The bone and cartilage interplay in osteoarthritis: key to effective treatment strategy

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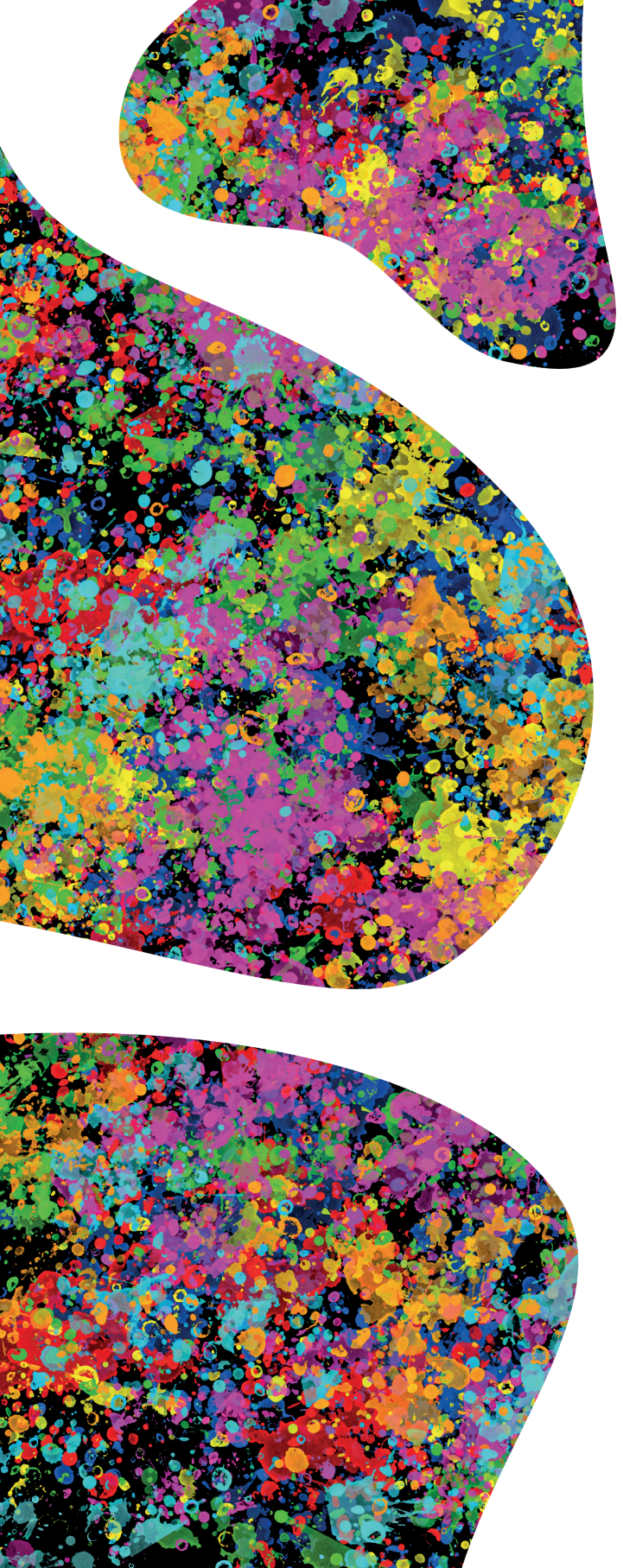

CHAPTER 1

General introduction

Burden of Osteoarthritis

Osteoarthritis (OA) is a prevalent degenerative, yet irreversible, disease of the articular joints. Globally, 7% percent of the population is affected by OA and in 2019 OA was the 15th highest cause of years lived with disability (YLDs) [1]. Prevalence of OA increases significantly with increasing age and incidence rate is higher in women than in men, especially between 55 and 59 years of age [2]. OA pathophysiology is characterized by progressive and heterogeneous deterioration and loss of articular cartilage, remodeling of subchondral bone, osteophyte formation, and inflammation (**Figure 1**) [3]. Clinical symptoms of OA are pain, (morning) stiffness, crepitus, and reduced range of motion [4, 5]. Therefore, OA has a negative impact on patient quality of life and with progression of the disease it could even result in complete disability. So far, no disease modifying treatments are available, except for costly total joint replacement surgery at end-stage disease. This results in high social and economic burden to society [2, 6, 7]. OA pathophysiology is a complex process in which initiation and progression of the disease is mostly multifactorial [8]. Risk factors for OA include age, sex, metabolic health, aberrant loading, trauma, and genetics [9, 10].

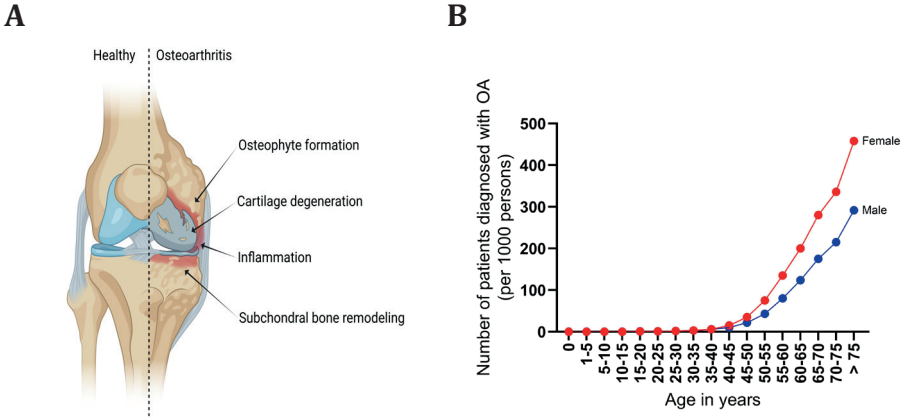

Figure 1 – Overview of osteoarthritis pathophysiology as age-related disease.
(A) Schematic overview of OA pathophysiology including cartilage degeneration, subchondral bone remodeling, osteophyte formation, and inflammation (created with Biorender.com). (B) Overview of number of patients diagnosed with OA in the Netherlands in 2020 according to CBS, stratified by age.

