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Bach1 deficiency reduces severity of osteoarthritis through upregulation of heme oxygenase-1

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Abstract

Introduction: BTB and CNC homology 1 (Bach1) is a transcriptional repressor of Heme oxygenase-1 (HO-1), which is cytoprotective through its antioxidant effects. The objective of this study was to define the role of Bach1 in cartilage homeostasis and osteoarthritis (OA) development using *in vitro* models and Bach1^{-/-} mice.

Methods: HO-1 expression in Bach1^{-/-} mice was analyzed by real-time PCR, immunohistochemistry and immunoblotting. Knee joints from Bach1^{-/-} and wild-type mice with age-related OA and surgically-induced OA were evaluated by OA scoring systems. Levels of autophagy proteins and superoxide dismutase 2 (SOD2) were determined by immunohistochemistry. The relationship between HO-1 and the protective effects for OA was determined in chondrocytes treated with small interfering RNA (siRNA) targeting HO-1 gene.

Results: HO-1 expression decreased with aging in articular cartilages and menisci of mouse knees. Bach1^{-/-} mice showed reduced severity of age-related OA and surgically-induced OA compared with wild-type mice. Microtubule-associated protein 1 light chain 3 (LC3), autophagy marker, and SOD2 were increased in articular cartilage of Bach1^{-/-} mice compared with wild-type mice. Interleukin-1 β (IL-1 β) induced a significant increase in *Adams-5* in wild-type chondrocytes but not in Bach1^{-/-} chondrocytes. The expression of SOD2 and the suppression of apoptosis in Bach1^{-/-} chondrocytes were mediated by HO-1.

Conclusions: Bach1 deficiency reduces the severity of OA-like changes. This may be due to maintenance of cartilage homeostasis and joint health by antioxidant effects through HO-1 and downregulation of extracellular matrix degrading enzymes. These results suggest that inactivation of Bach1 is a novel target and signaling pathway in OA prevention.

Introduction

Osteoarthritis (OA), the most prevalent aging-related joint disease, is characterized by degradation of articular cartilage and alterations in other joint tissues. The most important risk factors are aging, obesity, mechanical stress, and inflammation, and these factors impair tissue homeostasis through dysregulation of intracellular signaling mechanisms and extracellular matrix (ECM) remodeling [1, 2]. The interaction of aging-associated changes in cartilage and molecular mechanisms of OA pathogenesis remains to be elucidated. Oxidative stress has been

established as an important factor as it is elevated in joint tissues including articular cartilage in aging and OA [3–5].

Increased oxidative stress results from increased reactive oxygen species (ROS) generation and from reduced antioxidants, and is accompanied by a progressive accumulation of damaged molecules and organelles, leading to activation of catabolic factors such as inflammatory cytokines and extracellular matrix (ECM)-degrading proteases [1, 2]. Antioxidant enzymes such as Heme oxygenase-1 (HO-1) and superoxide dismutase 2 (SOD2) are an important defense against ROS-mediated damage [6, 7]. HO-1 promotes iron recycling by degrading heme into ferrous iron, carbon monoxide and biliverdin, and protects cells from various stresses [6, 8]. Previous studies revealed

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that induction of HO-1 has beneficial effects in several diseases [9–11]. The expression of HO-1 gene (*Hmox-1*) is negatively regulated by BTB and CNC homology 1 (Bach1), a transcriptional repressor, which binds to *Hmox-1* enhancers. Bach1 deficient mice thus have high constitutive HO-1 expression in various tissues under physiological conditions [12]. These mice have reduced disease severity in several injury models [13–15] and age-related degeneration of the meniscus [16]. However, potential beneficial effects of HO-1 in OA development have not been determined. The objectives of this study were to investigate the impact of Bach1 deficiency on two different animal models of OA, an aging model, primary OA, and a surgical model, posttraumatic OA.

Methods

Animal models of OA

Bach1^{-/-} mice on C57BL/6 background were described previously [12]. Only male mice were used in this study. All animal experiments were performed according to protocols approved by Hiroshima University Animal care and Use Committee. Knee joints were harvested at 6 months (n = 7), 12 months (n = 11), and 22 months (n = 14) to monitor spontaneous age-related OA. Experimental OA was induced in 10 week-old, wild-type mice (n = 13) and Bach1^{-/-} mice (n = 11) by transection of the medial meniscotibial ligament (MMTL) and the medial collateral ligament (MCL) in the right knees [17]. Mice were sacrificed 8 weeks after surgery, and the knee joints were collected for histological analysis.

Histological assessments

All knee joints were embedded intact in paraffin after fixation in 4 % Paraformaldehyde Phosphate Buffer Solution and decalcification in K-CX (FALMA, Tokyo, Japan). Knee joints sectioned (4.5 μm) in the sagittal plane through the central weight-bearing region of the medial and lateral femorotibial joint. The sections were stained with Safranin O/fast green and at least two different sections per sample were analyzed microscopically. In this study, we applied multiple separate scoring systems for articular cartilage, meniscus, synovitis, osteophyte formation and subchondral bone thickening. Osteoarthritic damage of articular cartilage was scored using a modified Mankin system [18, 19]. Meniscus degeneration was evaluated using a scoring system [20] which included the following 6 criteria, meniscus integrity (0 = smooth surface, 1 = irregularity of superficial layer or slight fibrillation, 2 = moderate fibrillation, 3 = severe fraying, tear or disruption), collagen structure (0 = normal, 1 = slight disturbance, 2 = moderate disturbance, 3 = severe disturbance or mucoid substances), cellular abnormalities (0 = normal, 1 = hypercellularity, 2 = cloning tendency, 3 = hypocellularity), stainability of safranin O

staining (0 = normal, 1 = slight reduction, 2 = moderate reduction, 3 = severe reduction), calcification and cyst formation (0 = normal, 1 = slight, 2 = moderate, 3 = severe). The maximum possible score per meniscus was 18. Osteophyte formation and subchondral bone thickening were scored on a scale of 0–3, where 0 = normal, 1 = mild, 2 = moderate and 3 = severe changes, and the average scores for tibia and femur were recorded. The severity of synovitis was evaluated according to a previously described histopathological classification system [21]. The parameters of synovitis included hyperplasia/enlargement of synovial lining layer, degree of inflammatory cell infiltration and activation of resident cells/synovial stroma. All parameters were graded from 0 (absent), 1 (slight), 2 (moderate) to 3 (strongly positive) and summarized ranging from 0–9, where 0–1 corresponds to no synovitis (grade = 0), 2–3 to a slight synovitis (grade 1), 4–6 to moderate synovitis (grade 2), and 7–9 to severe synovitis (grade 3).

