Osteoprotegerin: a double-edged sword in osteoarthritis development

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CHAPTER 1

General introduction

OSTEOARTHRITIS

Osteoarthritis (OA) is a chronic age-related degenerative disease of the joints characterized by degradation of the cartilage extracellular matrix (ECM), osteophyte formation, synovitis, and alterations in subchondral bone **(Figure 1A)**. OA affected joints are usually hands, hips and especially knees (1). Multiple risk factors of OA, such as gender, body mass index (BMI), bone mineral density (BMD), injury, and genetics influence the severity, course, and age of onset (2) **(Figure 1B)**. Due to current higher life expectancy, and an increase in metabolic factors such as obesity (3), a steep increase in OA prevalence is anticipated (4, 5). As such, according to the world health organization, in 2050, 15% of the world population over 60 years old will suffer from OA, of whom one third will be severely disabled. Moreover, with increasing OA patients, comorbidities such as stroke, peptic ulcer, and metabolic syndrome will rise in parallel (6).

Clinically, OA is marked by chronic pain, stiffness, and disability of patients (1, 7). Despite debilitating symptoms, no effective therapy is available except for joint replacement (1). Joint replacement, however, does not guarantee complete recovery since almost 25% of patients still experience pain and disability one year after surgery (8). Moreover, costly surgical procedures and lengthy rehabilitation is commonly accompanied with a decline in productivity. Hence, OA has a vast impact on economy, with health care costs accounting for 1-2.5% of gross national products (9, 10). In absence of effective disease modifying treatment strategies, patient care is mainly focused on controlling symptoms and minimizing disability, e.g. by non-steroidal anti-inflammatory drugs (NSAIDs) or life-style interventions, respectively (11, 12). To advance development of effective disease modifying OA treatments a better understanding of its pathophysiological mechanisms is necessary.

Radiographically, OA is assessed by Kellgren and Lawrence grading (0-4), which is based on a combination of characteristics such as joint space narrowing, osteophytosis and scleroses **(13)**. Radiographic OA score, however, does not accommodate emerging information about OA pathophysiological processes, becomes only visible once irreversible damage of joint tissues is a fact, and is insensitive to change (14). A more sensitive technique is magnetic resonance imaging (MRI). This method allows visualization of all joint tissues including cartilage, and is more sensitive to changes in disease over time, hence suitable to test efficacy of novel disease-modifying therapies (15). To score OA severity of hips, hands and knees with MRI the 'hip OA MRI scoring system' (HOAMS), Oslo hand OA MRI (OHOA-MRI), or MRI OA knee score (MOAKS) are applied respectively. These systems evaluate OA progression (0-4) by measuring osteophyte and cyst formation, cartilage loss or bone marrow lesions, among other parameters (16, 17).

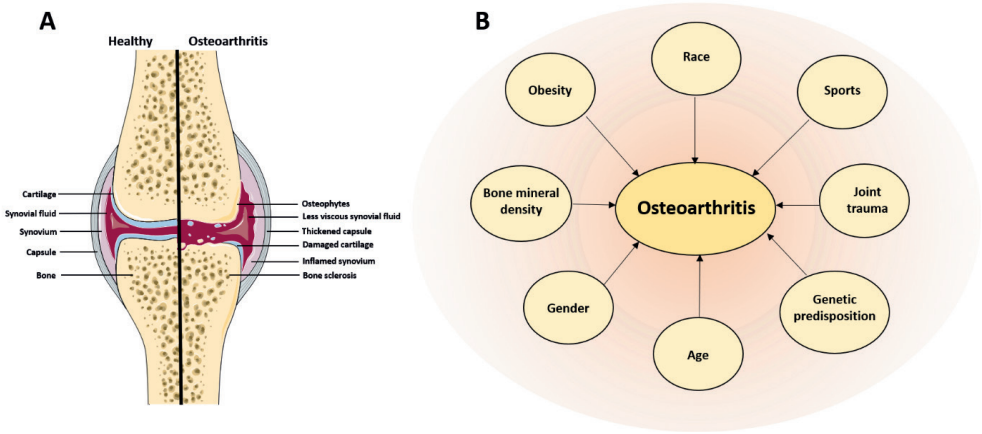

Figure 1. Osteoarthritis development and common risk factors contributing to disease progression. A) Healthy and osteoarthritic knee. **B**) Most common risk factors contributing to OA. Figure adapted from Wieland et al (18).

FORMATION OF OSTEOCHONDRAL COMPARTMENT OF JOINTS

During embryonic development and until young adulthood, bone is generated in a process termed endochondral ossification. Mesenchymal stromal cells (MSCs) condensate and differentiate into chondroprogenitor cells and further differentiate towards chondrocytes that deposit ECM **(Figure 2)**. Subsequently, the ECM network is invaded by osteoprogenitor cells that generate centres of ossification which gradually become hypertrophic and mineralized, while chondrocytes undergo terminal maturation with cartilage breakdown. This is reflected in the higher expression of metalloproteinase 13 (MMP13), that degrades the ECM, and of collagen type I and X that results in its mineralization. Additionally, hypertrophic chondrocytes can also transdifferentiate into osteoblasts, contributing to bone formation upon increased signaling e.g. from the RUNX Family Transcription Factor 2 (RUNX2) (19). In parallel with this process, vascular endothelial growth factor (VEGF) signals induce vessel formation and osteoclast, MSC and osteoprogenitor cells migration to the ECM. Moreover, a secondary ossification centre is formed at the extremes of long bones. This generates a cartilage growth plate, responsible for longitudinal growth of bone. For this, osteoclasts remove the previous cartilage matrix while osteoblasts deposit a novel bone matrix, lengthening bone tissue and ultimately replacing cartilage (20-22). When the primary ossification centre reaches the secondary centre of ossification, the growth plate closes and skeletal maturity is achieved.

