Mutation in the CCAL1 locus accounts for bidirectional process of human subchondral bone turnover and cartilage mineralization

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Abstract

Objectives. To study the mechanism by which the readthrough mutation in TNFRSF11B, encoding osteoprotegerin (OPG) with additional 19 amino acids at its C-terminus (OPG-XL), causes the characteristic bidirectional phenotype of subchondral bone turnover accompanied by cartilage mineralization in chondrocalcinosis patients.

Methods. OPG-XL was studied by human induced pluripotent stem cells expressing OPG-XL and two isogenic CRISPR/Cas9-corrected controls in cartilage and bone organoids. Osteoclastogenesis was studied with monocytes from OPG-XL carriers and matched healthy controls followed by gene expression characterization. Dual energy X-ray absorptiometry scans and MRI analyses were used to characterize the phenotype of carriers and non-carriers of the mutation.

Results. Human OPG-XL carriers relative to sex- and age-matched controls showed, after an initial delay, large active osteoclasts with high number of nuclei. By employing hiPSCs expressing OPG-XL and isogenic CRISPR/Cas9-corrected controls to established cartilage and bone organoids, we demonstrated that expression of OPG-XL resulted in excessive fibrosis in cartilage and high mineralization in bone accompanied by marked downregulation of MGP, encoding matrix Gla protein, and upregulation of DIO2, encoding type 2 deiodinase, gene expression, respectively.

Conclusions. The readthrough mutation at CCAL1 locus in TNFRSF11B identifies an unknown role for OPG-XL in subchondral bone turnover and cartilage mineralization in humans via DIO2 and MGP functions. Previously, OPG-XL was shown to affect binding between RANKL and heparan sulphate (HS) resulting in loss of immobilized OPG-XL. Therefore, effects may be triggered by deficiency in the immobilization of OPG-XL. Since the characteristic bidirectional pathophysiology of articular cartilage calcification accompanied by low subchondral bone mineralization is also a hallmark of OA pathophysiology, our results are likely extrapolated to common arthropathies.

Key words: hiPSCs, CRISPR/Cas9, TNFRSF11B, osteomorph, matrix mineralization

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Introduction

Joint tissue degeneration during OA is a complex multistep process characterized by a pathogenic bidirectional process of subchondral bone turnover and cartilage mineralization [1, 2]. It has been suggested that this characteristic inverse mineralization process has shared mechanisms with the frequently observed concurrent pathogenic bone turnover and vasculature mineralization [2, 3]. Key proteins likely involved are osteoprotegerin (OPG), a decoy receptor of osteoclastogenesis [4], and matrix Gla protein (MGP), a vitamin K-dependent inhibitor of ectopic bone formation [5], since overexpression or knockdown of their genes in murine models results in such an inverse pathological mineralization process [6–8]. The inverse causal role of dysfunctional OPG in human joint tissue mineralization was demonstrated by identification of a readthrough mutation at the chondrocalcinosis locus 1 (CCAL1; c1205A>T; p. Stop402Leu) [9]. The mutation in TNFRSF11B, encoding OPG was identified in multiple families worldwide [10–12]. In these families, the CCAL1 phenotype is defined by early onset OA with different levels of articular cartilage calcification, i.e. chondrocalcinosis [13] and low subchondral bone mineralization [12].

OPG is a well-known soluble decoy receptor that competes with RANK expressed in osteoclasts for binding to RANK ligand (RANKL) [14]. Binding of RANKL to RANK drives osteoclastogenesis and hence bone turnover, while binding to OPG inhibits this process [15]. Pleiotropic functions of OPG and RANKL were more recently suggested by showing that RANKL stimulates osteoclast fission to produce transcrionally distinct osteomorphs, which in turn recycle towards large multinucleated osteoclasts or polykaryons by fusion under tight control of OPG [15]. Although binding of OPG to RANKL, established by N-terminal domains of OPG, is frequently studied, less is known about the interaction of OPG via its C-terminus with membrane bound heparan sulphate (HS) on osteoblasts [16]. This binding appears indispensable for RANKL-mediated inhibition of osteoclastogenesis due to immobilization of secreted OPG on the osteoblast membrane and formation of a stable HS–OPG–RANKL complex [16, 17]. In line with this, the CCAL1 readthrough mutation, adding an additional 19 amino acids to the C-terminus of OPG, denoted OPG-XL, has been shown to hamper OPG–HS binding hence permitting osteoclastogenesis and bone turnover [11].

This explains characteristic low subchondral bone density in affected CCAL1 family members [12] and sporadic cases [18].

