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Author: Bömer, N.
Title: Thyroid hormone signalling in Osteoarthritis: early life events in late life disease
Issue Date: 2017-01-17
Chapter 5

Aberrant Calreticulin expression in articular cartilage of Dio2 deficient mice

Nils Bomer1,*, Frederique M.F. Cornelis2, Yolande F.M. Ramos1, Wouter den Hollander1, Nico Lakenberg1, Ruud van der Breggen1, Lies Storms1, P. Eline Slagboom1, Rik J.U. Lories2,3, Ingrid Meulenbelt1

1Dept. of Molecular Epidemiology, LUMC, Leiden, Netherlands; 2Laboratory of Tissue Homeostasis and Disease, Skeletal Biology and Engineering Research Centre, KU Leuven, Leuven, Belgium; 3Division of Rheumatology, University Hospitals Leuven, Leuven, Belgium

Edited version of: PLoS ONE 11(5):e0154999
Abstract

**Objective:** To identify intrinsic differences in cartilage gene expression profiles between wild-type- and Dio2<sup>−/−</sup>-mice, as a mechanism to investigate factors that contribute to prolonged healthy tissue homeostasis.

**Methods:** Previously generated microarray-data (Illumina MouseWG-6 v2) of knee cartilage of wild-type and Dio2<sup>−/−</sup>-mice were re-analyzed to identify differential expressed genes independent of mechanical loading conditions by forced treadmill-running. RT-qPCR and western blot analyses of overexpression and knockdown of Calr in mouse chondro-progenitor cells (ATDC5) were applied to assess the direct effect of differential Calr expression on cartilage deposition.

**Results:** Differential expression analyses of articular cartilage of Dio2<sup>+/+</sup> (N = 9) and wild-type-mice (N = 11) while applying a cutoff threshold (P < 0.05 (FDR) and FC > |1.5|) resulted in 1 probe located in Calreticulin (Calr) that was found significantly downregulated in Dio2<sup>−/−</sup> mice (FC = -1.731; P = 0.044). Furthermore, overexpression of Calr during early chondrogenesis in ATDC5 cells leads to decreased proteoglycan deposition and corresponding lower Aggrecan expression, whereas knocking down Calr expression does not lead to histological differences of matrix composition.

**Conclusion:** We here demonstrate that the beneficial homeostatic state of articular cartilage in Dio2<sup>−/−</sup> mice is accompanied with significant lower expression of Calr. Functional analyses further showed that upregulation of Calr expression could act as an initiator of cartilage destruction. The consistent association between Calr and Dio2 expression suggests that enhanced expression of these genes facilitate detrimental effects on cartilage integrity.
Introduction

Osteoarthritis (OA) is a prevalent, complex, chronic and disabling disease among elderly and is characterized by progressive destruction of joint cartilage, remodeling of the subchondral bone, formation of osteophytes and synovitis [1,2]. The aetiology of OA is not entirely understood, yet multiple factors such as joint injury, obesity and mechanical stress have been found to significantly contribute to the onset and progression of the disease. Moreover, OA has a considerable genetic component and a variety of genetic studies have identified multiple genes that robustly confer risk to OA [3,4]. A notable OA risk gene is the deiodinase iodothyronine type-2 (D2) gene (DIO2) [5] that has multiple lines of substantiating functional follow up data indicating that risk allele carriers have a more vivid upregulation of DIO2 expression in articular cartilage. In vitro cell studies as well as a cartilage-specific transgenic rat study indicated that this resulted in cartilage destruction. In this in vitro model, inhibiting DIO2 action, resulted in prolonged cartilage homeostasis, as where Dio2-/--mice were shown to be protected against treadmill-running induced OA [6]. Moreover, genome wide expression analysis of articular cartilage of Dio2-/- and wild-type mice, before and after forced treadmill-running, demonstrated that particularly Calr, Hmgb2, Sox4 and Socs2 were differentially regulated in the Dio2-/- mice upon the mechanical applied stresses [6]. As Dio2-/- mice had less cartilage damage these genes likely contribute to enhanced adaptive capacity to environmental challenges such as mechanical overloading. In parallel to these finding, there is need for insight into intrinsic molecular pathways that assure articular cartilage tissue homeostasis independent of environmental challenges. In the current study, we therefore re-analysed our micro-array expression data of wild type and Dio2-/- mice independent of mechanical loading by treadmill-running and performed functional studies to validate results.

