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Chapter 4

The effect of forced exercise on knee-joints in Dio2<sup>−/−</sup>-mice; type II iodothyronine deiodinase-deficient mice are less prone to develop OA-like cartilage damage upon excessive mechanical stress

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Abstract

**Objective:** To further explore deiodinase iodothyronine type-2 (DIO2) as a therapeutic target in osteoarthritis by studying the effects of forced mechanical loading on in vivo joint cartilage tissue homeostasis and the modulating effect herein of Dio2 deficiency.

**Methods:** Wild-type and C57BL/6-Dio2+/−-mice were subjected to a forced running regime for one hour per day for three weeks. Severity of osteoarthritis was assessed by histological scoring for cartilage damage and synovitis. Genome wide gene expression was determined in knee-cartilage by microarray analysis (Illumina MouseWG-6 v2). STRING-db analyses were applied to determine enrichment for specific pathways and to visualize protein-protein interactions.

**Results:** In total, 158 probes representing 147 unique genes showed significantly differential expression with a fold-change ≥ 1.5 upon forced exercise. Among these are genes known for their association with OA (e.g. Mef2c, Egfr, Ctgf, Prg4 and Ctnnb1), supporting the use of forced running as an OA-model in mice. Dio2-deficient mice showed significantly less cartilage damage and signs of synovitis. Gene expression response upon exercise between wild-type and knockout-mice was significantly different for 29 genes.

**Conclusion:** Mice subjected to a running regime have significant increased cartilage damage and synovitis scores. Lack of Dio2 protected against cartilage damage in this model and was reflected in a specific gene expression profile, and either mark a favorable effect in the Dio2-knockout (e.g. Gnas) or an unfavorable effect in wild-type cartilage homeostasis (e.g. Hmbg2 and Calr). These data further support DIO2 activity as a therapeutic target in OA.
Introduction

Osteoarthritis (OA) is a prevalent, complex, chronic and disabling disease of articular joints, characterized by progressive destruction of joint cartilage, remodeling of the subchondral bone, formation of osteophytes and synovitis [1, 2]. It causes pain and disability to an increasing proportion of the population and is associated with the obesity pandemic, aging of the population and not in the least by improved survival of patients with cardiovascular or oncological health problems. All together this imposes a large and growing social and economic burden [3, 4]. The development of novel therapeutic approaches is therefore urgently needed and should be based on insights into the underlying disease mechanisms [5-7]. Several genetic studies identified robust signals for OA susceptibility [8-13], that suggest specific genes, involved in cartilage development and growth, to play a key role in the OA disease process [14, 15]. Notable example of such OA susceptibility alleles, are the C-variant of the rs225014 single nucleotide polymorphism (SNP) located in the coding region of the deiodinase iodothyronine type-2 (D2) gene (DIO2) [8, 16] and the rs945006 SNP in the deiodinase iodothyronine type-3 (D3) gene (DIO3), another deiodinase with a counter regulatory function for DIO2 [16]. For together, D2 and D3 primarily regulate the bio-availability of intracellular thyroid hormone in specific tissues such as the growth plate, but not systemically. The deiodinase type 2 protein (D2) catalyzes the conversion of intracellular inactive thyroxine (T4) to active thyroid hormone (T3). During skeletal development, this conversion plays a critical role in the process of endochondral bone formation by facilitating terminal maturation of hypertrophic chondrocytes subsequently leading to breakdown of the cartilage matrix and replacement by bone. This process is essential in skeletal development and growth but loss of the chondrocyte's maturational arrested characteristics is considered deleterious for postnatal articular cartilage.[17, 18] DIO2 mRNA and D2 protein levels are highly upregulated in human osteoarthritic cartilage as compared to healthy cartilage [19-21], suggesting that in disease DIO2 contributes to the loss of the highly specialized maturational arrested state of articular chondrocytes [17]. Cartilage-specific over-expression of human DIO2 in rats was associated with increased damage to the articular cartilage in a surgical OA model. However, this was without clear evidence that hypertrophy of chondrocytes plays an essential role and rather pointing towards increased tissue destructive enzyme activity and enhanced expression of IL-1 target genes [22]. Upregulation of DIO2 expression in a human in vitro model resulted in a marked reduction of the capacity of chondrocytes to deposit ECM components, including type II and type X collagen, while inducing OA-specific markers of cartilage matrix degeneration and mineralization [18]. In contrast, pharmalogical inhibition of DIO2 increased the expression of collagens and aggrecan without clear effect on hypertrophy or tissue destructive enzymes. These accumulating data suggest that D2 inhibition and/or modulation may become a therapeutic target, but the in vivo impact of D2 loss of function in joint biology and disease remains largely unknown in particular at the molecular level.

