Osteoprotegerin: a double-edged sword in osteoarthritis development

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CHAPTER 5

General discussion

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

Osteoarthritis disease management is hampered by a lack of translation of robust genetic findings to underlying disease mechanisms, drug target discovery and testing. In this thesis we established sustainable and reliable human 3D *in vitro* organoid models of cartilage and bone for in-depth study of *TNFRSF11B* encoding osteoprotegerin (OPG) in OA pathophysiology. OPG is a decoy receptor binding to the receptor activator of nuclear KB factor ligand (RANKL) to inhibit osteoclastogenesis. It is highly expressed in cartilage and subchondral bone, and was previously found to be consistently upregulated in transcriptome wide studies of OA affected cartilage while no specific function for OPG in cartilage was known. Additionally, a high impact mutation at the CCAL1 locus was identified by our group (1) resulting in a 19 amino acid elongation of OPG and referred to as OPG-XL. The CCAL1 phenotype is characterized by early onset osteoarthritis with different degrees of articular cartilage calcification (chondrocalcinosis), while low subchondral bone mineralization was reported in other identified CCAL1 families (2). Together, by applying next generation human organoid models of cartilage and bone we here set out to study how dysfunctional OPG is associated to OA pathophysiology and particularly for OPG-XL with respect to the bi-directional pathogenic phenotype of bone loss and articular cartilage calcification.

In **Chapter 2** we mimicked OA related expression of *TNFRSF11B* in a 3D *in vitro* model of primary chondrocytes to investigate its role in OA pathophysiology. Hereto, chondrocytes obtained from the Research Arthritis and Articular Tissue (RAAK) study were modified to overexpress *TNFRSF11B*. RNA sequencing data of preserved and lesioned cartilage from the RAAK study was used to identify differentially expressed genes correlating with *TNFRSF11B* expression. This allowed us to study a co-expression network of this gene with its associated genes in OA pathophysiology. Our study showed that *TNFRSF11B* upregulation affects chondrocytes to undergo trans-differentiation to osteoblasts.

In **Chapter 3**, we used human induced pluripotent stem cell (hiPSC) technology for an efficient directed differentiation into neo-cartilage organoids. Particularly, we focused on characterizing neo-cartilage generated from two differentiation protocols: a step-wise protocol towards generation of chondroprogenitors (hiCPCs), and a commercial protocol, following an initial transition to mesenchymal stromal cells (hiMSCs). Subsequently, neo-cartilage generated from both protocols was compared to human neo-cartilage from chondrocytes (hPACs) and bone marrow mesenchymal stromal cells (hBMSCs). Based on a set of 20 relevant genes, this showed a 65% and 53% similarity, respectively. Although still with potential to increase the similarities, our observations are

highly encouraging for the field of cartilage regeneration starting with a stable cell source such as hiPSCs.

In Chapter 4 we set out to perform in depth clinical phenotyping of OPG-XL carriers highlighting on one hand severe cartilage degeneration accompanied with severe foci of calcified cartilage, and on the other hand osteopenic subchondral bone phenotype. To obtain stable and sustainable isogenic cell sources for our model, we generated hiPSCs from an OPG-XL family member and applied CRISPR/Cas9 to repair the OPG-XL mutation. Upon employing our established OPG-XL hiPS cells and isogenic control hiPS cells to established organoid models of neo-cartilage and neo-bone tissue, the underlying disease mechanisms of OPG-XL resulting in familial early-onset OA (FOA) phenotype could be studied in detail. By doing so, we demonstrated that OPG-XL in chondrocytes resulted in excessive cartilage fibrosis marked by downregulation of *MGP*, while OPG-XL in osteoblasts showed high mineralized osseous tissue, accompanied by upregulation of *DIO2* gene expression. By differentiating monocytes from family members with OPG-XL and healthy aged matched controls to osteoclasts, and subsequently culturing them on bone, we showed a delayed osteoclastogenesis with a trend towards more active osteoclast formation in OPG-XL carriers.