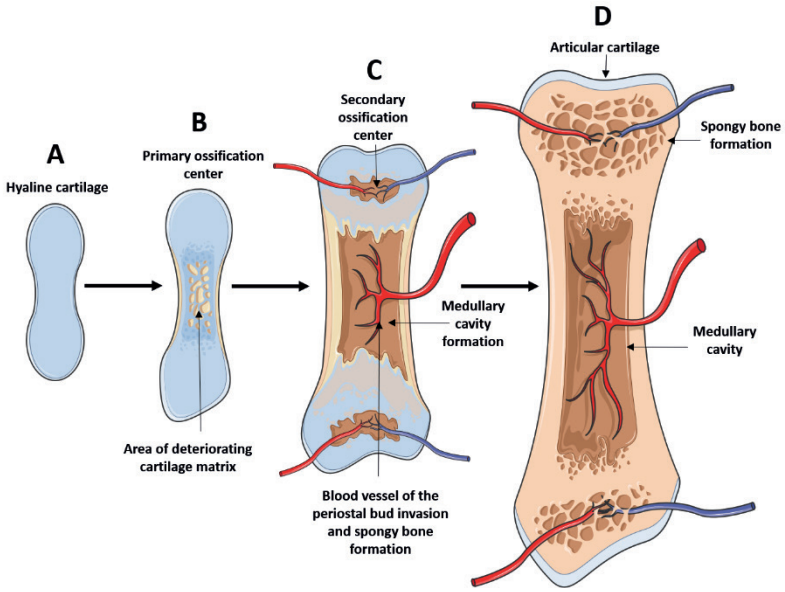

Figure 2. Endochondral ossification from the growth plate. (A) Bone collar forms in the diaphysis of hyaline cartilage. **(B)** Subsequently, cartilage calcifies in the centre of the diaphysis and develops an initial ossification centre where blood vessels of the periosteal bud invades its internal cavities. **(C)** Spongy bone starts forming and diaphysis elongates, generating a medullary cavity. **(D)** In parallel, a secondary ossification centre forms. The extremes of the bones ossify and hyaline cartilage remains on its surface. Adapted from Egawa et al (23).

Healthy (state of) osteochondral compartment

Articular hyaline cartilage is an avascular connective tissue that escapes the endochondral ossification process to cover and protect the end of long bones (24). It contains a structured network of dense ECM produced by a unique cell type, named chondrocyte. From a metabolic perspective, chondrocytes reside in a maturational arrested, near quiescent state in the articular cartilage (25). Nevertheless, during healthy cartilage remodelling, they can become metabolically active to allow ECM breakdown and novel cartilage formation (26, 27). Hence, it is essential that chondrocytes are able to return to their maturational arrested phenotype in order to maintain their healthy steady state. In healthy articular cartilage, different layers can be distinguished depending on its composition and cell morphology (28-30). The superficial zone is formed by flattened chondrocytes, and collagen fibres, and it represents between 10-20% of the total cartilage. The middle zone represents between 40-60% of the total cartilage layer, and has a lower chondrocyte density. The deep zone represents 30% of the total cartilage **(Figure 3A)**. In this layer, chondrocytes are organized in columns, showing the lowest cell density of all cartilage zones. Lastly, the calcified zone contains a reduced number of chondrocytes which are embedded

in a calcified matrix. Remarkably, between the deep and calcified zone there is an interface called tidemark which represents the border between uncalcified and calcified cartilage (24, 31).

Cartilage ECM consists of a mix of collagens, proteoglycans, glycosaminoglycans and glycoproteins responsible for its protective and viscoelastic properties (29, 32, 33). Collagen fibres constitute the primary support of the ECM, provide tensile strength and allow for cell adhesion in cartilage (34). Among all types of collagen, collagen type II (COL2) is the most abundant, reaching 90-95% of total collagen composition (33) and stabilizing the ECM together with proteoglycans. The largest proteoglycan, aggrecan, is distributed within the collagen matrix and forms a complex with the glycosaminoglycan hyaluronic acid. The resulting negative charge causes a strong retention of water in the tissue. The flow of water, upon repetitive loading cycles, distributes nutrients to chondrocytes and withdraws waste products. Additionally, the synovial fluid provides lubrication and osmotic properties necessary to hold mechanical loads and reduce friction on the joints.