The mechanism by which OPG-XL results in cartilage calcification remains, however, elusive. In fact, a robust role of TNFRSF11B and TNFSF11, encoding RANKL, particularly in cartilage (patho)physiology, has been highlighted by transcriptome-wide studies. Herein, TNFRSF11B and TNFSF11, but not TNFRSF11A encoding RANK, show high expression and are robustly responsive to OA cartilage pathophysiology as marked by consistent high upregulation in human OA affected relative to preserved [19–21] or healthy [22] cartilage. In contrast, differential expression of TNFRSF11B or TNFSF11 in subchondral bone underlying preserved and lesioned areas of OA cartilage was not observed [23]. Other than that, with TNFSF11 being a robust OA risk gene identified in the largest genome-wide association study to date [24], aberrant function of OPG/RANKL also underlies common OA aetiology.

Here, we set out to functionally characterize the effects of OPG-XL in joint tissues by employing mutated and control human primary chondrocytes, as well as human induced pluripotent stem cells (hiPSCs) from affected CCAL1 family members and CRISPR/Cas9 repaired hiPSC isogenic controls, to established in vitro organoid models of cartilage and bone [25]. Additionally, to study the effect of OPG-XL in human osteoclastogenesis, monocytes isolated from blood of carriers of the mutation were compared with monocytes of age- and sex-matched controls in osteoclastogenesis assays. Altogether, we aimed to decipher effects of OPG-XL on joint tissue mineralization that could explain the CCAL1 phenotype of articular cartilage calcification in concurrence with low subchondral bone mineralization. Given that subchondral bone turnover and cartilage calcification are general hallmarks of OA pathophysiology and at the molecular level involve TNFRSF11B and TNFSF11, our results are likely of relevance to common OA.

Methods

Study participants

Within the Familial early-onset OsteoArthritis (FOA) study, 13 family members were enrolled (six females and seven males aged 23–62 years with mean age 47 years; Supplementary Table S1, available at

### Rheumatology key messages

- OPG-XL mutation directly affects chondrocyte and osteoblast states towards matrix mineralization mediated by respectively MGP and DIO2.
- Expression of OPG-XL drives accumulation of large active osteoclasts with high number of nuclei.
- Interference with OPG–RANKL–heparan sulphate underlying concurrent cartilage calcification and subchondral bone loss likely extrapolates to common arthropathies.
*Rheumatology* online). Cartilage samples were collected within the ongoing Research Arthritis and Articular Cartilage (RAAK) study from five common OA patients and one family member undergoing total joint replacement surgery (RAAK: two females and three males aged 50–87 years with mean age 73 years; FOA: female aged 61).

The Medical Ethics Committee of the Leiden University Medical Center (LUMC) gave approval for the RAAK study (P08.239 and P19.013), the familial OA (FOA) study (P12-256), and for generation of hiPSCs from skin fibroblasts of healthy donors (P13.080). Informed consent was obtained from all participants and donors in our manuscript.

Genotyping and radiographic analyses

FOA family members were genotyped with an in-house genetic test developed by the Department of Clinical Genetics to determine presence or absence of a previously identified readthrough mutation in TNFRSF11B (OPG-XL; Fig. 1A) [9], and were characterized by dual energy X-ray absorptiometry (DEXA) as well as radiographs and MRI of the knees to respectively determine BMD and OA severity (Supplementary Tables S2 and S3, available at *Rheumatology* online; Fig. 1B). Further details are described in Supplementary Materials and methods, available at *Rheumatology* online.

Generation, characterization and CRISPR/Cas9 correction of OPG-XL patient hiPSCs

Human iPSCs were generated by the LUMC iPSC core facility as described before [26] from skin fibroblasts of a FOA participant, carrier of the mutation resulting in expression of OPG-XL (line LUMC0103iOPG). Pluripotency and spontaneous differentiation were assessed, and cells were karyotyped after 15 passages (Supplementary Fig. S1A–C, available at *Rheumatology* online). Cells were maintained under standard conditions (37°C, 5% CO₂) in TeSR-E8 medium (STEMCELL Technologies, Cologne, Germany).

To obtain two independent isogenic hiPSC controls without the mutation (lines B89 and C81), CRISPR/Cas9 correction of the mutation was performed for the OPG-XL hiPSCs (LUMC0103iOPG). For this, two single guide RNAs (sgRNAs), gRNA1 (5'-AAAAATAGCTGCTTATTA-3') and gRNA2 (5'-AAAAATAGCTGCTTATTA-3'), were cloned into a CRISPR/Cas9 plasmid (PX458), and co-transfected using Lipofectamine Stem Reagent (Thermo Fisher Scientific, Landsmeer, The Netherlands) with single-stranded oligo donor repair template (ssODN 5'-CAGCTGAAAGCCTCAAGTGCCTGG-3') and gRNA2 (5'-AGCTGCTTATTA-3'), were cloned into a CRISPR/Cas9 plasmid (PX458), and co-transfected using Lipofectamine Stem Reagent (Thermo Fisher Scientific, Landsmeer, The Netherlands) with single-stranded oligo donor repair template (ssODN 5'-CAGCTGAAAGCCTCAAGTGCCTGG-3') to achieve homologous directed repair. Further details are described in Supplementary Materials and methods and Supplementary Fig. S1D–F, available at *Rheumatology* online.

