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Role of RANKL (TNFSF11)-Dependent Osteopetrosis in the Dental Phenotype of Msx2 Null Mutant Mice

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Abstract

The Msx2 homeoprotein is implicated in all aspects of craniofacial skeletal development. During postnatal growth, Msx2 is expressed in all cells involved in mineralized tissue formation and plays a role in their differentiation and function. Msx2 null (Msx2²⁻/⁻) mice display complex craniofacial skeleton abnormalities with bone and tooth defects. A moderate form of osteoporotic phenotype is observed, along with decreased expression of RANKL (TNFSF11), the main osteoclast-differentiating factor. In order to elucidate the role of such an osteopetrosis in the Msx2²⁻/⁻ mouse dental phenotype, a bone resorption rescue was performed by mating Msx2²⁻/⁻ mice with a transgenic mouse line overexpressing Rank (Tnfrsf11a). Msx2²⁻/⁻ Rank²⁻ mice had significant improvement in the molar phenotype, while incisor epithelioma defects were exacerbated in the enamel area, with formation of massive osteolytic tumors. Although compensation for RANKL loss of function could have potential as a therapy for osteopetrosis, but in Msx2²⁻/⁻ mice, this approach via RANK overexpression in monocyte-derived lineages, amplified latent epithelial tumor development in the peculiar continuously growing incisor.


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Introduction

Mutations in muscle segment homeobox (MSX) transcription factors cause craniofacial malformations such as cleft palate for MSX1 and craniosynostosis (Boston type) for MSX2 [1]. An MSX2 mutation is associated with amelogenesis imperfecta [2], highlighting the importance of this protein in dental epithelial cell differentiation and function. The fact that MSX2 is required for normal dental epithelial cell fates is supported by the Msx2²⁻/⁻ mouse dental phenotype. These mutant mice present amelogenesis imperfecta and root dysmorphia associated with differentiation defects in epithelial cells (i.e., ameloblasts and defects in Hertwig epithelial root sheaths [HERS] and epithelial cell rests of Malassez) [3–5]. In addition, Msx2²⁻/⁻ mice display dentinogenesis imperfecta and regional and graded osteopetrosis from the first to the third molar, with inclusion of the mandibular third molar [3]. Molar inclusion can give rise to tooth ankylosis and odontogenic tumor formation [3–4], as described for other osteopetrosis mouse models [Src and nl mutants] [6–7]. However, the role of osteopetrosis in the multifaced dental phenotypes observed in Msx2²⁻/⁻ mice is unclear. Previous studies have suggested that osteopetrosis in Msx2²⁻/⁻ mutants could have two nonexclusive origins. First, because Msx2 is expressed during growth by a subpopulation of alveolar bone osteoclasts [3], this osteoclast subset may be missing in the null mutants. Second, gene expression of the key osteoclast differentiation factor RANKL is severely decreased in the dental epithelium and alveolar bone of Msx2²⁻/⁻ mice [3–5]. Therefore, in order to investigate the importance of osteopetrosis in Msx2²⁻/⁻ mouse dental defects, we developed a strategy to rescue bone resorption by overexpressing RANK in osteoclast precursors [8] of Msx2²⁻/⁻ mice. The phenotypes of different teeth were then analyzed in these mice.

Materials and Methods

Animal generation and sampling

Ethics statement: the Consultative Bioethics Committee for Health and Life Science has specifically approved the present study (CEEA-2011-32). Staff trained to perform in vivo studies did all of the experiments.

Msx2 knockout (KO) mice were generated by replacing the entire coding sequence of Msx2 with the bacterial LacZ gene [3]. Rank transgenic mice were generated by heterologous recombination of a cassette containing 3.2 kb of the human myeloid related
protein 8 (MRP8, also known as S100A8) gene promoter and the coding DNA sequence of the mouse Rank gene. Approximately 30 copies were inserted in tandem in the transgenic line [9].

Males that were heterozygous for the Msx2 gene mutation and overexpressed Rank were mated with females heterozygous for the Msx2 gene mutation in order to generate all possible Msx2 and Rank genotypes. The genetic background of all of the mice was CD1 Swiss. Mice were studied at 2, 3, 4, 8, 10, and 16 weeks, with at least three animals in each experimental group for a total of 147 animals.

