Thyroid hormone signalling in Osteoarthritis: early life events in late life disease

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Chapter 1

General introduction

Parts of this text are published as a review:
N. Bomer et al. / Best Practice & Research Clinical Rheumatology 29 (2015) 683-691

Introduction

Osteoarthritis (OA) is the most common arthritic disorder and is known as an age-related, prevalent, complex, chronic and disabling disease of articular joints. Disease progression is characterized by progressive destruction of joint articular cartilage, remodeling of the subchondral bone, formation of osteophytes and synovitis[1, 2]. Structural changes to OA cartilage include aberrant extracellular matrix (ECM) content and surface disruptions that range from fibrillation, clefting and delamination to complete articular surface erosion[3-5]. The complex nature of the disorder is likely based on interindividual variation in genetics, set-points in development, joint geometry and repair capacity. These inherited differences between individuals likely modulate the effects of secondary factors ranging from mechanical (over)loading[6, 7] and BMI[8, 9] to systemic inflammation[10, 11].

Currently, there is no adequate therapy to reverse or slow down the disease. Since over 20% of the Dutch population is OA affected, of which 80% have limitations in movement and 25% inhibition in major daily activities of life, OA puts a high social and economic burden on society. In the Netherlands the total costs for OA were estimated to be €1.1 billion in 2011, equivalent to 1.2% of the gross national health care costs (National Compass The Netherlands, 2011). Even more, OA is distinguished by its growing prevalence due to increasing life expectancy and the obesity epidemic. According to the United Nations, by 2050 people aged over 60 will account for more than 20% of the world's population.[12] This means that by 2050, 130 million people will suffer from OA worldwide, of whom 40 million will be severely disabled by the disease.[12]

Research into causal factors inducing OA focuses on genetics and cell-biological approaches. Comprehensive genome wide searches for genetic variants conferring risk for OA have resulted in robust genome wide significant signals.[13, 14] Functional follow up studies, to show biological relevance of the loci identified, have only recently started to be implemented as a logical next step. Consequently, little progress has been made in clinical translation of these findings, let alone identification of novel evidence based treatment options or disease modifying OA drugs. A more systematic dissection of underlying biological mechanisms of discovered OA susceptibility genes will deepen our fundamental understanding of the different OA disease pathways and respective disease drivers.

In this introduction we outline the disease processes and mechanisms that we face in OA research. We focus particularly on the role of local thyroid hormone signalling, and discuss which steps should be taken to go from a genetic association to functional genomics analyses in such a way that we obtain mechanistic understanding of genetic variation, gene expression and epigenetics underlying OA susceptibility. By subsequently finding ways to counteract identified aberrant disease processes, evidence based treatment options should be identified.

Articular cartilage

Articular cartilage is the connective tissue that covers the end of long bones. It has a smooth, wear-resistant lubricated surface that allows bones to glide over one another with minimal friction, yet eligible to absorb impact forces. Cartilage consists of only one cell type embedded in an extensive network of ECM and does not have nerves, blood vessels, or a lymphatic system[15]. The single cell type present in cartilage is the articular chondrocyte, yet contributes only ~5% to the total cartilage volume. Articular chondrocytes reside in a maturational arrested state without detectable proliferation and at a low metabolic activity.[16] Nonetheless, chondrocytes maintain cartilage tissue homeostasis throughout life and, as such, they need to become metabolically active and apply remodelling of the extracellular matrix upon micro-traumas.[17] To cope with these challenges, the chondrocyte is required to continuously and dynamically adjust expression of catabolic and anabolic genes, while securing its capacity to restore its maturational arrested steady state phenotype.[18] In OA affected cartilage, articular chondrocytes have seemingly lost their maturational arrested state as they proliferate and get a growth plate chondrocyte-like morphology, similarly as during endochondral ossification[19], thereby exhibiting articular cartilage debilitating activity.[20] Endochondral ossification is the developmental process of bone formation in which mesenchymal stem cells condensate and subsequently differentiate into chondroblasts in order to establish the cartilage anlage; a matrix rich in type II collagen and specific proteoglycans such as aggrecan[21]. The chondroblasts then become chondrocytes and organize into zones that form the growth plates of bone. The chondrocytes become hypertrophic and commence terminal differentiation, followed by mineralization of the cartilage anlage, apoptosis of the chondrocytes and vascular invasion[19]. In this process, local, intracellular active thyroid hormone (T3) is known to be essential in signalling terminal maturation of hypertrophic chondrocytes[22].