In this study, we set out to study the molecular network of Dio2 in the healthy and challenged joint. We performed genome wide expression analyses in aging wild-type
and Dio2−/− mice, including groups exposed to a moderately strenuous running regime. Our results indicate that Dio2 is effectively involved in specific gene networks that can be associated with osteoarthritis and provide further insights into the complex molecular interactions involved in healthy and diseases articular cartilage.

Materials and methods

Animal experiments
Dio2−/− mice were a kind gift of Dr. V. Galton (Dartmouth Medical School, NH, USA)[23] and were backcrossed onto the C57Bl/6 background. All experiments were approved by the Ethics Committee for Animal Research (KU Leuven, Belgium).

Four to 6 months old male Dio2−/− (n=22) and wild-type mice (n=30) ran for 3 weeks 1 hour/day, 5 days/week, at a speed of 11 m/min and with an inclination of 5°. For additional details, see the Online Methods.

Histological assessment of osteoarthritis
Right knees were fixed overnight at 4°C in 2% formaldehyde, decalcified for 3 weeks in 0.5M EDTA pH 7.5 and embedded in paraffin. Severity of disease was determined by histological scores on hematoxylin/eosin or Safranin O stained sections (5μm) throughout the knee (5 sections at 100μm distance). For additional details on histological assessment and statistical analyses, see the Online Methods.

RNA isolation
Snap frozen cartilage of the left knees was powderized using a Retsch Mixer Mill 200 under cryogenic conditions. RNA was isolated and washed using the RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s protocol. RNA quality was assessed using a Biocore lab-on-a-chip and quantity was assessed using a Nanodrop spectrophotometer. For additional details, see the Online Methods.

Microarray analysis
Complementary DNA synthesis, amplification, biotin labeling and hybridization onto the microarrays was performed using the Ambion TotalPrep-96 RNA amplification kit (Life Technologies, Bleiswijk, The Netherlands) according to manufacturer’s protocol. After hybridization on Illumina MouseWG-6 v2 BeadChip microarrays (Illumina, Eindhoven, The Netherlands) the slides were scanned with the Illumina Beadscanner 500GX. For additional details on microarray handling and data analyses, see the Online Methods.

Pathway analysis and protein-protein interaction networks
Gene enrichment among the genes with significant differential expression was performed with STRING (Search Tool for the Retrieval of Interacting Genes/Proteins 9.1) [24]. Pathways with a P-value ≤ 0.05 after FDR correction were considered significant. Enrichment in protein-protein interactions was also analyzed using the STRING database. For addi-
Quantitative RT-PCR assays (validation)
Validation of the microarray results was performed by quantitative real-time PCR. 500 ng of total RNA was processed with the First Strand cDNA Synthesis Kit according to the manufacturer’s protocol (Roche Applied Science, Almere, The Netherlands) upon which cDNA was diluted 5 times. RT-qPCR measurements were performed on the Roche Lightcycler 480 II, using Fast Start Sybr Green Master reaction mix according to the manufacturer’s protocol (Roche Applied Science). For additional details, see the Online Methods.

Results

Reduced severity of OA after forced running in Dio2\textsuperscript{-/-} mice
To study the role of Dio2 in joint homeostasis, Dio2\textsuperscript{+/-} and wild-type mice were studied in a forced-exercise setup and compared to non-running mice (Figure 1). No striking developmental skeletal phenotype appears present in the Dio2\textsuperscript{-/-} mice \cite{25} and our observations in our mouse colony are in agreement with these findings. Wild-type and Dio2\textsuperscript{-/-} mice displayed a similar running behavior. As shown in Figure 1, overall group analysis indicated a significant difference between wild-type- and knockout-mice with respect to cartilage damage ($P = 0.0006$) and synovial hyperplasia ($P = 0.0536$). No specific effects within wild-type- or knockout-mice of exercise were found, nor interaction between genotype and exercise. Sidak’s multiple comparison test indicated that the effect was determined by the difference in genotype in the exercise group ($P < 0.0001$ for cartilage damage – 95% CI of the difference between the means (0.1820 to 0.5820) and $P = 0.0184$ for synovial hyperplasia – 95% CI of the difference between the means (0.03661 to 0.4627)) (Figure 1).