Immunohistochemical analysis

Knee joint sections were immunostained with anti-HO-1 antibody (1:75, ab52947, Abcam, Austin, TX, USA) using Vectastain ABC-AP alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA) as described previously [22]. For anti-microtubule-associated protein 1 light chain 3 (LC3) antibody (1:100, AP1801a, ABGENT, San Diego, CA, USA), anti-manganese superoxide dismutase (MnSOD) antibody (1:100, SPC-117, StressMarq, Victoria, BC, Canada), sections in Immunoactive pH 6.0 (Matsunami Glass, Osaka, Japan) were heated in a microwave oven and kept at 85 °C for 1.5 minutes. Slides were cooled for 20 minutes at room temperature after antigen unmasking. After washing with PBS, 3 % H₂O₂ treated for 10 minutes, sections were blocked with 10 % serum for 20 minutes at room temperature. Antibodies were applied and incubated overnight at 4 °C. After washing with PBS, sections were incubated with biotinylated secondary antibody for 30 minutes at room temperature and then incubated using the peroxidase based Elite ABC system (Vector Laboratories) for 30 minutes. Slides were washed, and sections were incubated with 3,3'-diaminobenzidine (DAB) substrate.

Isolation and culture of mouse articular chondrocytes

Primary articular chondrocytes were dissected from the femoral heads of 1-month-old Bach1^{-/-} and wild-type mice by digestion with 0.3 % collagenase Type 2 (Worthington, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM) (Wako, Osaka, Japan) for 2 h. Isolated chondrocytes were cultured in DMEM with 10 % fetal bovine serum.

Transfection of small interfering RNA into mouse articular chondrocytes

Articular chondrocytes from wild-type mice and *Bach1*^{-/-} mice were seeded at 5×10^4 cells/well on a 24-well plate and were transfected with small interfering RNA (siRNA) for HO-1 using Lipofectamine RNAiMax Reagent (Invitrogen, Carlsbad, CA, USA). The sequences of the siHO-1 were: (sense) 5'-CAACAGUGGCAGUGGGAAUTT-3' and (antisense) 5'-AUUCCACUGCCACUGUUGTT-3' (Hokkaido System Sciences, Hokkaido, Japan). Control siRNAs were also prepared for the control group (siRNA negative control; siNeg #1, Invitrogen). At 24 h after transfection, articular chondrocytes were treated with IL-1 β (1 ng/ml; Pepro-Tech, Rocky Hill, NJ, USA) for an additional 24 h.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from chondrocytes using TRIzol Reagent (Invitrogen). Complementary DNA (cDNA) was synthesized using 500 ng of total RNA with the Super-Script VILO cDNA Synthesis Kit (Invitrogen). A real-time PCR assay was performed using TaqMan Gene Expression Assay probes (Applied Biosystems, Foster City, CA, USA) to amplify the *Bach1* (Mm01344527), *Hmox-1* (Mm00516005), *Col2a1* (Mm01309565_m1), *Acan* (Mm00545807), *Mmp-13* (Mm01168713), *Adamts-5* (Mm01344182_m1), and *Sod2* (Mm01313000_m1), and *Gapdh* (Mm99999915_g1) was used as the internal control to normalize the sample differences. Relative expression was calculated using the $\Delta\Delta C_t$ values, and results were expressed as $2^{-\Delta\Delta C_t}$.

DNA microarray analysis

DNA microarray (TORAY, Tokyo, Japan, 3D-Gene, Mouse Oligo chip 24 k) analysis was performed using total RNA from chondrocytes from wild-type mice and *Bach1*^{-/-} mice.

Immunoblotting assay

For immunoblotting, proteins were extracted from cultured chondrocytes using M-PERTM protein extraction reagent including protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Anti-HO-1 antibody (diluted 1:2000), anti-LC3 antibody (diluted 1:1000), and anti-MnSOD antibody (diluted 1:1000) were used as primary antibodies. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (sc-2030; Santa Cruz Biotechnology, Dallas, TX, USA) and anti-mouse IgG antibody (sc-2005; Santa Cruz Biotechnology) were used as secondary antibody. The signal was detected with chemiluminescence of enhanced immuno-enhancer (Wako, Osaka, Japan) using the ImageQuant LAS 4000 system (GE Healthcare, Uppsala, Sweden).

Apoptosis assay

Articular chondrocytes were seeded at 1.5×10^4 cells/well on 96-well plates and transfected with siHO-1 or siRNA negative control. Articular chondrocytes were treated with tert-butyl hydroperoxide (t-BHP) (200 μ M; Wako) for 5 h at 24 h after transfection. Apoptotic chondrocytes were quantitated by counting the numbers of cell nuclei stained with Cell Event Caspase-3/7 Green Detection ReagentTM and NucBlue Live cell stain Ready-ProbesTM (Invitrogen) in three random fields on each duplicate well at a magnification of $\times 10$ under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan).

Statistical analysis

The data were analyzed using the Mann-Whitney *U* test, Steel or Steel-Dwass to determine statistical differences. Differences were considered statistically significant at $P < 0.05$ (*) and $P < 0.01$ (**).

Results

Expression of HO-1 in articular cartilage

To examine the genes regulated by *Bach1*, DNA microarray analysis was performed on articular chondrocytes of wild-type mice and *Bach1*^{-/-} mice. The results showed that expression of 34 mRNAs was increased more than three-fold in *Bach1*^{-/-} articular chondrocytes, and only *Hmox1* was among the top 20 differentially expressed genes and highly expressed genes in *Bach1*^{-/-} chondrocytes (Table 1). The expression of HO-1 gene and protein were significantly increased in *Bach1*^{-/-} chondrocytes compared with wild-type chondrocytes (Fig. 1a, b). Studies to further evaluate the phenotype of *Bach1*^{-/-} articular chondrocytes from 1-month-old mice observed a decrease in *Col2a1* and *Acan* with a simultaneous increase in *Mmp-13*. However, these differences were not significantly different between *Bach1*^{-/-} and wild-type chondrocytes (Fig. 1c), and articular cartilages from *Bach1*^{-/-} mice at 1 month of age did not have reduced Safranin O staining (data not shown). Furthermore, we evaluated the expression of HO-1 in knee joints from 22-month-old mice. Articular cartilage and meniscus in *Bach1*^{-/-} mice had significantly more HO-1 positive cells than wild-type mice (Fig. 1d).

Expression of HO-1 in articular cartilage with aging

To examine aging-related changes in HO-1 expression in mouse knee joints, we performed immunohistochemical analysis. HO-1 was expressed in chondrocytes in the articular cartilage and the menisci in knee joints from wild-type mice at 3 months of age (Fig. 2a). By 12 months of age, HO-1 expression was substantially reduced to only a subset of cells in the superficial zone of cartilage (Fig. 2a). In 22-month-old mice that did not have structural cartilage defects, HO-1 staining in

