Osteomodulin down-regulation is associated with osteoarthritis development

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Article
Abstract

Abnormal subchondral bone remodeling leading to sclerosis is a main feature of osteoarthritis (OA) and Osteomodulin (OMD), a proteoglycan involved in extracellular matrix mineralization, is associated to the sclerotic phenotype. However, the functions of OMD remain poorly understood, specifically in vivo. We used knock-out and overexpressing male mice for Omd and mutant zebrafish to study its roles in bone and cartilage metabolism and in the development of OA. The expression of Omd is deeply correlated to bone and cartilage microarchitectures affecting the bone volume and the onset of subchondral bone sclerosis and spontaneous cartilage lesions. Mechanistically, OMD binds to RANKL and inhibits osteoclastogenesis; thus controlling the balance of the bone remodeling. In conclusions, OMD is a key factor in subchondral bone sclerosis associated with OA. It participates in bone and cartilage homeostasis acting on the regulation of osteoclastogenesis. Targeting OMD may be a promising new and personalized approach for OA.

Introduction

Osteoarthritis (OA) is a degenerative joint disease with a high prevalence affecting 527.8 million people worldwide in 2019. OA, being a major source of handicap, is a public health challenge and a rising societal burden due to the aging population and increasing life expectancy. OA is a heterogenous disease originating from multifactorial causes with different subtypes of patients linked to distinct phenotypes. It is associated with pathologic changes in all the joint tissues including subchondral bone, cartilage, meniscus and synovium. One of the main OA features is subchondral bone sclerosis that results from impaired subchondral bone remodeling driven by excessive mechanical loading. Bone sclerosis is associated with abnormalities to bone matrix biochemistry and mechanical properties that contribute to OA physiopathology. Among these abnormalities, loss of matrix elasticity, abnormal mineralization, modification of the proteomic landscape with impaired cytokine production such as increased Transforming growth factor β and Interleukin 6 levels, overexpression of proteases, and decreased synthesis of small proteoglycans are well documented. These changes are associated to the “bone-driven” OA phenotype.

The Small Leucine-rich Proteoglycans (SLRPs) are intricately related with bone's physical properties and can be used as a fingerprint of its health status. The majority of SLRPs control the organization of the collagen fibrils and, through the extracellular matrix (ECM), interact directly with cytokines acting as a reservoir and a regulator of their bioavailability. Mice deficient for the SLRPs biglycan, fibromodulin, epiphycan, lumican, and chondroadherin demonstrated the protective role of these proteoglycans on the bone and cartilage matrix or osteoblasts; many of the knock-out mutants showed premature or more pronounced OA. In contrast, the knock-out for opticin was associated with an inhibition of cartilage damage in an OA model. Until now, the role played by osteomodulin (OMD), also known as osteoadherin, in OA physiopathology is poorly documented.
OMD is a keratan sulfate proteoglycan, and a member of the SLRP family. OMD was originally isolated and characterized from bone and shown to be strongly expressed by osteoblasts\textsuperscript{26–28}. Even though OMD is considered to be mainly expressed in bone, its expression has been observed in other cell types such as articular chondrocytes and fibrochondrocytes\textsuperscript{29}. It is involved in the mineralization process by binding to osteoblasts through the $\alpha V\beta 3$ integrin and by stabilizing Bone morphogenetic protein 2 (BMP2) ligands on their membrane receptors\textsuperscript{28,30,31}. A secretome analysis comparing osteoblasts from sclerotic and non-sclerotic areas of OA patients performed in our laboratory has shown that OMD is one of the major proteins downregulated by sclerotic osteoblasts in culture\textsuperscript{8}. Mature osteoblasts show enhanced expression of $OMD$ when osteoclast activity is increased [29].

For the first time, knock-out mice for $Omd$, here referred to as KO, and mice with $Omd$ gain-of-function in osteoblasts, hereafter referred to as UP, were used to decipher the roles of $Omd$ on bone remodeling and OA physiopathology. We followed the development of OA in aging mice and after the destabilization of the medial meniscus (DMM). We have focused on the subchondral bone as a lack of OMD was reported to be related to bone sclerosis\textsuperscript{8}. In addition, we used the zebrafish model to study the role of $omd$ in development and in bone remodeling. Finally, using in vitro models we deepened our investigation on the relationship between OMD and osteoclastogenesis.

**Results**

**General growth characteristics of Omd KO and UP mice**

$Omd$ KO mice had a lower weight and body size compared to the WT, only at 4 months. UP mice had a smaller weight than the WT at 4 months while their body size was not significantly different (Supplemental Fig. 1A, 1B). At 8 and 16 months, weight and size were similar in all genotypes (Supplemental Fig. 1A, 1B). UP mice displayed a longer femur than the KO at 8 months. The femoral length evolved differently over time between the genotypes with the UP reaching the mature size the soonest. Their femoral length was significantly increased at 8 months compared to the length at 4 months. At 16 months, each genotype reached a similar femoral length (Supplemental Fig. 1C, 1D).

**OMD is mainly localized in bone and calcified cartilage in mouse knee joint tissues**

We have performed immunohistochemical detection of OMD in the knee joint of 4, 8, and 16-month-old mice. OMD was present at all ages in WT and UP mice but absent in KO mice indicating the specificity of the immunostaining (Fig. 1A). OMD was strongly localized in the calcified cartilage ECM, while heterogeneous and light staining was also observed in the deep zone of the uncalkified articular cartilage ECM and in some chondrocytes (Fig. 1A, 1B). In bone, the lining cells were strongly stained as well as the ECM, mostly the mineralization front (Fig. 1A, 1C, 1D). The ECM and some cells in the meniscus were stained (Fig. 1A). The cartilaginous ECM of the growth plate was not stained (Fig. 1C).
Omd influences bone and cartilage microarchitectures

Effect of Omd on articular cartilage structure

Histological analysis revealed that the tibial growth plate significantly decreased between 4 and 8 months in all genotypes, but further decreased between 8 and 16 months only in the WT but not in other genotypes. At 16 months, the growth plate of the KO was larger than in the WT (Fig. 2A, 2B).

