Cartilage endoplasmic reticulum stress may influence the onset but not the progression of experimental osteoarthritis

Louise H. W. Kung, Lorna Mullan, Jamie Soul, Ping Wang, Kazutoshi Mori, John F. Bateman, Michael D. Briggs and Raymond P. Boot-Handford

Abstract

Background: Osteoarthritis has been associated with a plethora of pathological factors and one which has recently emerged is chondrocyte endoplasmic reticulum (ER) stress. ER stress is sensed by key ER-resident stress sensors, one of which is activating transcription factor 6 (ATF6). The purpose of this study is to determine whether increased ER stress plays a role in OA.

Methods: OA was induced in male wild-type (+/+) and Atf6α−/− mice by destabilisation of the medial meniscus (DMM). Atf6α−/− mice have increased ER stress in chondrocytes via the collagen II promoter-driven expression of ER stress-inducing Tgα. Knee joints were scored histologically for OA severity. RNA-seq was performed on laser-micro-dissected RNA from cartilage of +/+ and c/c DMM-operated mice.

Results: In situ hybridisation demonstrated a correlation between the upregulation of ER stress marker, BiP, and early signs of proteoglycan loss and cartilage damage in DMM-operated +/+ mice. Histological analysis revealed a significant reduction in OA severity in c/c mice compared with +/+ at 2 weeks post-DMM. This chondroprotective effect in c/c mice was associated with a higher ambient level of BiP protein prior to DMM and a delay in chondrocyte apoptosis. RNA-seq analysis suggested Xbp1-regulated networks to be significantly enriched in c/c mice at 2 weeks post-DMM. Compromising the ER through genetically ablating Atf6α, a key ER stress sensor, had no effect on DMM-induced OA severity.

Conclusion: Our studies indicate that an increased capacity to effectively manage increases in ER stress in articular cartilage due either to pre-conditioning as a result of prior exposure to ER stress or to genetic pre-disposition may be beneficial in delaying the onset of OA, but once established, ER stress plays no significant role in disease progression.

Keywords: Endoplasmic reticulum (ER) stress, Osteoarthritis, Mouse, Medial meniscus destabilisation (DMM), ATF6α, Apoptosis, Histology, RNA-seq
The UPR is mediated by three ER-resident transmembrane stress sensors: pancreatic ER kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6), and results in attenuation of protein translation and upregulation of chaperones (including BiP/Grp78) and genes involved in ER-associated degradation. The transcriptional upregulation of BiP has been used extensively as a marker of ER stress [11, 12]. The initial responses to ER stress are aimed at alleviating the stress although should these responses fail to restore protein homeostasis, CHOP-mediated apoptosis may be triggered [13].

Chondrocytes, due to their high secretory load, are particularly sensitive to ER stress [10]. Indeed, elevated chondrocyte ER stress is not only associated with a number of chondrodysplasias but represents the principal disease mechanism in at least one of these forms of dwarfism [10, 14–16]. Over recent years, several studies have associated ER stress with OA in both man and mouse [17–22]. In all cases, an upregulation of the ER chaperone BiP was reported in osteoarthritic tissue. However, it remains unclear whether increased ER stress is merely a physiological response, for instance to the anabolic, increased ECM synthesis seen in OA, or it can influence the onset and progression of disease.

Our present study was therefore to determine whether exposure to increased ER stress can modulate OA onset or progression. We previously generated a transgenic mouse model (ColIIITgcog; [23]) in which ER stress was induced in chondrocytes by the Col2a1 promoter-driven expression of a misfolding protein, the cog form of thyroglobulin (Tgcog), in a similar fashion to that described previously [15]. Briefly, Tgcog misfolds in the ER inducing ER stress and an UPR [14, 23, 24]. We also investigated a possible connection between disturbing the cells ability to sense ER stress and disease progression through ablating one of the key ER stress sensors ATF6α. Atf6α−/− mice have no overt phenotype; however, their ability to respond to ER stress challenges, including through the induction of BiP, is severely compromised [25]. We induced OA by destabilisation of the medial meniscus (DMM) in ColIIITgcog and Atf6α-knockout mice [25] to examine the effects of either prior exposure to increased ER stress or a compromised UPR on disease onset and progression, respectively. Our results show increased ER stress is an early response in surgically induced OA. The ColIIITgcog mice, whose chondrocytes have been pre-conditioned to increased ER stress throughout development [23], are protected against the earliest stages of OA. Furthermore, Atf6α-knockout mice, which have a diminished ability to ameliorate ER stress, showed no significant differences in OA severity compared with control mice, suggesting ER stress is not a significant factor in disease progression.

Methods

Mouse strains

Generation of the ColIIITgcog mouse has been previously described [23]. Mice were bred to homozygosity, and breedings of wild-type (+/+) and homozygote (c/c) mice were maintained on a FVB/N/C57Bl6 background. Atf6α−/− knockout mice were generated as described previously [25].