Table 1 Gene expression in chondrocytes from *Bach1*^{-/-} mice

Top 20	Symbol	Ratio (<i>Bach1</i> ^{-/-} : wild)	Top 20	Symbol	Normalized expression level
1	LOC635992	5.82	1	Fth1	23704
2	Mllt3	5.79	2	Ftl2	21177
3	4930481F22Rik	4.69	3	mt-Nd1	18945
4	Unknown	4.31	4	Fn1	18837
5	Tnfrsf26	4.29	5	unknown	18423
6	Nlrp4d	4.19	6	Rps3a	18007
7	Ly9	4.06	7	Ubb	17696
8	V1re10	4.01	8	Vim	17167
9	V1rh21	3.64	9	unknown	17069
10	Hmox1	3.63	10	Rpl35	16832
11	Lefty2	3.6	11	Gapdh	16831
12	Cdkl3	3.58	12	Col2a1	16623
13	Unknown	3.55	13	Ubb	16615
14	4933407L21Rik	3.52	14	unknown	16589
15	Arhgap4	3.5	15	Hmox1	16534
16	Serpina1f	3.43	16	EG432768	16487
17	Xmr	3.38	17	Tpt1	16335
18	Gldn	3.35	18	Ftl1	15922
19	Ckt2	3.35	19	Rpl32	15662
20	Hmgb1l	3.34	20	Ppia	15642

Total RNA was isolated from articular chondrocytes in wild-type and *Bach1*^{-/-} mice. Left side: top 20 of differentially expressed genes are shown as the ratio of chondrocytes in *Bach1*^{-/-} mice to chondrocytes in wild-type mice. Right side: the 20 genes of the highest expression level in chondrocytes from *Bach1*^{-/-} mice

articular cartilage was no longer detectable, with the exception of a few cells at the joint margin (Fig. 2a). Thus, there is a significant reduction in HO-1 positive cells with aging in the articular cartilages (Fig. 2b).

Reduced severity of osteoarthritis in *Bach1*^{-/-} mice

Skeletal development in *Bach1*^{-/-} mice was normal. There were no differences in overall joint architecture or changes in the articular cartilage and growth plate. The body weight of *Bach1*^{-/-} mice was not significantly different at 4 and 10 weeks, however, at 22 months it was significantly lower than in wild-type mice (Fig. 3a). To examine whether *Bach1* affected age-related OA development, spontaneous osteoarthritic changes in the knee joints from *Bach1*^{-/-} and wild-type mice were evaluated at 6, 12 and 22 months of age. From 1 to 6 months of age, both strains of mice had intact articular cartilage and similar proteoglycan staining (data not shown). At 12 months of age, wild-type mice had a reduction in proteoglycan. OA severity in wild-type mice at 22 months of age ranged from minimal changes to cartilage fibrillation or partial and full-thickness defects with exposure of subchondral bone. These changes were significantly less severe in *Bach1*^{-/-} mice (Fig. 3b). Wild-type mice also exhibited meniscus degradation, inflammatory changes in synovium, osteophyte formation and

subchondral bone thickening. Semiquantitative scoring systems also indicated a significant decrease in the severity of OA-like changes in all joint tissues in *Bach1*^{-/-} mice at 22 months of age compared with the wild-type mice (Fig. 3c). Next, we used the surgical OA model with transection of MMTL and MCL in 10-week-old mice. Consistent with observations in the aging OA model, the surgical OA model demonstrated significantly reduced articular cartilage scores in *Bach1*^{-/-} mice (Fig. 3d). Although the severity of meniscus degradation was accelerated by transection of the MMTL, meniscus degradation was significantly different between the wild-type and *Bach1*^{-/-} mice. However, inflammatory changes in synovium were not significantly different (Fig. 3e).

SOD2 and LC3 expression in articular cartilage of aged mice

SOD2 and autophagy are essential cellular homeostasis mechanisms and protect against aging-related diseases [23]. To determine whether the increase in HO-1-positive cells in articular cartilage was associated with changes in SOD2 expression and autophagy, the expression of SOD2 and LC3, a main marker of autophagy, was characterized by immunohistochemistry. At 12 and 22 months of age, *Bach1*^{-/-} mice

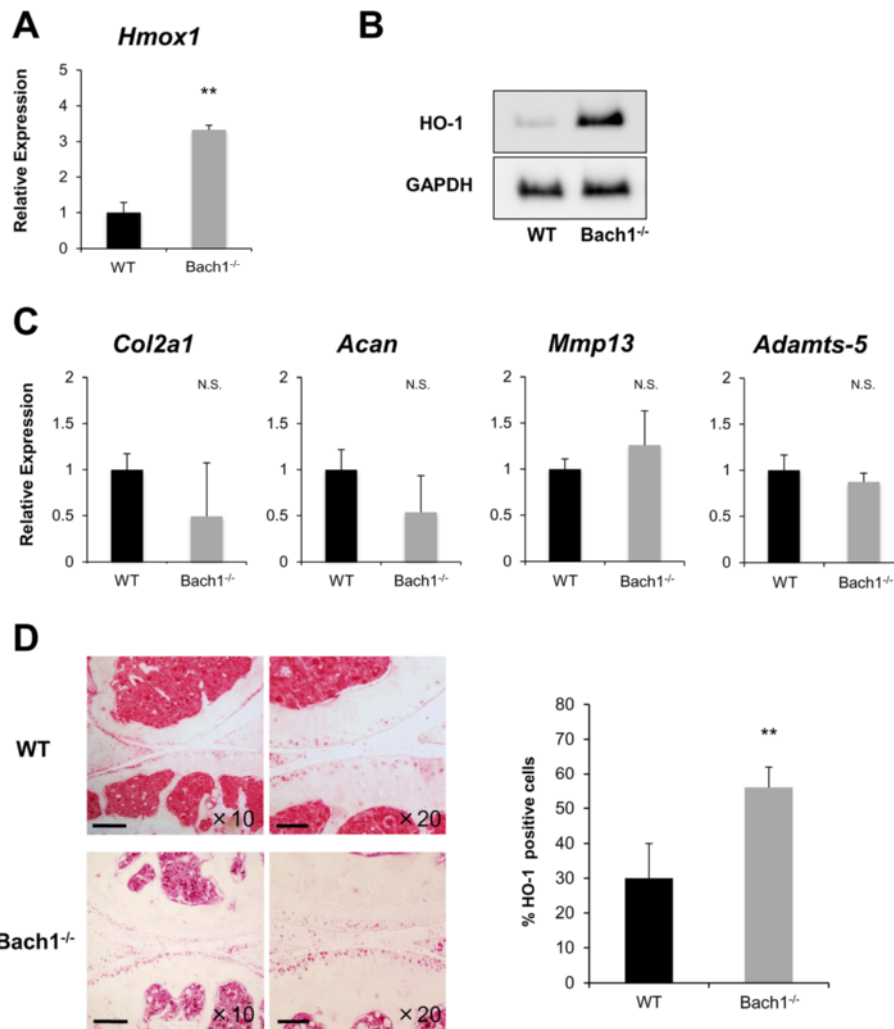


Fig. 1 Expression of heme oxygenase-1 (*HO-1*) in articular cartilages from wild-type (*WT*) mice and *Bach1*^{-/-} mice. Primary articular chondrocytes were isolated from mouse femoral head cartilage (n = 4 per group) at the age of 1 month. **a** Real-time PCR assay for *Hmox1*. **b** Western blot analysis of *HO-1* protein (n = 4). **c** Evaluation of the phenotype of articular chondrocytes in *Bach1*^{-/-} mice. Expression of cartilage-associated genes was determined by real-time PCR assay (n = 4 per group). **d** Comparison of *HO-1* expression in articular cartilage from 22-month-old wild-type mice and *Bach1*^{-/-} mice (n = 4 per group). Knee joints were assessed by immunohistochemistry using anti-*HO-1* antibody. Original magnification $\times 10$ (left; scale bars 200 μm , and $\times 20$ (right; scale bars 100 μm). Values in **a**, **c** and **d** were expressed as means \pm SD. Statistical analysis was performed with the Mann-Whitney *U* test; ***P* < 0.01

showed significantly increased numbers of SOD2- and LC3-positive chondrocytes compared with wild-type mice (Fig. 4a). *Bach1*^{-/-} mice also showed increased numbers of SOD2- and LC3-positive cells as compared to wild-type mice in the surgical OA model (Fig. 4b). These results suggest that articular cartilage in *Bach1*^{-/-} mice maintained not only *HO-1* but also expression of SOD2 and the autophagy marker LC3 in the surgical and the aging-related OA model.