(Patho-)physiology of the osteochondral unit

Development and growth of longitudinal bones relies on a process called endochondral ossification (**Figure 2**). During prenatal development a cartilage template is formed, which is pre- and postnatally replaced by bone tissue. During endochondral ossification, chondrocytes present in the cartilage template become hypertrophic and start to secrete factors such as runt-related transcription factor 2 (*RUNX2*), vascular endothelial growth factor (*VEGF*), and collagen type 10 (*COL10*) [11]. Subsequently, the cartilage template is invaded by osteoblast progenitors, blood vessels, endothelial cells, and hematopoietic

cells that give rise to formation of osteoclasts, together resulting in resorption of hypertrophic cartilage and deposition of trabecular bone and bone marrow tissue in the so-called primary ossification center [12]. This primary ossification center expands and a secondary ossification center appears in the epiphysis of the developing bone, leaving the epiphyseal growth plate in between. The epiphyseal growth plate is responsible for the longitudinal growth of bones. With age this growth plate gets thinner, until both ossification centers fuse.

The cartilage at the end of bones escapes the endochondral ossification process, forming an avascular load-bearing structure called articular cartilage (**Figure 2**) [13, 14]. Chondrocytes are thought to be the only cell type present in articular cartilage and they reside in a maturational arrest state and do not proliferate. Chondrocytes are responsible for structural integrity of cartilage extracellular matrix (ECM), which consists of four zones: superficial, middle, deep, and calcified zone, with each zone having its specific fiber and cell organization **(Figure 3)** [13]. Main cartilage ECM components are collagens, such as collagen type 2 (*COL2*), and proteoglycans, such as aggrecan (*ACAN*). With OA, chondrocytes lose their maturational arrested state and become hypertrophic-like, resembling growth plate morphology. Thereby, they start to actively produce catabolic enzymes, such as matrix metalloproteinases (*MMPs*) and disintegrin and metalloproteinase with thrombospondin motifs 4 and 5 (ADAMTS-4 and -5) [15-17]. These enzymes result in fragmentation and degradation of collagens and proteoglycans, respectively. Moreover, the reactivated chondrocytes secrete

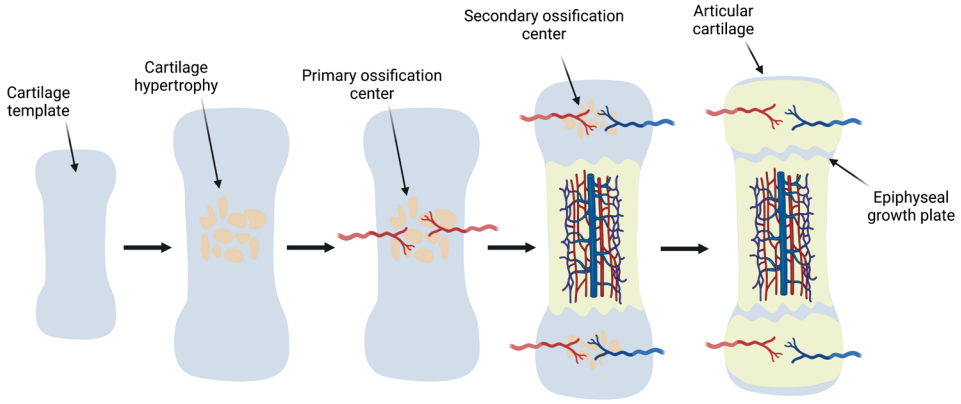

Figure 2 – Schematic overview of endochondral ossification process.
A cartilage template is pre- and postnatally replaced by bone tissue. First, chondrocytes become hypertrophic and a primary ossification center is formed. This primary ossification center expands and a secondary ossification center develops in the epiphysis of the cartilage template, leaving the epiphyseal growth plate in between. With age this growth plate gets thinner, until both ossification centers fuse (created with Biorender.com).

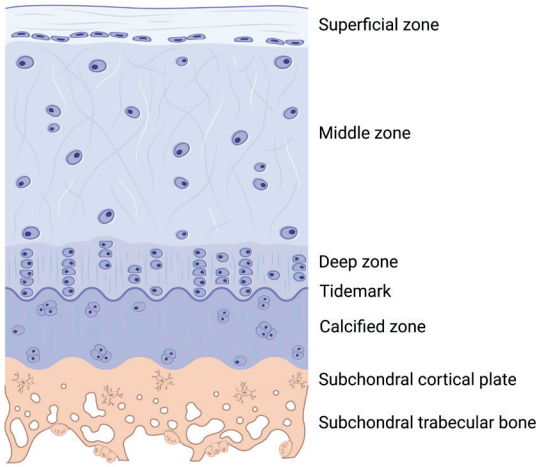

Figure 3 – Schematic overview of osteochondral structure.
Cartilage consist of multiple zones, including the superficial, middle, deep, and calcified zone. The subchondral bone can be divided in the subchondral cortical plate and subchondral trabecular bone (created with Biorender.com).

factors promoting calcification and vascularization of the ECM, such as runt-related transcription factor 2 (RUNX2), vascular endothelial growth factor (VEGF), and collagen type X (COL10A1) [18, 19]. The degeneration and mineralization of cartilage in OA is accompanied with alterations in the subchondral bone.