**Microradiographs, histological analyses, tartrate-resistant acid phosphatase (TRAP) activity assays, and keratin 14 immunohistochemistry**

After anesthesia of the mice, intracardiac perfusions were performed with a fixative solution containing 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) pH 7.4. Complete fixation was ensured by immersion of the heads in fixative solution overnight at 4°C. After rinsing in PBS, the head halves (cut along the sagittal axis) were microradiographed on a Faxitron X-ray System (Frederick, MD, USA) at a focal distance of 56 cm for 20 min (power setting: 12 mA and 15 kV). The head halves were then processed for histology by decalcification at 4°C for up to 2 months (depending on the age of the samples) in a pH 7.4 PBS solution that contained 4% EDTA (Sigma) and 0.2% paraformaldehyde. After extensive washing in PBS, the samples were dehydrated in increasing concentrations of ethanol and toluene and were finally embedded in paraffin (Paraplast plus, Sigma). Serial frontal sections of the head halves were sliced with a microtome (RM 2145; Leica, Rueil-Malmaison, France). The 7-μm-thick sections were deparaffinized and rehydrated before being either stained according to a modified van Gieson protocol [9], assayed for tartrate-resistant acid phosphatase (TRAP) activity as previously described [9], or immunolabeled for cytokeratin-14. Briefly, after saturation for 1 h with 10% horse serum in 1×PBS, sections were incubated overnight at 4°C with anti-keratin-14 rabbit primary antibody (PRB-1535; Covance, Paris, France). After washing in 1×PBS, an anti-rabbit biotinylated secondary antibody (BA-1100; Vector Laboratories, Burlingame, CA, USA) was applied for 1 h. Sections were then washed, treated with streptavidin–alkaline phosphatase conjugate (Roche, Meylan, France), and stained with nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate (NBT/BCIP, Roche).

**Micro-computed tomography scanner imaging**

A micro-CT scanner (desktop Skyscan 1172; Skyscan, Aartselaar, Belgium) was used to provide three-dimensional images of mouse mandibles. This system is based on a cone-beam X-ray source. A spatial resolution that produced voxels that measure 6.7 μm per side was used. Acquisition parameters were 80 kV anode voltage and 100 mA for an exposition time of 4 s. A 0.25° rotation step was performed between two exposures. A total of five exposures were obtained for each angle, and means were calculated. For each mode, a 0.5-mm aluminum filter was installed in the beam path to block the softest X-rays and to increase the accuracy of the beam-hardening correction (BHC). Cross-sectional images were reconstructed with a classical Feldkamp cone-beam algorithm with NRecon (Skyscan). Three-dimensional reconstructions were achieved with the software package CTAn (Skyscan). A threshold between 40 and 140 was selected, because it provided the best image of the mandible and suppressed artifacts.

**RT-PCR and TaqMan array RT-qPCR analyses**

Dissections of 2-week-old mouse mandibles (five mice per group) were performed under a stereomicroscope in order to collect alveolar bones and incisor epithelia, as previously described [5]. Tissues were directly immersed in RNA extraction solution (Tri-React; Eurodexus, Soufflémétersheim, France), and the extraction was performed according to the manufacturer’s instructions. For classical RT-PCR, reverse transcription was performed on 1 μg of total RNA with Superscript II (Gibco, Cergy-Pontoise, France) and hexanucleotide random primers (Gibco), and PCRs were done with EurobioQia (Eurobio, Courtaboeuf, France), following the manufacturer’s instructions. The following sets of primers chosen in different exons were used: RANKL-Fw ATG TCT CTT GTC AGC TTG CT; RANKL-Rv GCT CAT AAT GCC TCT CCT G; Rank-Fw GCT CAT GAC CGT GA TGA AGA AG; Rank-Rv AGG GCC TTC GCC GAT GC; Rank-Fw GAG CAT CCG GCT TCT CCT GT; Rank-Rv TCG TGC TCC CTC TTC TCA TC; Opg-Fw GGA TGA TGT GGT GAT GTG CAG G; Opg-Rv CCG AGG CAA ACT GTC CAC CAA; Runx2-Fw GGA GCA GGA GAG AGG TTC TTG G; Runx2-Rv TGG TTC GGT GGT GCC TCT GT; Msx2-Fw CCA ACA AGG AAG CGG AGG A; Msx2-Rv TGG TGC TGC TGC TTC ACC ACC A.