The role of *HO-1* and *Bach1* in SOD2 and LC3 expression in mouse chondrocytes

The expression of both SOD2 and LC3 increased during aging in articular cartilage of *Bach1*^{-/-} mice compared

with wild-type mice, similar to *HO-1* (Fig. 4). Thus, to elucidate the relation between *Bach1*, *HO-1* and SOD2, LC3, articular chondrocytes from wild-type and *Bach1*^{-/-} mice were treated with siHO-1 or negative control siRNA. Expression SOD2 was significantly greater in chondrocytes from *Bach1*^{-/-} mice at 1 month of age (Fig. 5a). Furthermore, the increased SOD2 expression in *Bach1*^{-/-} mice was reduced by *HO-1* knockdown (Fig. 5a). LC3 expression was not significantly different between wild-type and *Bach1*^{-/-} chondrocytes with or without siHO-1 (data not shown). We also examined key enzymes in OA cartilage degradation, *Mmp-13* and *Adamts-5* in IL-1 β -treated *Bach1*^{-/-} chondrocytes. IL-1 β induced a significant increase in *Adamts-5* in wild-type

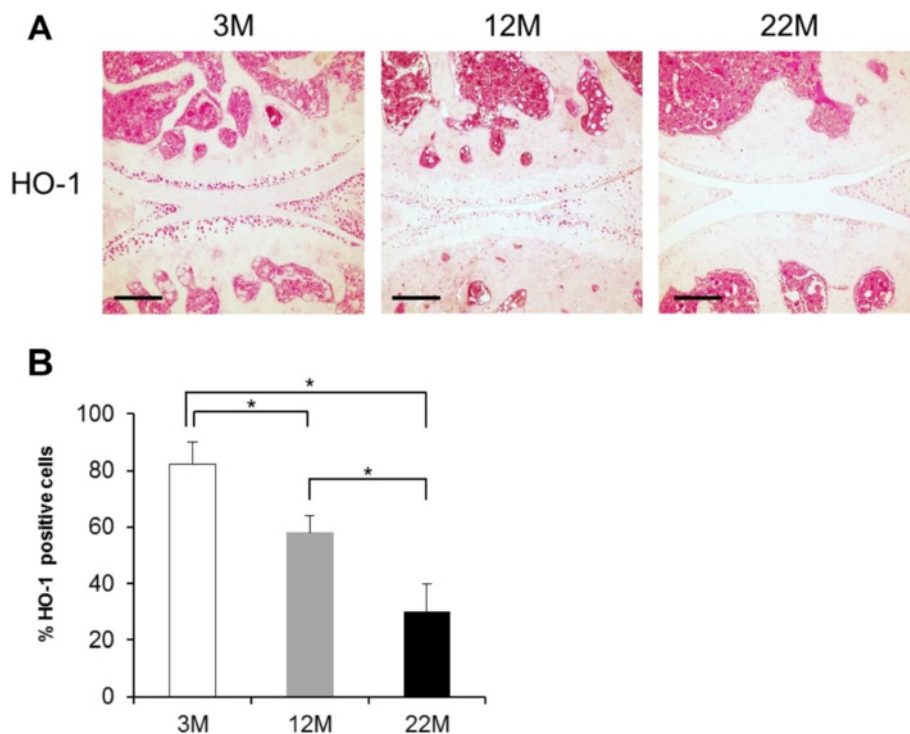


Fig. 2 Expression of heme oxygenase-1 (*HO-1*) in knee joints in aging and osteoarthritis (OA). **a** Knee joints from 3-month- (3 M), 12-month (12 M) and 22-month (22 M)-old wild-type mice ($n = 3-4$ per group) were collected and the sections were assessed by immunohistochemistry. Original magnification $\times 10$; scale bars 200 μm . **b** Values are expressed as means \pm SD. Statistical analysis was performed with the Steel–Dwass test; $*P < 0.05$

chondrocytes but not in *Bach1*^{-/-} chondrocytes. There were trends towards increased *Mmp-13* in IL-1 β -treated wild-type chondrocytes but not in *Bach1*^{-/-} chondrocytes. Furthermore, their expression was not directly regulated by HO-1 because the knockdown of HO-1 did not significantly affect expression of these genes (Fig. 5b). *Bach1*^{-/-} mouse chondrocytes had significantly decreased numbers of caspase-3/7-positive cells following treatment with the oxidant t-BHP. Compared with wild-type mice, the number of positive cells was significantly increased in chondrocytes treated with siHO-1 (Fig. 5c). These results suggest that upregulated SOD2 and the suppression of apoptosis in *Bach1*^{-/-} chondrocytes were mediated by HO-1.

Discussion

In aging and OA, oxidative stress is elevated in joint tissues including cartilage [4]. The antioxidant enzyme HO-1 mediates general adaptive responses and provides enhanced resistance to various stresses. HO-1 is negatively regulated by Bach1, a transcriptional repressor. Bach1 is induced by transforming growth factor β (TGF- β), and is inactivated by oxidative stress, binding of heme, and oxidation of cysteine residue [12, 24–26]. HO-1 was markedly decreased in articular cartilage with aging in

wild-type mice. These results suggest that maintenance of HO-1 expression has potential to protect against OA development and aging.