Disease mechanisms

As much as endochondral ossification is essential during skeletal development, as deleterious it is when taking place in mature articular cartilage (Figure 1).

Genetic susceptibility to OA

OA has a considerable, but complex, genetic component[24]; many genetic variants (SNPs) with small effects are expected to influence the onset and course of the disease.[24, 25] Genetic studies in OA originally included mainly candidate gene association and genome wide linkage studies, yet extended in more recent years to large scale genome wide association studies (GWAS), including large case/control studies. Based on genome wide significance ($P < 5e\text{-}8$) and/or compelling functional follow up, these studies have resulted in a list of acknowledged susceptibility loci[26-29].

Despite the phenotypic complexity of OA, robust genetic evidence was produced for a common underlying mechanism involving the maturational process of growth plate chon-

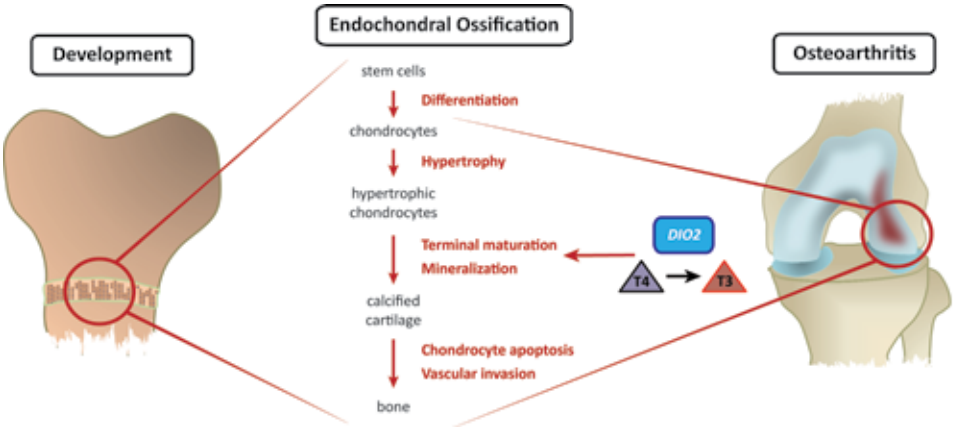

Figure 1. Reoccurrence of developmental mechanisms in OA pathophysiology.
Growth plate chondrocyte maturation in the process of endochondral ossification is essential during skeletal development (left). The occurrence of this process in adult articular cartilage forces maturational arrested chondrocytes to commence hypertrophic differentiation and terminal differentiation (right). The bio-availability of intracellular active thyroid hormone (T3) is known to be essential in this signaling cascade and recuperation of this growth plate morphology and signaling of articular chondrocytes is recognized as a hallmark of end stage OA pathophysiology[23].

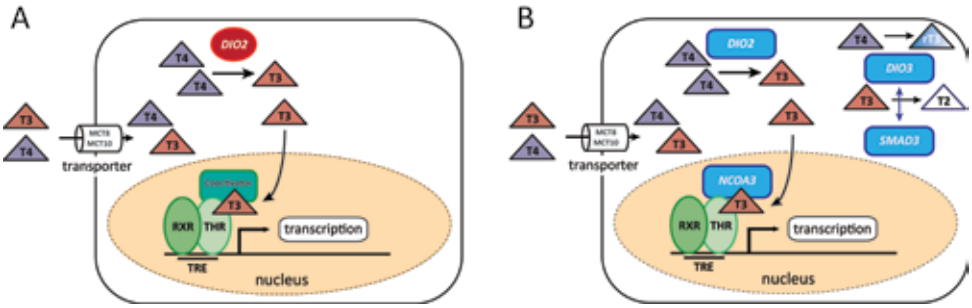

Figure 2. Graphical representation of the processes involved in intracellular thyroid hormone availability.
(A) Thyroid homone responsive cells, like chondrocytes, carry cell membrane transporters such as the monocarboxylate transporters 8 and 10 (*MCT8*, *MCT10*) admitting inactive (T4) and active (T3) thyroid hormone[47]. Intracellular 3,3'5,5'-tetraiodothyronine (T4) can subsequently be activated into 3,3',5-triiodothyronine (T3) by iodothyronine-deiodinase enzyme type 2 protein (D2)[48], encoded by *DIO2*[38]. Thereby, D2 regulates the local T3 bio-availability in specific target tissues such as the growth plate, but not systemically[48]. After entering the nucleus, T3 binds Thyroid Hormone Receptors (THRs). In general, THRs are located in the nucleus, where they homo- or hetero-dimerize with another nuclear receptor, predominantly RXR, before activating target gene expression. The formed dimers bind thyroid hormone response elements (TRE) in the promoters of target genes and recruit co-activators to start gene transcription upon T3-binding[49]. (B) Inclusion of the OA associated genes connected to thyroid hormone signalling (*NCOA3*, *DIO3* and *SMAD3*).