Human primary chondrocytes

Human primary chondrocytes were collected from OA patients (RAAK study; N = 5 donors) and carrier of OPG-XL (FOA study; N = 1 donor) undergoing joint replacement surgery. Collection, expansion and deposition of cartilage extracellular matrix of primary chondrocytes has been previously described [20].

Chondrogenesis and osteogenesis of iPSCs

Mesenchymal stromal cells were generated from hiPSC (hiMSCs) of the OPG-XL hiPSCs (LUMC0103iOPG) and of the two thereof derived CRISPR/Cas9 isogenic control hiPSCs (B89 and C81) using Stemcell Technologies' Mesenchymal Progenitor Kit following the manufacturer's instructions. Chondrogenesis and osteogenesis was performed in organoids following our established protocol employing 750,000 cells per organoid as described before [25].

Isolation of blood cells and osteoclastogenesis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of six FOA members with mutation and six sex- and age-matched controls (characteristics of donors in Supplementary Table S4, available at *Rheumatology* online) using Ficoll density gradient centrifugation as previously described [27]. Further details of osteoclastogenesis with CD14 positive monocytes are described in Supplementary Materials and methods, available at *Rheumatology* online.

CTX-1 measurement and resorption-pit assay

Concentration of C-terminal telopeptide of type 1 collagen (CTX-1) in conditioned medium following 14 or 21 days' culture on slices of human tibia bone was determined with ELISA (Immunodiagnostic System, Inc, Tyne and Wear, United Kingdom) according to the manufacturer's protocol. Measurements were performed for osteoclastogenesis assays of three FOA participants expressing OPG-XL and matched healthy controls. Relative activity per osteoclast was calculated by dividing the concentration of CTX-1 by the total number of osteoclasts. Bone resorption was analysed in cell cultures of two FOA participants expressing OPG-XL and matched healthy controls as previously described [28] with Coomassie Brilliant Blue (Sigma-Aldrich, Amsterdam, The Netherlands). Pre-defined areas of each bone slice, covering approximately one-fifth of the total area, were analysed with Image Pro-Plus software (Media Cybernetics, Rockville, MD, USA).

Histology and immunohistochemistry

Histology was performed as previously described [29]. Overall cellular and tissue structure was visualized with haematoxylin–eosin (H&E) staining. Glycerosaminoglycans were visualized by staining with 1% Alcian blue 8-GX (Sigma-Aldrich) and Nuclear Fast Red (Sigma-Aldrich). Calcium deposits were visualized with 2% Alizarin Red S (Sigma-Aldrich). Osteoclasts were stained with tartrate...
Fig. 1 Characterization of early-onset osteoarthritis family

A

B

C

D

E

F

(continued)
resistant acid phosphatase (TRAcP; Leucocyte acid phosphatase kit, Sigma-Aldrich).

Gene expression analysis
For osteoclast assays, RNA was extracted from the different cultures at day 7, 14 and 21. For each individual RNA isolation of neo-cartilage and neo-bone, we pooled two organoids for either OPG-XL or CRISPR/Cas9 repaired (wild type, WT) from several independent rounds of differentiations. This generated for neo-cartilage of OPG-XL a total of 5–7 datapoints and for WT a total of 9–12 datapoints in the gene expression plots. For neo-bone, this generated for OPG-XL and WT a total of respectively 10–16 and 7–14 datapoints. Further details and primer sequences can be found in Supplementary Materials and methods and Supplementary Table S5, available at Rheumatology online.

Statistical analysis
Generalized estimating equations (GEE) [30] as implemented in IBM SPSS 25.0 software (IBM Corp., Armonk, NY, USA) was applied to analyse association between phenotype and genotype. GEE methodology provides a method of analysing correlated data that otherwise could not be modelled in a generalized linear model. By applying this method, we were able to effectively adjust for familial dependencies of included participants to obtain β, s.e. and P-values for gene expression differences across neo-cartilage and neo-bone [2]. Differences between osteoclast categories defined by the number of nuclei and gene expression were tested using two-way ANOVA and Sidák’s multiple comparison test (GraphPad Prism 6.0 software, GraphPad Software Inc., La Jolla, CA, USA). P-values <0.05 were considered statistically significant.