In the 4-month-old KO mice, the calcified cartilage layer was thinner in the medial tibial plateau and thicker in the tibial lateral plateau than in the WT (Fig. 2C, 2E). The ratio of calcified cartilage/total cartilage for the medial tibial compartment of the KO was significantly lower than in the WT and UP mice at each time point (Fig. 2D). In the tibial lateral plateau, this ratio was higher in the 4-month-old KO and UP mice than in the WT and in the 16-month-old KO than in the UP mice (Fig. 2F). Further, this ratio decreased with age in the KO and UP genotypes while it remained stable in WT. The thickness of the cartilage (including non-calcified and calcified cartilage) was not different between genotypes except in the medial plateau of 8-month-old KO mice, in which cartilage was thinner than in WT (Supplemental Fig. 2).

Effect of Omd on bone structure

Metaphysis of the tibia

The total volume of the trabecular bone was lower in the KO than in other genotypes (Fig. 3A, 3B). The trabecular BV/TV ratio was not significantly different between genotypes at 4 months. In contrast, this ratio was significantly higher in the KO than in the UP mice at 8 and 16 months and lower in UP mice than the WT at 8 months (Fig. 3A, 3B). The number of trabeculae of the KO was higher at 8 and 16 months than the WT and UP while no difference was observed at 4 months (Fig. 3C). The UP had significantly fewer trabeculae at 16 months compared to the WT. The porosity was lower in the KO and higher in the UP than in WT at all ages. The porosity was significantly lower in the KO at 8 and 16 months than in the UP (Fig. 3C). The space between trabeculae was greater in UP than in KO mice at 16 months but no difference between genotypes was observed for the trabecular thickness. At 16 months, the structure model index of the UP mice was significantly higher than in another genotype which indicated a shift from a plate to rod-like geometry of the trabecular bone (Supplemental Fig. 3A).

In the cortical bone, the BV/TV ratio was higher in the KO compared to the UP mice at 8 and 16 months and to the WT at 16 months (Fig. 3D, 3E). The cortical bone thickness increased with age in all genotypes but was significantly higher in the KO relative to the UP at 8 months and then relative to the WT and UP at 16 months (Fig. 3F). Cortical bone porosity was also affected by Omd expression. The porosity was consistently the lowest in the KO and the highest in the UP mice. The cortical bone porosity was significantly lower in KO compared to the UP mice at 8 and at 16 months and then compared to the WT at 16 months (Fig. 3C, 3F). In addition, the tibial crest was longer in KO than in WT and UP (Supplemental Fig. 3B).
Epiphysis of the tibia

KO mice had a higher lateral subchondral bone BV/TV ratio than the UP mice at 8 and 16 months and compared to the WT at 16 months. In the medial tibial plateau, BV/TV was more elevated in KO mice than in UP mice but only at 16 months (Fig. 4A, 4B). Computed tomography illustrated that bone volume was higher in the KO than in the WT and the UP mice (Fig. 4C).

Omd deficiency leads to a thicker and less porous bone at the tibia epiphysis and subchondral bone sclerosis. In contrast, overexpression of Omd by osteoblasts was associated with a decrease in the trabecular number and an altered trabecular shape. The differences between genotypes were clearly exacerbated in older mice.

Omd prevents articular degradation and subchondral bone sclerosis

To study the role of Omd in the pathology of OA, we compared the spontaneous development of bone and cartilage structural changes in KO, WT and UP mice during their aging, but also after destabilization of the median meniscus. In unoperated mice, cartilage lesions appeared with aging and a higher OARSI score was observed in the medial tibial plateau of KO mice than in WT (Fig. 5A). This observation was consistent with the fact that the loss of proteoglycans was significantly different for the medial tibial plateau (Fig. 5B, 5C). No differences were observed in the lateral tibial plateau and femoral condyles in 16-month-old mice. In the DMM model, the lesions of the medial tibial plateau were severe and no difference between genotypes was observed (Fig. 5D, 5G). Cartilage lesions were less severe in the lateral tibial plateau and KO mice tended to have a greater OARSI score than the WT (Fig. 5D, 5G) but the difference was not significant. No significant difference was observed for the scored loss of proteoglycan (Fig. 5E).

In WT with DMM-induced OA, the BV/TV ratio of the subchondral bone of the medial tibial plateau was significantly higher than in non-operated mice while it was not affected in the lateral plateau. Comparison of the medial subchondral bone BV/TV ratio between genotypes showed that it was lower in UP mice than the WT (Fig. 5F, 5G). These results suggest that the expression of Omd helps to prevent the development of subchondral bone sclerosis associated with OA.

Loss of Omd expression induced gait abnormalities in mice

The gait pattern of mice was assessed at all ages using the CatWalk XT platform. We reported different types of gait parameters. At 4 months, the print area was smaller in the KO than in the WT, and at 8 months, the print area was smaller in the KO than in the WT and UP mice. The difference between KO and other genotypes was not significant anymore at 16 months. The swing was shorter in KO mice than in other genotypes at 8 and 16 months. Finally, the intensity of the contact of paws toward the glass platform was higher in the KO compared to the WT at 8 and 16 months and compared to the UP at 8 months (Fig. 6 & Supplementary Table 1).
**omd** is expressed in the zebrafish skeleton and its mutation induces articular cartilage lesions and impaired bone remodeling

The zebrafish genome presents a single homolog to the human *OMD* gene, the ortholog *omd* encoding a 401 amino acids protein presenting 46% identical and 63% similar amino acids.

We characterized the localization of *omd* expression in larvae zebrafish using whole-mount *in situ* hybridization at 48-hpf, 5-dpf and 8-dpf. We observed a strong expression of *omd* specific to craniofacial cartilages including the jaw joint during the development (Supplemental Fig. 4).

