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

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Chapter 7

Summary and general discussion

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Introduction

In the field of OA research the step from genetics to biological functionality, also named 'functional genomics', is necessary to allow valorisation of genetic findings[1-3], thereby augmenting the need for functional data of disease relevant tissues. Even so, it was estimated that pursuing druggable targets directed by genetic studies are twice as often successful as compared to those without it[4]. In this thesis we apply the functional genomics methodology, to proceed from a genetic association to mechanistic understanding of the effect of genetic variation on gene expression and epigenetic regulation contributing to OA susceptibility. Particularly we set out to characterize and validate the pathophysiological processes that underlie the role of *DIO2*/thyroid hormone signalling in the onset of OA after identifying the *DIO2* gene as a OA susceptibility locus. We hypothesized that upregulation of *DIO2* and thereby increased thyroid hormone signalling may be key in the etiology of OA, whereas mitigating *DIO2* expression could possibly prolong cartilage homeostasis. For that matter, disease specific tissue analysis, customized cell- and tailored mouse-models, were applied. Proper organisation and expansion of a disease-specific tissue- and cell-bank (RAAK) was executed as part of this thesis and resulted in a tailored toolbox, ideal to dissect molecular mechanisms underlying disease onset. Our progress in functional genomics was supported by this collection of cartilage tissue samples in a large number of OA patients in a design allowing for patient specific comparison of preserved and OA affected cartilage. Human bonemarrow-derived mesenchymal stem cells (hBM-MSCs) and human primary articular chondrocytes (hPACs) of multiple donors were eligibly subjected to *in vitro* micromass culture experiments, which allowed for functional validation and causal inference of identified susceptibility genes.

Translating genomics into mechanisms of disease; functional genomics in OA

A first important step from genetic susceptibility to underlying disease mechanism is understanding the functional consequences of the identified risk allele. The effectiveness of assessing allelic imbalanced expression (AIE), in OA research was shown previously for *DIO2*[5]. In the current thesis AIE was implemented for the identified hand OA susceptibility gene *ALDH1A2* outlined in chapter 2. Besides assessing differential *ALDH1A2* expression levels in OA effected cartilage as compared to preserved cartilage isolated from the same joint, we provided proof for an allelic imbalance in expression of the gene, dependent on the genotypes of the rs3204689 SNP in the 3'UTR of the *ALDH1A2* transcript. We showed that the transcript with the risk-conferring C allele was expressed at a lower level than the corresponding normal transcript. Our expression data supported a role for *ALDH1A2* in mediating the effect of the osteoarthritis risk variants in the associated locus, indicating likely causality for the genetic variant. Nevertheless, to understand the effect of the identified AIE and differential gene expression, further

research is needed to dissect molecular effect on articular cartilage. The use of tailored cell-models to do so is exemplified in this thesis for *DIO2*.

Functional genomics of *DIO2*; intracellular thyroid hormone signalling

DIO2 expression and DNA methylation in disease specific tissues

As mentioned before, *DIO2* expression was shown absent in healthy cartilage, but was found highly upregulated in both macroscopically normal and lesioned OA cartilage[6-8]. Carriers of the *DIO2* rs225014 risk allele (chapter 3) were found to have increased *DIO2* gene expression in affected- compared to preserved cartilage. A consistent positive correlation was further demonstrated between methylation at the CpG site 2031 base pairs upstream of the *DIO2* transcription start site (CpG-2031) and *DIO2* expression in articular cartilage among carriers of the rs225014 risk allele. These regulatory properties of DNA methylation on *DIO2* expression was subsequently confirmed by applying *5-aza-2'-deoxycytidine* (AZA) treatment eliciting general demethylation of the DNA, concomitant with a decrease in methylation at CpG -2031 and *DIO2* expression[9]. Given that the CpG-2031 maps within an active CCCTC-transcription factor (CTCF) binding site, we hypothesized that methylation dependent binding of CTCF could act as a positional isolator of *DIO2* expression[9]. Albeit that we found partial proof for this hypothesis, we were not able to confirm three-dimensional chromatin conformations to underlie the relation between the rs225014 tagged allelic imbalance and methylation-dependent upregulation of *DIO2* among rs225014 risk-allele carriers. Nonetheless, our data provided evidence in humans that genetic predisposition combined with OA-related changes of the articular cartilage resulted in loss of epigenetic silencing of *DIO2*. The re-initiated expression of *DIO2* in adult OA affected articular cartilage due to the increase of DNA methylation could be part of the observed unfavourable recuperation of early life signalling of chondrocytes in OA affected cartilage[10, 11]. The fact that the presence of increased *DIO2* expression in preserved cartilage by itself does not initiate OA, shows that a secondary trigger is needed. Since OA lesions are predominantly found at the loading 'hotspots' of the OA-joints, it was hypothesised that high *DIO2* expression combined with biomechanical (over-)loading could be the combined trigger to commence cartilage degradation marking the onset of OA. This hypothesis was further addressed in chapter 4.