Results
Carriers of OPG-XL are characterized by severe OA and osteopenia
TNFRSF11B was genotyped in study participants, identifying seven carriers and six non-carriers of OPG-XL among 13 members of a family with early-onset OA (FOA; Fig. 1; Supplementary Table S1, available at Rheumatology online). Whole body DEXA scans showed that BMD of non-carriers was similar to that of the general population (Fig. 1C; Supplementary Table S2A, available at Rheumatology online). In contrast, carriers of OPG-XL had significantly lower BMD specifically of the femoral neck, narrow neck and total hips, confirming incidence of osteopenia (Fig. 1C; Supplementary Table S2B, available at Rheumatology online). No significant difference, however, was observed for lumbar spine BMD. Furthermore, OA features were scored for FOA members based on the semi-quantitative MRI OA knee score (MOAKS). This showed that severe osteophytosis, bone marrow lesions (BML) and cysts have significantly higher prevalence in carriers of the mutation than in non-carriers (Supplementary Table S3, available at Rheumatology online). Analysis of the knee radiographs confirmed presence of chondrocalcinosis in three participants expressing OPG-XL. Altogether, this demonstrated that the CCAL1 phenotype in the FOA family is characterized by low BMD and severe cartilage loss, osteophyte formation, and presence of cysts and BML. High mineralization of cartilage was also observed in knee joint of a carrier of the mutation undergoing joint replacement surgery (Fig. 1D, specific regions with calcified cartilage indicated with dashed line).

Primary chondrocytes with OPG-XL deposit hypertrophic neo-cartilage with low glycosaminoglycan content
We first examined, in an established 3D in vitro chondrogenesis model of human primary chondrocytes [29] of a carrier of the OPG-XL mutation (N = 1 patient, n = 8 replicates), formation of neo-cartilage in comparison with primary chondrocytes from participants of the RAAK study undergoing joint replacement surgery (N = 5 patients, n = 1 replicate). As shown in Fig. 1E, Alcian blue staining in neo-cartilage with OPG-XL appeared less homogeneous as compared with neo-cartilage deposited by RAAK chondrocytes. Furthermore, H&E staining showed less dense matrix deposition towards the edges of the organoid. Gene expression of common
extracellular matrix (ECM) genes and degradation markers highlighted significantly higher expression of COL1A1, COL10A1 and MMP13 (Fig. 1F; Supplementary Table S6, available at Rheumatology online). Moreover, low MGP expression indicated a higher mineralization in the OPG-XL neo-cartilage organoids. Together, these data indicated deposition of low-quality neo-cartilage matrix with a fibrotic (COL1A1) and/or hypertrophic (COL10A1) and mineralized (MGP) phenotype in the presence of OPG-XL.

Neo-cartilage and neo-bone expressing OPG-XL exhibit altered mineralization and fibrotic phenotype

To study effects of OPG-XL on deposition of extracellular matrix in the joint, patient hiPSCs carrying the mutation were generated from skin fibroblasts (Supplementary Fig. S1, available at Rheumatology online), and both neo-cartilage and neo-bone organoids were created from these hiPSCs and two independent isogenic, CRISPR/Cas9-corrected control (WT) hiPSCs. Structure of deposited neo-cartilage ECM was visualized by histological staining with Alcian blue and H&E (Fig. 2A; Supplementary Fig. S2, available at Rheumatology online). This showed ECM deposition across both groups. Matrix, however, was more homogeneous in the two isogenic control organoids as compared with organoids from hiPSCs expressing OPG-XL. Additionally, H&E staining visualized fibrotic ECM particularly towards the outer rim of the OPG-XL neo-cartilage organoids.

In line with the histological observations, quantitative gene expression analysis showed no difference between the two isogenic controls (Fig. 2B, grey- and purple-filled circles in WT boxes). Therefore, samples were analysed together in comparison with OPG-XL samples, which revealed no significant differences in expression levels of TNFSF11 encoding RANKL. TNFRSF11A encoding RANK, or in expression levels of TNFRSF11B in neo-cartilage in the presence of OPG-XL. Fibrotic character of neo-cartilage deposited in presence of OPG-XL was confirmed by significantly higher expression of COL1A1 and lower expression of the cartilage specific COL2A1 and aggrecan (ACAN). Concurrently, strong upregulation of alkaline phosphatase (ALPL) and osteoblast characteristic RUNX family transcription factor 2 (RUNX2) was detected together with significantly lower expression of SRY-box transcription factor 9 (SOX9). Notably, lower expression of MGP was highly significant, which was also observed in neo-cartilage deposited by primary chondrocytes with OPG-XL (Fig. 1F). These data indicated a strong shift in cartilage metabolism towards mineralization and transdifferentiation towards osteoblasts. This was in contrast with lower expression levels of secreted phosphoprotein 1 (SPP1) and matrix metalloproteinase 13 (MMP13).

Structure of deposited neo-bone ECM was visualized by histological staining with Alizarin red and H&E (Fig. 2A; Supplementary Fig. S2, available at Rheumatology online). Alizarin red staining was more intense and more homogeneously distributed in the OPG-XL organoids compared with isogenic controls. In the control group, H&E showed a higher concentration of cells within the core of the organoid and an outer rim characterized by fibrosis.