In the present study, *Bach1*^{-/-} mice exhibited highly increased HO-1 expression even in articular cartilage of aged mice and reduced severity of age-related OA-like changes. However, *Bach1* deficiency and accompanying overexpression of HO-1 did not influence aging and life span [27]. Inflammation and obesity generally increase with aging. Our study showed that weight of *Bach1*^{-/-} mice were lower than wild-type mice at 22 months old. Thus, although the decreased weight of aged *Bach1*^{-/-} mice may contribute to joint health through metabolic changes, the level of pro-inflammatory cytokines in serum was not significantly different between wild-type mice and *Bach1*^{-/-} mice at 22 months old (data not shown). Previously, it was reported that inflammatory diseases are less severe in *Bach1*^{-/-} mice, in part through increased HO-1 in macrophages [28]. In a similar fashion, bone destruction is attenuated in *Bach1*-deficient mice via altered osteoclastogenesis [29]. The induction of HO-1 also results in protective effects against inflammatory and degradative responses in OA chondrocytes and OA synoviocytes [30–32]. Thus, the reduced severity of

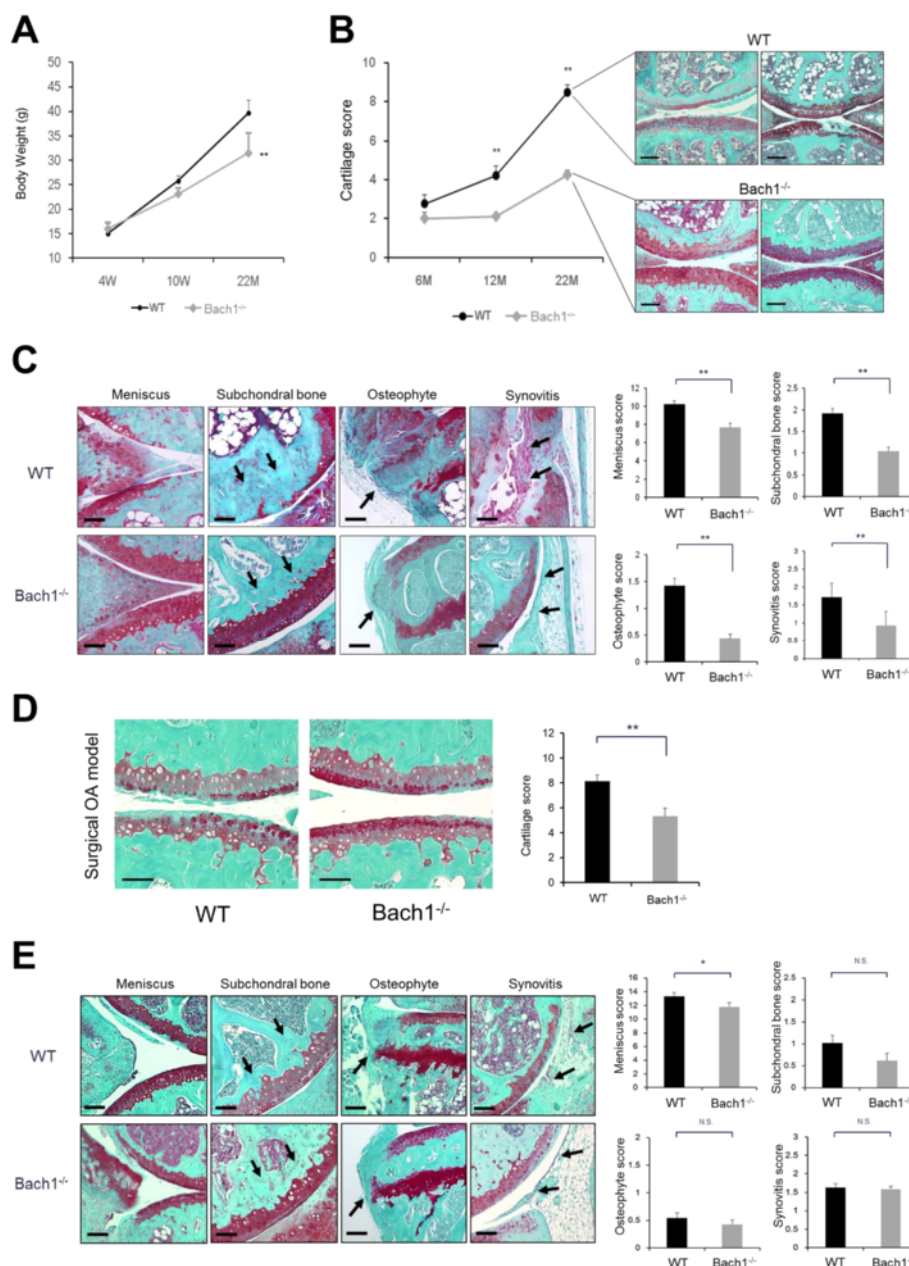


Fig. 3 Bach1^{-/-} mice have reduced severity of osteoarthritis (OA) in an aging model and a surgical OA model. **a** Body weight of wild-type mice and Bach1^{-/-} mice at 4 (4 W) and 10 weeks (10 W) and 22 months (22 M) (n = 5–9 animals per time point). **b** Histological assessment using modified Mankin scoring system of spontaneous cartilage degeneration exacerbated with aging in wild-type mice and Bach1^{-/-} mice. Photographs of a knee from a representative mouse at 22 months old, from each group are also shown. Original magnification × 10; scale bars 200 μm. **c** Analyses of OA pathology using the semiquantitative scoring system in mice at 22 months old, including synovitis, osteophyte formation, subchondral bone thickening and meniscus degradation. Original magnification × 20; scale bars 100 μm. **d** Analyses of cartilage pathology in a surgically induced OA model. Original magnification × 20; scale bars 100 μm. **e** Analyses of OA pathology in a surgically induced OA model, including synovitis, osteophyte formation, subchondral bone thickening and meniscus degradation. Original magnification × 20; scale bars 100 μm. Values are the mean ± SD. Statistical analysis was performed with the Mann–Whitney U test; *P < 0.05, **P < 0.01 versus wild-type mice

OA-like changes in Bach1^{-/-} mice might occur through suppression of inflammation by higher constitutively expressed HO-1 in various cells, including chondrocytes. In aged mice and the surgical OA model, however, the level of proinflammatory

cytokines in serum was not significantly different between wild-type mice and Bach1^{-/-} mice. In addition, the level of proinflammatory cytokines in serum from Bach1^{-/-} mice was increased post-surgically compared with pre-surgical levels in the same mice (data not