The subchondral bone consists of subchondral cortical plate and subchondral trabecular bone. The subchondral cortical plate is defined as a thin cortical bone structure beneath the calcified cartilage, which is invaded with blood vessels and nerves. The subchondral trabecular bone is more porous, contains even more blood vessels and nerves compared to the cortical plate and is important in shock-absorbing [20]. Cell types residing in the subchondral bone are osteoblasts, osteocytes, and osteoclasts. Osteoblasts and osteocytes are responsible for production and maintenance of bone matrix, while osteoclasts are responsible for bone resorption in response to environmental factors, such as mechanical loading [21]. Main constituent of subchondral bone is collagen type 1 (COL1), which forms a network that serves as a scaffold for hydroxyapatite crystal deposition [22, 23]. In healthy bone, there is a balance between bone ECM production and resorption. However, with OA, this balance gets disturbed, resulting in increased subchondral bone plate thickness and decreased bone mineral density (BMD) in end-stage OA [22, 24, 25]. Together, increased subchondral bone plate thickness and mineralization of articular cartilage result in joint space narrowing, a typical characteristic of OA [5]. Another feature commonly seen in OA is the formation of bony

structures along the joint margins, called osteophytes. Osteophytes are formed through endochondral ossification in presence of growth factors transforming growth factor beta (TGF-β) and bone morphogenic protein 2 (BMP2) and they are hypothesized to increase joint stability in response to the enlarged mechanical load applied [26, 27].

Genetics

Although development of OA is multifactorial, genetic predisposition is one of the strongest determinants of the disease [10]. To identify genetic variants and/or genes conferring risk to OA, comprehensive genome-wide association studies (GWASs) have been performed [28-33]. In GWASs genetic variants, called single nucleotide polymorphisms (SNPs), are being statistically associated to a specific disease or trait [34]. Since OA is a polygenic disease, with multiple causal genes showing small effects, effect sizes of OA susceptibility SNPs are generally low and large sample sizes are required to identify these SNPs [35]. The largest GWAS meta-analysis so far identifying OA risk SNPs is performed recently by Boer and colleagues [28]. This study included 826,690 individuals, of which 177,517 were diagnosed with OA and resulted in identification of 100 independent SNPs being associated with OA. These variants were located near genes including WW domain containing E3 ubiquitin protein ligase 2 (*WWP2*), interleukin 11 (*IL11*), transforming growth factor alpha (*TGF-α*), and aldehyde dehydrogenase 1 family member A2 (*ALDH1A2*) (**Table 1**). These genes are involved in maintenance processes in both bone and cartilage, confirming that both tissues have a substantial role in initiation and development of OA and stressing the importance of including both tissues and their interaction in OA research.

Functional genomics

Next to identification of OA susceptibility genes, better understanding of molecular OA pathogenesis is required towards development of disease modifying treatments. A valuable tool for this is transcriptomic data, such as RNA-sequencing data, as it can be used to identify genes that mark OA pathophysiology, identify OA subtypes, and it can be used to determine the direction of effect of compelling OA risk genes.

Differential expression analysis

To identify underlying genes and pathways that mark OA pathophysiology, multiple studies have been performed comparing healthy or macroscopically preserved and lesioned OA areas of the joint on transcriptomic level [61-63]. In this respect, RNA-sequencing (RNA-seq) was performed on articular cartilage from patients who underwent total joint replacement surgery due to OA as part of the Research in Articular osteoArthritis Cartilage (RAAK) study. Upon comparing gene expression levels of macroscopically preserved and lesioned OA articular cartilage, 2387 genes

Table 1 – Notable examples of OA susceptibility SNPs, identified in genome wide association studies, with their mapped genes and hypothesized direction of effect.
These genes are all involved in maintenance processes of both articular cartilage and subchondral bone.

Mapped gene	Function of protein	SNP	Risk allele	Trait	Annotation	Gene expression risk	References
IL11	Regulates bone formation, remodeling, and resorption	rs4252548	T	Hip OA	Missense	Decreased expression	[28, 31, 32, 36]
SMAD3	Prevents chondrocyte hypertrophy	rs12908498	C	Hip OA	Intron	Decreased expression	[28, 37-39]
DIO2	Induces chondrocyte hypertrophy	rs225014	T	Symptomatic OA	Exon	Increased expression	[40, 41]
TNC	Promotes cartilage repair	rs1330349	C	Hip OA	Intron	Decreased expression	[28, 32, 42, 43]
COL27A1	Involved in transition from cartilage to bone	rs72760655	A	Knee and hip OA	Upstream gene	Decreased expression	[28, 38, 44, 45]
GDF5	Involved in joint development and cartilage repair	rs143384	A	Knee OA	5' UTR	Decreased expression	[28, 31, 32, 44, 46-48]
WWP2	Plays a role in chondrogenesis	rs34195470	A	Knee OA	Intron	Increased expression	[28, 31, 38, 42, 49]
MGP	Regulates cartilage calcification	rs1800801	C	Hand OA	Exon	Decreased expression	[50-52]
TGF-α	Involved in transition from cartilage to bone	rs3771501	T	Hip OA	Intron	Increased expression	[28, 32, 53, 54]
ALDH1A2	Involved in maintenance of cartilage and bone	rs11071366	A	Hand OA	Intron	Decreased expression	[28, 55]
CHADL	Negative modulator of chondrocyte differentiation	rs117018441	T	Hip OA	Intron	Increased expression	[29, 31]
COL11A1	Involved in endochondral ossification and microarchitecture of developing bone	rs3753841	T	Hip OA	Missense	Decreased expression	[31, 56]
FRZB	Involved in endochondral ossification	rs288326	G	Hip OA	Exon	Decreased expression	[57-60]