In line with the findings in cartilage organoids, quantitative gene expression analysis of neo-bone showed no significant differences in levels of TNFSF11B and TNFRSF11A expression. Unlike neo-cartilage, neo-bone carrying the OPG-XL mutation did show lower expression of TNFSF11 (Fig. 2B; Table 1; Supplementary Fig. S3, available at Rheumatology online). Moreover, neo-bone expressing OPG-XL had significantly higher expression of COL1A1 and COL10A1, but particularly of DIO2. Altogether, the results indicate that both neo-cartilage and neo-bone ECM in the presence of OPG-XL is characterized by increased fibrosis and strong mineralization, respectively, indicating a modulatory role for OPG in cartilage and bone formation.

Restained osteoclastogenesis and large polykaryons of monocytes from FOA patients expressing OPG-XL

Given that OPG plays a key role in osteoclast formation, osteoclastogenesis assays were performed with monocytes from six carriers of OPG-XL mutation and six healthy controls (Fig. 3A). The number of osteoclasts and their respective nuclei was determined at day 14 and day 21 of culture (Supplementary Table S7, available at Rheumatology online). As shown in Fig. 3B and Supplementary Table S7, available at Rheumatology online, the total number of osteoclasts (cells with >3 nuclei) formed at day 14 in OPG-XL (n=90) is delayed relative to controls (n=296). At day 21, however, we showed a significant accumulation of osteoclasts with high number of nuclei formed in OPG-XL (93% >6 nuclei) compared with controls (15% >6 nuclei; Fig. 3C; Supplementary Table S7, available at Rheumatology online). Together, these data indicate that after initial restrained osteoclastogenesis, a relative larger fraction of osteoclasts with large number of nuclei accumulate in the presence of OPG-XL as compared with controls.

Osteoclasts expressing OPG-XL display similar bone resorptive activity as compared with controls

Following morphological characterization of osteoclasts, resorptive activity was assessed. To that end, formation of resorption pits by osteoclasts generated from controls and from carriers of OPG-XL was determined. As shown in Fig. 4, despite lower numbers of osteoclasts in the presence of OPG-XL, overall bone resorption was comparable, with at least equal surface areas of resorption pits after 21 days (Fig. 4A) and amounts of CTX-1 released at days 7, 14 and 21 (Fig. 4B-D). These data indicate that, despite the fact that fewer osteoclasts develop in the presence of OPG-XL, the total bone resorption activity was comparable to controls.

Finally, well-known markers of osteoclast bone resorption activity were analysed by RT-qPCR. As shown
FIG. 2 Characterization of OPG-XL neo-cartilage and neo-bone organoids

(A) Alcian blue, Alizarin red and H&E staining of neo-cartilage and neo-bone. (B) Boxplots for $-\Delta C_t$ values of relevant genes for CRISPR/Cas9 control (WT) and OPG-XL organoids following 6 weeks of chondrogenesis with additional 2 weeks of osteogenesis to generate neo-bone (scale bars: 50 μm; neo-cartilage: WT $n=9–12$ and OPG-XL $n=5–7$ samples; neo-bone: WT $n=7–14$ and OPG-XL $n=10–16$ samples). $P$-values determined with generalized estimation equation while including every independent gene as dependent variable, and mutation status as covariate (*$P<0.05$; **$P<10^{-4}$; ***$P<10^{-6}$). ALPL: alkaline phosphatase; DIO2: type 2 deiodinase; H&E: haematoxylin and eosin; MGP: matrix Gla protein; MMP3: matrix metalloprotease 3; MMP13: matrix metalloprotease 13; OPG-XL: C-terminal extended osteoprotegerin encoded by TNFRSF11B readthrough mutation; RUNX2: RUNX family transcription factor 2; SPP1: secreted phosphoprotein 1; TNFSF11: gene encoding RANK ligand; TNFRSF11A: gene encoding RANK; TNFRSF11B: gene encoding osteoprotegerin or OPG.
in Fig. 4E and Table 2, at day 21 OPG-XL-expressing osteoclasts showed significantly higher levels of NFATc1 (Nuclear factor of activated T-cells, cytoplasmic 1), CTSK (Cathepsin K), TRAcP (Tartarate-resistant acid phosphatase) and DC-STAMP (Dendrocyte Expessed Seven Transmembrane Protein). This suggests that monocytes carrying the OPG-XL mutation, despite an initial delay in osteoclastogenesis, are prone to differentiate towards osteoclasts with potential for higher bone resorption activity based on gene expression levels.