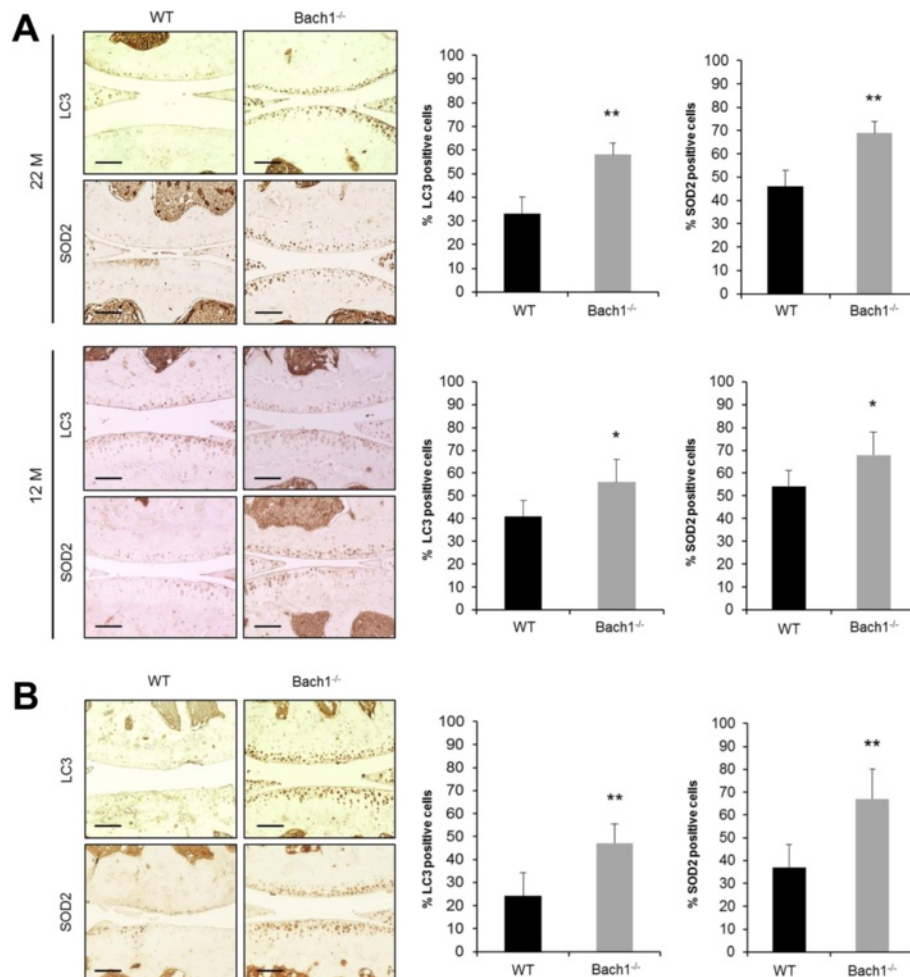


Fig. 4 Expression of microtubule-associated protein 1 light chain 3 (LC3) and superoxide dismutase 2 (SOD2) in articular cartilage. Knee joints from wild-type (WT) mice and Bach1^{-/-} mice were analyzed by immunohistochemistry for LC3 and SOD2. Total number of LC3- and SOD2-positive cells in six fields were counted and the percentage of positive cells was calculated. **a** Representative immunostaining of articular cartilage from 12-month- (12 M) and 22 month (22 M)-old mice (aging model) (n = 4 per group). **b** Representative immunostaining of articular cartilage from a surgically induced osteoarthritis (OA) model (n = 4 per group). Original magnification $\times 20$; scale bars 100 μm . Values are the mean \pm SD. Statistical analysis was performed with the Mann-Whitney *U* test; **P* < 0.05, ***P* < 0.01 versus wild-type mice

shown). Thus, in spite of increased proinflammatory cytokines in Bach1^{-/-} mice with surgical OA, the OA-like changes were attenuated, indicating that the reduction of OA-like changes in Bach1^{-/-} mice may be not only due to the anti-inflammatory effect of HO-1 upregulation.

SOD2 and autophagy also are key mediators of aging-related diseases and prevent the accumulation of defective mitochondria that produce high levels of ROS in chondrocytes [23]. SOD2 is the main antioxidant enzyme that scavenges superoxide anions in the inner mitochondrial matrix [33]. Autophagy has important anti-aging functions and reduces aging-associated cell death, dysfunction, and disease [34]. Recently, it was reported that SOD2 is reduced during OA development and

in aging [5, 35], and autophagy decreased in articular cartilage of human OA, aging-related and surgically-induced OA in mice [36]. Autophagy is involved in maintenance of articular cartilage homeostasis, and reduces severity in mouse OA models [22, 37]. In the present study, Bach1^{-/-} mice maintained the expression of SOD2 and LC3 in articular cartilage, as well as HO-1. Although the upregulated LC3 expression in Bach1^{-/-} mice was not significantly reduced by HO-1 knockdown, SOD2 was regulated by HO-1. Chondrocyte-specific deletion of SOD2 in human cells and mice accelerates OA-like changes accompanied by oxidative damage and mitochondrial dysfunction [38]. Thus, maintenance of SOD2 in chondrocytes also may be important for OA prevention. While increased *Mmp*-