were identified as being false discovery rate (FDR) significantly differentially expressed [63]. These differentially expressed genes were enriched for processes involved in extracellular matrix organization, characterized by upregulation of periostin (*POSTN),* TNF receptor superfamily member 11b (*TNFRSF11B*) and secreted phosphoprotein 1 (*SPP1*) and processes involved in skeletal system development, characterized by upregulation of bone morphogenic protein 3 (*BMP3*) and 6 (*BMP6*) and downregulation of frizzled related protein (*FRZB*) and growth differentiation factor 10 (*GDF10*). In another large transcriptomic analysis study, RNA-seq was performed on paired preserved and lesioned cartilage of 124 OA patients [64]. Differentially expressed genes found in this study were enriched for, amongst others, cytokine activity, characterized by upregulation of cytokine receptor like factor 1 (*CRLF1*), *IL11*, and *IL1-β*, suggesting OA-related inflammation is driven by the interleukin 6 (IL6) super family (**Table 2**).

While valuable extensive effort has been made to characterize the pathophysiological process in articular cartilage, the pathophysiology of underlying subchondral bone is less explored. This despite the fact that there is accumulating evidence that subchondral bone, in interaction with articular cartilage, contributes to both OA onset and progression [24, 27, 65, 66]. Chou and colleagues used microarray analysis to identify differentially expressed genes between OA and non-OA subchondral bone [62]. Among the differentially expressed genes were *TNF,* collagen type 12 alpha 1 (*COL12A1*), sclerostin (*SOST*), bone morphogenic protein 7 (*BMP7*), and chordin-like 2 (*CHRDL2*) (**Table 2**). Another study used microarray analysis to identify differential expression of genes between OA bone marrow lesion and control bone samples [67]. They found genes involved in osteochondral turnover, neurogenesis, and inflammation. However, both of these studies only included knee samples and in both studies microarray analysis was performed. The disadvantage of microarray analysis is that it only profiles predefined genes, while RNA-seq, for example, results in transcriptome-wide gene expression profiling. Therefore, valuable information might be missed by microarray analysis

Characterization of OA subtypes

Recently, OA is more recognized to be a heterogeneous disease with variable characteristics across OA patients. For that matter, transcriptomic analysis of articular cartilage can also be used to identify OA subtypes to better understand heterogeneity of the underlying molecular disease process. Yuan and colleagues identified four subtypes of knee OA by performing unsupervised clustering based on top 4000 genes that showed highest variation across patients [70]. These four subtypes represented GAG metabolic disorder, collagen metabolic disorder, activated sensory neurons, and inflammation. In another study, two OA subtypes were identified also in knee OA samples [71]. These two subtypes were associated to chondrocyte hypertrophy and immune response,

Table 2 – Differential expression between macroscopically preserved and lesioned OA articular cartilage and subchondral bone.

Articular Cartilage

Pathway	Gene	Fold change	FDR	Reference FC and FDR	Similar direction of effect shown by:
Extracellular matrix	*POSTN*	2.04	3.4E-02	[63]	[64, 68]
	TNFRSF11B	3.01	7.1E-12	[63]	[61, 64, 68]
	SPP1	3.14	9.0E-07	[63]	[61, 64]
Skeletal system development	*BMP3*	0.28	2.9E-03	[63]	[69]
	BMP6	2.43	1.6E-09	[63]	[61]
	FRZB	0.27	1.9E-09	[63]	[61, 64, 68]
	GDF10	0.35	1.0E-08	[63]	[61, 64, 68]
Cytokine activity	*IL11*	22.79	1.6E-20	[63]	[64, 68]
	CRLF1	3.04	3.0E-10	[63]	[61, 64, 68]

Subchondral bone

Pathway	Gene	Fold change	FDR	Reference FC and FDR	Similar direction of effect shown by:
Bone mineral density	*SOST*	2.51	2.59E-04	[62]	-
Abnormal bone morphology	*COL12A1*	2.26	6.55E-08	[62]	-
	TNF	0.32	1.17E-08	[62]	-
Mineralization of cells	*BMP7*	0.40	1.65E-09	[62]	-
	CHRDL2	0.25	1.40E-04	[62]	-

respectively. Recently, Coutinho de Almeida and colleagues also identified two OA subtypes using RNA-seq data of both hip and knee OA samples, representing similar processes [72]. More importantly, they showed that these subtypes were associated with phenotypic differences. Identification of these OA subtypes enables better predictions of clinical outcomes of OA treatments [70]. However, to distinguish OA subtypes in clinical practice, non-invasive biomarkers are necessary to stratify patients on OA subtype before treatments start.

Allelic imbalanced expression

While some OA risk variants are missense mutations located in the protein-coding region of a gene and thereby directly affecting protein structure, most SNPs conferring risk to OA are located in non-coding regions. Functional follow-up studies have shown that SNPs in non-coding regions frequently act via altered expression of positional genes in *cis*, also known as allelic imbalanced expression (AIE) [73, 74]. Transcriptomic data can also be used to screen for allelic imbalance. In this respect, den Hollander and colleagues used RNA-seq data of preserved and lesioned OA articular cartilage to screen for transcriptome-wide AIE [42]. As a result, 2,070 SNPs were identified marking AIE of 1,031 genes, including 18 genes that were also identified as OA susceptibility genes in GWASs. Among these 18 genes were *WWP2, FRZB*, and matrix gla protein (*MGP*) identified as highly significant. More recently, Coutinho de Almeida and colleagues also screened for AIE in both articular cartilage and subchondral bone OA samples [75]. In this study, 26 SNPs were identified being subjected to AIE in cartilage, and 7 SNPs were identified in subchondral bone. These studies on AIE are extremely valuable as they can be used to make firm hypothesis on the direction of effect of identified compelling OA risk genes. However, for translation of these OA risk genes towards development of disease modifying OA treatments, functional follow-up studies are required to elucidate molecular mechanisms and targets of these genes [76-78].