To gain first insights into the function of Omd in zebrafish cartilage development, we studied the overexpression of *omd* by microinjecting 0.4ng and 0.8ng of its mRNA into zygotes. At 24-hpf, *omd* induced a ventralization of embryos that was not observed upon microinjection of control GFP mRNA (Supplemental Fig. 5A, 5B). Further, larvae injected with *omd* developed deformities at 4-dpf, mostly affecting the axial symmetry. Larvae presenting axial deformities demonstrated evident cartilage defects with abnormal development of the craniofacial cartilage (Supplemental Fig. 5C). As *omd* overexpression was inducing developmental defects, we designed a zebrafish *omd* mutant line (*omd*−/−) for further characterization in adult. *in situ* hybridization of *omd*−/− individuals revealed that the mutation led to the absence of *omd* mRNA in the craniofacial structures (Supplemental Fig. 4), indicating that no Omd protein was produced in the mutants.

We then compared the jaw joints in one-year-old *omd*−/− zebrafish to those in WT to detect cartilage damage. For the palatoquadrate, the OARSI score of mutants (ranging from 1 to 3) was greater than in the WT, and clefts on their articular cartilage were observed (Fig. 7A). We then studied cathepsin k expression by osteoclasts in the regenerating caudal fin at 7 days post-amputation. The cathepsin K production was significantly higher in *Tg(ctsk:Citrine); omd*−/− indicating that more osteoclasts were generated in the absence of *omd* expression (Fig. 7B). Furthermore, osteoclast activity was studied in elasmoid scales through TRAP staining. More TRAP staining was present on elasmoid scale of the *omd*−/−. The staining appeared to be more evenly distributed throughout the scales of the mutant and particularly localized on the edges and along the grooves of the scale. The circularity of the scales was also impacted. The scales of the *omd*−/− were more circular than in the WT (Fig. 7C).

**OMD inhibited osteoclastogenesis by binding to RANKL**

We investigated the effects of the treatment of OMD on gene expression in cultured human primary trabecular osteoblasts. The RNA-seq revealed that only 35 genes (with padj < 0.05) were differentially expressed after the OMD 10 ng/ml treatment, and with relatively modest fold-changes to expression (Supplemental Fig. 6 & Supplementary Table 2). GSEA analysis using webgestalt on GO terms revealed an increase of some genes linked to the response to acid chemicals (genes *AKR1C1, AKR1C2, AKR1C3*) and a decrease of few genes involved extracellular structure organization (GO:0043062) and ossification (genes *ACAN* and *IBSP*), and of the molecular function of actin binding. The Reactome database revealed
an up-regulation of genes responsible of collagen network degradation and a down-regulation of ECM proteoglycans, ECM organization, collagen formation, and integrin cell-surface interactions.

These observations led us to explore the interaction of OMD with RANKL, which is the regulator of osteoclast differentiation, using a solid phase binding assay. These experiments revealed a clear interaction between the two proteins, which correlated to the increasing amounts of both OMD and RANKL, demonstrating that they bind directly to each other (Fig. 8A). The potential biological effects of this interaction were tested on primary murine osteoclasts culture. We showed that OMD added at 10ng/ml and 40ng/ml reduced osteoclast number. No difference between 10 and 40 ng/ml of OMD was observed, both reducing the osteoclast number by 50% on average (Fig. 8B). We propose a model where osteoblasts secrete OMD in the ECM to trap RANKL and prevent it to bind to the pre-osteoclast receptor RANK in order to inhibit their differentiation into fully committed osteoclasts (Fig. 8C).

Discussion

OMD is a small proteoglycan involved in bone and dental matrix mineralization but also in ectopic mineralization of other tissues, such as in arteries, suggesting it could be involved in cartilage mineralization and degradation during aging and OA.

We have previously demonstrated that osteoblasts located in the sclerotic area of OA subchondral bone produced less OMD than neighboring osteoblasts coming from non-sclerotic area. Interestingly, OMD levels were also lower in the serum of OA patients. To study the impact of Omd expression on bone remodeling, skeletal development and architecture, we followed mice deficient for Omd and mice overexpressing Omd during 16 months.

While the presence of OMD in bone has been previously reported, we showed for the first time that OMD is localized in mineralized tissues of the murine knee joints and is identified in calcified cartilage and tidemark. Interestingly, we observed that the calcified cartilage layer was thinner in the medial tibial compartment but thicker in the lateral tibial compartment of KO mice than in other genotypes indicating that OMD plays a key role in cartilage mineralization. The consequences of calcified cartilage thickness on cartilage degradation in OA remain controversial. One study showed that the calcified cartilage was thinning with OA, resulting in the reduction of the cartilage elastic modulus. However, other studies showed that the calcified cartilage thickness increased with the progression of OA. The presence of the more severe cartilage lesions in the medial tibial plateau of aging KO mice, where the calcified cartilage was thinner, supports the hypothesis that a thinner layer of cartilage calcified is a factor promoting cartilage degradation. Of course, this theory needs to be confirmed in other models. In the DMM OA model, there was no significant difference in cartilage damage between genotypes. This finding contrasted with the observation performed in the aging KO mice in which the cartilage lesions severity were higher in KO mice than in other genotype. This observation can be explained by the higher severity of the lesions in DMM model reflecting more of a late stage of OA. We can anticipate a floor effect in DMM-induced OA model because the cartilage lesions were too severe.
At the bone level, 8 and 16-month-old KO mice had more trabecular and cortical BV/TV than the WT while, inversely, UP mice had a reduced ratio. This finding highlights that \textit{Omd} plays a key role in bone remodeling. More precisely, keeping the homeostatic expression of \textit{Omd} helps preserve its volume and structure. \textit{Omd} overexpression not only reduced BV/TV but also increased the structure model index, which is an indicator of the altered shape of trabeculae, and higher bone porosity. Over time, aging was aggravating those observations. This indicates that, when overexpressed, \textit{Omd} may turn to cause detrimental effects on skeletal tissues. In the KO, the global bone morphology was affected. Their tibia was narrower and their tibial crest longer, this morphological change may affect muscle insertion and, by so, the muscle to bone relationship. Further, KO mice were more prone to spontaneously develop subchondral bone sclerosis, as indicated by higher BV/TV, like in sclerotic subchondral bone in OA.