Surgical induction of osteoarthritis

OA was induced in 10–12-week-old male mice by DMM of the right knee as described [26]. Briefly, under isoflurane anaesthesia and following standard surgical site preparation, the medial menisco-tibial ligament was exposed by gentle blunt dissection and transected with a 5-mm micro-surgical blade using sterile surgical techniques. Joints were flushed with sterile saline prior to separate closure of the joint capsule, subcutaneous tissue (8/0 polyglactin 910 suture) and skin (cyanoacrylate). The left knee was the non-operated control. Operated mice received post-operative pain relief (buprenorphine) for 48 h. For RNA-seq analysis, sham operations where the medial menisco-tibial ligament was visualised but not transected were performed in independent animals. Mice were randomly allocated to groups/time points prior to study commencement using their individual ID numbers.

Histology

Animals were sacrificed at 2 (n = 6/group), 4 (n = 13/group) and 8 (n = 14/group) weeks post-surgery. Knee joints were dissected, fixed in parafomaldehyde overnight, demineralised using 0.8 M EDTA pH 7.4 for 1–2 weeks, paraffin-embedded, sectioned coronally at 5 μm thickness, stained with safranin O and fast-green and photographed as previously described [23]. OA severity was determined using the Osteoarthritis Research Society International (OARSI) scoring system [27]. At least five sections across the whole joint, spaced ~80 μm apart, were scored by three separate scorers in a blind fashion. The joint score was calculated by combining the individual scores from all four quadrants of the joint. Statistical differences between groups were evaluated using ANOVA (GraphPad Prism).

Immunohistochemistry (IHC)

IHC was performed as described [23]. Briefly, antigen unmasking was carried out in citrate buffer pH 6.0 heated to > 85 °C for 10 min followed by washes in PBS. Primary antibody used was anti-Grp 78 goat polyclonal (Santa Cruz, SC-1051) diluted 1/300. Sections were then incubated with ABC reagent (Vector Laboratories, PK-
6100) for 30 min and developed using the Vector VIP kit (Vector Laboratories, SK-4600). Negative control sections for IHC were performed with the appropriate serum minus the primary antibody. Tissue sections that were known to express the protein of interest were included as positive controls. Images were captured using the Carl Zeiss Axiovision microscope fitted with an Axiocam colour CCD camera and associated Axiovision software.

**In situ hybridisation (ISH)**
DIG-labelled colourimetric in situ was performed as described [15] with the following riboprobes: Col2a1: 600 bp insert encoding the 3’ UTR from L.M.A.G.E clone ID 735113; BiP: 350 bp fragment from L.M.A.G.E clone ID6334883; thyroglobulin: 700 bp cDNA fragment amplified using primers F:TTGTGAGATCCATCCCATCAAGC and R:GTGACTACGATGAAGTTGC.

**Terminal transferase dUTP nick end labelling (TUNEL) assay**
TUNEL assays were performed using a fluorometric TUNEL kit (Promega, G3250) following the manufacturer’s instructions. Images were collected using the CoolSNAP ES Olympus BX51 camera and associated Metaview software. Two slides containing sections spaced at least 80 μm apart, per animal, were assayed. Separate cell counts throughout the entire lateral and medial tibial plateau were assessed as described previously [23] and analysed by ANOVA (GraphPad Prism).

**Laser capture microdissection of articular cartilage, RNA preparation and RNA sequencing**
Dissection, laser microdissection and RNA extraction from mouse articular cartilage were performed as previously described [28, 29]. Each mouse articular cartilage yielded ~ 20–30 ng of total RNA. +/- SHAM, +/- DMM and c/c DMM mice at 2 weeks post-surgery (n = 3/group) were used in this experiment. The integrity of total RNA was assessed on the Agilent Technology Bioanalyzer 2100 using RNA6000 Pico-Chip (Agilent) and quantified using a QuBit assay (Life Technologies). RNA sequencing cDNA libraries were generated using the NuGen Ovation® Single Cell RNA-Seq system (NuGEN Technologies Inc., Part No.0342) according to the manufacturer’s instructions. Adapter indices were used to multiplex libraries, which were pooled prior to cluster generation using a cBot instrument. The loaded flow-cell was then paired-end sequenced (100 + 100 cycles, plus indices) on an Illumina HiSeq2500 instrument. Demultiplexing of the output data (allowing one mismatch) and BCL-to-Fastq conversion was performed with CASAVA1.8.3. The read counts for each sample (at least 10 M) were normalised to the total number of reads and analysed using DESeq2 at a 10% FDR (adjusted for multiple testing using the Benjamini-Hochberg correction: $p_{adj}$ value reported by DESeq). Differentially expressed genes were used in pathway and functional analyses (Ingenuity Systems).

**Quantitative polymerase chain reaction (qPCR)**
Due to the limited amounts of tissue RNA available, cDNA libraries from the same pools used for RNA sequencing analysis were used for qPCR. Reactions were performed in duplicate using the SYBR Green kit (Applied Biosystems) following the manufacturer’s instructions on a StepOne Plus detector system (Life Technologies). Primer sequences (Additional file 1: Table S1) used had been previously validated [29] or were designed for this study using Primer-Blast. Relative gene expression levels were normalised to β-actin and calculated using the $2^{-ΔΔCt}$ method. To calculate fold changes of gene expression, the $2^{-ΔCt}$ method was used.