Materials and methods

Dio2-/- treadmill-running animal model and microarray assay

Animal experiments

As described previously [6], Dio2-/--mice were a kind gift of Dr. V. Galton (Dartmouth Medical School, NH, USA) and were backcrossed onto the C57Bl/6 background. In the experiments reported here, mice were in the 6th generation of backcrossing. Wild-type C57Bl6 mice were purchased from Janvier (Le Genest St Isle, France). All experiments were approved by the Ethics Committee for Animal Research (KU Leuven, Belgium).

Four to 6-months old male Dio2-/- and wild-type-mice were subjected to a forced treadmill-running regimen on a four lane modular treadmill (Columbus Instruments, Columbus, OH, USA). These mice ran for 3 weeks 1 hour/day, 5 days/week, at a speed of 11 m/min and with an inclination of 5°. All animals, including mice that were used as non-running controls, were littermates and were caged together and housed in the same facility [6]. Mice were group housed in standard mouse cages with sawdust as bedding material.
and under conventional laboratory conditions; constant room temperature (22 ± 2 °C), humidity level (55 ± 5 %), a 12-h light:12-h dark cycle (lights on at 8 AM) and standard food (Sniff, Soest, Germany) and water available ad libitum. Mice were euthanized by decapitation under sedation.

**RNA isolation of murine knee cartilage**

For gene expression studies, cartilage was micro-dissected, snap frozen in liquid nitrogen, and stored at -80° Celsius upon isolation. Cartilage of left-side knee-joints of Dio2<sup>-/-</sup> (n=6 NoRun and n=12 Run) and wild-type-mice (n=6 NoRun and n=16 Run) was used for RNA isolation. To gain isolation efficiency and thereby mRNA quality, articular cartilage of knee-joints of two mice from the same sub-group were pooled resulting in n=9 Dio2<sup>-/-</sup> and n=11 wild-type samples, analyzed in the manuscript [6].

**Microarray analysis**

Sample homogenization, RNA isolations, complementary DNA synthesis, amplification, biotin labeling and hybridization onto the Illumina MouseWG-6 v2 BeadChip microarrays (Illumina, Eindhoven, The Netherlands) were performed as described [6]. Samples were dispersed for experimental condition over the arrays of 6 chips from a single batch, and each chip contained a replicate sample that was also present on a second chip, to exclude batch effects across chips. Slide scanning, basic quality control and data normalization were performed as described [6]. Replicates measured over the different chips were taken into account when performing normalization. For further analysis, the replicate with the lowest number of calls was discarded from the dataset. Raw probe-level data were exported for analyses using the Limma R-package [7]. We checked the data for large-scale batch effects between chips using principal component analysis. Probes with P-value ≤0.05 after FDR correction for multiple testing were considered significant.

**Gain and loss of function of **Calr** in ATDC5, chondrogenic induction and histological evaluation**

The murine chondrogenic cell line ATDC5 (kindly provided by H.C.M. Sips, LUMC) was maintained in DMEM/Ham’s F-12 (1:1 Gibco) supplemented with 5% FBS and antibiotics at 37°C in a humidified 10% CO2 / 90% atmosphere. To induce chondrogenesis, ATDC5 cells were plated in the medium described above supplemented with ITS+ (6.25 μg/ml insulin, 6.25 μg/ml transferrine, 6.25 ng/ml sodium selenite, 5.33 linoleic acid, and 1.25 μg/ml bovine serum albumine; Becton&Dickinson, Breda, The Netherlands). The medium was replaced every other day. The total cell culture time was 4 days.