Together, our studies demonstrated that generating neo-cartilage from an hiPSC source is possible and, depending on the protocol, this can result in neo-cartilage highly similar to primary chondrocytes or neo-cartilage with more hypertrophic characteristics. This highlights the strength of hiPSC technology not only for regenerative therapies but also for modelling cartilage diseases. Additionally, identified OPG co-expression network can now be exploited to further follow-up on possible novel routes underlying OA pathophysiology. Moreover, by studying the OPG-XL mutation and the common functions of OPG, we can now certainly emphasize that OA, a disease traditionally defined by cartilage loss, is heavily driven by an interplay of chondrocytes, osteoblasts and osteoclasts. The pleiotropy that OPG-XL showed in each cell indicates how OA treatments can be beneficial for one tissue while detrimental for another one. This makes OPG a double-edged sword in OA development that should be carefully monitored when treating this disease.

OSTEOPROTEGERIN; A ROLE IN COMMON OSTEOARTHRITIS

TNFRSF11B encoding for osteoprotegerin (OPG) is one of the most consistent and highly upregulated genes in lesioned OA cartilage (2, 3). Additionally, a readthrough mutation in this gene introducing 19 extra amino acids in OPG (OPG-XL) was found in an early onset OA family characterized by chondrocalcinosis.

High impact causal mutations in extended families with an early onset disease phenotype are especially relevant since they can direct towards underlying pathways that might be sharing the same etiology, hence translating its insights into common OA phenotypes (4-7).

Effects of increased OPG expression in cartilage matrix

By measuring changes of conventional matrix homeostasis and mineralization genes in **Chapter 2** we showed that upregulation of *TNFRSF11B* resulted in an increase in cartilage anabolism as measured by the higher expression of *COL1A1* and *COL2A1* while the fact that *COL10A1* was not increased suggested absence of hypertrophy (8). Nonetheless, a high *MMP13* expression combined with high *RUNX2*, *POSTN*, *ASPN* and *OGN* indicated a likely chondrocyte to osteoblast transition, commonly observed in OA pathophysiology (9, 10). We also showed that, in cartilage, the well-known *TNFRSF11B*, *TNFRSF11* and *TNFSF11A* triad is not responsive to upregulation of *TNFRSF11B.* This, together with the fact that *TNFSF11* is actually lowly expressed in cartilage and we did not find high correlation, determined by values higher than $r \geq 0.75$, between *TNFRSF11B* with *TNFRSF11A* or *TNFSF11* expression in cartilage, suggests that the interaction among the triad in cartilage may not play the same role as in bone.

This is in line with the finding of Komuro *et al* (11) and Tat *et al* (12), showing no alterations in RANK and OPG expression upon adding exogenous RANKL to chondrocytes. Interestingly, *TNFSF11* was revealed as an OA susceptibility gene in the largest OA GWAS to date (13), but this was not the case of *TNFRSF11B* and *TNFRSF11A*. This confirmed once more that the three partners of the triad have additional independent functions. Nevertheless, a higher expression of *TNFRSF11B* and of *TNFSF11* is associated with a worsening of OA status, while *TNFSF11* is one of the higher co-expressed genes to *TNFRSF11B* in OA bone (14).

Gene expression profiling with co-expression analysis has previously been used as a powerful method to identify novel co-expression networks of genes (15). This allows to determine unknown relations between shared pathways that might be of high relevance to the studied gene and to better study complex diseases such as OA (16). Moreover, these signature pathways can serve as potential candidates for diagnosis and the development of novel therapies against biological processes that might be impaired in the diseased condition (15). In our RNA sequencing data, we found 51 genes that highly correlated with *TNFRSF11B* ($r \geq 0.75$), representing the co-expression network of *TNFRSF11B* in OA pathophysiology. From this network, the expression of 30 genes was compared between control hPACs and hPACs overexpressing *TNFRSF11B*. Despite the high correlations with *TNFRSF11B*, notably only eight genes (27%) were found to be responsive to *TNFRSF11B* upregulation in our *in vitro* model (*CDON*, *BMP6*, *CDH19*,

P3H2, WNT16, SLC16A7, SLC15A3 and *FITM2*). This may be explained partly by the fact that some of the genes are upstream of *TNFRSF11B*. Alternatively, genes may be correlated to *TNFRSF11B* as a general result of ongoing OA disease processes, since *TNFRSF11B* is one of the highest upregulated genes in lesioned OA cartilage (3). This illustrates that *in vitro* models are representative for the *in vivo* situation, but also have their limitations.