We also observed sclerosis of the subchondral bone following the DMM procedure, in all the genotypes. Yet, the subchondral bone of the medial tibia of UP mice was thinner than the KO and WT mice suggesting that \textit{Omd} could prevent subchondral bone sclerosis in OA. These data suggest that \textit{Omd} is deeply involved in subchondral bone sclerosis in OA, a key feature of OA involved in cartilage degradation. Therefore, we can hypothesize that the impact of \textit{Omd} on cartilage degradation could be secondary to its effect on bone.

The gait analysis with the Catwalk XT identified different motor patterns between the genotypes. The gait pattern of KO mice, including the print area, the swing, and the intensity of the contact of paws toward the glass platform, were modified. More precisely, for their hind paws, KO mice had a lower print area, a shorter swing, and a higher intensity of the contact of the paw. This may result in pain, discomfort, or of mechanical disorders associated with joint damage or with skeletal tissue abnormalities. It is important to highlight that a decreased hind print area is considered the best predictor for spontaneous OA. On the other hand, the different gait patterns of the KO could be the result of OA development leading to abnormal loading on affected limbs.

To corroborate our findings from the mice model, we studied mutant adult zebrafish which did not express \textit{omd}. In zebrafish, we found cartilage lesions in the articular cartilage in the jaw joint. Again, this suggests that \textit{omd} prevents spontaneous cartilage lesions during aging and that a decrease of \textit{omd} production by osteoblasts and hypertrophic chondrocytes could be deleterious for cartilage. Altogether, these findings support that a loss of OMD contributes to OA development. We have then investigated by which mechanism of action OMD could regulate bone and cartilage metabolism. Our transcriptomic data revealed that OMD is unlikely to perform its function on osteoblasts through direct gene expression regulation as very few genes were modified and with a low magnitude. It remains noteworthy to specify that among the regulated genes, \textit{IBSP} was downregulated by OMD. \textit{IBSP} overexpression by hypertrophic chondrocytes is associated with OA. Consequently, OMD could control cartilage calcification in OA by downregulating IBSP production.

Direct binding of OMD to key bone regulatory factors is another possible mechanism of action. SLRPs are known to bind cytokines, growth factors, and ligands like RANKL. Herein, we showed that OMD is...
not only enhancing the differentiation of osteoblasts \textsuperscript{31}, but is also able to bind directly to RANKL and block its biological activity on osteoclasts. The mutant zebrafish model confirmed the role of \textit{omd} in osteoclastogenesis. The number of cathepsin K positive osteoclasts increased in the regenerating caudal fin of the mutants. Furthermore, observations of elasmoid scales, which share similar transcriptomic profile with the mammalian skeleton including genes related to human diseases \textsuperscript{43}, highlighted higher TRAP staining and more circular scales in zebrafish lacking \textit{omd}. As osteoclasts are inducing OMD expression in mature osteoblasts \textsuperscript{44}, OMD presents a negative feedback activity on them. Furthermore, sulfated GAGs are known to inhibit the differentiation of osteoclasts and the sulfation level of OMD is higher during the ECM mineralization process \textsuperscript{45,46}. Those observations are crucial since we know that bone remodeling plays a key role in the bone-driven OA phenotype and that an elevated number of osteoclasts are found in sclerotic subchondral bone \textsuperscript{47}. Our findings present OMD as a novel regulator of the bone remodeling process able to prevent subchondral bone sclerosis in pathological conditions like OA.

In conclusion, alterations of the \textit{OMD} expression modify the bone and cartilage metabolism and structure. OMD helps to preserve bone and cartilage integrity and a local decrease of its production leads to the development of OA mainly by increasing subchondral bone sclerosis and thinning the calcified cartilage while its overexpression alleviate the subchondral bone sclerosis. OMD is able to directly bind to RANKL and inhibit osteoclastogenesis to regulate bone remodeling and limit subchondral bone sclerosis. Our previous and current researches, making use of both \textit{in vitro} and \textit{in vivo} experiments either with human, mouse or zebrafish models, build a strong and compelling body of evidence of OMD being a key factor in OA associated with subchondral bone sclerosis.

\textbf{Methods}

\textbf{Mouse strains and housing}

The mutant mouse strain deficient for \textit{Omd} used for this research project, C57BL/6 \textit{Omd}^\textit{tm1Lex}/Mmucd, RRID: MMRRC\textunderscore011749-UCD, was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at the University of California at Davis, a NIH-funded strain repository, and was donated to the MMRRC by Lexicon Genetics Incorporated. The mutation targeted the coding exons 1 and 2 by homologous recombination. The genotyping protocol from MMRRC was applied. The overexpressing mouse strain for \textit{Omd} used for this research project, C57BL/6 \textit{Tg(Bglap-Omd)1Kieg}, EMMA ID EM:02120, was obtained from the European Mouse Mutant Archive (EMMA), a repository supported by the national research programs and by the EC's Research and Innovation programme Horizon 2020. The transgenic line expressed \textit{Omd} under the osteocalcin promoter in addition to its natural expression, hence \textit{Omd} overexpression is only osteoblast specific. Strains were crossed with the WT C57BL/6 to maintain the line. Transgenic, WT, and mutant mice were maintained on a 12-hour-light/dark cycle with food and water supplied \textit{ad libitum}. To ease the nomenclature in the paper, we refer to the \textit{Omd} deficient mice as “KO”
and to the \textit{Tg(Bglap-Omd)} as “UP”. The ethical committee of the University of Liège approved all experimental procedures (reference no. 19-2090).

**Mouse model of OA**

Post-traumatic OA was induced by DMM on UP, WT, and KO strains at 16 weeks. The surgical transection of the medial menisco-tibial ligament of the right knee was performed in order to induce mild instability of the knee \(^{48}\). The mice were euthanized 12 weeks after surgery and their knees histologically analyzed.