Epigenetics in osteoarthritis

Epigenetics refers to changes in heritable phenotype without alterations in the genetic code. Epigenetic regulation provides cells with a mechanism to respond to environmental cues such as mechanical stress and microtraumas by changing gene and protein expression levels temporarily [79]. Epigenetic mechanisms include DNA methylation, histone modification, and non-coding RNA expression, all being extensively associated to OA pathophysiology [80].

DNA methylation and histone modifications

DNA consist of a sequence of adenine, thymine, cytosine, and guanine and cytosine followed by guanine, is called a CpG site. In a CpG site, the cytosine can be converted

to 5-methylcytosine (5mC) by methylation catalyzed by methyltransferases. This processes is called DNA methylation and this process alters the binding of proteins, such as transcription factors, to the DNA and therefore it changes gene expression levels (**Figure 4A**) [81]. DNA is condensed around histone proteins (H3, H4, H2A, and H2B). To modulate gene expression, histone proteins undergo modifications such as methylation and acetylation (**Figure 4B**) [82, 83]. Histone methylation mainly inhibits gene transcription by blocking binding of transcription factors, while histone acetylation is associated with increased gene transcription. Histone modifications are executed by histone methyl transferases, histone acetyl transferases, histone deacetylases, and histone demethylases [80].

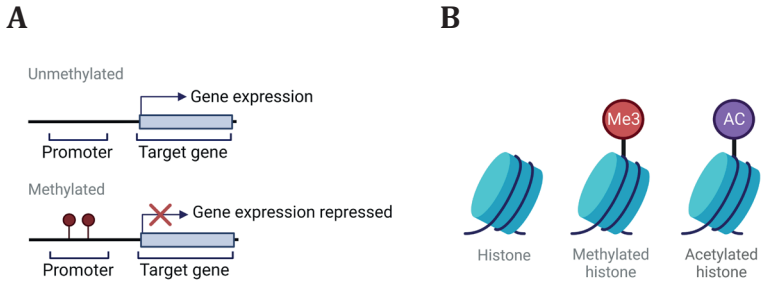

Figure 4 – Overview of epigenetic processes.
(A) overview of DNA methylation. Methylation of the DNA alters the binding of proteins, such as transcription factors, to the DNA (created with Biorender.com) (B) Schematic overview of histone modifications. Methylation of histones is associated with decreased gene transcription, while acetylation of histones is associated with increased gene transcription (created with Biorender.com)

MicroRNA expression

While DNA methylation and histone modifications are mainly regulating transcription of genes, non-coding RNAs are a class of transcriptional and (post-)translational regulators. Non-coding RNAs are classified based on their size in micro-RNAs (miRNAs) and long non-coding RNAs (lncRNAs). MiRNAs are typically between 18 and 25 nucleotides in length and they negatively regulate translation of mRNA to protein. Most miRNAs bind to the 3' untranslated region (UTR) of their target mRNA, thereby inhibiting translation and/or reducing mRNA stability [84, 85]. The number of base pairs that overlap between miRNAs and their target mRNA determine whether the mRNA is degraded via Argonaute RISC Catalytic Component 2 (Ago2) or repressed via Argonaute RISC Catalytic Component 1 (Ago1). The target mRNA will be degraded when there is (almost) a perfect overlap between miRNA and target mRNA, while translation of the target mRNA will be repressed when there is only partial overlap (**Figure 5A**) [86].

As dysregulated miRNAs mark complex diseases, such as OA, multiple studies focused on characterization of miRNA expression and identification of their mRNA targets in OA pathophysiology. To date, the role of miRNAs in OA has mainly been studied in articular cartilage. For example, Iliopoulos and colleagues compared expression levels of 365 miRNAs in cartilage of 33 OA joints and cartilage of 10 non-OA joints [87]. This resulted in the identification of 16 differentially expressed miRNAs, including upregulation of miR-22 and downregulation of miR-140 in OA cartilage. In another study, miRNAs were identified being differentially expressed between OA and non-OA cartilage and bone, including miR-9 and miR-98[88]. Upon gene targeting prediction and pathway analysis, these miRNAs seem to play a role in inflammation. More recently, integration of transcriptome-wide miRNA-seq and mRNA-seq of OA articular cartilage resulted in identification of 143 miRNAs differentially expressed between macroscopically preserved and lesioned OA cartilage [63]. possible mRNA target was identified for 62 of these differentially expressed miRNAs, including *RGS4*. *RGS4* expression was found to be regulated by mir-140, which is abundantly expressed in articular cartilage and known to be involved in chondrogenesis and osteoarthritis [89, 90]. Mir-140 is co-transcribed with its host gene *WWP2* and regulated by *SOX9*. Moreover, miR-140 is shown to be involved in endochondral ossification, as loss of miR-140 expression in mice results in bone defects and malformations [91].