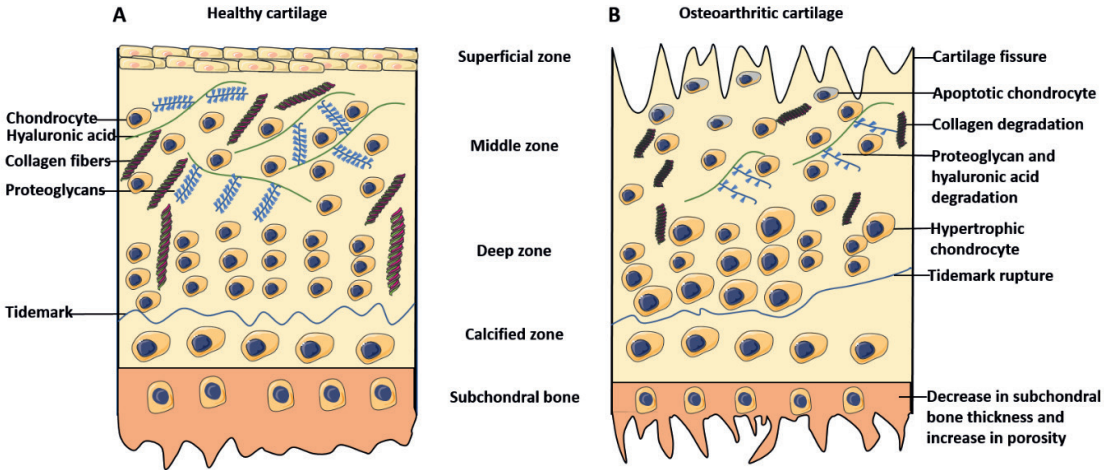

Figure 3. The different zones and cell composition in healthy (A) and osteoarthritic (B) articular cartilage.

Subchondral bone is the rigid tissue underlying calcified cartilage. It is divided in the subchondral cortical plate, with a more compact bone, and the subchondral trabecular bone with a porous composition (35, 36). Subchondral bone distributes mechanical loads across joint surfaces and provides nutrient supply to cartilage (37). It is composed of different cell types that coordinate a highly responsive remodelling process. Osteoblasts initiate mineralization of the ECM, where collagen type I acts as a scaffold for hydroxyapatite crystal deposition (38). The majority of

osteoblasts (90%) becomes embedded in lacunae within the mineralized matrix and forms the osteocytes. Osteocytes are responsible of sensing mechanical loads and controlling bone formation and remodelling processes (39). To accomplish bone resorption, the osteoclasts, a group of haematopoietically-derived cells, ensure that 10% of the total bone mass gets replaced every year (30, 40, 41).

Osteoarthritic osteochondral compartment

OA can severely affect organization of the cartilage ECM. As such, in OA, the superficial cartilage zone starts to present fissures and erosions that slowly progress to the middle zone. Later, loss of proteoglycans and glycosaminogycans follow, all accompanied with a higher chondrocyte proliferation (4, 42). As OA develops, the fissures extend into the deep cartilage zone causing chondrocyte hypertrophy, further proliferation, and ultimately apoptosis. Altogether, OA creates an unbalanced tissue remodeling towards breakdown (42, 43). This is also accompanied by an increase in collagen and proteoglycan breakdown enzymes such as metalloproteinases (44, 45) and aggrecanases. In parallel, there is a reduction of collagen type II, with an increase in collagen type I and collagen type X (46). Subsequently, the calcified zone extends and a rupture of the tidemark can occur, resulting in a new calcified/uncalcified boundary while the physical barrier preventing vessel growth is lost **(Figure 3B)** (47). Remarkably, there is a strong resemblance between OA pathophysiological processes and the terminal differentiation observed in growth plate chondrocytes during endochondral ossification (48-50). As such, both processes result in a regain of growth-plate morphology and end-stage mineralization. To quantify these differences at a histological level, a grading system such as the Mankin score can be applied. For this, joint tissues are scored from zero (normal) to fourteen (most severe) based on cartilage structure, cellularity, proteoglycan content and tidemark disruption (51).

OA in subchondral bone is characterized by an increase in bone remodelling which leads to a thinning of the subchondral plate **(Figure 3B)**. Progression of the OA process results in a reduction of bone turnover, followed by subchondral bone sclerosis and a reduced thickness of the trabeculae (52, 53). Moreover, there is an increase in subchondral bone volume concomitant with reduced subchondral bone mineralization and stiffness due to a lower calcium to collagen ratio (37). To quantify OA progession in subchondral bone, a four-stage evaluation scale (1-4) was developed by Aho et al (54). This method was based on subchondral bone remodelling and increase in subchondral bone volume. Nevertheless, there is no consensus yet about a standardized method to grade subchondral bone OA progession. Besides the cartilage and bone independent processes in OA, a strong interaction between both tissues exists (55, 56). To better understand this interaction and their role in OA pathophysiology further research at a molecular level is necessary (38).