For spontaneous OA, UP, WT, and KO mice were euthanized at 16 months and their knees histologically analyzed.

**Knee joint histology and histomorphometry**

Knee joints of the mice, at 4, 8, 16 months, and 28 weeks from the DMM model, were fixed for 24 hours in 4\% paraformaldehyde at 4\°C, followed by decalcification in hydrochloric acid (DC2 medium; Labonord) for 2 hours and 30 minutes at 4\°C then washed in Milli-Q water overnight at 4\°C before embedding in paraffin. Coronal sections of 5 \(\mu\)m were cut within the central area with 3 sections of at least 80 \(\mu\)m apart selected for the analysis with the Safranin-O Fast-Green staining. An additional central section was used for the Toluidine Blue staining. Each compartment of the knee joint was scored by two readers following OARSI guidelines for the mouse model as described in \(^{49}\) and the mean score from the 3 sections was calculated.

Histomorphometry of the sections was performed with the software QuPath version 0.3.2 \(^{50}\). The sections were photographed at the magnification of 10X. The cartilage histomorphometry analysis was performed on the section stained with Toluidine Blue. The total cartilage, the calcified cartilage, the plate length, and the growth plate area were measured. For the growth plate, the area was measured inside a consistent circle of a fixed size under the articular plateau. The subchondral bone area analysis was performed on the 3 sections stained with the Safranin-O Fast-Green. The bone area was measured under the tibial plateau according to its length and the bone marrow area was removed. The measured region of interest (ROI) is explained in Supplemental Fig. 7.

**Immunohistochemistry**

Epitope retrieval was performed using the chondroitinase ABC (50 units/ml, Sigma-Aldrich) in 60mM sodium acetate and 100mM Tris (pH 8) for 30 min at 37\°C. Animal-Free Blocking Solution (Cell Signaling Technology, dilution 5X) was used to block the sections prior overnight incubation with the primary polyclonal goat antibodies anti-mouse OMD (R&D systems, AF3308, 0.8 \(\mu\)g/mL) in Antibody diluent (Dako, S2022). The sections were then incubated for 30 minutes with the secondary polyclonal rabbit antibody anti-goat coupled with HRP (DakoP0449, dilution 1:400) diluted in Antibody diluent. The revelation of the secondary antibody was determined by using DAB (Cell Signaling Technology, 8059) for 2 minutes. Sections were counterstained by the Hematoxyline of Carazzi (Sigma-Aldrich) for 4 minutes.

**Micro-computed tomography (\(\mu\)CT) and image analysis**
Tibiae from mice were dislocated and fixed for 24 hours in 4% paraformaldehyde at 4°C and transferred into phosphate-buffered saline (PBS) for storage, at 4°C. Samples were imaged using the Phoenix NanoTom M (GE Measurement and Control Solutions, Germany). A target of diamond was applied, and scans were operated at a voltage of 60 kV and a current of 170 µA, voxel size of 3µm. A filter of aluminum of 0.2 mm was used to reduce beam hardening during the acquisition. The exposure time was 500 ms, and 1800 images were acquired over 360° using the fast scan mode (frame averaging = 1; image skip = 0). During reconstruction ( Datosix, GE Measurement, and Control Solutions), we applied a beam hardening correction of 8. After reconstruction, scans were oriented in the same plane, using DataViewer (Bruker MicroCT, Kontich, Belgium). Images were analyzed using CTAn (Bruker MicroCT, Kontich, Belgium).

For assessment of the trabecular architecture, we selected 150 images (450 µm height) starting at 30 µm below the growth plate level. Using 3D analysis, the trabecular volume (BV), total ROI volume (TV), number of trabeculae, porosity, and the structure model index (SMI) were calculated. For analysis of the cortical architecture, we selected 100 images (300 µm height) starting at 1500 µm below the growth plate level and corresponding to the mid-shaft. Using 3D analysis, the BV, TV, cortical thickness (Ct.Th), porosity and the tibial crest length were calculated. 3D visualization was performed using CTVox (Bruker MicroCT, Kontich, Belgium). The subchondral bone of the tibia, showing a coronal view of the medial and lateral plateau, was also visualized in 3D using CTVox and a 2-D visualization was generated using DataViewer.

**CatWalk XT**

The gait analysis of the mice was performed using the CatWalk XT System (Noldus, Netherlands; software version XT 10.5). The CatWalk XT platform was placed in a dark and silent environment to enhance the quality of the recording and reduce animal stress. The same detection settings were used for each mouse: camera gain of 18.99 dB, green intensity threshold of 0.1, detection threshold of 0.1 a.U, red ceiling light of 17.2 V, and green walkway light of 16.5 V. The gait was recorded, and the CatWalk XT software automatically labeled the footprint and generated the various associated gait parameters for the compliant runs. A compliant run was defined as a run where the mouse did not stop while going through the walkway with at least 12 footprints, the maximum variation was set at 60% and the speed was comprised between 10 and 45 cm/s. At least 3 runs for each mouse were recorded and the data represent the mean value. The data of the left and right paws were pooled for the front and hind paws to simplify the run parameter visualization.