Microarrays: Differential expression in knee-joints upon forced exercise
To identify genes responsive to the forced running regime in mice, genome wide gene expression in knee-cartilage was studied by microarray. Gene expression was detected before and after the running regime in interaction with the genetic background (wild-type and Dio2\textsuperscript{-/-}-mice). Figure 2 shows a schematic overview of the study strategy. After quality control and normalization, 20872 of the 45281 probes of the BeadChip array were used for analyses. Microarrays on human articular cartilage samples showed corresponding numbers for specific expression \cite{26}. In order to detect all articular cartilage genes that are responsive to the applied running regime, we performed differential expression analyses in 3 strata: total (A), Dio2\textsuperscript{-/-} (B) and wild type (C) group (Figure 2). For each strata significance was adjusted for multiple testing according to the “Benjamini and Hochberg” method. In the total and wild type strata, we independently detected respectively 1862 and 892 probes representing 1699 and 830 genes (558 overlapping) that were significantly differentially expressed between the forced exercise and the control group. In contrast, in the Dio2\textsuperscript{-/-}-stratum, this comparison did not result in any significantly differentially expressed probes after multiple testing. Among the differentially ex-
Figure 1. Histological scoring of osteoarthritis.
Comparing wild-type and Dio2−/− mice as well as the effect of forced running. (A) Frontal Haematoxylin-Safranin O stained sections of C57Bl/6 wild-type and Dio2−/− knees (medial) of mice subjected to a running regime (run) and control mice (no run) (magnification 10x). (B) Cartilage damage was increased in wild-type mice as compared to Dio2−/− mice (2-way ANOVA p = 0.0006) and (C) a similar trend was observed for synovitis (2-way ANOVA p=0.0536). None of the other comparisons (e.g. ‘WT no run’ versus ‘KO no run’) were found significantly different. Data are shown as individual values, mean and 95% confidence intervals.
Following microarray analysis, data were statistically analyzed. Secondary analyses using gene enrichment analysis and interaction analysis will lead to a set of genes which will be technically validated.
pressed probes in the total and wild-type groups, we observed respectively 102 and 97 probes with a fold-change of 1.5 and higher that together consisted of 158 significant unique probes, representing 147 unique transcripts that were responsive to the running regime (Figure 2 and Supplement Table S1). Notably, of the 158 differentially expressed probes, 31 were up-regulated (20%) and 127 showed down-regulation (80%). Among the 147 unique transcripts, we observed genes known for a potentially role in cartilage homeostasis and disease, such as Proteoglycan 4, also known as Lubricin (Prg4, 1.95-fold down; \( P=4.44 \times 10^{-2} \)) [27], myocyte enhancer factor 2C (Mef2c, 1.9-fold down; \( P=3.05 \times 10^{-2} \)) [28, 29] and connective tissue growth factor (Ctgf, also known as Ccn2, 1.79-fold down; \( P=1.32 \times 10^{-2} \)) [30].

Technical validation of our microarray results was carried out by RT-qPCR in the discovery cohort (20 samples previously included in the microarray analyses). Genes to be validated were selected based on P-value and fold difference (differential reaction on mechanical stress). Here, 19 out of the 20 genes tested showed similar effect sizes and direction as the original data, only Pfn1 did not show similar effect of expression (see Supplementary Table S2).

**Pathway analyses: Protein-protein interactions and gene enrichment analyses**

To identify functional connections between the genes that were found as differentially expressed (Table S1) we determined the protein-protein interaction prediction using STRING-db. Analyzing the 147 differentially expressed genes, representing 124 unique proteins, showed enrichment of interaction in the subset of proteins (\( P=1.61 \times 10^{-4} \); 63 interactions; Figure S1), indicating that the gene-products that were identified are closely interacting as a response to the forced running regime.