Notable among the *TNFRSF11B* correlated and highly responsive genes was *BMP6*. *BMP6* encodes the bone morphogenetic protein 6 and is a member of the transforming growth factor superfamily. It is known to have a pleiotropic role that ranges from increasing chondrogenic differentiation potential and proteoglycan deposition, chondrocyte maturation, osteoclast inhibition, induction of bone ECM deposition, and OA development (17-22). By performing our unique co-expression approach we were able to show that BMP6 is strongly upregulated in our model. Given the known functions of *BMP6* in bone formation we hence hypothesize that it might play a role in driving OA by enhancing chondrocyte to osteoblast transition. This highlights the strong potential of our approach to detect novel target genes that otherwise would go unnoticed, and addresses BMP6 and the other osteogenic markers as possible cartilage OA therapeutic targets.

In conclusion, the particularly high upregulation of *MMP13* in combination with the upregulation of characteristic osteogenic genes *RUNX2, POSTN, BMP6, ASPN,* and *OGN* and in absence of differential expression of the hypertrophic and mineralization markers *COL10A1* and *ALPL,* demonstrated that *TNFRSF11B* affects OA pathophysiology by advancing late stage terminal maturation (23). This would urge for novel therapies aiming at directly reducing undesired osteogenic transitions, such as vitamin K supplementation. For that matter, the use of vitamin K antagonist as anticoagulation therapy has shown an increase in progression of hip and knee OA, highlighting the importance that vitamin K pathways can have in OA (24). Therefore, it could be speculated that vitamin K may also be considered as a possible treatment in the OPG-XL family members, since the function of MGP is dependent on this vitamin. Likewise, these highly OPG responding genes might arise as attractive targets for researching its molecular effect and bring into play novel drug treatments. On the other hand, we were not able to visualize a mineralization effect at immunohistochemistry or histology levels. This might be due to the selected early timepoint (day 7) for evaluating chondrocyte neo-cartilage deposition towards mineralization of the ECM (25). A later timepoint might have shown a stronger mineralization at protein level (23, 26). An alternative approach would be to upregulate of *TNFRSF11B* in MSCs (27) with subsequent generation of neo-cartilage. This would likely show an even stronger mineralization effect including matrix calcification. Ultimately, the lack of changes in expression in the *TNFRSF11B* triad in cartilage suggests it functions differently as in bone, highlighting more research is needed to understand their function in cartilage.

hiPSC TECHNOLOGY IN CARTILAGE REGENERATION AND DISEASE MODELING

hiPSCs provide sustainable and reliable OA relevant tissue organoids while reduce dependence on joint tissue availability. Additionally, they address the societal need to reduce, refine and replace the use of animals models (28). When combined with CRISPR/Cas9 genetic engineering, we can precisely repair or introduce disease mutations and generate isogenic controls with identical genetic background to study their particular effect. Nonetheless, hiPSCs have also shown challenges due to the strong variation in differentiation efficiencies between lines and a tendency to generate hypertrophic and fibrous matrix (29-31). In **Chapter 3** we therefore first assessed the efficiency of hiPSCs neo-cartilage organoid production as well as its quality as compared to the autologous tissue by following a step-wise protocol to generate chondrocytes from human iPSC-derived chondroprogenitor cells (hiCPCs) (32) and a commercial protocol via human iPSC-derived mesenchymal stromal cells (hiMSCs).

By prioritizing on *COL2A1* neo-cartilage gene expression (33), we observed a 79% success rate for the step-wise protocol via hiCPCs and a 54% success rate for the commercial protocol via hiMSCs. For that matter, hiMSCs displayed high levels of heterogeneity in cell morphology and proliferation with respect to each other whereas classical hBMSC characterization markers associated to trilineage differentiation potential did not correlate to chondrogenesis success. Although a 79% success rate is considerable, more research is necessary to improve expansion of hiCPCs. It could be questioned if prioritization for *COL2A1* was the optimal strategy since *COL2A1* is a late chondrogenic marker also expressed in a wide variety of cells (33).