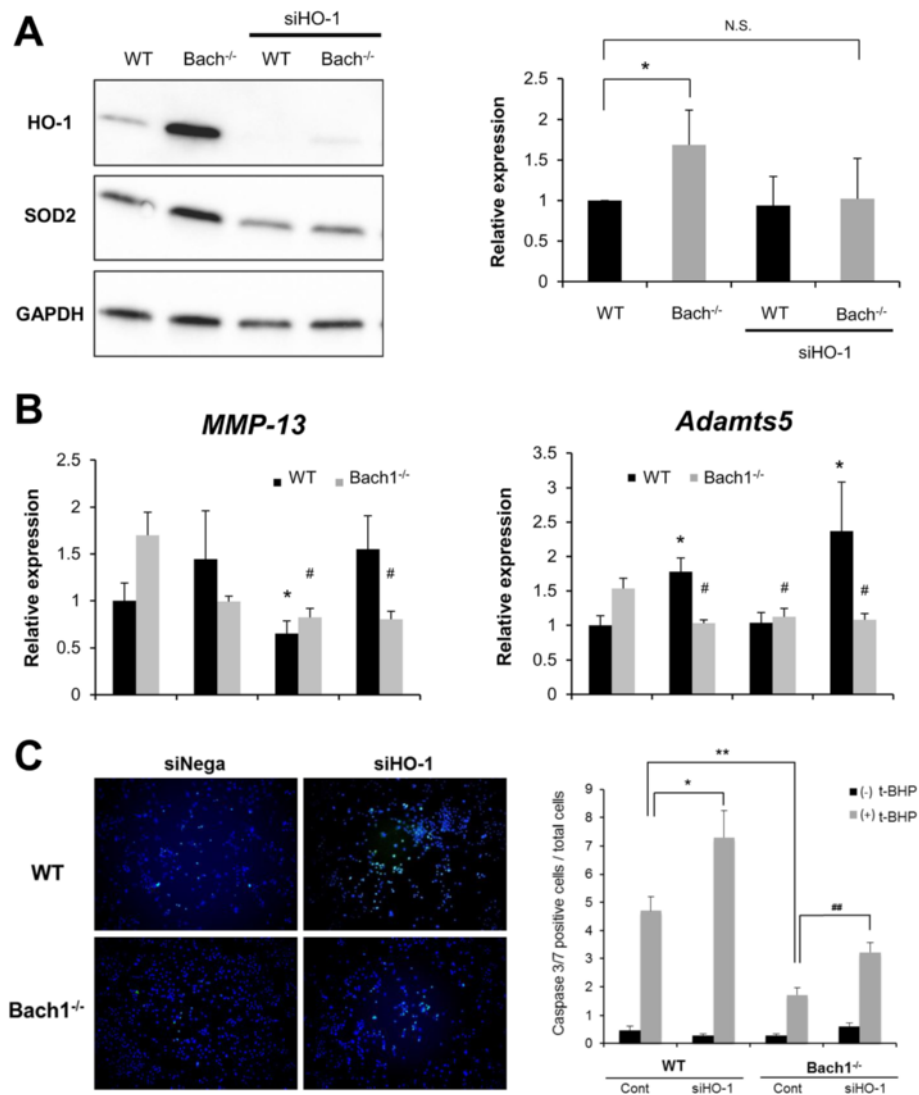


Fig. 5 The involvement of heme oxygenase-1 (*HO-1*) in changes observed in *Bach1*^{-/-} articular chondrocytes. Small interfering *HO-1* (*siHO-1*) or control siRNA (10 μ M) was transfected into chondrocytes from wild-type (*WT*) and *Bach1*^{-/-} mice at 1 month of age ($n=4$ per group). **a** The expression of superoxide dismutase 2 (*SOD2*) protein was detected by immunoblot analysis. Values are the mean \pm SD. Statistical analysis was performed with the Steel-Dwass test; * $P < 0.05$ versus wild-type chondrocytes. **b** The expression of *Mmp-13* and *Adamts-5* in *Bach1*^{-/-} chondrocytes with IL-1 β (1 ng/ml) for 24 h. Values are the mean \pm SD. Statistical analysis was performed with the Steel test; * $P < 0.05$ versus wild-type chondrocytes (-IL); # $P < 0.05$ versus *Bach1*^{-/-} chondrocytes (-IL). **c** Caspase-3/7 was detected in wild-type and *Bach1*^{-/-} mouse chondrocytes with or without the oxidant tert-butyl hydroperoxide (*t-BHP*) (200 μ M) for 5 h. Values are the mean \pm SD. Statistical analysis was performed with the Steel-Dwass test; * $P < 0.05$, ** $P < 0.01$ versus wild-type chondrocytes with *t-BHP*; ## $P < 0.01$ versus *Bach1*^{-/-} chondrocytes treated with *t-BHP*. *GAPDH* glyceraldehyde-3-phosphate dehydrogenase

13, *Adamts-5* and apoptosis, key OA-related factors, were suppressed in *Bach1*^{-/-} mouse chondrocytes under stress, only apoptosis was significantly regulated by *HO-1*. Our findings indicate that the protective effects against OA development in *Bach1*^{-/-} mice appear to be antioxidant activity and cytoprotective effects through *HO-1* in chondrocytes and downregulation of ECM-degrading enzymes. Thereby, *Bach1* deficiency coordinates maintenance of cartilage homeostasis and joint health.

Our observations suggest that inducers of *HO-1* may be effective therapeutic agents for OA prevention. Local gene delivery of *HO-1* into the mouse knee joint results in transduction of the joint tissues such as synovium, except for cartilage, however, it does not reduce OA-like changes [39]. This result suggests that the expression of *HO-1* in cartilage is important for the protection and the treatment of OA. Thus, the pharmacological inhibition of *Bach1* and induction of *HO-1* in cartilage might have potential in the prevention or treatment of OA.

Conclusions

Bach1^{-/-} mice have reduced severity of OA in aging and surgical models and this is at least in part mediated through regulation of HO-1, ECM degrading enzymes and chondrocyte viability. These findings suggest that approaches to reduce Bach1 expression have potential as a novel therapeutic approach.

Abbreviations

Acan: aggrecan; Adamts-5: a disintegrin and metalloproteinase with thrombospondin motifs-5; Bach1: BTB and CNC homology 1; cDNA: complementary DNA; Col2a1: collagen type II alpha 1; DMEM: Dulbecco's modified Eagle's medium; ECM: extracellular matrix; HO-1: heme oxygenase-1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HRP: horseradish peroxidase; IgG: immunoglobulin G; IL-1 β : Interleukin-1 β ; LC3: microtubule-associated protein 1 light chain 3; MCL: medial collateral ligament; Mmp13: matrix metalloproteinase 13; MMTL: medial meniscotibial ligament; MnSOD: manganese superoxide dismutase; OA: osteoarthritis; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; ROS: reactive oxygen species; siRNA: small interfering RNA; SOD2: superoxide dismutase 2; t-BHP: tert-butyl hydroperoxide.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TT conceived of the study, carried out the experimental work using mice, the histological assessment and the statistical analysis, drafted the manuscript and revised the manuscript. SM participated in its design and coordination, interpreted the data, drafted the manuscript and revised the manuscript. HI carried out the gene expression assay, the immunoassays and the apoptosis assay, and helped to revise the manuscript. YH and TN carried out the immunoassays and participated in histological assessment, and helped to revise the manuscript. KI provided Bach1-deficient mice and information about the mouse, and helped to draft the manuscript. MKL interpreted the data, helped to draft the manuscript and revised the manuscript. MO participated in its design, coordination and interpretation of the data, and contributed to drafting and revising the manuscript. All authors made substantive intellectual contributions to the study and read and approved the final manuscript.