For *Calr* overexpression purposes, a plasmid vector containing untagged mouse *Calr* (pCMV3-*mCalr*) was purchased (Sino Biological Inc., Beijing, P.R. China). As a control for the *Calr* overexpression experiments, ATDC5 cells were transfected with an empty pCMV3 vector. For knockdown of *Calr* expression, 3 unique 27mer siRNA duplexes, specific for *Calr*, were purchased (OriGene technologies, Rockville, MD, USA). Together with the 27mer siRNA duplexes a universal scrambled negative control siRNA duplex (OriGene technologies) was purchased to be used as a control for the knockdown experiments.
The day before transfection, ATDC5 cells were passaged 1:6 from a confluent 6-well plate into a new 12-wells plate. Fugene® 6 transfection reagent was used to transfect ATDC5 cells with either the mCalr-vector or control vector, or the mixed siRNA duplexes or control duplex, according to the manufacturers protocol. One day after transfection, regular proliferation medium was changed for chondrogenesis medium. After 4 days of chondrogenesis, the cells were harvested for RNA and histology.

ATDC5 cells were fixed in 4% formaldehyde and stained for acidic polysaccharides (glycosaminoglycans (GAGs)) with Alcian Blue (8-GX pH=1; Sigma-Aldrich). To measure the staining intensity, the Alcian Blue staining was washed with 6M guanidine hydrochloride (Sigma-Aldrich) to decolorize the extracellular matrix. Intensity of the supernatant was measured using a photospectrometer at 620 nm wavelength. Experiments were performed with biological triplo’s.

**DIO2 overexpressing hBMSCs, 3D chondrogenic induction and mRNA evaluation**

Previously, we performed in vitro 3D chondrogenesis using lentiviral DIO2 transduced hBMSCs [8]. Chondrogenesis was initiated in 0.5 ml serum-free chondrogenic differentiation medium (DMEM, supplemented with Ascorbic acid (50 µg/ml; Sigma-Aldrich; Zwijndrecht, The Netherlands), L-Proline (40 µg/ml; Sigma-Aldrich), Sodium Puryvate (100 µg/ml; Sigma-Aldrich), Dexamethasone (0.1 µM; Sigma-Aldrich), ITS+, antibiotics, and TGF-β1 (10 ng/ml; PeproTech)). Lentiviral constructs containing C-terminal FLAG-tagged cys-D2 (kindly provided by Prof. Dr. Bianco[9]) were constructed and transduced into hBMSCs as described before [8]. Here we used 3D pellets that were snap frozen in liquid nitrogen and stored at -80 degrees Celsius.

**RNA isolation**

RNA from the ATDC5 cells was isolated by lysing the cells in 500 µl of TRIzol® reagent (Life Technologies, Bleiswijk, The Netherlands), and 200 µl of chloroform was added before centrifugation (15 min at 14.000g). After addition of 1 v/v 70% ethanol/DEPC-treated water to the aqueous upper layer total RNA was isolated using Qiagen RNeasy mini columns following the manufacturer’s protocol. RNA isolation from the hBMSC pellets was performed by pooling two pellets for every given condition, respectively. The isolation of RNA was performed as described previously[10]. RNA quantity was assessed using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA).

**Quantitative RT-PCR assay (validation)**

Isolation of RNA was performed as described previously[6]. Approximately, 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). Relative gene expressions of the Roche Lightcycler 480 II data were calculated by using the $2^{\Delta\Delta C_T}$ method[11]. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a single refer-
ence gene for qPCR\cite{12,13}. Primer efficiencies were verified by performing a concentra-
tion curve experiment (primer sequences used are listed in S1 Table). The student T-test
was used to calculate the significance. All P-values < 0.05 were considered statistically
significant.