drocytes which coordinates the formation of cartilage and bone during endochondral ossification.[30-35] But how do the OA susceptibility genes, known for coordinating early life events, influence the onset of OA at a later stage in life? Our group proposed, 7 years ago, two possible hypothesis regarding this question. One hypothesis was that the susceptibility genes influence early suboptimal skeletal morphogenesis, thereby increasing OA susceptibility. The second hypothesis was that the genes contribute to chondrocyte dedifferentiation later in life as a consequence of aging[36, 37].

In order to answer these hypotheses, translation of these gene deviations towards underlying biological mechanisms is necessary. Nevertheless, the number of published genetic studies that lack experimental data showing the biological relevance of identified genetic variation is still large, it was advocated that enhanced implementation of functional genomics is needed to substantially augment translation to drug development and disease management[26].

DIO2 susceptibility to OA

By applying genome wide linkage analyses and replication by association to symptomatic OA, the deiodinase iodothyronine type-2 (D2) gene (*DIO2*)[38] was identified by our group as an OA susceptibility gene. The finding comprised consistent associated risk of the C-allele of the nonsynonimous rs225014 single nucleotide polymorphism (SNP) located in the coding region of *DIO2*[38]. Multiple follow up studies comparing OA affected and healthy articular cartilage in both mice[39] and humans[40-42] demonstrated significant upregulation of *DIO2* expression in OA affected cartilage, as well as a marked upregulation of D2 protein that positively correlated with increasing Mankin-score[43], a histology based marker of OA severity. It was investigated whether the *DIO2* risk alleles confer risk to OA via non-optimal shape of the bones in the joint consequently leading to recurrent damage of the cartilage and eventually triggering the OA onset[44, 45]. With respect to skeletal formation it was shown that the *DIO2* OA risk allele did not directly affect hip geometry. More likely, the cartilage structure or metabolism was affected leading to OA susceptibility as result of a non-optimal hip morphology and respectively induced biomechanical stresses[46].

Thyroid hormone and OA; a more common OA

Identification of *DIO2* as OA-susceptibility gene and the presence of *DIO2* mRNA expression and D2 protein in articular cartilage pointed at the importance of local thyroid hormone availability in the aetiology of symptomatic OA. The identified *DIO2* gene encodes an enzyme that regulates intracellular bioavailability of active thyroid hormone T3 (Figure 2A).

In thyroid hormone responsive cells like growth plate chondrocytes, T3 initiates the coordinated progression of endochondral ossification during pre-natal development and post-natal linear growth, facilitating the terminal maturation of hypertrophic chondrocytes[22, 50-52]. As mentioned before, there are striking parallels between chondrocyte signalling occurring in the downstream events of the growth plate and the behaviour of chondrocytes in OA affected articular cartilage (Figure 1)[23]. The relevance of intracellular thyroid signaling for the onset/development of OA was underscored by the suggested

modulation of osteoarthritis disease risk by the *DIO3* gene[53]. Candidate gene association studies indicated the protective effect of the G-variant of the rs945006 SNP located in the 3'-UTR of the deiodinase iodothyronine type-3 (D3) gene (*DIO3*)[53]. D3 enzymatically generates inactive metabolites (rT3 and T2) by removing a 5-iodine atom from T4 or T3, thereby diminishing levels of active T3[48, 54] and together with its counterpart D2, providing an elegant homeostatic mechanism to ensure intracellular T3 bio-availability locally, not systemically[48, 54]. A broader network of genes, involved in T3-signalling, confer OA-susceptibility by reversing T3 bio-availability (*DIO3*)[50, 53], influencing the T3 bio-availability machinery (*SMAD3*)[55, 56] and by transcriptional co-activation of thyroid responsive genes under influence of T3 (*NCOA3*)[57, 58] (Figure 2B). However, to understand how increased *DIO2* expression/T3 bio-availability affects articular cartilage and its relevance for the onset/development of OA we needed to commence functional analyses.