Next, we compared the quality of the neo cartilage deposited by hiCPCs relative to human primary chondrocytes (hPACs) and of hiMSCs relative to human bone marrow mesenchymal stromal cell (hBMSCs) based on a similarity panel of 20 genes related to chondrogenesis, hypertrophy and degradation of cartilage ECM. For hiCPCs relative to hPAC neo-cartilage we observed a 65% similarity. For the hiMSCs relative to hBMSCs we observed a similarity of 53%.

These results suggest that the matrix generated by hiMSCs had a hypertrophic phenotype defined by a high gene expression of *COL1A1*, *ALPL,* and *MMP13* and low *COL2A1*. The expression of *MMP13* and *ALPL* would suggest a higher collagen degradation with a subsequent calcification, characteristic of terminal chondrogenic differentiation and endochondral ossification (34, 35). A follow-up strategy could be based on the selection of an earlier chondrogenic marker such as *SOX9*. This was recently performed for immortalized adipose-derived stem cells resulting in an enhanced chondrogenic potential in these cells

accompanied with low hypertrophy (36, 37). Hence, at its current state, it could be advocated that hiMSCs are an ideal candidate for studying skeletal diseases in which endochondral bone formation and hypertrophy are driving mechanisms (38, 39). Nonetheless, for obtaining high quality neo-cartilage an improvement of this protocol is still required.

With respect to the step-wise protocol, we can certainly claim that hiCPCs are an excellent tool for efficiently (79%) producing high quality neo-cartilage (65%). The main differences between hPAC and hiCPC neo-cartilage are based on a higher expression of chondrogenic markers and lower hypertrophy in the hiCPCs. This would make them suitable candidates for production of neo-cartilage implants as regenerative therapy and drug testing applications. To have a further translation into the clinic, neo-cartilage generated from hiCPCs should be initially tested by implantation in animal models (40), and subsequently translated into humans. Nevertheless, the lack of expansion capacity of hiCPCs arises an issue that urgently needs to be solved for further translational applications.

Neo-cartilage generation with the current hiMSCs differentiation protocol requires an optimization of the initial chondrogenic selection process. In this respect, we observed that during the experiments of **Chapter 4**, higher concentrations of cells (750000 cells/pellet) resulted in a more stable cartilage deposition, similarly to results observed by Diederichs et al (37). Hence, we advocate that high quality neo-cartilage deposition from hiMSCs requires a higher cell density (41). Another decisive factor to determine chondrogenic potential was the cell proliferation rate. This parameter was not quantitatively measured in our study. Yet, when analyzing the time frame required for cell passaging, we observed that fast proliferating cells generated a more homogenous neo-cartilage deposition (unpublished data). This was similar to results shown by Dexheimer et al (42) and Mareddy et al (43) in BMSCs. Finally, several compounds can be administered to enhance mesodermal differentiation and improve successful chondrogenesis yield (44). For instance, Kreuser et al (45) noticed that an initial WNT/β-catenin pulse by CHIR99021, an essential process for primitive streak induction, strongly enhanced ECM-related gene expression markers and chondrogenic pellet formation. On the other hand, WNT inhibition during hiPSC derived-chondrogenesis showed a better cartilage deposition (46), altogether, suggesting the timing of activation and inhibition is key. Due to other hiMSC characteristics such as expansion capacity and trilineage and immunoregulatory potential, their applications in the regenerative medicine field will quickly develop. A summary of the different advantages and disadvantages in hiCPC and hiMSC differentiation and improvement strategies can be found in **Table 1**. In the meantime, by virtue of their predisposition for hypertrophy and endochondral ossification, hiMSCs were selected in this thesis to generate neo-cartilage from the OPG-XL carriers.

Table 1. Summary of iMSC and iCPC advantages and disadvantages for regenerative medicine and disease modeling, and strategies to improve them.