**Results**
**DMM increases ER stress in cartilage as OA damage becomes apparent**
Lateral compartments of DMM joints and non-operated control joints from +/- mice showed no significant OA-related changes (Fig. 1). DMM-operated +/- mice developed OA progressively over the 8-week period with cartilage damage specifically localised to the medial compartments of the joint (Fig. 1a, b). Two weeks post-DMM, +/- mice exhibited focal loss of proteoglycans and mild fibrillation at the articular surface. By 4 weeks post-DMM, there was cartilage structural damage and vertical cleft formation. At 8 weeks, DMM joints exhibited a significant degree of cartilage degradation. OARSI scoring revealed a significant increase in the severity of OA with time (Fig. 1b).

mRNA expression of Col2a1 and the ER stress marker BiP in articular cartilage of +/- non-operated knee joints was below the level of detection by ISH (Fig. 1a). In comparison, the +/- DMM-operated knees exhibited increases in Col2a1 and BiP expression that were evident at 2 weeks and most intense at 4 weeks post-DMM. The spatial localisation of increased expression of both BiP and Col2a1 expanded in tandem with the progression of cartilage damage, especially evident between 2 and 4 weeks post-DMM. The most intense upregulation of expression localised to chondrocytes immediately adjacent to the OA lesion, particularly apparent at 4 weeks post-DMM (Fig. 1a). The upregulation of BiP mRNA indicates that increased ER stress is an early feature of disease onset in this mechanically
induced OA and is coincidental with first signs of proteoglycan loss and structural damage.

ColIITgcog mice are protected against the initial stages of surgically induced OA

To determine whether prior exposure to increased ER stress can influence the onset or progression of OA, we conducted DMM surgery on ColIITgcog (c/c) mice which experience chondrocyte ER stress when the mutant thyroglobulin transgene, driven by the Col2a1 promoter, is expressed [23]. We hypothesised that following DMM surgery, the added increased ER stress experienced by chondrocytes as they upregulated collagen II (and therefore Tg cog) synthesis would increase either the rate of onset or the severity of disease in c/c mice.

We first established that the expression of the ColIITgcog transgene per se during development and early adulthood did not predispose the animals to OA or other articular cartilage abnormalities (Additional file 2: Figure S1A&B). The articular cartilage of ColIITgcog mice at 3–9 weeks and 18 months of age were normal with no signs of degeneration and were indistinguishable from
+/+ mice (Additional file 2: Figure S1A&B). Next, DMM surgery was conducted on c/c mice as part of the same experiment that generated the +/+ (control) data described above (Fig. 1). It should be noted that in the cartilage of non-operated knees in c/c mice, the expression of Col2a1, Tgcog and BiP was below the level of detection (Fig. 2a). The lack of detectable expression demonstrates that in an unchallenged adult knee joint Col2a1 expression (and thus Tgcog and BiP) is very low in contrast to the high levels seen in younger animals. Increased ER stress indicated by the upregulation of BiP mRNA accompanying Tgcog and Col2a1 expression was apparent in chondrocytes following DMM surgery in c/c mice compared to non-operated c/c controls (Fig. 2a). Furthermore, DMM-operated c/c mice developed signs of osteoarthritic damage (Fig. 2a) in a similar fashion to the controls (+/+) described above (Fig. 1). However, direct comparison of the level and type of damage revealed that

![Image](image_url)

**Fig. 2** ColIITgcog mice are protected against the initial stages of surgically induced OA. a Safranin O staining and in situ hybridisation (ISH) for Col2a1, Tgcog and BiP mRNA on sections from non-operated and DMM-operated knee joints from c/c mice at 2, 4 and 8 weeks post-DMM. The presence of each transcript is indicated by the blue staining and depicted by the arrows. c/c DMM-operated knee joints exhibited an upregulation in all three transcripts, Col2a1, Tgcog and BiP in chondrocytes around the OA-affected region compared to c/c non-operated joints. Histological scores of the SO-stained images using a semi-quantitative scoring system are shown. c/c mice exhibited a significant increase in the severity of OA with time. **p < 0.01, ***p < 0.001, ****p < 0.0001. b Safranin O staining of sections from non-operated and DMM-operated knee joints from +/+ and c/c mice at 2 weeks post-DMM. The insert shows an expanded view of the highlighted black box. Histological scores of the safranin O-stained images using a semi-quantitative scoring system are shown. c/c mice show significantly reduced OA severity compared with +/+ mice. Data shown as average score ± SEM. ****p < 0.0001 +/+ 2 week post-DMM versus c/c 2 week post-DMM. LFC = lateral femoral condyle; LTP = lateral tibial plateau; MFC = medial femoral condyle; MTP = medial tibial plateau. Scale bar = 100 μm.
At 2 weeks post-DMM, c/c mice had significantly less damage than the +/+ controls (Fig. 2b). The loss of proteoglycan and cartilage damage in the medial tibial plateau was significantly less in c/c mouse joints (Fig. 2b). However, at later time points there were no differences in the levels of damage seen in the two groups (cf historical scores for 4 and 8 weeks +/+ (Fig. 1) and c/c mice (Fig. 2)).