PCR products were separated by 2% agarose gel electrophoresis and were photographed with a Bio-Rad Gel Doc XR camera (Bio-Rad, Marnes-la-Coquette, France).

For TaqMan quantitative RT-PCR arrays, reverse transcription was performed on 1 μg of total RNA with the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) and PCR was performed with a 7900HT Fast system real-time PCR apparatus using Taqman mouse immune arrays (Applied Biosystems) according to the manufacturer’s instructions.

**Statistical analyses**

Data were analyzed using a one-factor analysis of variance to assess the effects of genotype. As appropriate, post-hoc testing was performed using Fisher’s Protected Least Significant Difference (PLSD). Differences were considered significant at p<0.05. Data are presented as means ± standard error of the mean (S.E.M).

**Results and Discussion**

Analyses of Msx2+/− mouse molars revealed delayed tooth eruption and shortened roots (Fig. 1a, d, g) [3,9]. RANK overexpression on an Msx2+/− background (Msx2+/− Rank+) resulted in significant recovery of all molar eruption and root elongation processes, as revealed by the relative positions of the teeth and alveolar bone crest (arrows in Fig. 1a–c), full eruption of the third molar (square in Fig. 1g–h), and the greater length of the molar roots at day 14 comparatively to Msx2+/− mouse molar (square in Fig. 1d–f; Fig. 2a, b; Fig. S4c). Measures of Msx2+/−, Msx2+/− Rank+ and WT mouse mandible first molar mesial root length and width at 2 and 3 weeks, performed on histological...
sections (three animals by group) using Image-J software, confirmed that, at 2 weeks, roots are significantly longer (p<0.05) in Msx2<sup>2/2</sup> Rank<sup>Tg</sup> molars comparatively to Msx2<sup>2/2</sup> molars but remained shorter (p<0.05) than WT molars (Fig. 1J). Moreover, the root width was significantly reduced in Msx2<sup>2/2</sup> Rank<sup>Tg</sup> molars comparatively to Msx2<sup>2/2</sup> molars (p<0.01) as previously described [9] but was superior (p<0.01) to WT molars (Fig. 1J).

At 3 weeks, mandible first molar mesial root length and width were not significantly different (p>0.5) in Msx2<sup>2/2</sup> Rank<sup>Tg</sup> and Msx2<sup>2/2</sup> mice (Fig. 1J) but were respectively significantly lower (p<0.001) and higher (P<0.05) than WT mouse ones (Fig. 1J).

RANK overexpression resulted in a significant (p = 0.0014) increased osteoclast numbers at 2 weeks (Fig. 2), a better commitment of HERS cells in the labial area (Fig. 3g, h, j, k), and a normalization in the size (volume measured using Image-J software) of most of the epithelial cell rests of Malassez (Fig. 3a, c, d, f, Fig. S1). However, the root morphology of Msx2<sup>2/2</sup> mice was not completely restored. The roots remained shorter than in wild-type (WT) mice (Fig. 1g–j) [9]. Moreover, epithelial cyst-like structures that were occasionally observed in the lingual area of the mandibular first molar mesial root in Msx2<sup>2/2</sup> mice (Fig. S2a) were also present in the Msx2<sup>2/2</sup> Rank<sup>Tg</sup> mutants (asterisk in Fig. 2d), at an approximately similar frequency, suggesting that the origin of these cyst-like structures was associated with MSX2 loss.
of function in epithelial cells. Keratin-14 immunostaining showed that these structures were associated with apparent continuity between dental and oral epithelia (Fig. 3j) and the formation of a periodontal pocket (square in Fig. 3j enlarged in 3l). Interestingly, cyst-like structures were only observed in the lingual part of the root. This asymmetrical localization may be associated with a labial-lingual gradient of transcription and growth factor expression during tooth morphogenesis and initial histogenesis [10–11]. Indeed, MSX2 loss may affect the expression or function of other factors; for example, DLX2 is known to be a key MSX2 partner [12].