DIO2 perturbation in customised in vitro chondrogenesis models

Furthermore in chapter 3, the *in vitro* micromass chondrogenesis model of human bone-marrow derived mesenchymal stem cells (hBM-MSCs), was applied to investigate the direct effect of *DIO2* upregulation on cartilage homeostasis and integrity in the development of human OA. We showed that in this OA model, upregulation of *DIO2* expression as well as subsequent increased T3 bio-availability, was sufficient to directly induce detrimental expression of the OA catabolic markers of cartilage destruction. A marked reduction of the chondrogenic capacity of the cells to deposit ECM components was found, concur-

rent with induction of OA specific markers of cartilage matrix degeneration (*ADAMTS5* and *MMP13*) and mineralization (*ALPL*). Given their concurrent upregulation, it was hypothesized that this process is likely mediated via *HIF-2*α/*RUNX2* signalling, an important pathway in the OA disease process. ChIP-seq data covering thyroid hormone receptor (THR) binding supported this hypothesis by showing that the *HIF-2*α locus harbours specific THR binding sites and is directly transcriptionally reactive to thyroid hormone[12]. In view of these data, we advocate that active thyroid hormone availability, likely by local *DIO2* action, in the *in vitro* OA-model of hBM-MSCs has an important impact on *HIF-2*α upregulation, a hallmark in the pathophysiology of OA.

Although we found a direct detrimental effect of increased *DIO2* expression and subsequent T3 availability on matrix deposition in this *in vitro* model, this finding was not in line with the observations made earlier in OA and preserved cartilage tissue. Preserved cartilage was shown to express high levels of *DIO2*, similar to OA affected cartilage, nevertheless being macroscopically unaffected. Additionally, we found that increased T3 bioavailability did not have a similar profound, detrimental effect on cartilage explant cultures, nor on *in vitro* 3D micromass cultures of primary chondrocytes, both isolated from preserved regions of the OA joints (unpublished data). In line with this, cartilage-specific h*DIO2* overexpressing (hD2Tg) rats did not show any inherent defects on the femoral or tibial articular cartilage surfaces[13]. However, submitting the hD2Tg rats to an injury-induced OA model (DMM), thereby applying an acute biomechanical burden on articular cartilage, resulted in more cartilage damage as compared with their wild-type littermates[13]. These findings, again, indicated that although *DIO2* is upregulated in an OA affected joint, such chondrocytes are not necessarily in an OA like state and additional perturbation is necessary to initiate terminal maturation and irreversible breakdown. With respect to the distinctive feature of BM-MSCs, to undergo all subsequent steps of the endochondral ossification program, ending in terminal maturation and apoptosis of the chondrocyte, we advocate that the tendency of these cells to commence a growth plate chondrocyte-like identity, BM-MSCs are ideal for pathophysiological OA research. Even though hBM-MSCs are mechanistically prone to do so, intervention with the general deiodinase inhibitor, Iopanoic Acid (IOP), contributed to prolonged 'healthy' cartilage homeostasis by virtue of attenuated upregulation of matrix degrading enzymes, a constant *COL2A1*/*COL1A1* ratio, denser cartilage matrix structure with significant less cellular lacunae, thereby indicating a reduced propensity of chondrogenically differentiated hBM-MSCs to enter the terminal maturational process. This suggested that in a fully activated OA process of endochondral ossification the deiodinase inhibitor IOP was able to arrest terminal maturation. These findings showed promising results for future therapeutic intervention, regarding thyroid hormone signalling and were encouraging further research in an *in vivo* model.

