osteoclast activity modulations may lead the transitions from primary to secondary dentinogenesis and from crown to root growth.

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S.S. performed experiments; N.A., S.X.D., S.B., A.E.C., B.C., S.S., and A.B., analyzed data; and N.A., A.E.C., S.S., and A.B. wrote the manuscript.

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