Another defect observed in the lingual root of Msx2−/− mice, independent of RANK overexpression, was the presence of a lacuna-like structure in the dentine at the crown-root transition site (squares in Fig. 3a, d enlarged in 3b, e, respectively). These structures were maintained in the adult (asterisks in Fig. S2b, c).

Similar to the molars, the defect in the root analog region of the incisors was improved by RANK overexpression, as reflected by a better commitment of HERS cells and the more typical size of the epithelial cell rests of Malassez (Fig. 3m–r). Strikingly, however, by 2 weeks, in the crown equivalent area of all incisors, the dental epithelium had converted into a massive osteolytic tumor (Fig. 4a–h). The tumor caused a deformation in the dentin (double arrows in Fig. 4f–h) and was associated with substantial resorption of the surrounding bone, as shown by the increased osteoclast numbers (Fig. 4b, f). The tumor caused total destruction of the mandible within 4 months (Fig. 5c). The increased osteoclasts around the incisor seemed to have a positive impact on tumor growth. This scenario is reminiscent of a previously described amplification loop between tumor cells and osteoclasts, which may occur in bone metastasis of several tumor types [13].

The MSX2 homeoprotein is a critical factor for epithelial cell commitment in various organs, including skin and skin appendages [3,14]. MSX2 misexpression was reported in tumors of these epithelial tissues in the context of bone metastasis [15] and osteolysis [16]. During bone resorption, MSX2 may positively regulate RANKL expression, as suggested by a reduction in Rankl expression in the dental epithelium of Msx2−/− mice (Fig. 6a).
[3–5] and similar expression in odontogenic tumors [17]. This regulation is of particular importance, because increased RANKL expression in tumor cells is directly correlated with hyperactive bone resorption [18]. To further elucidate how RANK overexpression promotes the conversion of Msx2−/− mouse incisor epithelium into massive tumors, expression levels of Rankl, Opg (Tnfrsf11b), Rank, and various inflammation markers were comparatively analyzed in the epithelium of 14-day-old WT and Msx2−/− mice that lacked or expressed the Rank transgene (Fig. 6a, b). Rankl, Rankl, and Opg expression were detected in WT mouse incisor epithelium. In contrast, in Msx2−/− mouse incisor epithelium, Rankl and Rank expression decreased but Opg expression increased (Fig. 6a), in accordance with the previously described osteopetrotic phenotype [3]. In Rank−/− mouse incisor epithelium, Rankl and Opg expression was increased and Rankl expression decreased compared to WT epithelium (Fig. 6a), as previously reported [9]. These variations are explained by the more advanced stage of tooth eruption [9]. In the Msx2−/− RankTg mouse epithelium, Rankl and Rank expression was increased and Opg expression decreased compared to Msx2−/− mouse epithelium (Fig. 6a). These variations are consistent with the observed augmentation in the surrounding alveolar bone resorption at 2 weeks (Fig. 2a, b). Interestingly, transgene expression was observed only in Msx2−/− RankTg mouse epithelium (Fig. 6a), suggesting that cells within the tumor mass were expressing the transgene; these cells might correspond to monocyte-derived cells. To further characterize the immune cells infiltrating the epithelial tumor, TaqMan inflammation/cancer array analyses were performed. Elevated signals for P2y1, Nos2, and Tbx21 (Fig. 6b; Fig. S3) are indicative of intra-tumoral T helper type 1 cytotoxic cells, in