DIO2 deficiency in tailored in vivo animal models; Exercise induced OA

The beneficial effect of inhibiting *DIO2* was translated to a mouse model in chapter 4, in collaboration with the Skeletal Biology and Engineering Research Centre (KU Leuven,

Belgium). We assessed the effect of forced exercise, in a treadmill running model of OA, on knee-joints in *Dio2*$^{-/-}$-mice and their wild-type littermates. No inherent histological differences were found between articular cartilage of *Dio2*$^{-/-}$-mice and their wild-type littermates. Nonetheless, we showed that *Dio2*$^{-/-}$-mice were protected against exercise induced OA as compared to wild-type-mice. Upon forced mechanical loading, only the wild-type-mice showed clear signs of cartilage damage and synovitus, supporting the use of forced running as an OA-model in mice. Biomechanical stress resulted in 147 transcripts to be significantly differentially expressed between the challenged (running) and unchallenged (non-running) mice in a genome wide expression analysis. However, cartilage from *Dio2*$^{-/-}$-mice was found to exhibit significant stress-induced expression. This absence and the non-appearance of histological cartilage damage in *Dio2*$^{-/-}$ mice suggested that degenerative pathways were not activated in this knockout strain despite the biomechanical burden imposed. Moreover, we found that *Dio2*-deficiency was reflected in a specific expression profile of genes showing significant interaction between *Dio2*-genotype and biomechanical stress that either mark a favourable effect in *Dio2* knockout- (eg. *Gnas*) or an unfavourable effect in wild-type-cartilage homeostasis (eg. *Hmbg2* and *Calr*).

How histological similar cartilage of *Dio2*-deficient, as compared to wild-type mice, was able to withstand the detrimental effects of mechanical stress is not clear. It was brought forward that the effects of systemic *Dio2*-deficiency could have an impact on general metabolism of these mice, and thereby regulating the effect as seen in the OA model. Of note here, it was shown that *Dio2*-deficient mice had a normal circulating T3 concentration as compared to wild-type-mice[14], disqualifying the effect to be attributed to systemic alteration of circulating T3. Furthermore, results presented in a concurrently supporting paper, correspondingly showed that articular cartilage of *Dio2*-knockout as compared to wild type littermates did not have intrinsic histological alterations in joint cartilage, nor in subchondral bone area[15]. This again showed, like for the hD2Tg rats, that aberrant *DIO2* expression by itself does not initiate OA-like macroscopical changes in the articular cartilage. Nonetheless, mineral content of the subchondral bone was found to be markedly increased in *Dio2* knockout-mice, resulting in bones with reduced toughness and increased susceptibility to fracture. How this bone abnormality in *Dio2*-deficient mice reflects its role in OA pathogenesis is unknown and it stays unclear whether alterations in subchondral bone and articular cartilage occur simultaneously or whether subchondral bone changes result in cartilage damage or vice versa[15, 16]. Overall, these findings further support the hypothesis that the combination of *DIO2* expression and mechanical stress is essential to commence OA. Furthermore, it provided additional *in vivo* support that interfering with intracellular thyroid hormone levels could be a powerful way to oppose the pathological events that are occurring in OA as a result of biomechanical burden, in particular since no striking developmental skeletal phenotype appears present in the *Dio2*$^{-/-}$-mice.

Intracellular thyroid hormone; a master switch to joint destruction?

In summary, articular chondrocytes may lose their healthy maturational arrested state

throughout life as a result of loss of epigenetic regulation due to mechanical stress, injury or age and convert into a hypertrophic state as reflected by up regulation of *DIO2* in preserved and lesioned OA articular cartilage[6, 7, 17, 18]. By applying functional follow up studies to the OA susceptibility gene *DIO2*[19] (Box 1) it was demonstrated that aberrant intracellular T3 signalling, likely modified by genetic variations in recognised OA susceptibility genes such as *DIO2*, *NCOA3*[20], *ALDH1A2*[21], *SMAD3*[22] and *DIO3*[23], disturbs adult articular cartilage homeostasis and provokes irreversible articular cartilage damage, particularly upon external stresses (Figure 1). These results showed that intracellular thyroid hormone signaling could be underlying a more common OA, and we therefore hypothesize that the induction of intracellular thyroid hormone signalling may be a master-switch that forces hypertrophic chondrocytes to initiate terminal maturation and inflict irreversible articular cartilage damage upon external stresses. Moreover, upon exploring ways to counteract the aberrant *DIO2* effect it was indicated that local T3 signaling could be mitigated by IOP. The data in this manuscript builds on a dedicated ongoing functional genomic OA research-line. By making use of the functional genomics pipeline, we[24-27] and others[6, 28] have provided mechanistic insight in the role of *DIO2*[19] in OA and provided an evidence based treatment option. These insights strongly suggest that particularly intracellular levels of thyroid hormone should be controlled tightly, especially under biomechanical perturbations, to maintain joint tissue homeostasis.