OA PATHOPHYSIOLOGY AT THE MOLECULAR LEVEL

To characterize OA pathophysiology, genome-wide differential gene expression analyses can be performed, while comparing macroscopically intact (preserved) with lesioned OA joint tissues (57). By performing RNA sequencing of macroscopically preserved and lesioned OA cartilage of 35 paired samples of OA patients in the RAAK (Research Arthritis and Articular Cartilage) study, (58) it was revealed that the OA pathophysiological process in cartilage is marked by 2387 differentially expressed genes. Consistent differentially expressed (DE) genes in OA-lesioned cartilage in this study (58) can be found in **Table 1**. These DE genes are enriched in pathways involved in skeletal development, cell adhesion, and extracellular matrix organization. Notable dysregulated genes involved in matrix mineralization that are highly consistent in lesioned versus preserved OA cartilage are *POSTN*, *MGP* and *TNFRSF11B* (58). Moreover, as marked by the consistent differential expression of genes *RUNX2*, *MMP13*, *SOX9*, *DIO2*, *COLX* and *ALPL* with OA, (43, 59) all orchestrating the endochondral ossification process, it was confirmed that chondrocytes entering an OA state recapitulate a growth plate morphology and may be subject to trans-differentiation to osteoblasts. As demonstrated by methylome wide studies of preserved and lesioned OA cartilage, the propensity of articular chondrocytes to undergo terminal maturation is associated with loosening of epigenetically controlled transcription (29, 60, 61) and further supports a shared route between endochondral ossification and OA (59, 62-64).

Additionally, RNA sequencing analyses in preserved versus lesioned subchondral bone of 26 OA patients, resulted in 1569 DE genes that mark the OA pathophysiological process in bone. Some of the most significantly DE genes in OA-subchondral bone can be found in **Table 2**. Of those genes, 305 showed the same direction of effect in cartilage and in subchondral bone, 14 of which were among the 25 highest expressed genes in both tissues (65), indicating crosstalk between articular cartilage and subchondral bone. These genes showed an enrichment in processes related to the extracellular matrix, characterized by up-regulation of *WNT16* and *OGN*, and of the proteinaceous extracellular matrix, characterized by up-regulation of *POSTN* and *ASPN*. Notably, *TNFRSF11B,* a gene involved in bone remodelling, was shown to be consistently and highly upregulated in OA cartilage while it was not differentially expressed in OA bone.

Table 1. Highest upregulated genes in OA cartilage in the RAAK study obtained from RNA sequencing of preserved against lesioned cartilage in 35 OA patients (58).

Gene	Name	Protein function	Effect in OA cartilage
IL11	**Interleukin 11**	**Cytokine stimulator of hematopoietic T cells**	**UP**
P3H2	Prolyl 3-Hydroxylase 2	Enzyme necessary for collagen chain assembly and stability	UP
ISM2	Isthmin 2	Type 1 thrombospondin domain, present in extracellular matrix proteins.	UP
CISH	Cytokine Inducible SH2 Containing Protein	Inhibitor of cytokine signaling	DN
CXCL14	C-X-C Motif Chemokine Ligand 14	Cytokine involved in immunoregulatory and inflammatory processes	UP
CD55	CD55 Molecule (Cromer Blood Group)	Glycoprotein involved in the regulation of the complement cascade	UP
NGF	Nerve Growth Factor	Protein involved in regulation and growth of sympathetic and sensory nervous systems	UP
WNT16	**Wingless-Type MMTV Integration Site Family, Member 16**	**Signaling protein involved in cell fate, developmental processes and patterning in embryogenesis**	**UP**
RIPK4	Receptor Interacting Serine/Threonine Kinase 4	Serine/ threonine protein kinase	UP
LRRC1	Leucine Rich Repeat Containing 1	Gene associated with epilepsy	UP
LAMB3	Laminin Subunit Beta 3	Laminin that belongs to basement membrane proteins	UP
R3HDML	R3H Domain Containing Like	Peptidase inhibitor	UP
PTGES	Prostaglandin E Synthase	Glutathione-dependent prostaglandin E synthase involved in collagen-induced arthritis	UP
TNFRSF11B	TNF Receptor Superfamily Member 11b	Osteoblast-secreted decoy receptor and negative regulator of bone resorption	UP
RELN	Reelin	ECM matrix protein involved in cell-cell interaction in brain development	DN
NPR3	Natriuretic Peptide Receptor 3	Peptide receptors involved in endocytosis of natriuretic peptides	UP
ERFE	Erythroferrone	Protein involved in lipid uptake in adipocytes and hepatocytes	UP
RCAN2	Regulator Of Calcineurin 2	Protein involved in endothelial cell function and angiogenesis	DN
PPP1R14C	**Protein Phosphatase 1 Regulatory Inhibitor Subunit 14C**	**Phosphatase involved in neuronal activity, metabolism, cell division and muscle contraction**	**UP**
SERPINE2	Serpin Family E Member 2	Serine protease inhibitor	UP

Table 2. Highest upregulated genes in OA subchondral bone in the RAAK study obtained from RNA sequencing of preserved against lesioned cartilage in 26 OA patients (65).