**Apoptosis is delayed in DMM-operated ColIITg<sup>cre</sup> mice compared with +/+ mice**

To investigate the mechanism by which the ColIITg<sup>cre</sup> transgene appeared to have a chondroprotective role during the onset of OA (2 weeks DMM), we examined the levels of apoptosis in the cartilage of +/+ and c/c mice following DMM (Fig. 3a). The lateral compartments of both +/+ and c/c mice 2 weeks post-DMM exhibited few apoptotic cells (Fig. 3b). In comparison, significant numbers of apoptotic cells were detected in the medial compartments of DMM-operated joints (Fig. 3a) although there was no significant difference in the proportion of apoptotic cells in +/+ and c/c cartilage (Fig. 3b). Nevertheless, close inspection of the OA lesion revealed a subtle but clear delay in apoptosis in 2-week DMM-operated joints of c/c compared to +/+ mice. Chondrocytes within the OA lesion of +/+ mice did not stain with DAPI (blue), nor were they labelled as apoptotic (green), indicating that these cells were already dead and their genomic DNA degraded (Fig. 3a). It was the chondrocytes immediately adjacent to the OA lesion that were TUNEL-positive in the +/+ medial compartment (Fig. 3a—see arrows). However, in c/c mice, the cells within the lesion were TUNEL-positive, indicating that apoptosis in these cells was still in progress and therefore delayed compared to the equivalent cells in +/+ controls (Fig. 3a).

**ColIITg<sup>cre</sup> mice articular chondrocytes exhibit a higher level of BiP protein prior to DMM surgery**

As described above for both +/+ and c/c mice, BiP mRNA levels in chondrocytes of non-operated joints were below the level of detection by in situ analyses and only became detectable in OA-affected chondrocytes 2 weeks post-DMM. We next looked to see whether BiP protein levels in the cartilage prior to DMM induction were equivalent in +/+ and c/c mice (Fig. 3c). Surprisingly, immunodetectable BiP was much more intense in chondrocytes of c/c mice compared to +/+ mice even though in both lines, mRNA levels for BiP were very low (Figs. 1 and 2). This increase in BiP protein was still apparent in the c/c mouse cartilage 2 weeks after DMM (Additional file 2: Figure S2). These data indicate that chondrocytes in the ColIITg<sup>cre</sup> line have a higher ambient concentration of BiP protein, presumably due to the prior exposure to increased levels of ER stress during development [23]. The increased concentration of BiP chaperone and the implicit increased capability to neutralise the damaging effects of elevated ER stress could explain why the onset of OA and chondrocyte apoptosis was slightly delayed in the c/c compared to +/+ line.

**RNA sequencing analysis identifies dysregulated genes 2 weeks after DMM surgery**

To investigate the gene expression changes associated with the chondroprotective effects in the c/c mice, we performed RNA sequencing on articular cartilage from 2 weeks DMM and SHAM knee joints. The expression profiles of 6 genes were assessed by PCR and compared with the RNA-seq data in the 3 groups to validate the expression data (Additional file 2: Figure S3). A total of 511 dysregulated genes were identified in +/+ DMM versus SHAM (Additional file 3: Table S2) and 2090 in c/c DMM versus SHAM (Additional file 3: Table S3). Comparing c/c DMM vs +/+ DMM revealed 633 genes that were either up- or downregulated (Additional file 3: Table S4).

The 50 genes most significantly dysregulated by DMM in +/+ mice were compared with the equivalent expression profiles in c/c DMM mice (Tables 1 and 2). Expression of 49 of the 50 genes were detected in c/c DMM mice. All but one of these 49 genes changed expression in the same direction in both +/+ and c/c mice; 39 of which were also statistically dysregulated in c/c DMM cartilage (p < 0.05; Table 1). In addition, the genes indicated by an asterisk (Table 1) were also found to be differentially regulated in a concordant fashion in an independent microarray analysis of 2-week DMM-induced changes [29]. Ingenuity analysis suggested the upstream regulators of differentially expressed genes in +/+ DMM cartilage compared with SHAM to include TGFβ1 & 3, EGF, FGF2 and TNF (Additional file 4: Table S5).

Ingenuity-generated upstream regulator analyses of the full list of differential genes suggested CDKN2A (a cell cycle regulatory gene), Xbp1 (a transcription factor governing key aspects of the UPR), Rictor (a regulatory element of MTORC2) and TGFβ1 as being potentially involved in producing the chondroprotective role seen in c/c mice at 2 weeks post-DMM (Table 3).

**Compromising the ATF6α arm of the UPR does not alter the severity of OA**

Given that we (Fig. 1) and others have shown that chondrocyte ER stress is a feature of cartilage damage in OA, we were surprised to find that the ColIITg<sup>cre</sup> mice did not develop more severe damage following DMM compared to controls. We therefore decided to probe the potential link between increased ER stress and OA disease in a different setting in which the capacity of cells to
manage increases in ER stress is compromised due to the ablation of a key ER stress sensor. ATF6α is one of the three key ER stress sensors, and although Atf6α−/− mice have no overt phenotype, their ability to respond to ER stress challenges is compromised [25]. For instance, the chondrocytes’ ability to induce BiP and CHOP in response to ER stress caused by the expression of mutant type X collagen in the metaphyseal chondrodysplasia-type Schmid mouse is much impaired and the clinical phenotype more severe on the Atf6α−/− background [30].