Long non-coding RNA expression

In contrast to miRNAs, lncRNAs are less frequently investigated mainly because of the poor evolutionary conservation between species and because of their generally low expression levels [92, 93]. LncRNAs are typically over 200 nucleotides in length and while lncRNAs lack protein-coding ability, they share similarities with mRNAs, as most lncRNAs have a 5' 7-methylguanosine cap and a 3' poly A tail and are transcribed by RNA polymerase II [94]. LncRNAs are involved in various transcriptional and (post-)translational processes, including chromatin remodeling, mRNA translation, transcription factor activity, and mRNA and protein stability (**Figure 5B**) [95, 96]**.** Moreover, lncRNA expression can be highly tissue- and disease specific [97]. Multiple lncRNAs have been reported to be involved in chondrogenesis and osteogenesis [93, 98]. Similar to miRNAs, in OA pathophysiology currently lncRNAs have been exclusively studied in articular cartilage. Upon comparing macroscopically preserved and lesioned OA cartilage, 191 lncRNAs were identified to be differentially expressed [99]. Among these differentially expressed lncRNAs was prolyl 3-hydroxylase 2 antisense RNA 1 (*P3H2-AS1*), which was shown to regulate expression levels of its sense gene prolyl 3-hydroxylase 2 (*P3H2*). In another study comparing OA and non-OA articular cartilage, maternally expressed 3 (*MEG3*) was found to be downregulated in both OA hips and knees [100]. As lncRNAs tend to be tissue- and disease specific, identification of

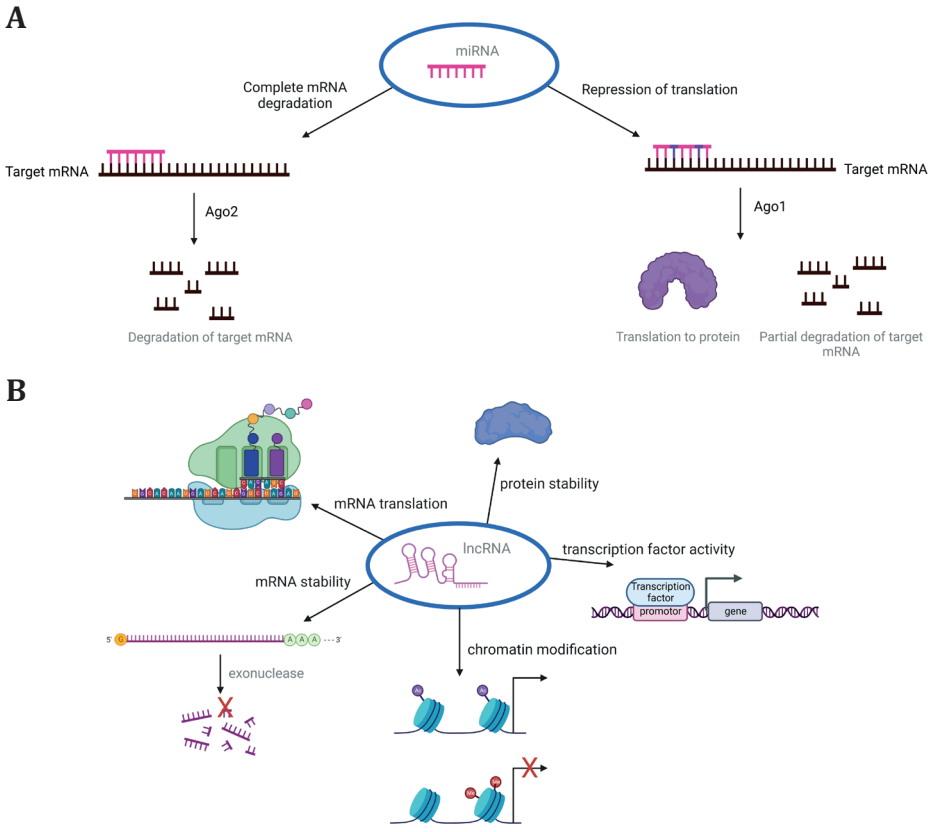

Figure 5 – Overview of non-coding mode-of-actions.
(A) Most miRNAs bind to the 3'UTR of their target mRNA, thereby (partly) inhibiting translation to protein (created with Biorender.com). (B) LncRNAs have various mode-of-actions both on a transcriptional, translational, and post-translational level (Created with Biorender.com).

lncRNAs that mark OA pathophysiological processes might bring new opportunities in development of joint tissue- and disease specific therapeutic strategies. Although multiple lncRNAs are identified marking OA in articular cartilage, studies on lncRNAs marking OA pathophysiology in subchondral bone are still lacking.

Biomarkers in osteoarthritis

To date, there are no reliable biomarkers that reflect ongoing processes in joint tissues. Classification and/or diagnosis of OA is therefore only based on imaging (radiography, MRI) and clinical symptoms, such as pain and stiffness of the affected joint [101]. Consequently, early diagnosis of OA, information on OA prognosis, and ability to predict treatment outcomes are still lacking [102]. To overcome this knowledge gap, research started focusing on identification of potential OA biomarkers using relatively easily accessible sites, such as synovial fluid, urine, and blood. For example, Soul and