Gene	Name	Function	Effect in OA bone
WNT16	**Wingless-Type MMTV Integration Site Family, Member 16**	**Signaling protein involved in cell fate, developmental processes and patterning in embryogenesis**	**UP**
IL11	**Interleukin 11**	**Cytokine stimulator of hematopoietic T cells**	**UP**
GDF6	Growth Differentiation Factor 6	Protein involved in bone formation and activation of SMAD signaling	UP
OGN	Osteoglycin	Regulator of Osteoblast differentiation and ectopic bone formation	UP
ASPN	Asporin	Chondrogenesis regulator and inducer of collagen mineralization	UP
MYO3A	Myosin IIIA	Protein involved in hearing	UP
CRLF1	Cytokine Receptor Like Factor 1	Involved in survival of neuronal cells	UP
GPR158	G Protein-Coupled Receptor 158	Protein highly expressed in the brain	UP
PPP1R14C	**Protein Phosphatase 1 Regulatory Inhibitor Subunit 14C**	**Phosphatase involved in neuronal activity, metabolism, cell division and muscle contraction**	**UP**
MT1G	Metallothionein 1G	Protein related to metal ion transporter pathways	UP
ALX4	ALX Homeobox 4	Transcription factor expressed in the mesenchyme of developing bones limbs, hair, teeth and mammary tissue	UP
P4HA3	Prolyl 4-Hydroxylase Subunit Alpha 3	Component of an enzyme involved in collagen synthesis	UP
FAP	Fibroblast Activation Protein Alpha	Protein involved in fibroblast growth and epithelial mesenchyme interactions	UP
POSTN	Periostin	Protein involved in tissue development being essential for skeletal, dental and cardiac development	UP
HIF3A	Hypoxia Inducible Factor 3 Subunit Alpha	Protein involved in hypoxia	DN
GPC5	Glypican 5	Protein involved in control of cell division and growth regulation	DN
FGF14	Fibroblast Growth Factor 14	Protein involved in embryonic development, cell growth and tissue repair.	DN
KIF1A	Kinesin Family Member 1A	Transporter of organelles along axonal microtubules	DN
SPOCK3	SPARC (Osteonectin), Cwcv And Kazal Like Domains Proteoglycan 3	Calcium-binding protein involved in MMPs inhibition	DN
CHRDL2	Chordin Like 2	Protein expressed in osteoblasts and OA cartilage	DN

GENETIC STUDY DESIGN FOR IDENTIFICATION OF CAUSALITY UNDERLYING DEVELOPMENT OA

The strong genetic component of OA was traditionally identified by studying segregation of OA affected members in families, twin studies, and by exploring early onset families with a Mendelian inheritance pattern of OA (associated) phenotypes (66). Hence, identification of genes that explain the heritable component of OA is a powerful tool to highlight underlying disease pathways.

Genome-wide association studies

Genome-wide association studies (GWAS) are performed to identify single nucleotide polymorphisms (SNPs) that confer risk to common age related OA, as it occurs in the population. **Table 3** shows a selection of genetic variants and their positional genes that have been robustly identified in large comprehensive genome wide association studies in OA up to date (67-71). The functions of these genes confirm that deviations in both cartilage and bone maintenance processes, are major pathways underlying OA pathology in humans. Moreover, follow-up studies have shown that risk SNPs frequently modulate pathology due to altering transcription of the genes in *cis* both in bone and cartilage (72-74). A notable recent example is Matrix Gla Protein (MGP) that regulates extracellular calcium levels via high affinity to its γ-carboxyglutamic acid (Gla) residues. As the OA risk allele (rs1800801; **Table 3**) has been associated with a reduced *MGP* gene expression (75) and with increased vascular calcification (76), this would suggest increased cartilage calcification in carriers of the OA risk allele. Another example was found in the deiodinase iodothyronine type II (D2) gene (*DIO2*). D2 is an enzyme that converts intracellular thyroxine (T4) into triiodothyronine (T3) in specific tissues such growth plate cartilage. Herein, T3 initiates terminal maturation of hypertrophic chondrocytes leading to breakdown and mineralization of cartilage to allow transition to bone (77). In bone, *DIO2* is essential for bone formation and mineralization.

Linkage analysis

Linkage analyses is a powerful tool to identify high impact causal mutations in extended families with an early onset disease phenotype, preferably inherited in a Mendelian inheritance pattern. The value of such familial high impact variants is that identified gene functions and underlying pathways can have shared etiology, hence giving insight into common OA phenotypes (78-81). By applying next generation sequencing of whole genomes or exomes of well selected (definitively affected) family members, genetic variants can be assessed. Subsequently, likely damaging high impact mutations can be prioritized based on for example the amino acid change or location of the mutation (82). Herein,

synonymous variants, tolerated missense variants, intergenic variants and intron variants would likely result in a less strong disease phenotype. On the other hand, variants producing a stop codon or a missense mutation can result in a truncation of the translated protein. To determine the likely effect of a mutation, tools such as Sorts Intolerant From Tolerant (SIFT) and/or polymorphism phenotyping version 2 (PolyPhen) can predict their damaging or neutral effect (83) by using physical and comparative evolutionary considerations.

Table 4 highlights compelling high impact mutations identified in early onset families with OA related phenotypes. Notably, high impact mutations causal to the early onset OA phenotypes are found in relevant extracellular matrix genes such as *COL11A1*, *COL11A2*, *COL2A1* as well as genes functioning in chondrogenic differentiation such as *GDF5* and *SMAD3* (84-87). A compelling example and subject of this thesis, was the identification of a readthrough mutation (c1205A=T; p.Stop402Leu) in *TNFRSF11B* encoding osteoprotegerin (OPG) localized at the chondrocalcinosis locus 1 (CCAL1) (88) in multiple families worldwide (89-91). In these families, the CCAL1 phenotype is defined by early onset OA with characteristic articular cartilage calcification i.e. chondrocalcinosis (92) and low subchondral bone mineralization (91). Being a readthrough mutation resulting in 19 additional amino acids at the C-terminal end of the protein, the mutation was named OPG-XL.