Firstly, histological examination of the articular cartilage of 6–7-month-old Atf6α−/− mice revealed no signs of degeneration (Fig. 4a) indicating that these mice do not spontaneously develop OA. We performed DMM surgery on Atf6α−/− and Atf6α+/+ controls at 10–12 weeks of age and collected knee joints 4 weeks later which is the usual first time point examined in this type of study.
Table 1 Fifty most dysregulated genes in articular cartilage of +/− DMM compared to SHAM and the equivalent expression in the ColIITgcog DMM group

| WT DMM v SHAM | Gene name | Fold change | padj  |
|---------------|-----------|-------------|-------|
| Inhba*        | 3.42      | 2.13E−10    |       |
| Adams18*      | −3.93     | 5.81E−08    |       |
| Bmp7*         | 6.93      | 3.89E−24    |       |
| Gpx3          | 7.45      | 8.48E−36    |       |
| Pappa         | 6.26      | 4.43E−13    |       |
| Fni*          | 5.35      | 6.87E−22    |       |
| Ank*          | 4.51      | 1.70E−27    |       |
| Crth*         | 3.05      | 2.73E−06    |       |
| Penk*         | 8.26      | 4.71E−27    |       |
| Prkg2*        | 5.09      | 6.04E−13    |       |
| Gas1          | −1.01     | NS          |       |
| Fibin         | −1.82     | 1.03E−06    |       |
| Scara3*       | −1.30     | NS          |       |
| Hbegf*        | 8.52      | 1.14E−17    |       |
| Pmepa1        | 3.65      | 9.44E−31    |       |
| Tms4f1*       | 4.13      | 3.90E−46    |       |
| Galtn1*       | 2.90      | 3.33E−21    |       |
| Hspad*        | 4.20      | 2.41E−38    |       |
| Comp          | −1.42     | 0.04857489  |       |
| Col6a3*       | 2.56      | 2.20E−10    |       |
| Bmp2*         | 4.52      | 2.87E−12    |       |
| Mgp           | 10.33     | 1.91E−17    |       |
| Adamts2*      | −3.04     | 0.020992517 |       |
| Npr3*         | 2.82      | 0.00016205  |       |
| Fgf1*         | 1.32      | NS          |       |
| Blhle40*      | 4.46      | 3.58E−18    |       |
| Chad*         | −1.68     | 0.000147026 |       |
| Serpine1      | 3.47      | 3.56E−07    |       |
| Serpina3n*    | 22.32     | NA          |       |
| Abi3bp        | −2.12     | 8.81E−08    |       |
| Ppp1r3c*      | −2.52     | 0.000331801 |       |
| Gcnt4         | 5.37      | 1.97E−08    |       |
| Olmd*         | −2.47     | 0.0055615   |       |
| Uosca*        | 2.84      | 3.35E−31    |       |
| Fhd2*         | 3.45      | 1.43E−08    |       |
| Ltbp2*        | 7.81      | NA          |       |
| Cyr61*        | 3.39      | 1.54E−07    |       |
| Ptds2*        | 15.18     | NA          |       |
| Cpiol2        | 1.42      | 0.03383883  |       |
| Cyt1l*        | −1.04     | NS          |       |
| Atp1a1*       | 2.44      | 4.53E−22    |       |
| Tsc22d1*      | 3.35      | 1.32E−14    |       |

Data compiled from Additional file 2: Table S2 & S3. Italics—genes significantly dysregulated in both WT DMM and ColIITgcog DMM compared to SHAM. *Gene also significantly dysregulated in similar direction in 2-week post-DMM wild-type cartilage samples assessed by microarray [29]. ND not detected, NA not available due to only one value in data set, NS not significant (false discovery rate/adjusted P value > 0.1).

Non-operated knee joints from Atf6α−/− and Atf6α+/+ mice showed no signs of cartilage degradation as expected (Fig. 4b). In contrast, DMM joints from Atf6α−/− and Atf6α+/+ mice displayed signs of proteoglycan and cartilage loss (Fig. 4b). Histological scoring revealed no differences in the severity of OA between Atf6α−/− and Atf6α+/+ mice (Fig. 4c). The lack of significant differences in the pathology between Atf6α−/− and Atf6α+/+ mice suggests ER stress does not play an important role in DMM-induced OA pathology.

Discussion

We aimed to establish the role of ER stress in modulating OA disease onset and progression. Firstly, we demonstrated that increased ER stress, indicated by elevated BiP mRNA, is a feature of disease onset in the DMM model, being present in chondrocytes at or adjacent to the initial site of damage (Fig. 1a). Cells expressing increased BiP had also upregulated Col2a1 mRNA which indicates these chondrocytes were undergoing an anabolic response to DMM surgery (Fig. 1a). The association of increased BiP with increased collagen synthesis in human OA was previously noted by Nugent et al. [18].