colleagues identified a set of proteins, including POSTN, TNC, and MGP, that were predicted to be secreted in the synovial fluid. This set of proteins in synovial fluid could reflect whether a patient is subjected to inflammation-driven or chondrocyte hypertrophy-driven OA [71]. Another study identified six proteins in measured in synovial fluid that were in association with synovial inflammation, severity of cartilage loss, and joint pain [103]. These synovial fluid proteins included MMP3 and soluble vascular cell adhesion molecule 1 (sVCAM1). Nonetheless, urine and blood are more easily accessible and therefore less invasive compared to synovial fluid. OA biomarkers that can be measured in urine are mostly based on breakdown products of main cartilage components collagen type 2 (COL2) and aggrecan (ACAN) [104]. For instance, urinary levels of C-terminal crosslinking telopeptide of type II collagen (CTX-II) are shown to be associated with radiographic signs of OA in multiple studies [105, 106]. Moreover, CTX-II were higher in OA patients compared to healthy controls [107]. Nevertheless, these levels are solely reflecting collagen type II breakdown and do not provide insight in other ongoing OA-related processes. Recently, circulating miRNAs gained interest and Ramos and colleagues showed for the first time that miRNA expression levels in plasma could reflect changes in mRNA expression patterns in articular cartilage [108]. They identified 7 miRNAs, including miR-140-3p, miR-181a-3p, and miR-4443, that were able to predict OA progression. In another study, circulating miR-140-3p, miR-33b-3p, and miR-671-3p were identified in serum as OA biomarker and reflecting metabolic processes in articular cartilage [109]. Finally, Murata and colleagues identified miR-132 being predictive for rheumatoid arthritis and OA [110].

In vitro osteoarthritis disease models

To study compelling OA risk genes appropriate *in vitro* human OA disease models are required that incorporate disease relevant tissues, e.g. bone and cartilage [111]. To date, available *in vitro* model systems for osteochondral tissues include 2D cell cultures, 3D pellet cultures, 3D multi tissue co-cultures (**Figure 6**).

2D cell cultures

The simplest *in vitro* models are 2D cell cultures of OA relevant cells, such as chondrocytes, bone-marrow derived mesenchymal stromal cells (hBMSCs), osteoblasts, osteocytes, and osteoclasts. These 2D cell cultures can be exposed to OA-related cytokines or to conditioned media to study their cellular response [112]. For example in the study of Van Geffen and colleagues [113], human chondrocytes were cultured in 2D and exposed to IL1-β, TNF-α, or human OA synovium-conditioned medium to study the effect of inflammation on interleukin 37 (*IL37*) expression levels. To incorporate intercellular communication in 2D cell cultures co-cultures can be performed in Transwells, for example to study intercellular communication between chondrocytes and bone

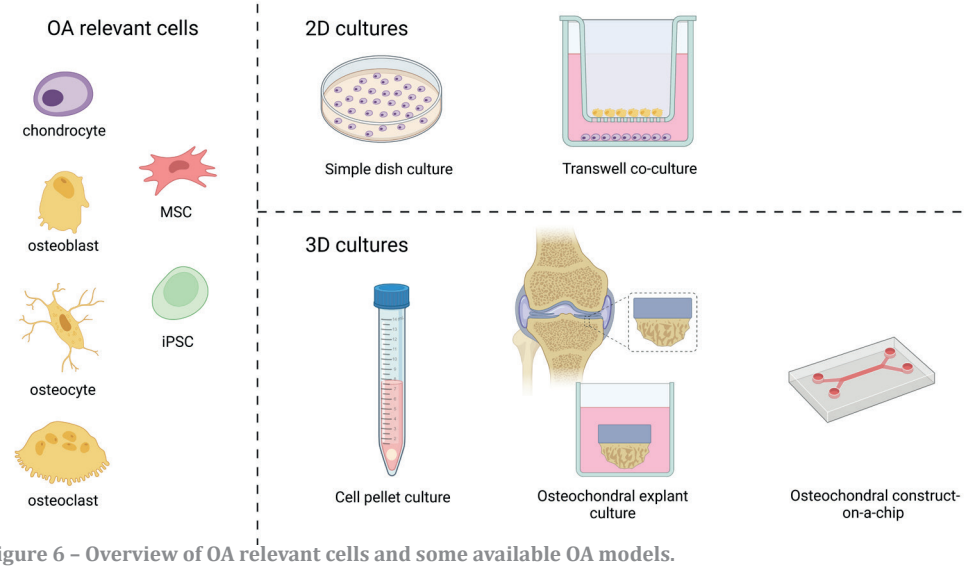

Figure 6 – Overview of OA relevant cells and some available OA models.

cells [114]. While being a useful tool, Transwell co-cultures still lack complexity and interaction of the ECM, and it is known that cells are prone to lose their specific phenotype on 2D surfaces [115].

3D pellet cultures

To include the effect of extracellular matrix and minimize dedifferentiation of cells, 3D cell pellet cultures or micro mass cultures are extensively used to model cartilaginous and osseous tissue [116, 117]. Caron and colleagues showed that chondrocytes in 3D pellet cultures are less prone to become hypertrophic compared to 2D cell cultures [115]. On another level, Bömer and colleagues showed that DNA methylation profile was 99% similar between 3D human chondrocyte pellet cultures and autologous articular cartilage [118]. Subsequently, these 3D human chondrocyte pellet cultures were used to study the effect of silencing OA risk gene fibronectin (*FN1*) [119]. In this study, it was shown that downregulation of *FN1* had detrimental effects on cartilage matrix deposition. These changes in cartilage matrix deposition can only be shown in 3D structures as no ECM is produced by 2D cell cultures, further stressing the advantage of using 3D model systems. In another study, lentiviral particle-mediated overexpression of *TNFRSF11B* in 3D human chondrocyte pellet cultures resulted in enhanced chondrocyte to osteoblast transition, thereby underscoring the role of *TNFRSF11B* in OA development [120]. Altogether, these studies show that 3D chondrocyte cell pellet are a suitable and valuable model for OA articular cartilage.