Figure 3. Effect of transgenic Rank on lower first molar and incisor root formation in Msx2−/− mice. Van Gieson histology staining (a–f) and keratin immunohistochemistry (g–r) were respectively performed on mandibular frontal sections of 3- and 2-week-old Msx2−/− mice either overexpressing or not expressing transgenic Rank. At 3 weeks, Rank overexpression had induced a normalization in the size of most epithelial cell rests of Malassez (Ma) (a and c versus d and f), and at 2 weeks it had induced a better commitment of Hertwig epithelial root sheath (HERS) cells, specifically in the labial area (j and k versus g and h). Occasionally and independently of Rank overexpression, epithelial cyst-like structures were observed in the lingual area of Msx2−/− mandibular first molars (j, i). Cytokeratin-14 immunolabelling revealed that these cyst-like structures were associated with abnormal continuity between dental and oral epithelia (j) and the presence of a periodontal pocket (square in j enlarged in l). Another defect observed at 3 weeks in the lingual root of Msx2−/− mice, also independent of Rank overexpression, was a lacuna-like structure in the dentine facing the site of transition between crown and root epithelia (squares in a and d enlarged in b and e, respectively). In the incisor root equivalent, normalization of the size of the epithelial cell rests of Malassez (Ma) was observed in Msx2−/− RankTg mice (squares in m versus p enlarged in n and o and q and r, respectively).

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Figure 4. Effect of *Rank* overexpression on lower incisors of *Msx2*−/− mice. Mandibular microradiographs (a, e) and TRAP activity assays (b, f) or van Gieson histology staining (c, d, g, h) of mandibular frontal sections were performed to characterize the effect of *Rank* overexpression on the lower incisors of 2-week-old *Msx2*−/− mice. Substantial enlargement of the area between the basal bone and the dentin was observed in *Msx2*−/− *RankTg* mice (e) compared to *Msx2*−/− mice not expressing *RankTg* (a). This enlarged area, which corresponds to the incisor epithelial compartment, was associated with abnormal curvature in both basal bone (red dotted line) and dentin (D). Mandibular frontal sections through the first (M1), second (M2), and third (M3) molar planes revealed that the enlargement corresponds to an epithelial hypertrophy with the presence of an internal necrosis-like area (asterisks in f–h). In *Msx2*−/− incisors, no hypertrophy of the epithelium was visible, but this tissue was disorganized and lacked the ameloblastic palisade structure (arrows in b–d). There was also a substantial increase in the number of osteoclasts around the incisors of *Msx2*−/− *RankTg* mice (f) compared to *Msx2*−/− mice not expressing *RankTg* (b). Moreover, the thickness of the mandibular basal bone in the *Msx2*−/− *RankTg* mutants appeared highly reduced compared to *Msx2*−/− mice not expressing *RankTg* (arrowheads in f–h versus b–d).

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Figure 5. Combined effects of loss of *Msx2* and *Rank* overexpression on mouse mandibular bone phenotype. Microradiographs and scans of 16-week-old WT (a), *Msx2*−/− (b), and *Msx2*−/− *RankTg* (c) mouse skulls were performed to compare characteristics of the bone of the mandible. While the *Msx2*−/− mouse mandibular features (b) presented no major alterations compared to WT animals (a), *Msx2*−/− *RankTg* mice had marked disruptions in the architecture of the mandibular bone (c). These disruptions were either mono- or bilateral and were associated with conversion of the incisor epithelium toward massive osteolytic tumors (asterisks in c). Basal bone around these tumors was thinner (arrows in c), porous (arrow in c), and displaced, as seen in the upper view of the mandibular scan (c).