**Zebrafish husbandry and strains**

Zebrafish (*Danio rerio*) were raised in standard conditions as described in 51. Mutant lines deficient for *omd* were generated using CRISPR-Cas9 mutagenesis with the guide RNA 5’-CAA-GAG-CTG-CGC-CAA-TG-TCA-3’. The gRNAs targeting *omd* were incubated with Cas9 protein (Thermo Fisher Scientific) before microinjections into 1-cell stage zygotes. The mutation targeted the START codon. The reporter line used to visualize osteoclasts is the transgenic line *TgBAC(ctsk:Citrine)* 52 and was kindly provided by Prof. Stefan Schulte-Merker. The ethical committee of the University of Liège approved all experimental procedures (references no. 16-1961 and 19-2133).
Injection of mRNA of omd in the Zebrafish

For omd overexpression, zebrafish omd mRNA and GFP mRNA, serving as a control, were microinjected into 1-cell stage zygotes. The following primers were used to generate the omd mRNA: forward 5'-CGA GAG AGA TAT TCA ATC CCA CAG-3' and reverse 5'-TCA ACC AAC AAG GAA TGG AAG-3'. The T7 promoter sequence for in vitro mRNA synthesis with the kit mMessage mMACHINE®T7 Ultra (Invitrogen) was added afterward with a nested PCR- using the forward primer: 5'-GCG AAT TGT AAT ACG ACT CAC TAT AGG GCC ACC ATG ACA TTG GCG CAG-3'. Fertilized eggs were injected with either 0.4 ng or 0.8 ng of mRNA. Phenotypic characterization was performed at 24-hpf and 4-dpf. At 4-dpf, the larvae were fixed with 4% PFA ON at 4°C and then stained with Alcian blue as described in 53.

Whole-mount in situ hybridization in the Zebrafish

Zebrafish larvae at 48-hpf, 5-dpf and 8-dpf were used for whole-mount in situ hybridization. Larvae were raised in presence of 0.003% of 1-phenyl-2-thiourea until 5-dpf to avoid pigmentation development. Larvae were fixed overnight in 4% of PFA at 4°C and stored in 100% methanol at -20°C until use. Visible in situ hybridizations were performed as described in 54 with a digestion step with the Proteinase K (Thermo Scientific) at 40 µg/ml during 30 minutes at 37°C for the 48-hpf larvae, at 50 µg/ml during 30 minutes at room temperature, at 40 µg/ml during 50 minutes at room temperature for the 8-dpf larvae.

Histology of the zebrafish jaw joint

1-year-old zebrafish were fixed with 4% PFA at 4°C for a minimum of 24 hours and were decalcified in 1 M EDTA solution for 20 days. Zebrafish were dehydrated in ethanol, embedded in paraffin, and sagittally sectioned at 5 µm. Sections showing the jaw joint were stained with Toluidine Blue. OARSI score was attributed to 1 section per jaw joint as described in 55.

Zebrafish osteoclast assay in the caudal fin

The mutants omd x TgBAC(ctsk:Citrine) were used at 1 year for the osteoclast analysis. Their caudal fins were cut and the fin was allowed to regenerate for 7 days. Regenerated caudal fins were cut for analysis and were incubated for 20 minutes with 0,01% alizarin red S (Sigma-Aldrich) to stain the mineralized bone matrix. Quantification of fluorescence from regenerated rays was performed using ImageJ software 56.

TRAP staining of the zebrafish scales

Ontogenetic scales of 1.6-year-old fish were plucked from the flank of the zebrafish and fixed with 4% PFA at room temperature for 30 minutes. Scales were incubated for 2 hours in the TRAP staining solution as described in 57. Quantification of the TRAP staining was performed using the ZFBONE software on FIJI 58.

Human trabecular osteoblast culture and RNA-seq analysis
Tibial bones were obtained from 6 male and 5 female patients undergoing total knee replacement surgery for OA. The age of the patients ranged from 58 to 89 years. All tissue samples used in this study were obtained after receiving approval from the University of Liege Medicine Faculty ethics committee (No. B70720108313, reference 2010/43) and written informed consent was obtained from each subject. Non-sclerotic trabecular bone was easily removed from the tibia with surgeon pliers and enzymatically processed to get digested bone pieces cultured as described in 59. At confluence, osteoblasts were collected by trypsinization, seeded (22,000 cells/cm²) in 12-well plates (Nunc). Osteoblasts were cultured until confluence, then switched into differentiation media as described in 59 during three days in the presence of 10ng/ml of the human recombinant OMD (R&D systems, 2884-AD) or in its absence for the same patient, serving as its own control.

Total RNA was extracted from osteoblast cultures, with RNA quality indicator scores (RIN) of 9.3; and RNA-seq for differential gene expression analyses was performed with a false Discovery Rate (FDR) of 0.01 to assess the statistical significance, as described in 60.

**Solid phase binding assay**

Human recombinant RANKL (OriGene, Germany) was bound for 2 hours under constant agitation to Well-Coated™ Nickel (G-Biosciences) previously washed with PBST. Unbound protein was removed by repeated washing with PBST. RANKL-coated plates were incubated overnight at 4°C with human recombinant OMD (R&D systems). The OMD bound to the coated plate was detected using the primary biotinylated polyclonal goat antibodies anti-human OMD (R&D systems, ref: BAF2884, 0.4 µg/mL). Plates were incubated with streptavidin-POD (Roche, dilution 1:25000) for 30 minutes to allow the detection. Finally, plates were read at 450 nm after applying TMB (TMBplus2, D-Tek, Denmark) for 8 minutes. The direct binding between OMD and RANKL was assessed with a fixed concentration of RANKL (0,2 µg/ml) and decreasing concentrations of OMD (1000 to 15,65 ng/l by serial 2X dilution), with the negative control missing RANKL; and with decreasing concentrations of RANKL (800 to 6,25 ng/ml by serial 2X dilution) and fixed concentration of OMD (0,5µg/ml), with the negative control missing OMD.

**Mouse osteoclast culture**

WT mice of at least 4 months of age were used to collect bone marrow cells. The bone marrow of the femur and the tibia was flushed with 10ml of αMEM containing 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were strained through a 70 µm filter and then centrifuged at 1200 rpm for 7 minutes at 22°C. After centrifugation, cells were suspended in 12 ml of media containing 5ng/ml M-CSF in a petri dish and incubated overnight at 37°C. The non-adherent cells were centrifuged at 1200 rpm for 7 minutes at 4°C the day after. The cells were suspended in osteoclast differentiation medium αMEM containing 10% FBS, 100 U/ml penicillin,100 mg/ml streptomycin, 30 ng/ml M-CSF and 10 ng/ml RANKL. For the treated conditions, 10 and 40 ng/ml of recombinant mouse OMD (R&D systems) were pre-incubated for at least 15 minutes with RANKL and M-CSF prior to adding to the suspension of the cells. Cells were seeded (525 000 cells/cm²) in 24-well plates. Cells were maintained until 4 days of
differentiation and were stained with a TRAP staining kit (Sigma Aldrich) according to the manufacturer’s instructions.