We next examined whether exposure to ER stress is a critical factor in OA onset or progression using the ColIITgcog (c/c) transgenic mouse [23]. ISH revealed undetectable levels of Col2a1 expression in the cartilage of unchallenged, non-operated joints of both +/+ and c/c adult mice (Figs. 1 and 2) demonstrating that the collagen II-driven expression of Tggtog which was high in actively growing animals [23] was not expressed at significant levels in adult ColIITgcog mice. Indeed, Tggtog mRNA was not detected by ISH in articular cartilage of...
Table 2 Fifty most significantly dysregulated genes in articular cartilage of ColIITgcog DMM compared to +/- DMM mice

| Gene name | Fold change | \( \rho_{\text{adj}} \) |
|-----------|-------------|-----------------|
| Enpp2     | 3.80        | 7.729E-24       |
| Apobr     | -5.38       | 1.581E-18       |
| Gas1      | 3.90        | 1.581E-18       |
| Serpina3n | 5.76        | 3.413E-16       |
| Chil1     | 3.32        | 9.585E-15       |
| Ltf       | -5.29       | 1.778E-14       |
| Igfgh2b   | -13.55      | 4.240E-13       |
| Fam111a   | -5.01       | 5.242E-13       |
| Tnfrsf1ib | 2.07        | 1.495E-12       |
| Lyz2      | -3.05       | 2.941E-12       |
| Mgp       | 3.22        | 3.442E-12       |
| Itga2b    | -7.20       | 1.080E-11       |
| Lox       | 2.43        | 7.576E-11       |
| Cpq       | 2.78        | 1.069E-10       |
| Eno2      | 6.51        | 2.247E-10       |
| Aph1b     | -9.03       | 8.851E-10       |
| Scara3    | 2.37        | 1.834E-09       |
| Camp      | -4.11       | 2.179E-09       |
| Rcan1     | 3.15        | 5.282E-09       |
| Alox5     | -9.00       | 6.821E-09       |
| Sema3c    | 3.29        | 1.006E-08       |
| Pabpc1    | -1.83       | 2.472E-08       |
| Pcsk5     | 2.35        | 2.829E-08       |
| Hells     | -5.99       | 6.300E-08       |
| Hbb-bt    | -4.42       | 1.544E-07       |
| 2810474O19Rik | -2.22 |        |
| Magt1     | 2.26        | 1.727E-07       |
| Gda       | -3.70       | 2.385E-07       |
| S100a8    | -3.75       | 2.393E-07       |
| Plekha2   | -2.69       | 4.021E-07       |
| Clmp      | 2.27        | 4.102E-07       |
| Pkd2      | 2.19        | 4.102E-07       |
| Dcn       | 2.07        | 4.102E-07       |
| Ank2      | 2.29        | 1.365E-06       |
| Fkbp9     | 1.84        | 1.590E-06       |
| Rps2-ps13 | -6.65       | 1.822E-06       |
| Tm4sf1    | 1.65        | 2.902E-06       |
| Vill      | -5.60       | 3.656E-06       |
| Ncf1      | -3.48       | 3.656E-06       |
| Cenpf     | -3.95       | 4.238E-06       |
| Mefd2     | 2.69        | 4.270E-06       |
| Ccd125    | -6.25       | 4.462E-06       |
| Spp1      | 2.95        | 4.873E-06       |

(Continued)
demonstrate the potential benefit of an adapted, pre-conditioned ER response, perhaps induced for instance by controlled heating and cooling of joints, in protecting against subsequent insults and thus delaying disease onset.

RNA-seq analysis revealed the most significantly dysregulated genes in +/- DMM vs SHAM controls were also dysregulated in c/c DMM cartilage (Table 1) and in an independent microarray-based study [29]. The RNA for sequencing in the current study was collected from the whole medial tibial condyle rather than the tissue in the immediate vicinity of the OA lesion, and it is therefore not surprising that significant elevations in mRNAs encoding genes such as BiP and Col2a1, which at 2 weeks post-DMM were highly localised to the edge of the damaged cartilage (see Figs. 1 and 2), were not detected in +/- DMM vs SHAM (Additional file 3: Table S2). More surprising was the absence of any significant increase in BiP mRNA in the cartilage of ColII Tgcog (c/c) DMM (Additional file 3: Table S3) despite the increased ambient BiP protein levels seen in the chondrocytes of the ColII Tgcog mice (Fig. 3 and Additional file 2: Figure S2). The increased BiP protein in the adult ColII Tgcog mouse chondrocytes is capable of activating all three arms of the UPR; IRE1, ATF6 and PERK [15, 39]. Moreover, in the current study, when looking for upstream regulators of genes dysregulated in c/c vs +/- mice 2 weeks post-DMM, the Xbp1-driven UPR network was significantly activated in ColII Tgcog OA cartilage (Table 3), consistent with evidence of increased Xbp1 splicing during cartilage development of ColII Tgcog mice [23]. These Xbp1–activated pathways downstream of the UPR sensor Xbp1 are considered a legacy of the ColII Tgcog chondrocytes having been conditioned to an elevated level of ER stress throughout development. XBP1 splicing is upregulated in human OA [19, 20], and it was also identified as an upstream regulator in mouse osteoarthritic tissue [29]. Additionally, the spliced active form of XBP1 is a negative regulator of apoptosis in OA [20]. Accordingly, DMM-operated c/c mice displayed a delay in chondrocyte apoptosis although our studies do not rule out the possibility that the delayed apoptosis is the result of less tissue damage rather than due to the increased BiP protein. Altogether, prior exposure to increased ER stress may have a chondroprotective role against the onset of OA perhaps through Xbp1-regulated pathways/networks together with the inherently higher ambient level of BiP protein present in the ColII Tgcog chondrocytes.