Multi-tissue culture systems

Given the tissue cross-talk, however, translation of strong OA risk genes towards their underlying mechanism is ideally performed in *in vitro* models that incorporate at least functional bone and cartilage tissue units. Therefore, human osteochondral explants might be an alternative. Osteochondral explants are directly derived from patient material and the main advantage is that cells maintain their natural aged 3D environment [121]. Houtman and colleagues explored the response of osteochondral explants upon exposure to IL1-β, triiodothyronine (T3), and 65% mechanical strain, and confirmed suitability of osteochondral explants as OA models for inflammation, hypertrophy, and posttraumatic OA, respectively [122]. Subsequently, the posttraumatic OA model was used to study potential pharmacological OA treatment with deiodinase inhibitor iopanoic acid (IOP), an FDA approved medication [123]. OA susceptibility gene *DIO2* encodes Iodothyronine deiodinase type 2 enzyme (D2), which is known to convert thyroxine (T4) to T3, thereby inducing hypertrophy [40]. IOP is known to inhibit D2 activity and therefore IOP was hypothesized to be a potential OA treatment. Upon exposing osteochondral explants to 65% mechanical strain to induce posttraumatic OA, with and without IOP treatment, Houtman and colleagues showed that IOP treatment was able to prevent posttraumatic OA-related changes in articular cartilage [123]. Together, these studies show that osteochondral explants provide major advantages in studying potential disease modifying OA treatments using a reliable human biomimetic model and complying to the principle of reduction, refinement, and replacement of animal models. Yet, use of osteochondral explants limits scalability as collection of explants is dependent on patients undergoing joint replacement surgery. Moreover, long-term cultures of osteochondral explants might be challenging, as their properties change over time [112]. Finally, genetic manipulation such as upregulation or silencing of genes cannot be performed in osteochondral explants, limiting these models to study OA related perturbations and treatment options. Henceforth, more state-of-the-art model systems are needed that are based on microfluidic tissue-on-chip principles.

Lin and colleagues developed a microfluidic osteochondral system that consists of a chondrogenic and osteogenic microenvironment [124]. Human bone marrow derived stem cells (hBMSCs) were seeded in hydrogels in these two compartments and chondrogenesis and osteogenesis was induced. More recently, to overcome the limited availability of hBMSCs, the same system was used to create osteochondral tissues using induced pluripotent stem cells (iPSCs) [125]. These iPSCs were first differentiated towards induced mesenchymal stem cells (iMSC) and these iMSCs were seeded in hydrogels. Upon culturing these hydrogels for 28 days within the microfluidic chip, the two compartments showed a chondrogenic and osteogenic phenotype, respectively. Subsequently, joint inflammation was mimicked by exposing the chondrogenic compartment to IL1-β and this inflammation was then treated by addition of anti-

inflammatory drug Celecoxib. Even though this system represents an elegant manner to study disease mechanisms and response to disease modifying OA drugs, the use of hydrogels has some disadvantages. Hydrogels require crosslinking methods, such as temperature changes, UV exposure, or enzymatic crosslinking, to form a stable network [126]. These crosslinking methods often are known to negatively affect cells, adding an uncertainty to the model. Moreover, hydrogels still fail to accurately mimic the 3D joint environment and reoccurring problems using hydrogels are formation of matrix islands and limited cell proliferation within hydrogels, which occur because of the elastic nature of the material [127]. Furthermore, tissue damage cannot be studied using hydrogels. Consequently, there are still shortcomings to bridge towards development of osteochondral constructs-on-a-chip consisting of biological ECM instead of hydrogels.

Outline of this thesis

In this thesis, we tried to make a step forward in transition from bench-to-bedside in OA by combining transcriptomic data from OA articular cartilage, subchondral bone, and plasma, with previously reported genetic studies, and OA disease modelling. In **chapter 2** and **chapter 3** we used RNA-sequencing data of subchondral bone to identify genes and lncRNAs that mark OA pathophysiology, by comparing macroscopically preserved and lesioned OA subchondral bone. Subsequently, we integrated these findings with previously reported findings on articular cartilage (partially of same patients) and genetics to identify potential druggable targets with possibly effects in both tissues.

In **chapter 4** and **chapter 5** we gained more insight in previously identified OA molecular endotypes in articular cartilage. To make OA molecular endotypes applicable to clinical practice, we first identified non-invasive biomarkers in plasma that allow stratification of patients based on their endotype before treatment (**chapter 4**). These OA molecular endotypes were identified based on articular cartilage, leaving the underlying subchondral bone unexplored. Therefore, we used RNA-sequencing data of the underlying subchondral bone to characterize these OA molecular endotypes in bone by performing differential expression analysis between these endotypes (**chapter 5**).

To translate genetic findings towards OA drug development, functional investigation is necessary to unravel underlying biological mechanisms of how these OA risk genes affect articular cartilage and/or subchondral bone matrix deposition. As proof-of-concept, in **chapter 6** and **chapter 7** we functionally investigated *WWP2* and *IL11* in two different models of joint tissue. The effect of *WWP2* upregulation on cartilage matrix deposition was explored using 3D human chondrocyte pellet cultures (**chapter 6**), while the effects of hrIL11 on both articular cartilage and subchondral bone were explored using osteochondral explant cultures (**chapter 7**).

Finally, we developed a new *in vitro* biomimetic model system representing functional articular cartilage and subchondral bone to study OA-related perturbations and/or OA susceptibility genes (**chapter 8**). This osteochondral-unit-on-a-chip allows in depth investigations of underlying mechanisms of OA risk genes in both tissues.

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