Results

After quality control and normalization of micro array expression data of articular cartilage
of N=9 Dio2\(^{-/-}\) and N=11 wild-type mice, 18226 of the 45281 probes were found to have a
detectable expression level, representing 12312 unique genes (~49\% of the mouse tran-
scriptome) and 2707 RIKEN sequences (S2 Table). Differential expression analyses while
applying a cutoff threshold (P < 0.05 (FDR) and FC > |1.5|\cite{6}) resulted in only 1 probe lo-
cated in Calreticulin (Calr) that was found significantly downregulated in Dio2\(^{-/-}\) mice (FC =
-1.731; P = 0.044). Technical validation by RT-qPCR in N=20 samples (N=9 Dio2\(^{-/-}\) and N=11
wild type mice) of the discovery cohort confirmed the expression difference (S1 Figure).
Notably, our previous data showed that forced treadmill-running had a profound effect on
Calr expression (FC = -1.58, P = 0.0418\cite{6}) in cartilage of wild-type-mice. However, strat-
fying expression of Calr by both mice type (Dio2\(^{-/-}\), Wildtype) and exposure (Run, Norun)
shows that the here identified Calr-effect appears to be independent of the mechanical
challenge (Figure 1).

Next, we investigated the effect of altered Calr expression on the chondrogenic capacity
of ATDC5 cells. As measured by photospectrometry, overexpressing Calr during early 2D
chondrogenesis resulted in significant lower Alcian Blue staining, indicating less glycos-
aminoglycan deposition (Figure 2). Concurrently, RT-qPCR confirmed significant lower
mRNA expression levels of Acan (FC = -2.32) (Figure 3). Of note was the 1.8-fold upregu-
lation of Dio2 in the cells overexpressing Calr, albeit that this effect by definition was not
significant (P = 0.08). On the other hand, knockdown of Calr, by siRNA duplexes in this
model, did not show an effect on the amount of deposited glycosaminoglycans stained by
Alcian Blue (Figure 2) nor on the mRNA expression levels of Acan (Figure 3). However, a
significant 4.7-fold downregulated effect was observed for Dio2 mRNA expression levels
(P = 0.017).

Finally, to confirm the transcriptional link between of DIO2 and CALR expression, we ex-
amined CALR expression in cartilage constructs previously generated from human BM-
SCs in vitro 3D chondrogenesis and compared them to those generated while applying
overexpression of DIO2 by lentiviral transduction\cite{8}. As shown in Figure 4, also in this
human BMSCs in vitro 3D chondrogenesis model overexpressing DIO2 coincided with sig-
nificant higher CALR expression.
Figure 1. Calreticulin expression stratified for treadmill-running and knockout.
Representation of the Calr expression values of all individual samples plotted by genotype-group (Wild-type and Dio2⁻/⁻) and treadmill-running-group (No Run and Run). Showing the significant reduction of Calr expression upon forced treadmill-running in wild-type-mice ($P = 0.005$) and the absence of change in the knockout-mice. P-values depicted are derived from Student T-Test.

Figure 2. Overexpressing Calr resulted in significantly lower Alcian Blue (AB) staining intensities.
Staining intensities of Alcian Blue staining measured with a photospectrometer (620 nm). Values are displayed as the average±SEM, relative to the control sample. Differences were analyzed with Student T-Test ((*) $P < 0.05$).
**Figure 3.** Expression analyses in Calr overexpressing ATDC5 cells.
RT-qPCR expression of Calr, Dio2 and Acan in ATDC5 cells transfected with either a Calr-containing vector for overexpression (Calr+) or 3 unique 27mer siRNA duplexes, specific for Calr, for knockdown (Calr-). Values of the RT-qPCR are displayed as the average±SEM, normalized for GAPDH expression and relative to the control samples (dashed line). Differences were analyzed with Student T-Test (**) P < 0.05).