**Table 1 Gene expression analyses of neo-cartilage and neo-bone organoids**

| Gene   | Extracellular matrix FD | s.e. | P-value* | Mineralization Gene FD | s.e. | P-value* |
|--------|-------------------------|------|----------|-------------------------|------|----------|
| Neo-cartilage                      |                  |      |          | Neo-cartilage            |      |          |
| COL1A1 | 1.7                     | 0.8  | 0.3      | 7.4 x 10^{-3}            |      |          |
| COL2A1 | -6.7                    | -2.8 | 0.5      | 3.9 x 10^{-7}            |      |          |
| COL10A1 | -1.2                   | -0.3 | 0.7      | 6.4 x 10^{-1}            |      |          |
| MMP13  | -2.4                    | -1.6 | 0.3      | 1.4 x 10^{-8}            |      |          |
| MMP3   | -3.4                    | -1.8 | 0.7      | 1.5 x 10^{-2}            |      |          |
| TNFRSF11B | 1.6               | 0.7  | 0.8      | 4.0 x 10^{-1}            |      |          |
| TNFRSF11A | 1.8             | 2.0  | 1.9      | 2.9 x 10^{-1}            |      |          |
| TNFRSF11 | 1.0                   | 0.1  | 1.6      | 9.7 x 10^{-1}            |      |          |
| ACAN1  | -3.9                    | -2.0 | 0.8      | 1.5 x 10^{-2}            |      |          |
| SOX9   | -2.0                    | -1.0 | 0.3      | 4.5 x 10^{-3}            |      |          |
| COMP   | -1.2                    | -0.3 | 0.2      | 1.8 x 10^{-1}            |      |          |
| ADAMTS5 | 1.6                    | 0.6  | 1.1      | 5.6 x 10^{-1}            |      |          |
| SMAD3  | -2.7                    | -1.4 | 0.4      | 4.0 x 10^{-4}            |      |          |
| Neo-bone                      |                  |      |          | Neo-bone                |      |          |
| COL1A1 | 1.7                     | 0.7  | 0.4      | 3.9 x 10^{-2}            |      |          |
| COL2A1 | 3.3                     | 1.7  | 0.7      | 1.2 x 10^{-2}            |      |          |
| COL10A1 | 4.8                    | 1.5  | 0.6      | 5.6 x 10^{-3}            |      |          |
| MMP13  | -3.3                    | -2.1 | 0.4      | 7.8 x 10^{-9}            |      |          |
| MMP3   | -14.9                   | 2.6  | 1.2      | 2.8 x 10^{-2}            |      |          |
| TNFRSF11B | 1.5               | 0.6  | 0.6      | 3.3 x 10^{-1}            |      |          |
| TNFRSF11A | 1.8             | 0.9  | 1.0      | 3.8 x 10^{-1}            |      |          |
| TNFRSF11 | -3.2                   | -1.7 | 0.7      | 2.0 x 10^{-2}            |      |          |

*Results presented are the average of seven samples for neo-cartilage and 16 samples for neo-bone in OPG-XL carriers and 12 samples for neo-cartilage and 14 samples for neo-bone in CRISPR/Cas9-corrected controls (WT) at week 6 following chondrogenesis and additional 2 weeks following osteogenesis. P-values determined with generalized estimation equation while including every independent gene as dependent variable, and mutation status as covariate (P-values >0.05 are indicated in bold). FD: fold difference.

Discussion

In the current study we explored the mechanism by which OPG-XL causes the characteristic bidirectional phenotype of subchondral bone turnover accompanied by cartilage mineralization in CCAL1 patients. Notably, OPG-XL, displaying 19 additional amino acids at the C-terminal end, was previously found to hamper the formation of a stable HS–OPG–RANKL complex on the osteoblast membrane [17], permitting RANKL mediated osteoclastogenesis in a murine model [11]. Here we show that human osteoclastogenesis with monocytes from OPG-XL carriers relative to sex- and age-matched controls, after an initial delay, indeed have enhanced osteoclastogenesis towards prominent large and active osteoclasts. By further characterization of OPG-XL by employing hiPSCs from a carrier of the mutation and two isogenic CRISPR/Cas9-corrected controls in cartilage and bone organoids, we demonstrated for the first time that likely due to interference with RANKL–HS–OPG, the mutation at the CCAL1 locus directly affects healthy osteoblast and chondrocyte states towards mineralization via respectively DIO2 and MGP functions. The fact that OPG/RANKL as well as DIO2 [29] and MGP [2, 31] are intrinsically involved in joint tissue (pathophysiology might indicate a link to common age-related osteoarthritis).

The hiPSC-derived neo-cartilage tissue deposited by OPG-XL chondrocytes, relative to isogenic controls, revealed a fibrotic histological phenotype with marked downregulation of COL2A1: COL1A1 expression ratio and most notable, downregulation of MGP expression. MGP is a well-known inhibitor of ectopic bone formation [5] and MGP is a robust OA risk gene [24, 31] with the risk allele associated with lower gene expression levels [2, 31]. Hence, our data, showing lower expression of
MGP in OPG-XL neo-cartilage organoids, suggest that OPG-XL directly affects propensity of chondrocytes to enter a mineralized OA state. On a different note, we showed that in OPG-XL neo-cartilage organoids the OPG–RANKL–RANK triad was not changed. Neo-osseous tissue deposited by osteoblasts from hiPSCs carrying the mutation relative to their isogenic controls did display high calcification as reflected by the prominent Alizarin red staining concurrent with not-able high expression of DIO2. DIO2, encoding type 2 deiodinase enzyme, essentially facilitates bone formation and mineralization [32]. Together, results from our human OPG-XL cartilage and bone organoids demonstrate that the mutation directly affects chondrocyte and osteoblast gene expression profiles marking matrix mineralization processes. We hypothesize that this is due to the impaired binding of OPG with HS, likely in interaction with RANKL as recently shown [11]. Since the prominent Alizarin red staining of neo-osseous tissue, concurrent with DIO2 upregulation could explain the extensive phenotypic foci of calcified cartilage observed in affected articular cartilage tissue of CCAL1 family members, we hypothesize that the chondrocalcinosis observed in OPG-XL carriers is likely not preceding OA onset in cartilage but arises merely during ongoing OA pathophysiology, i.e. when