Furthermore, we assessed whether the differentially expressed genes occurred more frequently in a specific pathways in mice. Gene enrichment analyses revealed enrichment for biological processes (GOTERM_BP_FAT) concerning, amongst others, “regulation of metabolic process” \( (P=0.0063; \text{GO:} 0019222; N=46, \text{e.g. Calr, Igfbp5, Sox4 and Tctf4}), \) “skeletal system development” \( (P=0.00395; \text{GO:} 0001501; N=12, \text{e.g. Ctnnb1, Ctgf, Sox4 and Mef2c}), \) “anatomical structure development” \( (P=0.039; \text{GO:} 0048856; N=36, \text{e.g. Sox4, Notch3, Egfr, Ctnnb1 and Mef2c}), \) and “regulation of response to stress” \( (P=0.0171; \text{GO:} 0008134; N=14; Setd8, Ankrd1, Mef2c, Egfr and Ctgf) \) with application of a false discovery rate algorithm.

To connect thyroid hormone signaling with the effects of forced mechanical stress, we added genes involved in intracellular thyroid hormone signaling (Dio2, Thra, Thrb and Rxra) into the protein-protein interaction prediction. The network that is formed (Figure 3) shows thyroid signaling genes/proteins to be incorporated into the large network, as previously seen (Figure S1). Thyroid signaling was found to interact directly with the differential expressed genes through, Ctgf (A) and Egfr (B), via thyroid receptor alpha (Thra) and retinoid x receptor (Rxra), both known factors in the development of OA[30-32].

**Genes differentially expressed in Dio2^-/- mice compared to wild type mice upon a forced running regime**

Since we found differences in differential expression patterns between knockout and wild
type mice upon the forced running regime, we assessed which of the 147 differentially expressed genes (Figure 1 and Supplementary Table S1) showed significant interaction between running regime and genetic background based on nominal P-values. In total 29 probes, representing 29 genes were found be significantly different expressed in knockout-mice as compared to wild type-mice when undergoing a forced running regime (Table 1). The significant differences in effect (beta-values), as a result of the running regime, between the knockout- and the wildtype-group can be devided in 3 sub-groups. Each group shows a different effect-size based on “genotype” for the 29 genes found to show a significant interaction. We identified 4 genes that showed no differential expression upon running in wild-type mice, but are differentially expressed (down-regulated) upon running in Dio2\(^{-/-}\) mice (Group 1; e.g. Gnas and Rhbd12). In total 16 genes only showed differential expression in wild-type mice and not in the Dio2\(^{-/-}\) mice (Group 2; e.g. Hmgb2, Calr and Lbh) whereas, 9 genes showed significant differential effect-sizes between wild-type and knockout mice, but having the same direction of effect (Group 3; e.g. Sox4 and Socs2). Depending on the gene-expression pattern the genes in the different groups could mark a favorable effect in the Dio2-knockout mice (Group 1) or an unfavorable effect in wild type cartilage homeostasis (Group 2).

Discussion

Dio2-deficient mice showed less cartilage damage and reduced severity of synovitis in a treadmill running model of OA. The absence of significant differential gene expression between the running and no-exercise group in Dio2\(^{-/-}\) mice suggests that degenerative pathways are not activated in this knockout strain despite the biomechanical burden that is imposed. These data provide novel support for inhibition of DIO2 as a therapeutic strategy in OA, in particular since no striking developmental skeletal phenotype appears present in the Dio2\(^{-/-}\) mice (results not shown). Upon forced mechanical loading, wild type mice showed clear signs of OA and differential expression of genes associated with the disease, supporting the use of forced running as an OA-model in mice. A subset of the genes was found directly interacting with thyroid signaling through Thra, Rxra and Dio2, depicted in Figure 3. This indicates the importance of thyroid hormone signaling as a regulatory system in the response to stress and, when suppressed, for the maintenance of cartilage homeostasis.