Methodology: studies and approaches used in this thesis

To comprehend how causality is explained by genetic variants, such as for *DIO2*, we need to understand the molecular consequences of these variants for gene expression and epigenetic regulation. Besides the study of disease-relevant tissues, which will contribute to a deeper insight into ongoing pathophysiological processes as result of the gene deviations, the study of respective cells will be key to establish *in vitro* models to further characterize genetic variants at a transcriptional and functional level and ultimately the use of an *in vivo* model organism will be needed.

RAAK-Biobank

Of great value to reach this aim is the availability of the RAAK-biobank, in which multiple disease relevant tissues (OA affected- and macroscopically unaffected cartilage, bone and ligament) and cells (bonemarrow-derived mesenchymal stem cells (hBM-MSCs) and primary articular chondrocytes (hPACs)) were collected from patients (N=400) who underwent total joint arthroplasty as result of end stage OA at the LUMC[59]. A disease-specific biobank is tailored for dissecting molecular mechanisms underlying disease onset.

Allelic Imbalanced Expression

A possible mechanism by which genetic variation could confer to OA susceptibility is the selective transcription of alleles in heterozygous carriers of disease associated SNPs[60]. Such allelic imbalanced expression (AIE) as marked by the intragenic OA susceptibility SNP rs225014 could be assessed in *DIO2* mRNA in OA cartilage. The OA risk allele 'C' being approximately 1.3 times more abundantly present than the wild-type allele 'T' (Figure 3).

Regarding the hypothesis postulated in 2008, this subtle difference in allelic expression throughout life could be detrimental to the articular cartilage and may underlie OA disease onset and progression. AIE was investigated by several groups for multiple susceptibility loci, such as *GDF5*[61], *SMAD3*[62], *NCOA3*[63], *GNL3* and *SPCS1*[64], *COL11A1*[65] and

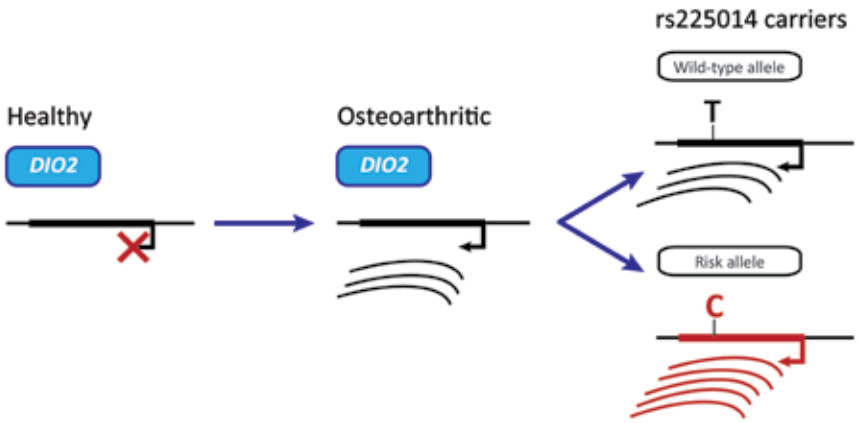

Figure 3. Graphical representation of the AIE mechanism of *DIO2* susceptibility.
Loss of silencing of *DIO2* expression may affect the propensity of maturational arrested chondrocytes to adopt an OA like state.

DIO2[66]. Indeed for most of the loci, AIE of OA risk alleles associated to disease susceptibility[67], as is also thought true for complex traits in general.[68,69]

Epigenetic regulation of gene expression

Epigenetics involves regulatory mechanisms that can switch genes on or off and determine which proteins are transcribed by factors other than an individual's DNA sequence. The most studied epigenetic mechanism is DNA methylation; the phenomenon in which the cytosine nucleotide that is located in cytosine-guanine (CpG) residue pairs is methylated. Genetic variations[70] and stochastic events due to injury or during ageing have been shown to cause changes in epigenetic marks resulting in subtle differential (allelic) gene expression. Moreover, such changes have frequently been found to be associated to pathological conditions[71], including OA[72]. The effect of the *DIO2* risk allele on local DNA methylation and subsequent *DIO2* gene expression, however, remains elusive.

