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Intracellular Localization of Galectin-3 has a Protective Role

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ABSTRACT

Objective: Although galectin-3 (gal-3) is expressed during arthritic disorders, its roles have never been described. The aim of the study was to determine the intracellular roles of gal-3 in chondrocytes and cartilage.

Methods: Following treatment with sodium nitroprusside, a cell death inducer, intracellular levels of total and phosphorylated gal-3 were measured by immunoblots in human osteoarthritic (OA) chondrocytes. Cell viability was also assessed by the lactate dehydrogenase activity in conditioned media from OA chondrocytes or from ATDC5 cells transfected with a gal-3-expressing vector. After generating an OA model by intra-articular injection of 0.5% mono-iodoacetate (MIA), histologic evaluation of articular cartilage and subchondral bone was performed in wild-type (WT) and gal-3 knockout (KO) mice aged 6 weeks and 4 months.

Results: In vitro experiments demonstrated that intracellular gal-3 had a protective role in chondrocyte survival which involved its phosphorylation. In contrast to 6-week old mice, 4-month-old gal-3 KO mice, compared to WT mice, presented OA-like cartilage modifications. OA induction via MIA injection in WT mice generated cartilage lesions similar to those found in gal-3 KO animals. Moreover, OA induction showed a significant decrease in subchondral bone surface in the gal-3 KO mice in contrast to the WT group.

Conclusion: Altogether these findings indicate that intracellular gal-3 has a beneficial effect in articular cells, as its absence in KO mice led to cartilage lesions.

INTRODUCTION

Due to their capacity to express several proteins found during endochondral bone formation, osteoarthritic (OA) chondrocytes are often compared to hypertrophic chondrocytes. Indeed, OA chondrocytes re-express annexins II and V [1,2], collagenase-3 [3,4] osteopontin [5], type X collagen [6], and galectin-3 (gal-3) [7].

Galectin-3 is an animal lectin that belongs to the galectin superfamily. Galectins, like other lectins, recognize a glycosylation structure with neither enzymatic nor immune activity [8]. The gal-3 gene was mapped on human chromosome 14 (14q21-22) and codes for a soluble protein of 30 kd [9,10]. Although first discovered in macrophages, gal-3 has been found to be more widely distributed in tissues, including the gut, brain, kidneys, and skeleton [11]. Data from the literature also suggest that gal-3, depending on its subcellular localization, is involved in a variety of processes such as RNA splicing [12], differentiation [13], apoptosis [14-16] and cell-cell or cell-matrix interactions [17-20].

In bone development, gal-3 is found in osteoblasts [21,22], osteoclasts [22] and in chondrocytes [21,22]. Specifically, gal-3 is expressed in the early hypertrophic chondrocytes of the growth plate; however, it is rarely found in the late hypertrophic chondrocytes undergoing terminal maturation in the calcified zone [23,24]. In our recent investigation on the capacity of human normal and OA chondrocytes to synthesize gal-3, we found that the level of gal-3 expression was increased in human OA articular cartilage [7]. A study by Colnot et al using a gal-3 knock-out (KO) mouse model suggested that gal-3 was implicated in chondrocyte death and in the vascular invasion coupling process [25].

In the present study, we further investigated the potential roles of intracellular gal-3 in articular tissues. For this purpose, we used human OA chondrocytes and wild type (WT) and gal-3 KO mice with or without OA induction.

MATERIALS AND METHODS

Human chondrocyte culture

Femoral condyles and tibial plateaus were obtained from 17 OA patients (F/M: 15/2; aged 67 ± 15) following total knee arthroplasty. All patients were evaluated by a certified rheumatologist and, based on the criteria developed by the American College of Rheumatology Diagnostic Subcommittee for Osteoarthritis [26], were diagnosed as having OA. Chondrocytes were released and cultured as previously described [3,27].

Total and phosphorylated gal-3 levels following sodium nitroprusside-induced chondrocyte death

Total galectin-3 levels were assessed in OA chondrocytes treated with sodium nitroprusside (SNP; 0 to 1 mM) for 2 or 5 hours. Then, the medium was removed replaced with fresh medium without SNP for a total incubation of 18 hours. At the end of the incubation, cells were lysed in a 0.5% sodium dodecyl sulfate (SDS) solution and ten µg of total proteins were loaded onto SDS polyacrylamide gel electrophoresis (SDS-PAGE).

Gal-3 phosphorylation determination was performed after treatment of OA chondrocytes with 1 mM SNP for up to 2 hours after which cells were immediately harvested. As the phospho-serine antibody (monoclonal 1C8; Calbiochem, San Diego, CA) did not discriminate gal-3 from other phosphorylated proteins having a similar molecular weight, we purified gal-3 from cell lysates according the following protocol. Cells were sonicated twice at 20 V (Vibra cell™; Sonics & Material Inc.) for 10 s and then centrifuged at 16,000 g for 15 min at 4°C. An aliquot of 20 µl of supernatant was saved for the immunoblotting of gal-3 (rabbit polyclonal anti-gal-3 antibody; dilution 1/40,000; Covalab, Lyon, France) and actin (rabbit polyclonal anti-actin antibody,

dilution 1/20,000; Sigma-Aldrich, Oakville, ON, Canada). This fraction will serve as a loading control. The remaining gal-3 was purified from the supernatant through incubation under agitation for 1 hour at 4°C with 20 µl lactose-agarose beads (Sigma-Aldrich) and centrifuged at 16,000 g for 2 min at 4°C. Beads were thrice rinsed with 500 µl cold phosphate-buffered saline (PBS) alone and thrice with 25 mM sucrose-containing PBS for 1 min using the centrifugations described above. Gal-3 was recovered by directly adding 20 µl of Laemmli's buffer under reducing conditions. Phosphorylated gal-3 was investigated by immunoblotting with the monoclonal anti-phospho-serine. For the evaluation of gal-3 phosphorylation, membranes were blocked in Tris-Tween buffered saline (TTBS) containing 3% bovine serum albumin (BSA), whereas the first and second antibodies were diluted in TTBS containing 0.3% BSA. The other steps were performed as described in the immunoblotting section.