Model	Advantages	Disadvantages	Strategies to improve models	References
iMSCs	Differentiation potential into chondrocytes, osteoblasts and adipocytes	Cell heterogeneity	Higher cell density in neo-cartilage organoids	(37)
	Higher expansion capacity	Longer differentiation protocol	Step-wise iMSC generation: Wnt/B-catenin	(45)
	Cell storage	Hypertrophic cartilage	Chondrogenic protocol adaptation to iMSCs (Wnt inhibition)	(46)
	Immunoregulatory potential		Optimization of a defined step-wise protocol; for instance with an early chondrogenic reporter line	(37)
	Exosome applicabilities		Selection of highly chondrogenic-osteogenic markers on iMSCs based on for instance cell proliferation capacity	(43)
			Selection of highly chondrogenic-osteogenic gene expression markers during chondrogenesis and osteogenesis	(47)
iCPCs	Direct and highly reproducible chondrogenic differentiation	Low expansion capacity	Improvement of iCPC maintenance protocol	(33)
	Similar neo-cartilage to primary chondrocytes neo-cartilage	Quick loss of chondrogenic potential	Filter aggregates depending on size	
		Inability to be stored	Use of an early chondrogenic reporter line to select for chondrogenic lineages	(32)
		Heterogeneity in visual aggregate selection and cell generation	Low cell heterogeneity might be beneficial for chondrogenesis generation	

EMPLOYING DISEASE MODELLING USING AN EARLY ONSET OA MUTATION

In **Chapter 4** we applied hiPS-cell derived cartilage and osseous organoid modelling and CRISPR/Cas9 technology to study the underlying disease mechanism of a high impact mutation at the CCAL1 locus resulting in a 19 amino acid elongation of the C-terminal end of OPG (OPG-XL). Specifically, we focused on the bidirectional pathogenic phenotype of bone loss and articular cartilage calcification observed in carriers of the mutation (1, 2).

Clinical examination by DEXA scans of the OPG-XL carriers confirmed osteopenia while MRI revealed a severe OA phenotype characterized by different degrees of chondrocalcinosis, osteophytosis, bone marrow lesions and cysts. In order to explain the bidirectional effect of OPG-XL, osteoclastogenesis was performed with monocytes from six OPG-XL carriers and matched controls. This revealed a delayed osteoclast primed state with a tendency towards increased bone resorption activity in time. Due to the rarity of the mutation, primary chondrocytes and osteoblasts are not easily obtained, demanding other strategies to investigate OPG-XL in these cells. For this, hiPSCs were generated from a carrier of the OPG-XL mutation by the LUMC iPSC core facility. Subsequently, hiPSCs were differentiated into hiMSCs and further towards chondrocytes to explore neo-cartilage deposition or towards osteoblast for neo-bone formation. Results were further confirmed in neo-cartilage of hPACs derived from a carrier of the mutation that during the course of this thesis had a joint replacement surgery, thus serving as a validation of our hiPSC model.

OPG-XL effect in neo-cartilage and neo osseous organoids

hiPSC-derived neo-cartilage tissue formed by OPG-XL chondrocytes, relative to isogenic controls, showed a fibrotic histological phenotype without obvious mineralization but with marked downregulation of *COL2A1* and, most notable, of *MGP* gene expression. As *MGP* is an inhibitor of ectopic bone formation (48) and a robust OA risk gene (13, 49) our data demonstrated that OPG-XL directly affects propensity of chondrocytes to enter a mineralized OA state. hiPSC-derived neo-osseous tissue formed by OPG-XL mutated osteoblasts relative to isogenic controls displayed a high calcification as reflected by the prominent Alizarin red staining concurrent with notable high gene expression of *DIO2* and low of *TNFSF11* encoding for RANKL. *DIO2*, encoding type 2 deiodinase enzyme, is essentially facilitating bone formation and mineralization (50), while the lower expression of *TNFSF11* indicates a direct interaction between OPG-XL and RANKL in bone which is missing in chondrocytes. By performing RNA sequencing between lesioned and preserved subchondral bone in our lab (14), *TNFSF11* was shown as one of the genes with highest correlated with *TNFRSF11B*. This was confirmed in our OPG-XL model. The strong Alizarin red staining of neo-osseous tissue, concurrent with *DIO2* and *MGP* upregulation could therefore explain the extensive phenotypic foci of calcified cartilage observed in OPG-XL carriers. This would suggest that chondrocalcinosis arises during ongoing OA pathophysiology (51).