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addition to monocyte-derived cells that are likely recruited and maintained by CSF1 (Fig. 6b). Cytotoxic cells may also be natural killer (NK) cells, which would correspond with the observed unaltered CD8α expression levels (Fig. S3) and increased transcription of genes encoding factors such as IL12A and CXCL10 (Fig. 6b; Fig. S3), which are known to stimulate NK cell chemotaxis and differentiation [19]. In response, NK cells produce IFNG, TNF, CSF2, CCL3, and CCL5 [19], which are all up-regulated in Msx2<sup>2−/−</sup> RankTg<sup>+</sup> mouse epithelium (Fig. 6b; Fig. S3). Further studies will be necessary to unravel the mechanistic relationship between inflammatory cell recruitment and epithelial tumor activation, and the relationship between tumor growth and RANKL expression. Keeping in mind that cells of the monocyte/macrophage lineage were present in the epithelial tumor (Fig. 6a), the recent finding that monocytes control NK cell differentiation in the context of antitumor immunity [20] constitutes an interesting basis for future studies. The epithelial tumor growth resulted in marked resorption of the surrounding alveolar bone, likely due to increased osteoclast numbers (Fig. 4f). Interestingly, Runx2 and osteocalcin transcripts remained stable, indicating unaltered bone apposition (Fig. 6c). The rise in osteoclast numbers is likely the result of the marked increase in Rankl expression (Fig. 6c). Analysis of different immune cell-lineage markers (Fig. 6e) suggested that cells of the myeloid lineage were increased in Msx2<sup>2−/−</sup> RankTg<sup>+</sup> mouse alveolar bone. There also appeared to be an increase in the cytotoxic T lymphocyte population, as suggested by increased CD8α and CD68 expression (Fig. 6d, e). These data and the high expression levels of B1a, B6, B17 Ccl5, Ccr4, and Ccr7 (Fig. 6d) provide evidence for enhanced bone loss through inflammation, as described in other pathologies [21]. CXCL10 functions as a chemokine for monocytes and is implicated in osteoclastogenesis [22–25], with possible crosstalk with RANKL [24]. Thus, CXCL10 production may constitute a key element in the massive osteolytic epithelial tumor development observed in Msx2<sup>2−/−</sup> RankTg<sup>+</sup> mice by fostering an amplification cycle between tumor growth and alveolar bone resorption. CXCL11 was shown to inhibit osteoclastogenesis by a mechanism independent of its CXCR3 receptor [26]. CXCL11 should therefore interfere with increased bone resorption and tumor growth. On the other hand, CXCL11 is also known to activate T lymphocytes [27], which could amplify inflammation of the bone environment and adjacent epithelia, where increased Cxcr3 transcriptional activity was observed (Fig. S3).

In addition to above described effects of RANK over-expression on Msx2<sup>2−/−</sup> dental phenotype, benefic effects of such over-expression have also been observed in other skeleton sites known
to be affect in Msx2−/− mouse (Fig. S4). For instance, the characteristic open foramen of Msx2−/− mouse skull was partly closed in RANK over-expressing mutant (Fig. S4a). Similarly, the Msx2−/− mouse tibia that presented features of soft osteopetrosis switched to rather osteopenic bone in RANK over-expressing mutant (Fig. S4b). Nevertheless, other skeleton defects associated to MSX2 lost were poorly improved by RANK over-expression as the tibia length that remained shorter than WT mouse one (Fig. S4b).

**Conclusion**

In conclusion, resorbing bone resorption in Msx2−/− mice by overexpressing RANK in the osteoclastic lineage allowed for the correction of a substantial portion of the molar abnormalities, most likely by counteracting the decrease in RANKL expression, which is correlated with Msx2−/− osteopetrosis. From a more general viewpoint, our results indicate that functional compensation may be a promising approach for the treatment of osteopetrosis. However, in this mouse model, in which Msx2 was not expressed and RANK was overexpressed, and which features continuously growing incisors, precocious formation of a massive and osteolytic odontogenic epithelial tumor was observed.

**Supporting Information**

**Figure S1** Comparative analysis of epithelial rest of Malassez sizes in roots of wild type, Msx2−/−, and Msx2+/− RANK−/− mice. Whatever the age considered, the RANK over-expression in the Msx2−/− mouse normalized the size of the rest of Malassez. Measures were realized as previously described [5] using Image-J software. (TIF)

**Figure S2** Van Gieson staining of Msx2−/− mouse mandible first molar frontal sections at 3, 4 and 16 weeks. The presence of a cyst-like structure at the root lingual surface was observed at 3 weeks (a). At 4 and 16 weeks lacunae in the dentin area facing the site of transition between crown and root epithelium was present (asterisk in b–c). D: dentine; PDL: periodontal ligament; P: pulp. (TIF)

**Author Contributions**

Conceived and designed the experiments: BC CM AB FL. Performed the experiments: BC YS DF FL. Analyzed the data: BC CM AB FL. Contributed reagents/materials/analysis tools: DF BR CM. Wrote the paper: BC AB FL.

**References**