With the differential expressed genes in the wild-type stratum and the combined knockout/wild-type stratum, pathway enrichment was found for expected biological processes, such as “skeletal system development”, but also for processes involved in “regulation of response to stress”. Notably, genes overlapping between these enriched processes as well as being ‘nodes’ in the recognized protein interaction networks (Egfr (B) and Ctnnb1(C); Figure 3) are well known for their association with OA [30, 33, 34]. These could, therefore, point at important modulators affecting the propensity to develop OA upon mechanical stress and as such at potential drugable targets for novel therapeutic approaches.
**Table 1**: Genes that show significant differential expression upon the running regime between wild-type- and knockout-mice (interaction)

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Run vs NoRun in Knockout</th>
<th>Run vs NoRun in Wildtype</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta¹</td>
<td>St. Error²</td>
<td>P-value³</td>
</tr>
<tr>
<td><strong>Group 1: Genes with an effect of the running regime in knockout- but not in wild-type-mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hist1h2an</td>
<td>-1.024</td>
<td>0.2603</td>
<td>0.000</td>
</tr>
<tr>
<td>Gnas</td>
<td>-0.956</td>
<td>0.2318</td>
<td>0.000</td>
</tr>
<tr>
<td>Rhbd1</td>
<td>-1.135</td>
<td>0.2176</td>
<td>0.000</td>
</tr>
<tr>
<td>E130112E08Rik</td>
<td>-1.041</td>
<td>0.2981</td>
<td>0.000</td>
</tr>
<tr>
<td><strong>Group 2: Genes with an effect of the running regime in wild-type- but not in knockout-mice</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anxa11</td>
<td>0.02</td>
<td>0.0852</td>
<td>0.818</td>
</tr>
<tr>
<td>Setd8</td>
<td>-0.006</td>
<td>0.0864</td>
<td>0.943</td>
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<td>Phdla1</td>
<td>-0.152</td>
<td>0.1019</td>
<td>0.136</td>
</tr>
<tr>
<td>Hmgb2</td>
<td>0.131</td>
<td>0.1439</td>
<td>0.363</td>
</tr>
<tr>
<td>Calr</td>
<td>-0.086</td>
<td>0.1561</td>
<td>0.581</td>
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<td>Gdi1</td>
<td>0.313</td>
<td>0.2297</td>
<td>0.173</td>
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<td>0.0682</td>
<td>0.057</td>
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<td>0.1028</td>
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<td>Igsf4a</td>
<td>-0.171</td>
<td>0.0978</td>
<td>0.081</td>
</tr>
<tr>
<td>Fcho1</td>
<td>0.132</td>
<td>0.1144</td>
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<td>Serpinb1a</td>
<td>0.396</td>
<td>0.3687</td>
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<td>Mtpn</td>
<td>-0.082</td>
<td>0.1084</td>
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<td>LOC433464</td>
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<td>Rel1</td>
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<td>0.1714</td>
<td>0.281</td>
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<td>Hist1h4i</td>
<td>0.113</td>
<td>0.2763</td>
<td>0.684</td>
</tr>
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<td><strong>Group 3: Genes with an effect of the running regime in both wild-type- and knockout-mice</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Mll5</td>
<td>-1.378</td>
<td>0.2306</td>
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</tr>
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<td>Chd1</td>
<td>-1.178</td>
<td>0.2311</td>
<td>0.000</td>
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<td>Septin11</td>
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<td>0.0887</td>
<td>0.002</td>
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<td>Sox4</td>
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<tr>
<td>Gns1</td>
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<td>0.011</td>
</tr>
<tr>
<td>Socs2</td>
<td>-0.337</td>
<td>0.1132</td>
<td>0.003</td>
</tr>
<tr>
<td>C330023M02Rik</td>
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<td>0.040</td>
</tr>
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<td>Hgsnat</td>
<td>-0.029</td>
<td>0.1274</td>
<td>0.023</td>
</tr>
<tr>
<td>Rap2c</td>
<td>-0.385</td>
<td>0.0743</td>
<td>0.000</td>
</tr>
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</table>

¹The beta-value is representing the difference in fold change between the running group and the non-running group. The direction of the beta-value is how the running-group is different from the non-running group. ²The standard error is showing the variation of the beta-value between samples. ³The nominal P-value is the P-value of that the single probe that was calculated to tell whether based on this single probe, the expression levels between runners is different of that of non-runners. ⁴The Interaction P-value is a summarizing value that tells whether the difference caused by exercise is significantly different between the knockout- and the wild-type-stratum
In contrast, when assessing gene expression in the knockout-group alone, we found no significant differential gene expression upon severe mechanical loading, after multiple testing adjustment. Taken together with the pathology observations these data indicate that the repression of Dio2 is beneficial against the development of cartilage damage upon mechanical stress. This effect could theoretically also be caused by a difference in power between the different strata (wild-type and knockout). However, since the comparison made in knockout animals (6 running versus 3 controls) is comparable to the wildtype animals (8 running versus 3 controls), this is unlikely to explain the complete absence of a significant differentially expressed gene in the knockout stratum.