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References

- Lotz MK, Carames B. Autophagy and cartilage homeostasis mechanisms in joint health, aging and OA. *Nat Rev Rheumatol*. 2011;7:579–87.
- Miyaki S, Asahara H. Macro view of microRNA function in osteoarthritis. *Nat Rev Rheumatol*. 2012;8:543–52.
- Carlo Jr MD, Loeser RF. Increased oxidative stress with aging reduces chondrocyte survival: correlation with intracellular glutathione levels. *Arthritis Rheum*. 2003;48:3419–30.
- Loeser RF, Carlson CS, Del Carlo M, Cole A. Detection of nitrotyrosine in aging and osteoarthritic cartilage: Correlation of oxidative damage with the presence of interleukin-1beta and with chondrocyte resistance to insulin-like growth factor 1. *Arthritis Rheum*. 2002;46:2349–57.
- Scott JL, Gabrielides C, Davidson RK, Swingle TE, Clark IM, Wallis GA, et al. Superoxide dismutase downregulation in osteoarthritis progression and end-stage disease. *Ann Rheum Dis*. 2010;69:1502–10.
- Son Y, Lee JH, Chung HT, Pae HO. Therapeutic roles of heme oxygenase-1 in metabolic diseases: curcumin and resveratrol analogues as possible inducers of heme oxygenase-1. *Oxid Med Cell Longev*. 2013;2013:639541.
- Miriyala S, Holley AK, St Clair DK. Mitochondrial superoxide dismutase—signals of distinction. *Anticancer Agents Med Chem*. 2011;11:181–90.
- Morse D, Choi AM. Heme oxygenase-1: the “emerging molecule” has arrived. *Am J Respir Cell Mol Biol*. 2002;27:8–16.
- Benallaoua M, Francois M, Batteux F, Thelier N, Shyy JY, Fitting C, et al. Pharmacologic induction of heme oxygenase 1 reduces acute inflammatory arthritis in mice. *Arthritis Rheum*. 2007;56:2585–94.
- Brines R, Maicas N, Ferrandiz ML, Loboda A, Jozkovicz A, Dulak J, et al. Heme oxygenase-1 regulates the progression of K/BxN serum transfer arthritis. *PLoS One*. 2012;7, e52435.
- Devesa I, Ferrandiz ML, Terencio MC, Joosten LA, van den Berg WB, Alcaraz MJ. Influence of heme oxygenase 1 modulation on the progression of murine collagen-induced arthritis. *Arthritis Rheum*. 2005;52:3230–8.
- Sun J, Hoshino H, Takaku K, Nakajima O, Muto A, Suzuki H, et al. Hemoprotein Bach1 regulates enhancer availability of heme oxygenase-1 gene. *EMBO J*. 2002;21:5216–24.
- Ohta R, Tanaka N, Nakanishi K, Kamei N, Nakamae T, Izumi B, et al. Heme oxygenase-1 modulates degeneration of the intervertebral disc after puncture in Bach 1 deficient mice. *Eur Spine J*. 2012;21:1748–57.
- Omura S, Suzuki H, Toyofuku M, Ozono R, Kohno N, Igarashi K. Effects of genetic ablation of bach1 upon smooth muscle cell proliferation and atherosclerosis after cuff injury. *Genes Cells*. 2005;10:277–85.
- Yamada K, Tanaka N, Nakanishi K, Kamei N, Ishikawa M, Mizuno T, et al. Modulation of the secondary injury process after spinal cord injury in Bach1-deficient mice by heme oxygenase-1. *J Neurosurg Spine*. 2008;9:611–20.
- Ochiai S, Mizuno T, Deie M, Igarashi K, Hamada Y, Ochi M. Oxidative stress reaction in the meniscus of Bach 1 deficient mice: potential prevention of meniscal degeneration. *J Orthop Res*. 2008;26:894–8.
- Kamekura S, Hoshi K, Shimoaka T, Chung U, Chikuda H, Yamada T, et al. Osteoarthritis development in novel experimental mouse models induced by knee joint instability. *Osteoarthritis Cartilage*. 2005;13:632–41.
- Kuroki H, Nakagawa Y, Mori K, Ohba M, Suzuki T, Mizuno Y, et al. Acoustic stiffness and change in plug cartilage over time after autologous osteochondral grafting: correlation between ultrasound signal intensity and histological score in a rabbit model. *Arthritis Res Ther*. 2004;6:R492–504.
- Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. *J Bone Joint Surg Am*. 1971;53:523–37.
- Pauli C, Grogan SP, Patil S, Otsuki S, Hasegawa A, Koziol J, et al. Macroscopic and histopathologic analysis of human knee menisci in aging and osteoarthritis. *Osteoarthritis Cartilage*. 2011;19:1132–41.
- Krenn V, Morawietz L, Haupl T, Neidel J, Petersen I, König A. Grading of chronic synovitis – a histopathological grading system for molecular and diagnostic pathology. *Pathol Res Pract*. 2002;198:317–25.
- Carames B, Hasegawa A, Taniguchi N, Miyaki S, Blanco FJ, Lotz M. Autophagy activation by rapamycin reduces severity of experimental osteoarthritis. *Ann Rheum Dis*. 2012;71:575–81.
- Sasaki H, Takayama K, Matsushita T, Ishida K, Kubo S, Matsumoto T, et al. Autophagy modulates osteoarthritis-related gene expression in human chondrocytes. *Arthritis Rheum*. 2012;64:1920–8.
- Dohi Y, Ikura T, Hoshikawa Y, Katoh Y, Ota K, Nakanome A, et al. Bach1 inhibits oxidative stress-induced cellular senescence by impeding p53 function on chromatin. *Nat Struct Mol Biol*. 2008;15:1246–54.
- Okita Y, Kamoshida A, Suzuki H, Itoh K, Motohashi H, Igarashi K, et al. Transforming growth factor-beta induces transcription factors MafK and Bach1 to suppress expression of the heme oxygenase-1 gene. *J Biol Chem*. 2013;288:20658–67.

26. Ishikawa M, Numazawa S, Yoshida T. Redox regulation of the transcriptional repressor Bach1. *Free Radic Biol Med*. 2005;38:1344–52.
27. Ota K, Brydun A, Itoh-Nakadai A, Sun J, Igarashi K. Bach1 deficiency and accompanying overexpression of heme oxygenase-1 do not influence aging or tumorigenesis in mice. *Oxid Med Cell Longev*. 2014;2014:757901.
28. Harusato A, Naito Y, Takagi T, Uchiyama K, Mizushima K, Hirai Y, et al. BTB and CNC homolog 1 (Bach1) deficiency ameliorates TNBS colitis in mice: role of M2 macrophages and heme oxygenase-1. *Inflamm Bowel Dis*. 2013;19:740–53.
29. Hama M, Kirino Y, Takeno M, Takase K, Miyazaki T, Yoshimi R, et al. Bach1 regulates osteoclastogenesis in a mouse model via both heme oxygenase 1-dependent and heme oxygenase 1-independent pathways. *Arthritis Rheum*. 2012;64:1518–28.
30. Garcia-Arandis I, Guillen MI, Castejon MA, Gomar F, Alcaraz MJ. Haem oxygenase-1 down-regulates high mobility group box 1 and matrix metalloproteinases in osteoarthritic synoviocytes. *Rheumatology (Oxford)*. 2010;49:854–61.
31. Guillen M, Megias J, Gomar F, Alcaraz M. Haem oxygenase-1 regulates catabolic and anabolic processes in osteoarthritic chondrocytes. *J Pathol*. 2008;214:515–22.
32. Megias J, Guillen MI, Clerigues V, Rojo AI, Cuadrado A, Castejon MA, et al. Heme oxygenase-1 induction modulates microsomal prostaglandin E synthase-1 expression and prostaglandin E(2) production in osteoarthritic chondrocytes. *Biochem Pharmacol*. 2009;77:1806–13.
33. El Assar M, Angulo J, Rodriguez-Manas L. Oxidative stress and vascular inflammation in aging. *Free Radic Biol Med*. 2013;65:380–401.
34. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. *Cell*. 2011;147:728–41.
35. Ruiz-Romero C, Calamia V, Mateos J, Carreira V, Martinez-Gomariz M, Fernandez M, et al. Mitochondrial dysregulation of osteoarthritic human articular chondrocytes analyzed by proteomics: a decrease in mitochondrial superoxide dismutase points to a redox imbalance. *Mol Cell Proteomics*. 2009;8:172–89.
36. Carames B, Taniguchi N, Otsuki S, Blanco FJ, Lotz M. Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. *Arthritis Rheum*. 2010;62:791–801.
37. Zhang Y, Vasheghani F, Li YH, Blati M, Simeone K, Fahmi H, et al. Cartilage-specific deletion of mTOR upregulates autophagy and protects mice from osteoarthritis. *Ann Rheum Dis*. 2015;74:1432–40.
38. Gavriilidis C, Miwa S, von Zglinicki T, Taylor RW, Young DA. Mitochondrial dysfunction in osteoarthritis is associated with down-regulation of superoxide dismutase 2. *Arthritis Rheum*. 2013;65:378–87.
39. Koike M, Nojiri H, Ozawa Y, Watanabe K, Muramatsu Y, Kaneko H, et al. Mechanical overloading causes mitochondrial superoxide and SOD2 imbalance in chondrocytes resulting in cartilage degeneration. *Sci Rep*. 2015;5:11722.

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