**Figure 4.** Calreticulin expression in DIO2 overexpressing hBMSCs.
qPCR expression of Calr in hBMSCs transduced with either a control virus-vector (eGFP) or a DIO2-eGFP vector. Values of the RT-qPCR are displayed as the average±SEM, normalized for GAPDH expression and relative to the control sample at every timepoint. Differences were analyzed with Student T-Test (**) P < 0.05).
Discussion

In parallel to disease modifiers, there is a need for insight into intrinsic factors that enhance stability of healthy articular chondrocytes. We show that cartilage of the Dio2−/− as compared to wild type mice has an intrinsic difference in Calr expression. Since Dio2−/− mice were previously found to be less susceptible to mechanically induced damage, our data indicate that mitigated Calr and/or Dio2 expression, mark a beneficial homeostatic state of articular cartilage. Despite the, here outlined, consistent association between Calr and Dio2 expression, no thyroid responsive elements were identified near Calr[14] nor did Calr show any predicted interactivity with thyroid hormone signaling based on String-DB [15].

Calr is a multifunctional protein that was shown to have many putative roles. Some examples are: Ca2+-binding, storage and stress induced release[16], quality control molecular chaperone binding to misfolded proteins and preventing them from being exported from the endoplasmic reticulum to the Golgi apparatus, and modulator of steroid-sensitive gene expression[17–21]. For that matter, the amino terminus of Calr interacts with the DNA-binding domain of nuclear hormone receptors (the glucocorticoid receptor[17], androgen receptor[18], and retinoic acid receptor[22]) and prevents the receptors from binding to their specific response elements, influencing transcriptional activities in vitro and in vivo. Furthermore, Calr expression was shown to be associated with cartilage thinning of mandibular cartilage in a rat model that studied the effects of compressive mechanical loading[16], and has been implicated in the pathogenesis of rheumatoid arthritis (RA)[23]. Similarly in this respect, we showed that overexpression of Calr during early chondrogenesis in ATDC5 cells leads to decreased proteoglycan deposition and corresponding lower Aggrecan expression, whereas knocking down Calr expression does not lead to histological differences of matrix composition. It was shown before that increasing levels of Calr, sensitized the cells to apoptosis during mechanical-induced endoplasmatic reticulum-stress, while decreasing Calr levels protected cells from apoptosis[24]. It was hypothesised that by regulating the amount of calcium stored in the ER, and therefore the amount that can be released to the cytosol to trigger downstream events, Calr can affect apoptotic outcomes[25]. Given our data, we hypothesize that lower levels of Calr expression, as seen in the Dio2−/−-mice, could results 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 is supported by a recent study which reported that calcium antagonists might be efficient in preventing progression of OA[26].

Together, we here demonstrate that the beneficial homeostatic state of articular cartilage in Dio2−/− mice is accompanied with significant lower expression of Calr. Moreover, we showed that increased Calr expression could directly provoke cartilage destruction. Given their consistent association, we conclude that enhanced interactive expression of Calr and Dio2 contributes to detrimental effects on cartilage integrity. The precise molecular mechanism of Calr in the maintenance of cartilage homeostasis and its interplay with Dio2 signalling is, however, little explored and more research is therefore needed.
Data Availability
The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE79239 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79239).

Acknowledgements
The authors acknowledge support by Treat~OA and IDEAL, which are funded by the European Union’s Seventh Framework Program (FP7/2007-2011) under respective grant agreement nos. 200800 and 259679. Furthermore, this work was supported by a grant from FWO Vlaanderen (Flanders Research Foundation) G.0828.11N and by the Dutch Arthritis Association. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing interests
The authors have declared that no competing interests exist.

Author contributions
Conceived and designed the experiments: NB FMFC PES RJUL IM. Performed the experiments: NB FMFC LS RvdB NL. Analyzed the data: NB FMFC YFMR WdH RJUL IM. Contributed reagents/materials/analysis tools: NB FMFC YFMR WdH RJUL IM. Wrote the paper: NB FMFC YFMR WdH NL RvdB LS PES RJUL IM.

Supplemental Data
http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0154999#sec017
Reference List