In vitro cell-models for OA research

Generally, the next step in translating disease susceptibility loci towards biological relevance is applying *in vitro* cell models especially suitable to investigate the consequences of genetic and transcriptional variation of candidate genes. Different *in vitro* models can be employed to investigate the effects on chondrogenesis. When utilizing human cells (hBM-MSCs or hPACs), 2 types of cell models are applied: 2D monolayer cultures and 3D micromass cultures. Comparing monolayer (2D) versus micromass (3D) cultures showed that the basal gene- and protein-expression in these cultures where fundamentally different[77]. The use of monolayer cultures mainly resulted in complete loss of chondrogenic[78,79] and recovery of hypertrophic phenotype of the chondrocytes[77]. In particular 3D models have been shown to support chondrocyte differentiation and proliferation and to produce superior cartilage-like tissue with enhanced mechanical properties[80]. The 3D model of

hBM-MSCs, was proven to resemble, at consecutive weeks, chondrogenic differentiation, recapitulation, hypertrophy and terminal maturation processes thereby providing an elegant model of OA development, mimicking the re-couperation of the (unfavourable) process of endochondral ossification[81].

In vivo animal models for OA research

Animal models further improve our understanding of the molecular mechanisms underlying genetic association, driving the OA pathology[83]. With respect to cartilage destruction, different mouse strains have been generated or reused for known OA susceptibility genes. *Gdf5* knockout mice were generated long before the identification of the *GDF5* susceptibility locus and were used subsequently to study the development of OA[84]. The same holds for *Smad3*-deficient mice[85]. An exception is the generation of *Frzb* knockout-mice[86, 87] specific for OA purposes.

Dio2 knockout mice were readily available and used in studies regarding, thermogenesis in brown adipose tissue[88], pituitary resistance to T4[89] and insulin resistance and susceptibility to diet induced obesity[90]. No experiments were applied however to study joint pathology in OA. The only animal-model in which the functional effects of aberrant *DIO2* signalling on articular cartilage was assessed, was performed on cartilage-specific human *DIO2* overexpressing (hD2Tg) rats[76]. Upon submission to an injury-induced OA model (DMM), hD2Tg rats showed a significant higher level of cartilage damage as compared to their wild type littermates. These results further suggested that increased D2 activity may in fact be detrimental for articular cartilage maintenance. It would therefore be of great value to asses the effect of *Dio2* deficiency on cartilage in an *in vivo* OA model utilizing *Dio2* knockout-mice. Recently, the model of biomechanical burden by exercise was put forward to provide a more physiological model of disease induction and progression[91].

Aims of the thesis

In this thesis, we set out to elucidate and characterize the pathophysiological processes that underlie the re-occurrence of developmental signalling in a late life disease; Osteoarthritis.

In chapter 2, as part of a large international collaboration, we identified susceptibility loci for hand OA by GWAS and particularly provided functional evidence for OA susceptibility of the *ALDH1A2* gene.

In chapter 3, functional genomics analyses, including disease specific tissues and the *in vitro* micromass chondrogenesis model of hBM-MSCs, were applied as a tool to identify the underlying molecular mechanism of genetic variation, gene expression and epigenetics of the *DIO2* gene in the development of OA.

The beneficial effect of inhibiting *DIO2* in the *in vitro* model as found in chapter 3 was translated to a *Dio2*-knockout mouse model in chapter 4, in collaboration with the Skeletal Biology and Engineering Research Centre (KU Leuven, Belgium). The effect of forced

exercise was assessed, in a treadmill running model of OA, on knee-joints in *Dio2*$^{-/-}$-mice and their wild-type littermates.

In chapter 5, we focus on *Calreticulin* (*Calr*), the single transcript reaching the significance threshold when comparing cartilage of wild-type- and *Dio2* knockout-mice. In parallel to disease modifiers, there is a need for insight into intrinsic factors that enhance stability of healthy articular chondrocytes. We aimed to elucidate the effect of altered *Calr* expression on cartilage homeostasis, as a mechanism underlying OA onset and facilitating the detrimental effects of increasing *Dio2* expression.

Finally, in chapter 6, we focussed on the epigenetic landscapes of our *in vitro* micromass chondrogenesis models of hBM-MSCs and hPACs as compared to paired autologous articular cartilage, to get better understanding of the cell-type specific methylome for future OA research.

Chapter 7 provides a general discussion, in which the results and future perspectives are discussed.

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