**Statistical analysis**

Results were statistically analyzed using GraphPad Prism 6.0. Tests performed and statistical significance are indicated in the figure legends with \( p \text{ values} < 0.05 \) considered statistically significant.

**Declarations**

**AUTHORS CONTRIBUTIONS**

J.Z, C.L.H, C.S and Y.H were responsible for study design. J.Z supervised murine *in vivo* experiments. J.Z and F.M.F.C supervised the DMM model. J.Z and T.P.C supervised the CatWalk XT experiment. J.Z and M.M developed the mutant zebrafish line. J.Z, Q.T, J.G and E.K supervised zebrafish *in vivo* experiments. J.Z and R.V.d.C performed the *in vitro* experiments. J.Z wrote the manuscript. R.J.L, M.M, D.E and C.L.H. provided resources and advised on research studies. C.S and C.L advised on research studies. J.Z, C.S and Y.H analyzed the results. Y.H acquired funding, verified data and assisted in writing the manuscript and acts as guarantor. All authors approved the final manuscript.

**DATA AVAILABILITY**

Data generated and analyzed during this study are included in this published article (and its supplementary information files).

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**COMPETING INTERESTS**
None declared.

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References


Figures

Figure 1
(A) Immunostaining of OMD (in brown) in the knee joint (medial tibial plateau) of KO, WT, and UP male mice at 4, 8, and 16 months. Scale bar = 100 µm. (B-D) Zoom on specific areas from WT of 16 months. Scale bar = 50 µm. Representative pictures with n=3 for each group. (B) Uncalcified articular cartilage (ac) and calcified cartilage (cc), separated by the tidemark (td – dotted line); chondrocytes (arrowheads). (C) Subchondral bone (sb), growth plate (gp) and lining cells (lc). (D) Metaphysis of the tibia showing the cortical bone (cb), the outer medial tibial side and the inner tibial side facing the bone marrow are indicated with (*).
Figure 2

Histomorphometry of the cartilage was performed with QuPath at 4, 8, and 16 months. Knee joints of male mice were stained with Toluidine Blue and areas corresponding to the total cartilage, the calcified cartilage, and the growth plate were measured for the medial tibial plateau and the lateral tibial plateau. (A) Measured of the growth plate area of both medial and lateral tibial plateaus were plotted to display the evolution of the growth plate over time with n= 13 for the KO, n= 16 for the WT and UP at 4 months; n= 16 for the KO and WT, and n= 15 for the UP at 8 months; n= 16 for the KO, n=14 for the WT and n= 18 for the UP at 16 months. (B) Toluidine blue of the growth plate for the KO and the WT at 16 months are represented. Scale bar = 100 µm. (C, E) The thickness of the calcified cartilage was measured on the medial and lateral plateaus from the tibia. (D, F) The ratio between the calcified cartilage and the total cartilage two was reported for both the medial and lateral plateaus. For the medial plateau (C, D): n= 7 for the KO, n= 8 for the WT and UP at 4 months; n= 8 for the KO and WT and n= 7 for the UP at 8 months; n= 8 for the KO, n= 7 for the WT and n= 9 for the UP at 16 months. For the lateral plateau (E, F): n= 7 for the KO, n= 8 for the WT and UP at 4 months; n= 8 for each genotype at 8 months; n= 8 for the KO, n= 7 for the WT and n= 9 for the UP at 16 months. Two-way ANOVA was performed to evaluate the genotype effect (in black) and the time effect inside a genotype (in the corresponding color). The data were plotted as a box plot showing all points with error bars representing ±SD and differences being considered significant at p-values<0.05 (*p<0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001).
Figure 3

µCT analysis of the metaphysis of the tibia of the male mice at 4, 8 and 16 months. The trabecular bone (left) and the cortical bone (right) were analyzed separately. (A, D) The 3D rendering of each genotype is represented with a scale bar of 500 µm. Red arrows indicate the tibial crest on the 16 months cortical bone. The zoom on the 16 months cortical bone illustrates the lateral side of the tibia with a scale bar of 500 µm. (B, E) The bone parameters measured for the trabecular and cortical bone were the bone volume
(BV); the total volume (TV) and their ratio (BV/TV). The data were plotted as a box plot showing all points with error bars representing ± SD. One-Way ANOVA was performed with differences being considered significant at p-values<0.05 (*p<0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001). (C, F) The trabecular number, trabecular porosity, cortical thickness, and cortical porosity are represented over time. At 4 months: n=8 for each genotype; at 8 months: n=8 for the KO, n=10 for the WT and UP; at 16 months: n=9 for the KO, n=11 for the WT, and n=10 for the UP. Two-Way ANOVA was performed on the kinetic analysis with error bars representing ± SEM and differences being considered significant at p-values<0.05, * represents significant differences between the KO and the UP, / represents significant differences between the KO and the WT and Δ represents significant differences between the WT and the UP (*/ Δp<0.05, **/ ΔΔp≤0.01, *** p≤0.001).
Figure 4

(A) Histomorphometry of the subchondral bone on Safranin-O Fast Green of the knee joint of male mice at 8 and 16 months was performed with QuPath on the lateral and medial plateaus of the tibia separately. At 8 months: n=8 for each genotype; at 16 months: n=7 for the KO, n=7 for the WT, and n=9 for the UP. The data were plotted as a box plot showing all points with error bars representing ±SD. Two-Way ANOVA was performed with differences being considered significant at p-values<0.05 (*p<0.05,
(B) Representative picture of the Safranin-O Fast Green of the knee joint of male mice showing the subchondral bone area for the lateral and medial plateau of the tibia separately. Scale bar = 100 µm. (C) µCT of the subchondral bone of the tibia of the mice at 16 months. The pink asterisk indicates the lateral plateau and the blue asterisk indicates the medial plateau.
Analysis of the development of OA lesions in the different genotypes after spontaneously occurring with age (left) or after the DMM (right). The spontaneous OA lesions were considered in the 16-month-old male mice and the DMM was performed on 16-week-old male mice and they were stopped at 28 weeks.