Table 3. Single nucleotide polymorphisms consistently identified in association with OA.

Gene	Single nucleotide polymorphism	Risk allele	Effect	Function	Reference
DIO2	rs225014	C	Catalyzer of thyroid hormone activation	UP	(93)
MGP	rs1800801	T	Regulator of cartilage mineralization	DN	(72)
IL11	rs4252548	T	Cytokine stimulator of hematopoietic T cells	DN	(94)
GDF5	rs143383	T	Regulator of cell growth and differentiation	DN	(95)
SMAD3	rs12901499	G	Signal transducer and transcriptional modulator	UP	(96)
ASPN	Triplet repeat of the codon for aspartic acid	-	Cartilage extracellular protein involved in chondrogenesis and mineralization	UP	(97)

Table 4. High impact mutations in early onset OA families.

Gene	Name	Protein function	Mutation	Reference
COL2A1	Collagen type II α1	Major structural component in cartilage	p.Arg275Cys	(98)
			p.Gly204Ala	(86)
			p.Arg719Cys	(99)
COL11A1	Collagen type XI α1	Minor fibrillar collagen in cartilage, alpha chain 1	p.Pro446Gln	
COL11A2	Collagen type XI α2	Minor fibrillar collagen in cartilage, alpha chain 2	p.Arg53Trp	(100)
COMP	Cartilage oligomeric matrix protein	Extracellular matrix protein present in cartilage	p.Arg718Trp	(85)
TNFRSF11B	Tumor necrosis factor receptor superfamily member 11b	Extracellular regulator of osteoclastogenesis	p.Ter402Leu	(88)
GDF5	Growth differentiation factor 5	TGF-beta family member protein involved in skeletal development	p.Leu441Pro	
			p.Arg438Leu	(101)
SMAD3	Mothers against decapentaplegic homolog 3	Protein involved in TGF-beta signaling and skeletal development	p.Arg287Trp	
			p.Thr261I1e	
			p.Thr247fsX61	(102)

OSTEOPROTEGERIN; ROLE IN CARTILAGE AND BONE PHYSIOLOGY

OPG is a decoy receptor which competes for binding of nuclear factor KB ligand (RANKL) to the receptor activator of the nuclear KB factor (RANK). Together, this triad is well known for regulating osteoclastogenesis (103), hence playing a critical role in bone homeostasis, endochondral ossification, and bone remodelling (5, 6) **(Figure 4)**. More recently, by using a heparan sulphate (HS)-binding deficient mutant OPG mouse model, it was found that OPG not only binds to free RANKL but also to RANKL as a membrane-bound form on osteoblasts through interaction of its C-terminus with heparan sulphate. 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 (104-106). Mice with aberrations in this triad, generated several skeletal diseases ranging from spontaneous fractures and osteoporosis to osteopetrosis (103, 107). For instance, infant mice treated with OPG for 12 weeks, developed a denser bone phenotype, while OPG knock-out mice showed a decrease in BMD and high incidence of bone fractures (108). Moreover, it was shown that RANKL stimulates osteoclast fission to produce transcriptionally distinct osteomorphs, which in turn, recycle towards large multinucleated osteoclasts or polykaryons by fusion, under tight control of OPG (109, 110).

Despite the large body of literature on the OPG-RANK-RANKL triad in bone homeostasis, the role of OPG in cartilage remains elusive. Nonetheless, a specific role of *TNFRSF11B* and its ligand *TNFSF11* encoding RANKL 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 (57, 58) or healthy (111) cartilage. In contrast, differential expression of *TNFRSF11B* or *TNSF11* in subchondral bone underlying preserved and lesioned areas of OA cartilage was not observed (65). Other than that, with *TNFSF11* being a robust OA risk gene identified in the largest genome wide association study to date (112) aberrant function of OPG/RANKL clearly indicates its relevance in common OA pathology.

Next to the indicated separate roles of OPG in bone and cartilage, accumulating evidence suggests that OPG may also play a role in the dynamic interaction between articular cartilage and (subchondral) bone metabolism. The direction of changes of subchondral bone density and mineralization in osteoarthritis patients, however, remains unclear and possibly dependent on the subtype. This controversy is exemplified by a study of osteoarthritis patients that were shown to benefit from treatment with strontium ranelate, which is a drug licensed for osteoporosis and acts by increasing bone formation while decreasing bone resorption via stimulation of OPG (113). In absence of an effective OA therapy, these studies have gained a lot of attention but also elicited debate, since they contradict to epidemiological studies indicating that individuals with high systemic bone mass are at increased risk for the incidence of osteoarthritis. Additional studies, subsequently confirmed that indeed strontium ranelate administration resulted in an increase in cartilage deposition and a decrease in MMP release, with upregulation of osteoblast formation and reduced subchondral bone remodelling (114, 115) . On the other hand, in an OA guinea pig model, Chu et al (116) showed that upon strontium ranelate administration there was an increase in osteophyte size, which is a known characteristic of OA development. Taken together, these findings would suggest the involvement of OPG in other biological processes related to cartilage formation, independent from its known function in bone homeostasis (117, 118).