Timeline for DIO2 and Osteoarthritis; from genetic susceptibility to functional genomics

2008	Identification of *DIO2* (rs225014) as an OA susceptibility locus[19].
2008-2010	upregulated *DIO2* mRNA levels with disease progression[6, 7, 17, 18].
2012	Increased DIO2 protein in OA affected cartilage and allelic imbalance of OA risk SNP (rs225014) in OA tissues[25].
2013	*DIO2* upregulation causes cartilage destruction in an OA-rat model[28].
2014	Identification of underlying (epigenetic and transcriptomic) mechanisms of *DIO2* susceptibility in OA[24].
2014	Attenuating D2 activity with IOP results in prolonged 'healthy' cartilage homeostasis[24].
2014	Knocking out *Dio2* in a murine model protects against cartilage damage only upon biomechanical burden[26, 29].

Intrinsic factors for healthy cartilage; *DIO2* deficiency

In parallel to OA disease modifiers, there is a need for insight into intrinsic factors that enhance stability of healthy articular chondrocytes also upon environmental challenges such as biomechanical (over-) loading. In chapter 5, we focused on *Calreticulin* (*Calr*), the single transcript reaching the significance threshold when comparing cartilage of wild-type- and the protected *Dio2*$^{-/-}$-mice. Notably, our previous data showed that biome-