(A) Representative TRACP staining of osteoclasts generated from monocytes of OPG-XL carriers and sex- and age-matched controls after 21 days of culture with M-CSF and RANKL (Scale bar = 200 μm). (B) Osteoclasts were counted and separated according to the number of nuclei per osteoclast at day 14 and day 21. OPG-XL: C-terminal extended osteoprotegerin encoded by TNFRSF11B readthrough mutation; RANKL: RANK ligand; TRAcP: Tartrate-resistant acid phosphatase.

| TRACP positive cells/cm² at day 14 | Controls b | OPG-XL b | P value c |
|-------------------------------------|------------|-----------|-----------|
| Nuclei/osteoclast a                  |            |           |           |
| 3-5                                 | 263 (89%)  | 86 (96%)  | 5.9x10⁻²  |
| 6-10                                | 30 (10%)   | 4 (4%)    | 9.5x10⁻²  |
| 10-20                               | 3 (1%)     | 0         | ND        |
| >20                                 | 0          | 0         | ND        |
| Total                               | 296        | 90        |           |

| TRACP positive cells/cm² at day 21 | Controls b | OPG-XL b | P value c |
|-------------------------------------|------------|-----------|-----------|
| Nuclei/osteoclast a                  |            |           |           |
| 3-5                                 | 644 (85%)  | 290 (61%) | 2.5x10⁻¹⁰ |
| 6-10                                | 94 (12%)   | 114 (24%) | 1.1x10⁻⁷  |
| 10-20                               | 13 (2%)    | 53 (11%)  | 6.8x10⁻¹³ |
| >20                                 | 6 (1%)     | 16 (3%)   | 1.2x10⁻³  |
| Total                               | 757        | 473       |           |

a Strata of osteoclasts according to the number of nuclei per osteoclast. b Average number of osteoclasts from N = 6 OPG-XL carriers and N = 6 sex- and age-matched controls; 2–3 replicates each were counted. c P-value for each stratum was determined with χ² test relative to total number of osteoclasts in all other strata.

Fig. 3 Characterization of osteoclastogenesis
Fig. 4 Bone resorption and gene expression for osteoclasts from controls and OPG-XL carriers

(A) Representative image of bone resorption pits formed by osteoclasts from control and OPG-XL carrier on slices of human tibia bone after 21 days of culture (scale bars: 200 \(\mu\)m). (B) Percentage of bone resorption for controls \((N = 2)\) and OPG-XL carriers \((N = 2)\). (C) Concentration of CTX-1 (nM) in conditioned medium after 7, 14 and 21 days of culture (controls and OPG-XL carriers \(N = 3\)). (D) CTX-1 release in conditioned medium per osteoclast at 14 and 21 days of culture (controls \(N = 3\) and OPG-XL carriers \(N = 3\)). (E) Boxplots for \(-\Delta C_t\) values of genes related to osteoclast (continued)
chondrocytes have a tendency to undergo transdifferentiation to osteoblasts [33]. By performing osteoclastogenesis assays of OPG-XL carriers relative to age- and sex-matched controls, we showed that, although significantly lower in number, osteoclastogenesis in OPG-XL carriers resulted in high nucleated osteoclasts (Fig. 3). These multi-nuclear osteoclasts appear at least equally active as controls as demonstrated by the comparable resorbed bone surface and released levels of CTX-1 (Fig. 4). The fact that we were able to quantify the resorption pits only for two donors and controls has likely resulted in limited statistical power to detect small differences. This hypothesis could hence explain the bidirectional phenotype of the OPG-XL carriers characterized by higher cartilage mineralization and osteopenia. A similar case was previously reported by Zhang et al. [34], where MGP knockout mice have a characteristic phenotype of premature bone mineralization and osteopenia. Since RANKL was found to induce osteoclast fusion whereas OPG blocked fusion of highly mobile osteomorphs [15], it is tempting to link the role of immobilized OPG on the surface of osteoblasts to in vivo recycling of osteoclasts [35]. Nevertheless, our study does not allow us to distinguish whether the OPG-XL mutation results in highly nucleated and active osteoclasts due to enhanced RANKL availability or due to dysfunctional blocking of osteomorph fusion. Among genes significantly changed in the presence of OPG-XL we observed that MMP13 was higher in the primary chondrocytes while lower in the iPSC-derived chondrocytes. This suggests that MMP13 itself is not directly related to the changes resulting from OPG-XL, but rather, that the effect results from individual variation and/or differences in the maturity of neo-cartilage derived from hiPSCs as compared with that of primary chondrocytes.