| Table 3 | Top upstream regulators identified in ColII Tgcog DMM mice compared to +/- DMM mice |
|-----------------------------------------------|-----------------------------------------------|
| **Upstream Regulators** | **Activation z-score** | **p-value of overlap** | **Target Molecules in Dataset** |
| CDKN3A | 3.990 | 4.78E-08 | BIRC5, CD95, CTGF, CXCL14, DUSP1, E2F2, EPSP, FAM111A, FEN1, GNA5, GSK3B, HMGB1, IL11, TGFBI, JUN, KANK2, LAMP1, MCMA, MKK4, MAPK3, NRC1, ODC1, PHLAD2, PKM, RPA1, TGFBI, TMPO, VIM, VRK1 |
| XBP1 | 3.807 | 1.44E-08 | APP, CALR, COL1A1, CRP, CREB3, DR1, DNA, FXR1, FXR2, F11, F0L14, MDC2, NCF1, PDCD4, PI3K, PPARB, RASSF1B, RAP2B, SEC23A, SEC34A, SEC10A, SPRR1F, TRIP13, TRIM13, USO1 |
| RCTOR | 3.317 | 1.27E-01 | ATP6V1D1, CHARGE, DNM2, DOCK7, DUSP1, E2F2, EPSP, FAM111A, FEN1, GNA5, GSK3B, HMGB1, IL11, TGFBI, JUN, KANK2, LAMP1, MCMA, MKK4, MAPK3, NRC1, ODC1, PHLAD2, PKM, RPA1, TGFBI, TMPO, VIM, VRK1 |
| TGFBI1 | 3.198 | 2.98E-23 | ABCG1, ACVR1, ADAM1, ALC1, ANKH, APP, AQP2, ARPC2, ASPM, BACE1, BCLAF1, BIRC5, BMP9, BSG, CBF4, CAM1, CAM2, CAP1, CD38, CEP21, CEP51, CEP61, CEP71, CFH, CHS1, CHST1, COMP, CSG1, CREB3, CTGF, CCL12, CCR1, DNM2, DNAJB1, DNM1L, DUSP1, E2F2, EPSP, FAM111A, FEN1, F0L14, FOI1, GDI1, GEM, GPRF73, GNAS, GNAS2, HBEF2, HESA, HSPB1, ITPR1, ITPR1, JUN, LCN2, LIFR, LOC1, LPA4, TP1, TP1, TP1, TP1, UGT1A1, VCP, VCAN, VDAC3, XDH, XVL1 |
| TM4ST1 | 2.889 | 8.08E-07 | ASPM, BCLAF1, CHARGE, DNM2, DUSP1, E2F2, EPSP, FAM111A, FEN1, GNA5, GSK3B, HMGB1, IL11, TGFBI, JUN, KANK2, LAMP1, MCMA, MKK4, MAPK3, NRC1, ODC1, PHLAD2, PKM, RPA1, TGFBI, TMPO, VIM, VRK1, XDH, XVL1 |
| CTGF | 2.841 | 5.34E-05 | BIRC5, COL1A1, CTGF, DNA, FN1, HAPLN1, JUN, LCN2, LIFR, LPL, LTAH1, TP1, TP1, TP1, TP1, UGT1A1, VCAN, VDAC3, XDH, XVL1 |
| BMP6 | 2.830 | 2.02E-02 | BIRC5, COL1A1, CTGF, DNA, FN1, HAPLN1, JUN, LCN2, LIFR, LPL, LTAH1, TP1, TP1, TP1, TP1, UGT1A1, VCAN, VDAC3, XDH, XVL1 |
| FBN1C32 | 2.821 | 1.50E-02 | CADM1, CAUF1, DNAJC3, PDAC, PPF6C, ST3FA, VIMP |
| IL1A | 2.578 | 7.22E-04 | APP, BIRC5, COL1A1, CTGF, DNA, FN1, HAPLN1, JUN, LCN2, LIFR, LPL, LTAH1, TP1, TP1, TP1, TP1, UGT1A1, VCAN, VDAC3, XDH, XVL1 |
| POUSF1 | 2.598 | 2.21E-02 | ACVR1, BIRC5, BMP5, CRABP1, DUSP1, EPSP, FAM111A, FEN1, GNA5, GSK3B, HMGB1, IL11, TGFBI, TMPO, VIM, VRK1 |
| NUFB1 | 2.498 | 1.22E-06 | ASPM, BCLAF1, CASP3, CENP, DXCP, DUSP6, EDN1, ESR1, FAM111A, FAM14A1, FGF1, GFI1, GNAS, HBEF2, HSPB1, ITPR1, JUN, KANK2, LAMP1, MCMA, MKK4, MAPK3, NRC1, ODC1, PHLAD2, PKM, RPA1, TGFBI, TMPO, VIM, VRK1, XDH, XVL1 |
| REL | 2.449 | 1.01E-01 | APP, BIRC5, FN1, JUN, LCN2, LIFR, LPL, LTAH1, TP1, TP1, TP1, TP1, UGT1A1, VCAN, VDAC3, XDH, XVL1 |
| BNIP3L | 2.449 | 6.75E-03 | CENPF, CENP, KLF11, NUFB1, POUSF1, TP1, TP2A |