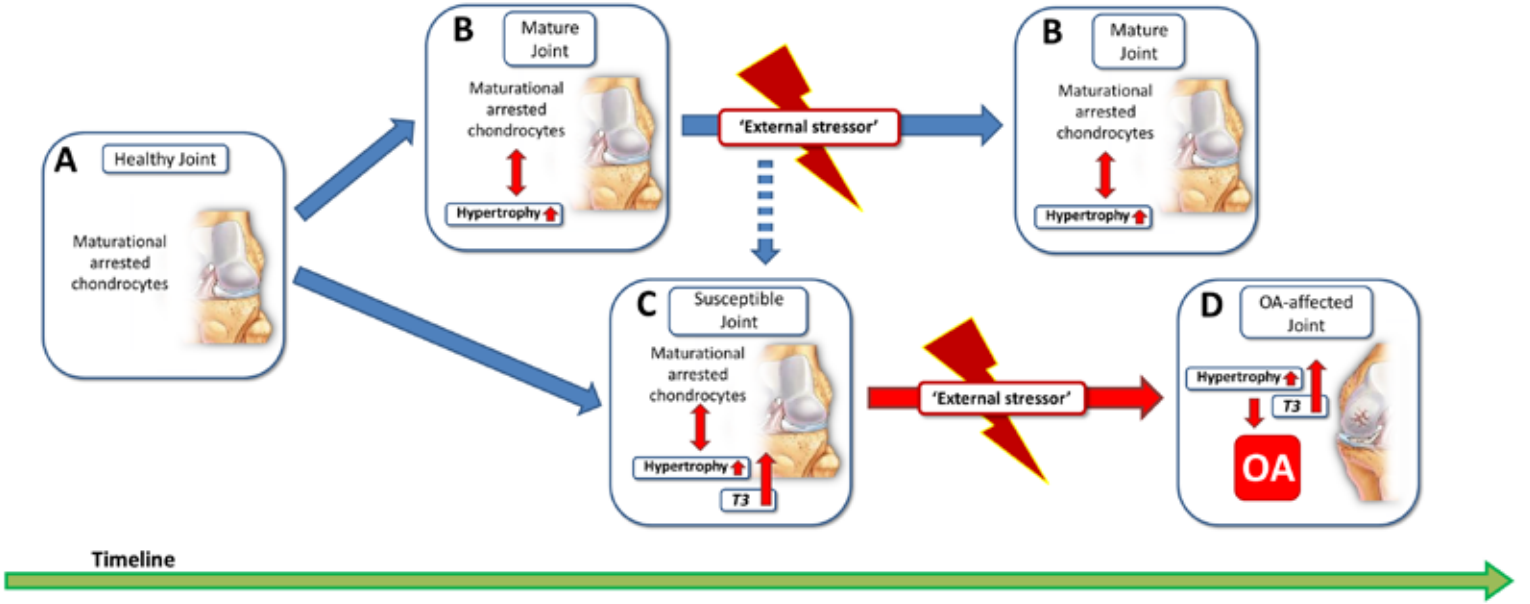

Figure 1: Graphical representation of hypothesized importance of thyroid hormone in the onset of OA. (A) In healthy joints, chondrocytes reside in the articular cartilage in a maturational arrested state. With ageing, chondrocytes of the adult joint can become hypertrophic (B) but this is hypothesised not to be crucial for the onset of OA. External stressors, like injury and joint damage, applied on these joint are not necessarily the tipping point towards irreversible cartilage damage, but can make the joint susceptible for OA. (C) The additional re-initiated bioavailability of active thyroid hormone, in these susceptible joints, is here proposed as the master-switch that initiates irreversible cartilage damage under the influence of mechanical stress (D) likely due to terminal maturation of the articular chondrocyte calcification of the matrix.

chanical stress had a profound effect on *Calr* expression in cartilage of wild-type-mice, whereas *Calr* expression was not altered in *Dio2*-knockout-mice upon challenge. However, independent of challenge, *Calr* was found significantly downregulated in cartilage of *Dio2*$^{-/-}$ mice and therewith accompanying the beneficial homeostatic state of the articular cartilage. We demonstrated that overexpression of *Calr* during early chondrogenesis lead to decreased proteoglycan deposition and corresponding lower Aggrecan expression. Knocking down *Calr* expression did not result in histological differences of matrix composition. In addition to this, expression levels of *Calr* and *Dio2* were found to be correlated. These data implicated that both lower levels of *Calr* and *Dio2* appear beneficial to cartilage homeostasis also upon mechanical perturbation.

The hypothesis on the underlying mechanism of *Calr*, resulting in the beneficial homeostatic state of articular cartilage, comes in twofold. First, by regulating the amount of calcium stored in the endoplasmatic reticulum, and therefore the amount that can be released to the cytosol to trigger downstream events, *Calr* could affect apoptotic outcomes[30]. Given our data, we could hypothesize that lower levels of *Calr* expression, as a result of *Dio2*-deficiency, resulted in a more subtle calcium-induced apoptotic signal upon stress, being favourable for the maintenance of cartilage tissue homeostasis. The beneficial effect of reduced calcium-levels for cartilage integrity was supported by a recent study which reported that calcium antagonists might be efficient in preventing progression of OA[31]. Second, *Calr* was shown to interact with nuclear hormone receptors, like the glucocorticoid, androgen and retinoic acid receptor. Although interaction with thyroid hormone receptors was not yet shown, it was made presumable, since the gene family of nuclear receptors was characterized by the presence of a typical, well conserved DNA-binding domain[32]. This implicated that *Calr*, possibly interacts with thyroid hormone receptor initiated transcription and thereby interfering with activation of detrimental pathways, like *HIF-2α* signalling upon increased T3 bioavailability. This showed the opportunities of *Calr* as a therapeutic target in parallel to *DIO2*, interfering in or as a result of the thyroid hormone signalling cascade, in the treatment of OA.

Functional genomics; *in vitro* 3D cell models compared to autologous cartilage

Being triggered by the difference in effect of T3 bioavailability on the chondrogenic capacity of hBM-MSCs and hPACs, we focussed in chapter 6 on the epigenetic landscapes of our *in vitro* micro-mass chondrogenesis models of hBM-MSCs and hPACs as compared to paired autologous articular cartilage. As discussed before, increased T3 bioavailability did not have a simmilar profound, detrimental effect on cartilage explant cultures, nor on *in vitro* 3D hPAC micromass cultures, both isolated from preserved regions of the OA joints, as in 3D hBM-MSCs micromass cultures. We suspected that this was due to an aberrant methylome in hBM-MSCs, making them more prone to commence OA initiating transcription. However, the exact underlying molecular mechanism is not understood and studies which explore comprehensive pathways in endochondral differentiation of

MSCs and chondrocytes had not been published in recent years[33]. Our study showed that *in vitro* engineered neo-cartilage tissue from hPACs, exhibited a DNA methylation landscape that is almost identical (99% similarity) to autologous cartilage, in contrast to neo-cartilage engineered from hBM-MSCs. This showed that even though the model of hBM-MSC micromass cultures resembled a model of OA development[34], the cells in this neo-cartilage did not represent adult articular chondrocytes, that reside in macroscopically normal articular cartilage, based on their epigenetic signature, nor did they represent the shift towards OA affected chondrocytes (data not shown). Of note, the vast methylation difference was ruled out to be a result of the time course of chondrogenesis, since only a minority of CpGs showed significant methylation changes over time in both hBM-MSCs (0.25% of all CpGs) and hPACs (0.06% of all CpGs). Moreover, the majority of these time course-dependant CpGs did not contribute to the difference with autologous cartilage. Having said all this, as to date, hBM-MSCs are widely used for cartilage engineering purposes, even though the effects of these vast methylation differences on cartilage regeneration and long term consequences of implantation, are not known.