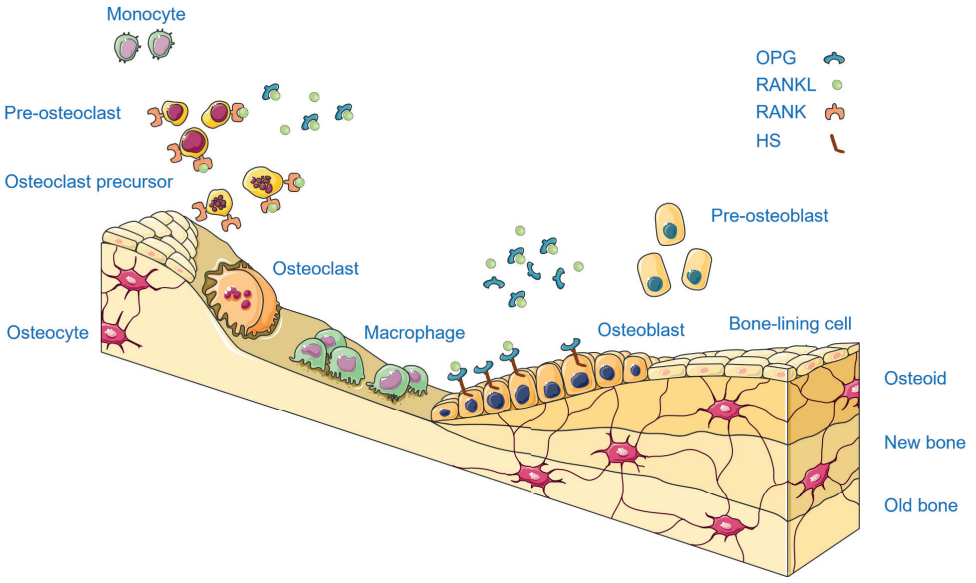

Figure 4. Role of Osteoprotegerin in bone resorption. OPG is a soluble decoy receptor that inhibits differentiation of osteoclast precursors by competing for its binding of RANKL to RANK. This results in less osteoclasts available and inhibition of bone resorption.

HUMAN DISEASE MODELS IN OA

To allow translation of identified strong OA risk genes towards underlying biological mechanisms, studies on target discovery, and drug testing; a human model system that incorporates disease relevant tissue units is necessary. Moreover, such models require the possibility to perturb the system with essential genetic and/or environmental cues to trigger OA-like changes. For instance, to study underlying biological mechanisms of OA risk genes, it is important to be able to change the expression levels of these genes and/or apply genetic engineering tools to introduce high impact OA mutations (119). Some of the current OA models that allow these perturbations will be described in the following paragraphs.

In vitro models

A two dimensional (2D) *in vitro* model allows culture of either primary cartilage or bone cells or immortalized cell lines on flat surfaces, hence representing a simplified osteochondral system. It allows high throughput screening, for instance, upon lentiviral induction or inhibition of a specific gene, or upon exposure to therapeutic compounds. Nevertheless, the lack of nutrient and oxygen gradients, fast chondrocyte dedifferentiation, or the lack of extracellular matrix production,

arise as important challenges for accurately reproducing OA. Three-dimensional (3D) *in vitro* models optionally employed with a variety of different cells such as human primary articular chondrocytes (hPACs), osteoblasts and mesenchymal stromal cells (MSCs) can better address these issues (120, 121). Yet, hPACs, osteoblasts and MSCs can only be obtained after invasive procedures, while there is a large heterogeneity in chondrogenic capacity between donors (122-124). Moreover, primary cells have a tendency to rapidly become senescent during *in vitro* expansion (124-126). Nonetheless, 3D in vitro models have highlighted relevant functions of some proteins. For instance, cartilage oligomeric matrix protein (COMP) overexpression in bovine chondrocytes resulted in a collagen formation with a smaller diameter (127). Another example can be found upon overexpressing DIO2 in human chondrocytes (128), resulting in a lower ECM deposition and a higher catabolic and mineralization response.

Ex vivo models

Ex vivo models culturing human osteochondral explants can also be used to study OA. This human model offers a reliable method to study the interaction between different cell types and the interplay between aged cartilage and bone (129). Moreover, osteochondral explants can be used to investigate joint mechanobiology. For instance Houtman et al showed a catabolic response in chondrocyte signaling upon loading human explants (130). Unfortunately in these models, specific medium requirements for each cell type are necessary to maintain the cell inherent phenotype over a longer time period, while genetic engineering strategies cannot be applied (120).