OPG-XL effect in osteoclasts

By studying the effects of the OPG-XL mutation in our osteoclast assay, a strong reduction in osteoclasts with few nuclei in the OPG-XL group was observed. Nevertheless, when osteoclasts were cultured for longer periods, they showed a

trend towards an increase in the number of nuclei per cell. Despite the significantly lower number of osteoclasts with few nuclei, a similar bone resorption activity was described, hence suggesting a higher osteoclast activity. This was also supported by expression levels of known osteoclast markers *NFATc1*, *CTSK*, *TRAcP* and DC-*STAMP*. Nevertheless, CTX-1 expression per osteoclast did not show differences between both groups. These data would confirm an initial priming state of OPG-XL that would delay osteoclast generation, and a tendency to increase osteoclast activity over time.

In addition to the fact that osteoclastogenesis data demonstrated important modulatory effects of OPG expression during maturation of monocytes to osteoclasts, we could link the role of OPG-XL to a dysfunctional *in vivo* recycling of osteoclasts via fission of polykaryons and fusion of the recently discovered osteomorphs during bone resorption (52). In this cycle, osteomorphs are mobile cells formed after fission of osteoclasts, whereas osteoclasts can be formed by (re-)fusion of such cells. Importantly, RANKL was found to induce osteoclast fission, whereas OPG blocked fusion of osteomorphs (53) that upon withdrawal results in their fusion and a higher osteoclast activity. Hence, it could be speculated that the higher amount of RANKL due to a dysfunctional binding of RANKL-HS-OPG-XL would result in a higher osteoclast formation and fission as observed in the lower numbers of osteoclasts during initial timepoints of osteoclastogenesis. Consequently, and as a feedback mechanism, RANKL would decrease, as observed in the neo-osseus organoids, allowing osteomorphs to fuse. At the same time, dysfunctional OPG-XL would result in a lower inhibition of osteomorph fusion which would generate osteoclasts with a higher activity and more nuclei, as observed in our data **(Figure 1)**.

Pleiotropy of OPG-XL

Here, we clearly showed the pleiotropy of OPG-XL in different tissues: cartilage, bone and osteoclasts. The priming effects caused in osteoclastogenesis and the lack of response of RANK and RANKL in chondrocytes indicate that OPG has a broader role than only the well-known common bone resorption processes. This is highlighted by the contradicting results of OA therapies promoting OPG overexpression such as strontium ranelate and the data shown in this thesis. Altogether this suggests a double-edged sword effect of OPG. A coculture with hiPSC derived osteoblasts, chondrocytes and osteoclasts in a model that allows crosstalk between the different cells would be essential to understand its function in the joint tissue. Moreover, a characterization of its binding properties to HS-RANKL should be investigated.