Our results suggested that the use of hPAC micromass cultures or autologous cartilage explant cultures should be utilized for OA research, since they displayed a more natural reflection of the autologous cartilage than the hBM-MSCs model. It is yet unclear, and unlikely, whether the processes as seen with OA occur spontaneous in this model. An external, though unnatural, stressor will be needed, such as e.g. biomechanical stress, predisposing the model for OA. Nonetheless, since an external stressor will be needed to commence onset of OA-like damage, the choice of external stressor used to imply this damage will influence the type of OA that is being investigated. For example, using IL-1β will lead to an inflammation induced OA, as will the use of reactive oxygen species lead to cellular stress induced OA. The same holds up for the choice of mouse-model for research on the onset and development of OA, or the development of new drugs for that matter. The widely used DMM-model will help to understand the changes occuring in post-traumatic articular cartilage, as will the collagenase-induced model explain the inflammatory-induced OA. The model of mechanical loading by forced exercise[35] was put forward to provide a more physiological model of disease induction and progression[36]. Nontheless, we should always keep in mind that these models are not natural and strain the development of OA-like cartilage damage.

Future perspectives

In recent years accumulating evidence showed that counteracting the deleterious effect of *DIO2* by attenuation of thyroid signalling may be key in securing joint tissue homeostasis irrespective of the OA disease subtype. Future endeavours should be designed to demonstrate that local inhibition of D2 by intra-articular admission of IOP, could be an effective therapy to alleviate the burden of OA thereby increasing mobility, well-being and quality of life particularly among elderly. The reoccurrence of developmental processes in adult cartilage exemplifies the dysdifferentiation theory of ageing[37]; methylation

changes cause transcriptional alterations with age, resulting in de-repression of genes in tissues where they are normally not expressed.

The availability of diseased, but unaffected cartilage explant-tissue allows us to establish a human *ex vivo* OA-model wherein different strains of mechanical loading induces different grades of OA-like cartilage destruction. It is generally accepted that biomechanical loading is necessary for the maintenance of cartilage homeostasis[38, 39]. However, abnormal, altered or injurious loading is associated with inflammatory and metabolic imbalances that may eventually lead to OA like damage[39]. Moreover, *ex vivo* cartilage explants subjected to these magnitudes of stress exhibit a significant suppression of metabolic activity and particularly affects the biosynthesis of aggrecan and collagen[40] similar to the *in vivo* situation[24]. This model is ideal to assess the ability of compelling novel druggable targets, such as IOP, to prevent, stop or reverse OA. Furthermore, by applying this *ex vivo* "bench-work" it will be relatively easy to translate effect to *in vivo* models and even to human pre-clinical trials comprising patients with OA. Gaining knowledge on how biomechanical stress and injury provokes intracellular thyroid hormone signalling in articular joints to bring about its detrimental effects in mature articular cartilage is essential to understand the mechanisms for future therapy. Given that *DIO2* does not affect circulating thyroid hormone levels in humans[24], these investigations should focus on and target the local adaptation to T3 availability of the articular cartilage. Testing the therapeutic effects of deiodinase inhibitor IOP, *ex vivo* and *in vivo,* will give us valuable information on the potential clinical use. Nevertheless, we need to keep notice of the fact that we are looking for molecular explanations for OA onset and development in artificial models, both in vitro and in vivo. Therefore our findings should always be placed in perspective and, like in the discovery and interpretation of genetic- and bio-markers, characterize OA-subtypes in the different *in vitro* and *in vivo* models that are used to dissect underlying molecular mechanisms[36, 41, 42].

Results of the studies performed during this PhD have led to the translational research proposal, supported by the Dutch Arthritis Foundation, in which the beneficial effects of attenuated *DIO2* expression by means of intra-articular IOP injection will be investigated on cartilage integrity in both ex vivo explant- and in vivo mouse-models of OA.

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