By precise genetic engineering of hiPSCs derived from affected CCAL1 family members, while applying established differentiation protocols towards human biominemic cartilage and osseous organoids [36], we could study in multiple biological replicates how expression of OPG-XL could result in the characteristic bidirectional phenotype of subchondral bone turnover accompanied by cartilage mineralization. Strength of using isogenic controls is that this allows us to study unbiased effects of the mutation, independent of variation between family members such as genetics, sex or age. As such, we are confident that our approach was able to create reliable data highly translating to the human in vivo situation while complying with the societal need to reduce animal studies [37, 38]. A potential drawback of our study is that we were not able to model direct interaction between cells populating cartilage and bone: chondrocytes, osteoblast and osteoclasts. In this context, using the isogenic pairs of our patient-derived hiPSCs in human joint-on-chip models, currently being developed, could further address the molecular mechanism underlying the bi-directional effect of OPG on hard and soft tissues [39, 40].

**TABLE 2** Gene expression analyses of osteoclasts from OPG-XL carriers and sex- and age-matched controls

| Marker of osteoclast activity | FD  | P-value* | Marker of osteoclast activity | FD  | P-value* |
|------------------------------|-----|----------|------------------------------|-----|----------|
| TNFRSF11B                    | Day 7 | 2.0 | 1.7 × 10⁻² | Day 7 | 1.0 | 9.9 × 10⁻¹ |
| Day 14                       | −1.9 | 1.5 × 10⁻² | Day 14 | 1.2 | 9.5 × 10⁻² |
| Day 21                       | 1.8 | 2.8 × 10⁻² | Day 21 | 1.5 | 1.0 × 10⁻⁴ |
| TNFRSF11B                    | Day 7 | 2.4 | 1.0 × 10⁻⁴ | Day 7 | −2.2 | 1.0 × 10⁻⁴ |
| Day 14                       | −1.2 | 6.4 × 10⁻¹ | Day 14 | −1.1 | 5.7 × 10⁻¹ |
| Day 21                       | 1.6 | 1.6 × 10⁻² | Day 21 | 1.5 | 6.2 × 10⁻³ |
| NFATc1                       | Day 7 | −1.3 | 1.2 × 10⁻² | Day 7 | −2.1 | 1.0 × 10⁻⁴ |
| Day 14                       | 1.1 | 1.2 × 10⁻¹ | Day 14 | 2.2 | 1.0 × 10⁻⁴ |
| Day 21                       | 1.6 | 1.0 × 10⁻⁴ | Day 21 | 2.1 | 1.0 × 10⁻⁴ |

*Results presented are the average of n = 2–3 replicates from N = 4–5 OPG-XL carriers compared with age- and sex-matched controls. P-values determined with two-way ANOVA with Šidák’s multiple comparison analysis. FD: fold difference; OPG-XL: C-terminal extended osteoprotegerin encoded by TNFRSF11B readthrough mutation.

**Fig. 4** Continued

formation and activity (n = 2–3 replicates from 4–5 controls and OPG-XL carriers). P-values determined with two-way ANOVA with Šidák’s multiple comparison analysis (*P < 0.05; **P < 10⁻²; ***P < 10⁻⁴). CTX-1: C-terminal telopeptide of type 1 collagen; DC-STAMP: endodcyte Expressed Seven Transmembrane Protein; NFATc1: Nuclear factor of activated T-cells, cytoplasmic 1; OPG-XL: C-terminal extended osteoprotegerin encoded by TNFRSF11B readthrough mutation; TNFRSF11B: gene encoding RANK ligand; TNFRSF11B: gene encoding osteoprotegerin or OPG; TRAcP: Tartrate-resistant acid phosphatase.
In conclusion, our data demonstrated that expression of OPG-XL in human cartilage and bone not only enhances RANKL-induced osteoclastogenesis, but also directly affects chondrocyte and osteoblast states towards matrix mineralization via functions of respectively MGP and DIO2. We advocate that the bidirectional phenotype of subchondral bone turnover accompanied by cartilage mineralization is a characteristic process that occurs in CCAL1 patients at early ages but which can be extrapolated to common age-related OA patients. Vice versa, our data suggest that in cartilage, proper binding of OPG to HS on chondrocytes intrinsically contributes to a healthy unmineralized tissue state while in bone it supports the steady state turnover with adaptive activity involving osteoblasts and osteoclasts.

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Data availability statement
The data that support the funding of this study are available from the corresponding author upon request.

Supplementary data
Supplementary data are available at Rheumatology online.

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