Looking at the effect of Dio2-deficiency on gene expression, we found that the gene expression response upon exercise between wild-type and knockout-mice was significantly different for 29 genes with nominal significance, of the total set of 147 differentially expressed genes. Genes categorized in group 1 (Table 1), showing no differential expression upon forced running in wild-type mice, but are differentially expressed with nominal significance in Dio2\(^{-}\) mice (E130112E08Rik, Gnas, Hist1h2an and Rhbdl2) could represent the respective favorable effects. Gnas, for example, is involved in skeletal development (GO:0001501) and complex skeletal disorders such as Albright Hereditary Osteodystrophy that can lead to early OA [35]. Gnas upregulation was found directly regulating hypertrophic differentiation of growth plate cartilage in vivo [36]. The knockout-specific down-regulation of Gnas upon forced running could be a protective mechanism within the Dio2 knockout mouse.

We hypothesize that the sixteen genes within group 2, only showing differential expression in wild-type mice and not in the knockout-group (e.g. Hmgb2 and Calr), represent the unfavorable, damaging effects of forced running in wild type mice. Hmgb2 was shown to be expressed at higher levels in human MSCs as compared to human articular chondrocytes and the expression declined during chondrogenic differentiation of MSCs [37]. The up-regulation of Hmgb2 seen in wild type-mice upon forced running could be explained as a marker that changes in the differentiation status of the chondrocytes are occurring. A second interesting member of this group is calreticulin (Calr). Calr interacts with the glucocorticoid receptor (GR). It may also interact with other steroid receptors or thyroid receptors in a similar way [38]. Furthermore, Calr was shown to be involved in cartilage thinning of mandibular cartilage in a rat model that studied the effects of compressive mechanical loading [39]. In our forced running experiment we see a similar induction of Calr expression upon stress, but not in the Dio2 knockout-mice, possibly giving the knockout-mouse an advantage against the formation of OA-like degradation or cartilage thinning.

Nine additional genes showed significant differential effect-sizes between wild-type and knockout mice, albeit the same direction of effect (e.g. Sox4 and Socs2). Notable within this third group (Table 1) is Sox4, which was previously found to be expressed very early during chondrogenesis [40], much earlier than the well defined Sox5 and Sox6 [41]. Furthermore, it was shown that expression of Sox4 could be stimulated by adding physiological concentrations of human parathyroid hormone (PTH), indicating involvement of the PTH/PTHrP receptor [42]. Here we show that Sox4 expression is influenced by the ab-
The absence of Dio2. In the knockout-mice the expression of Sox4 is 2 times less down-regulated upon running when compared with the control group. How this influences the structural integrity of articular cartilage remains unknown. Other genes in the list have no straightforward connection to OA based on current literature.

In conclusion, in the current paper we find that Dio2 deficiency has a protective effect on the homeostasis of articular cartilage in the knee-joints of mice undergoing a forced running regime. This is consistent with our earlier findings, showing that pharmacological inhibition of deiodinases in a human in vitro chondrogenesis model has a beneficial effect on the early formation and maintenance of articular cartilage ECM [18]. It is therefore hypothesized that control of thyroid hormone signaling, both during development and adult cartilage maintenance, is essential to ensure normal bone and cartilage homeostasis, and that it could act as the master-switch that forces maturational arrested chondrocytes to re-activate the endochondral ossification process, leading to articular cartilage destruction. Our results show that interfering with intracellular thyroid hormone levels could be a powerful way to oppose the pathological events that are occurring in OA.

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Competing interest
The authors declare no competing financial, personal, or professional interests.

Author contributions
Conceived and designed the experiments: NB; FMFC; PES; IM; RJLL
Performed the experiments: NB; FMFC; LS; RvdB; NL;
Analyzed the data: NB; FMFC; YFMR; WdH; IM; RJLL
Wrote the manuscript: NB; FMFC; IM; RJLL
Critically reviewed the manuscript: All authors

Supplemental Data
http://ard.bmj.com/content/75/3/571/suppl/DC1
Reference List