In vivo models

In vivo models of small animals, especially mice and rats, are frequently used to study OA. For this aim, surgically and chemically induced models can trigger OA development by disturbing joint biomechanics and/or by administering compounds such as collagenase, which is detrimental to joint health. Additionally, genetic manipulation such as a knock-in or knock-out experiments can be applied to prove gene causality in an *in vivo* complex system. For instance, Bomer et al showed the relevance of *DIO2* in a knock-out mouse model. Data of the study showed that DIO2 knock out mice relative to wild type littermates were protected against cartilage damage upon force running (131). In another study by Wu et al, OPG transgenic mice were generated and showed an increase BMD and trabecular tibia number and thickness when compared to wild type mice (132). Nevertheless, differences in joint structure and loading regimes, with an absence of spontaneous OA in some animal models, fail to represent to gradual development of OA in humans with aging. Moreover, animal research must progressively integrate the principle of 3Rs: replacement, reduction and

refinement, only performing research in animal models when strictly necessary.

Induced pluripotent stem cells

In 2006, Yamanaka generated embryonic stem cell-like cells by introducing four reprogramming factors (Oct-4, Sox-2, Klf4 and c-Myc) into fibroblast mouse cells and termed these cells as induced pluripotent stem cells (iPSCs). These cells were able to differentiate into the three germ layers while maintaining the genetic background of the donor. As a result, iPSC technology was later applied to human cells and became a method not only for disease modeling, follow up of genetic studies or regenerative medicine, but also for high-throughput drug screening and personalized medicine (133, 134). Additionally, iPS-cell production can be scaled, while the use of such immortal-like cells avoids the need for biopsies and repeated surgeries on patients. Moreover, iPSC technology easily allows to study the causative effect of point mutations (135) by performing genome editing tools such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (136). In this system, a RNA guided endonuclease induces double stranded breaks in the genome after recognition of a protospacer adjacent motive (PAM). When this technology is combined with a repaired or mutation template, an iPSC isogenic control or point-mutation line can be generated, making this system ideal for studying specific mutations (137). Nevertheless, issues can arise due to the strong variation in differentiation efficiencies between iPSC lines and clones (138, 139) and a tendency to generate fibrous cartilage matrix (124, 140). Hence, even though several protocols are available, the optimal method for generation of chondrocytes and osteoblasts from iPSCs remains to be established. Some studies comparing human bone marrow-derived mesenchymal stromal cells (BMSCs) and iPSC-derived mesenchymal stromal cells (iMSCs) suggest major functional and genetic differences, not only between cells but also between neo-cartilage and neo-bone derived from both cell types (141, 142). However, in these studies, iMSCs were generated via the formation of cell aggregates called embryoid bodies (EBs), often variable and with low efficiency (141, 142), while direct monolayer generation was shown to be more robust (143).

Alternatively, a stepwise approach can be taken to generate neo-cartilage from hiPSCs via chondroprogenitor cells (iCPCs) (144-146). Notably, differentiation of iPSCs with this protocol mimics each developmental step through anterior primitive streak formation and successive emergence of iCPCs, diminishing variability between independent differentiations. Unfortunately, a major disadvantage of this method is the inability to generate bone and its inefficiency to expand hiCPCs, due to the rapid loss of their chondrogenic potential (145). Therefore, heterogeneity in the resulting cell population, urges for efficient and cell expandable step-wise differentiation approaches where a better study of fundamental biological processes is required (147, 148). Only when these signals

and complex interactions are better understood they can be used as accurate follow up models.

AIMS AND CONTENTS OF THIS THESIS

The role of OPG, encoded by *TNFRSF11B*, in bone development is commonly known, however its function in cartilage remains elusive. Its high expression in OA lesioned cartilage and identification of a readthrough mutation in a family with early onset OA suggest a crucial role for OPG in the homeostasis of this tissue.

To address the role of OPG in cartilage, in **Chapter 2** we will overexpress *TNFRSF11B* in preserved chondrocytes with a lentiviral construct to determine its likely function as a trigger of OA. Additionally, gene expression levels of a unique RNA sequencing dataset of preserved against lesioned cartilage will be correlated to *TNFRSF11B* and used as a readout to determine downstream effects of this process.

Once the function of *TNFRSF11B* is determined in cartilage, we will investigate the effects of its readthrough mutation in the early onset OA family. Nevertheless obtaining cartilage and bone tissue from them is dependent on joint replacement surgeries of the few family members that carry the mutation. Remarkably, iPSC technology can be used to generate neo-cartilage and neo-osseous tissue that later can be researched in an *in vitro* model. With this aim in mind, in **Chapter 3** we will establish the optimal method for iPSC derived neo-cartilage generation while comparing two differentiation protocols with cartilage deposited by primary cells. Once the optimal method for neo-cartilage and neo-bone is achieved, an iPSC line from an early onset family member carrier of a mutation in *TNFRSF11B* at the CCAL1 locus will be generated in **Chapter 4**. The mutation will be rescued by applying CRISPR/Cas9. Subsequently, taking the established optimal approach for *in vitro* iPSC-derived OA modelling determined in **Chapter 3**, neo-cartilage and neo-bone will be generated from both lines. By doing so, we will get substantial understanding of the molecular background underlying their phenotypes. Finally, monocyte-derived osteoclasts of OPG-XL carriers will be generated and their osteoclast activity researched. In parallel with the *in vitro* experiments, family members with and without the mutation will take part on different tests to evaluate their joints status, BMD and OA stage. With this thesis we expect to determine the role of OPG in cartilage and discover the functional effects of the readthrough mutation, which will ultimately help to address the role of OPG in OA development.

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