(A, D): The cartilage degradation was assessed with the OARSI score (from 0 to 6) according to the OARSI recommendations. The score was attributed to the lateral and the medial tibial plateaus and to the lateral and medial condyles for the spontaneous model and the DMM model. (B, E): The score of the loss of proteoglycan (from 0 to 5) was assessed according to the OARSI recommendations for the lateral and medial tibial plateaus for the spontaneous model and the DMM model. For the OARSI score of the 16-month-old mice: n=8 for the KO and the WT and n=9 for the UP and for the loss of proteoglycan n=10 for the KO, n=8 for the WT and n=10 for the UP. For the DMM model: n=9 for the KO, n=10 for the WT, and n=8 for the UP. One-way ANOVA was performed with differences being considered significant at p-values<0.05(*p<0.05).

(C): Illustrations of the lateral and medial plateaus stained with Toluidine Blue of the 16-month-old mice with zooms on proteoglycan loss issued from the KO and indicated by the arrowhead. Scale bar = 100 µm.

(F): Histomorphometry of the subchondral bone on Safranin-O Fast Green of the knee joint of the DMM mice was performed with QuPath on the medial and lateral plateaus separately. Each genotype was compared to a similar age group of 8-month-old mice. At 8 months: n=8 for each genotype; for the DMM: n=9 for the KO, n=10 for the WT, and n=8 for the UP. The data were plotted as a box plot showing all points with error bars representing ± SD. Two-Way ANOVA was performed with differences being considered significant at p-values<0.05 (*p<0.05, **p≤0.01).

(G): Illustrations of the lateral and medial plateaus stained with Safranin-O Fast Green in the DMM. Scale bar = 100 µm.
**Figure 6**

Analysis of the gait of 4, 8, and 16-month-old male mice with the CatWalkXT. The intensity corresponds to the mean intensity at the maximum paw contact normalized with the mean of the maximum contact paw area, the speed, and the weight of the mouse. At 4 months: n=8 for each genotype; at 8 months n=8 for each genotype; at 16 months: n=14 for the KO, n=12 for the WT and n=10 for the UP. The data were plotted as a box plot showing all points with error bars representing ±SD. One-Way Anova was performed
when the distribution was gaussian and Kruskal-Wallis was performed when the distribution was not gaussian with differences being considered significant at p-values<0.05 (*p<0.05, **p≤0.01, ***p≤0.001).

Figure 7

The mutant deficient for omd was generated through CRISP/Cas 9. (A) Histology of the jaw joint was performed on 1-year-old zebrafish. The OARSI score of the palatoquadrate was attributed to the jaw joint
stained with Toluidine Blue with n = 4 for the WT and the mutant. The data were plotted as a box plot showing all points with error bars representing ±SD. Mann-Whitney test was performed with differences being considered significant at p-values<0.05 (*p<0.05).

(B) The mutant line was crossed with the Tg(ctsk:Citrine) for the osteoclasts analysis during the caudal fin regeneration. The caudal fin of 1-year-old zebrafish was cut and the regenerating fin was observed after 7 days. The osteoclasts are represented in yellow-green from the ctsk:Citrine signal and the mineralized ray were stained with Alizarin red. The data were plotted as a box plot showing all points with error bars representing ±SD. The pixel intensity of the regenerating rays is plotted and normalized by the background intensity with n = 14 for the WT and n = 16 for the mutant from two independent experiments which were pooled to perform the unpaired student t-test with differences being considered significant at p-values<0.05 (**p≤0.01).

(C) TRAP staining was performed on the elasmoid scales of 1.6-year-old zebrafish. The TRAP staining area was normalized with the total scale area. The TRAP staining and the circularity of the scales were assessed with ZFBONE - Fiji with n = 5 for the WT and the mutant and with 6 to 15 scales/zebrafish analyzed. Scale bar = 0.2 mm The data were plotted as a box plot showing all points with error bars representing ±SD. Unpaired student t-test with differences being considered significant at p-values < 0.05 (*p < 0.05, **p ≤ 0.01).
(A) Solid Phase binding assay on the capture of RANKL by OMD. RANKL was coated on a plate followed by OMD addition. Above: Binding assay with different concentrations of OMD (1000 to 15.65 ng/ml by serial 2X dilution), with 0.2µg/m of coated RANKL (red curve) and negative control without RANKL (blue curve). Below: Binding assay with different concentrations of coated RANKL (800 to 6.25 ng/ml) and 0.5µg/ml of given OMD (pink curve); negative control without OMD (purple curve). Wilcoxon test was
performed with differences being considered significant at p-values < 0.05 (*p<0.05, **p ≤ 0.01). (B) Assay of the effect of OMD on primary murine osteoclast culture. The peripheral blood mononuclear cells were collected from murine bone marrow and differentiated into osteoclasts with M-CSF and RANKL. Osteoclasts were counted after 4 days of differentiation following a TRAP staining. Each point represents a mouse, n = 7. The osteoclast count was represented in percentage of cells with the corresponding control set as 100%. Blue arrows point at osteoclasts. Scale bar = 50 µm. The data were plotted as a box plot showing all points with error bars representing ±SD. One-way ANOVA was performed with differences being considered significant at p-values < 0.05 (*p<0.05). (C) Schematic representation of the mechanism of OMD on osteoclastogenesis. Osteoblasts secrete RANKL which binds to the RANK receptor on the membrane of pre-osteoclasts to induce their differentiation into osteoclasts. In parallel, osteoblasts also secrete OMD which displays the ability to capture RANKL and would prevent it to bind to RANK.

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