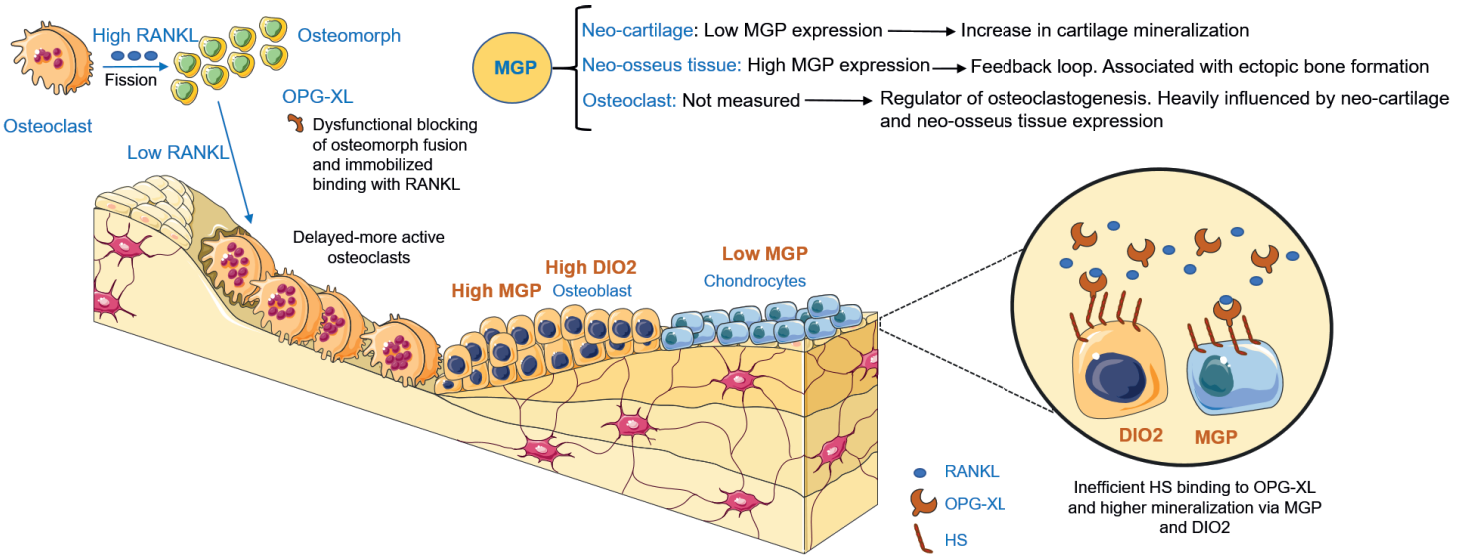

Figure 1. Proposed mechanism of OPG-XL in neo-cartilage, neo-osseus tissue and osteoclasts. Osteoclasts undergo a strong fission into osteomorphs and posteriorly accumulate for longer time periods after high RANKL expression due to inefficient OPG-XL-HS binding. Upon RANKL decrease as a feedback mechanism, and dysfunctional OPG-XL fusion blocking, osteoclast fusion resumes with a higher activity. *MGP* differences in expression in cartilage and bone through defective OPG-XL-HS binding result in a higher mineralization in cartilage and ectopic bone formation. Moreover *MGP* produced by both tissues deeply controls osteoclast activity, contributing to higher bone resorption.

With respect to osteoclastogenesis, accumulation of osteomorphs likely contribute to the FOA phenotype. A better understanding of these cells, together with a proper characterization of their markers is essential to understand their role in bone resorption in common OA and more specifically in the FOA family members. The here described characteristics of OPG are very similar to those of MGP and appear to be mediated via this protein. Therefore, in addition to further study OPG, we also propose to investigate the role of MGP in relation to osteomorph function and more specifically in other systems where MGP is involved such as in the vasculature system of the OPG-XL carriers. In the meantime, the robust MGP changes and its contribution to controlling mineralization and osteoclast generation indicate the potential suitability of this protein as a therapeutic target for the FOA family members.

FUTURE PERSPECTIVES

During the development of this thesis, hiPSC applicability into disease modeling and regenerative therapies has seen a sharpen rise. An increase of our understanding of hiPSC nature, clonal heterogeneity, and tissue differentiation is further developing by studying these cells and their differentiation potential at the single cell transcriptomic and epigenetic level (31, 54). Genetic studies of risk disease genes, especially in GWAS, offer a direct application when combined with hiPSC technology and genetic engineering strategies such as CRISPR/Cas9. Hence, particular risk SNPs could be further studied in big cohorts of hiPSC libraries with defined genetic make-up and translated from *in silico* approaches towards *in vitro* strategies (55). Nevertheless, when studying disease models, we should take into account the tissue immaturity that would have to be addressed for studying ageing diseases such as OA (56). Particularly here, the integration of environmental factors associated with OA, such as mechanical loading, should be researched (57). Moreover, a novel sense of disease perception in OA is necessary. As such, OA, a traditionally cartilage degenerative disease, is gradually progressing to be considered a whole joint disease where an interplay with osteoblasts and osteoclasts can play a pivotal role in its development. This thesis clearly shows this by studying OPG and its readthrough mutation. The effects shown in the likely chondrocyte to osteoblast transdifferentiation and OA development upon OPG overexpression are in contrast with positive results observed upon administering strontium ranelate. Additionally, the readthrough mutation identified in the OPG-XL family members clearly indicated an interplay between chondrocytes, osteoblasts and osteoclasts where vitamin K treatments arise as a possible treatment option. This highlights the need in future research for complex disease models, in which chondrocytes, osteoblasts, osteoclast and synovial cells can properly interact.

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