Tg Tgcog expression is capable of activating all three arms of the UPR; IRE1, ATF6 and PERK [15, 39]. Moreover, in the current study, when looking for upstream regulators of genes dysregulated in c/c vs +/- mice 2 weeks post-DMM, the Xbp1-driven UPR network was significantly activated in ColII Tgcog OA cartilage (Table 3), consistent with evidence of increased Xbp1 splicing during cartilage development of ColII Tgcog mice [23]. These Xbp1–activated pathways downstream of the UPR sensor IRE1 are considered a legacy of the ColII Tgcog chondrocytes having been conditioned to an elevated level of ER stress throughout development. XBP1 splicing is upregulated in human OA [19, 20], and it was also identified as an upstream regulator in mouse osteoarthritic tissue [29]. Additionally, the spliced active form of XBP1 is a negative regulator of apoptosis in OA [20]. Accordingly, DMM-operated c/c mice displayed a delay in chondrocyte apoptosis although our studies do not rule out the possibility that the delayed apoptosis is the result of less tissue damage rather than due to the increased BiP protein. Altogether, prior exposure to increased ER stress may have a chondroprotective role against the onset of OA perhaps through Xbp1-regulated pathways/networks together with the inherently higher ambient level of BiP protein present in the ColII Tgcog chondrocytes.
It is difficult to interpret the role of ER stress in disease progression in the Col17tg20g mouse given that the chondrocytes start off with elevated levels of BiP. We therefore adopted a second approach by performing DMM on the Atf6α−/− mouse, which has a compromised ability to respond to increases in ER stress in many tissues [25] including cartilage most evident through their severely compromised induction of BiP [30]. However, we were unable to detect any significant changes in OA severity in Atf6α−/− mice following DMM suggesting that whilst increased ER stress is apparent early during disease onset in OA chondrocytes, control of the level of ER stress by ATF6α is not a major determinant of disease progression in DMM.

CHOP-knockout mice had reduced chondrocyte apoptosis and disease progression in a mechanically induced model of OA similar to DMM [22]. CHOP is a pro-apoptotic transcription factor that acts downstream of the PERK pathway under ER stress. Interestingly, PERK has emerged as a potential novel target for OA therapy [40]. However, ER stress is one of several forms of cellular stress that can promote apoptosis. Others include oxidative stress, osmotic stress, metabolic stress and heat shock [41], many of which have also been implicated in...
the OA disease mechanism(s) [42]. These later causes may be of more significance than ER stress in OA progression specifically given the lack of effect of the ColII Tg<sup>−−</sup> or Atf6α<sup>−−</−</sup> genotypes on OA severity at 4 and/or 8 weeks post-DMM.

It should be borne-in-mind that much of the work described and discussed above is based on studies using mechanically induced OA in genetically homogeneous lines, coupled with gene knockouts. However, when interpreting the relevance of these findings for the human OA condition, the genetic heterogeneity of the population together with the complex pattern of environmental influences must be taken into account.

Conclusion
Our studies indicate that an increased capacity to effectively manage increases in ER stress in articular cartilage due either to genetic pre-disposition or pre-conditioning as a result of prior exposure to increased ER stress may be beneficial in delaying the onset of OA. Once significant tissue damage is established, ER stress does not play a significant role on disease progression.

Additional files

| Additional file 1: | Table S1. Primer sequences for qPCR. (DOCX 18 kb) |
|-------------------|-------------------------------------------------|
| Additional file 2: | Figure S1. ColIITg<sup>−−</sup> mice have normal articular cartilage with no signs of degeneration. Figure S2. Increased BiP protein in articular cartilage of DMM-operated ColIITg<sup>−−</sup> mice. Figure S3. Validation of RNAseq expression data by qPCR. Graphs displaying RNAseq data relative to housekeeping genes. Figure S4. ERα<sup>−−</sup> knockout mice. Figure S5. ERα<sup>−−</sup> knockout mice. |
| Additional file 3: | Table S2. Differentially regulated genes in +/+ DMM vs SHAM. Table S3. Differentially regulated genes in c/c DMM vs SHAM. Table S4. Differentially regulated genes in +/+ DMM vs c/c DMM. (DOCX 15459 kb) |
| Additional file 4: | Table S5. Top upstream regulators identified in +/+ DMM mice compared to SHAM mice. (DOCX 255 kb) |

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Authors’ contributions
RBH and MDB conceived and designed the study. LK, LM, JS, PW and RBH generated and analysed the data; JB collaborated on the articular cartilage microdissection; KM provided the Atf6α knockout mouse line; LK and RBH wrote the manuscript; all authors read and approved the manuscript.

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Author/s:
Kung, LHW; Mullan, L; Soul, J; Wang, P; Mori, K; Bateman, JF; Briggs, MD; Boot-Handford, RP

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