Transfection of ATDC5 cells with a gal-3-expressing vector

The chondrogenic cell line ATDC5 (cell no. RCB0565; Riken Cell Bank, Tsukuba, Japan) was cultured as previously described [28,29]. Cells were seeded at a density of 100,000 cells/cm^2, left to recover overnight, and transfected with 1 µg each of a vector construct containing a full-length sequence of gal-3 cloned in pcDNA4/HisMax (Invitrogen) or in an empty vector using lipofectamine and Plus Reagent (Invitrogen), according to the manufacturer's instructions. The efficacy of the transfection was assessed by cotransfecting cells with 0.25 µg of luciferase-expressing vector (pGL3-control; Promega, Madison, WI).

Immunoblotting

Proteins were electrophoresed in discontinuous 4-12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) according to the method of Towbin et al [30]. After blotting, immunodetection was performed as previously described [7].

Galectin-3 KO mice and osteoarthritis mouse model

WT 129c/c mice and gal-3 KO 129c/c mice [31] were housed in wire cages in animal rooms with controlled temperature, humidity, and light cycles. Mice used in these studies were either 6 weeks old or 4 months old. They were allowed food and water ad libitum. After anaesthetization with Somnotol, OA was induced through a single injection of mono-iodoacetate (MIA; Sigma-Aldrich) into each knee joint of the four-month-old mice [32,33]. A 5 mg/ml MIA solution was prepared using sterile physiological saline. Following a skin incision, the intra-articular injection (2 µl) was administered under the patellar ligament using a Hamilton syringe with a 26G$^{3/8}$ intradermal needle. The day of MIA injection was considered day 0; the animals were sacrificed 7, 14, or 21 days after injection. The animals used as controls in this study were not injected as it was previously shown that saline injection had no effect [32,33]. All mouse studies were performed according to regulations established by the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Montreal Hospital Centre.

Cartilage histological grading and subchondral bone morphometry

Histological evaluation and bone morphometry measurement were performed on the sagittal sections of the mouse knee joint as previously described [34,35].

Protein assay

Cells were lysed in 0.5% SDS to quantify proteins with the bicinchoninic acid (BCA) assay [36].

Statistical analysis

Data are expressed as mean ± SEM or median [range]. Two-tailed Student's t or Mann-Whitney U tests were performed to assess the differences between groups; results of $p<0.05$ were considered significant.

RESULTS

The intracellular role of galectin-3 in chondrocyte survival

In the present study, we examined the role(s) played by gal-3 in chondrocyte physiology and, more particularly, its intracellular role in chondrocyte survival. To this end we investigated whether gal-3 was a key element in the process of chondrocyte survival when cells were treated with SNP. As represented in Figure 1A, data showed that when human OA chondrocytes were treated with SNP for 2 or 5 hours and then incubated in fresh medium overnight, the intracellular level of gal-3 was dose dependently decreased. The 5 hours of treatment with SNP abrogated the gal-3 level. In contrast, addition of NOC-12, a pure generator of nitric oxide (NO), prevented the SNP-induced gal-3 decrease and maintained gal-3 levels as in basal condition (Figure 1B). As it is suggested that nuclear factor kappa B (NF-κB) positively regulates gal-3 [37], human OA chondrocytes were incubated with SNP in the presence of lactacystin or MG-132, inhibitors of the NF-κB activation. Data showed that reduction of NF-κB activation in the presence of SNP further reduced gal-3 levels (Figure 1C). These results were confirmed when using a more specific NF-κB inhibitor such as SN-50 (supplemental data). NOC-12, which restored gal-3 levels reduced by SNP, decreased the lactate dehydrogenase (LDH) activity whereas MG-132 and lactacystin could not counteract this effect (Figure 1D).

It was previously suggested [38] that gal-3 has anti-apoptotic properties when it is phosphorylated. Our data (Figure 2A) showed that gal-3 was rapidly dephosphorylated during the first 2h of incubation with SNP, whereas the total gal-3 and actin levels remained constant. To definitively demonstrate the gal-3 protective role in chondrocyte survival, the chondrogenic, ATDC5 cell line, was transfected with either a gal-3-expressing vector or an empty one. Immunoblotting (Figure 2B) showed that only ATDC5 cells, transfected with the gal-3-expressing vector produced gal-3. When these transfected cells were incubated in the presence of SNP, data (Figure 2C) showed that increasing SNP concentrations enhanced LDH activity. Interestingly, cells transfected with the gal-3-expressing vector showed a statistically significant decrease in LDH activity regardless of the SNP concentration. Altogether these results demonstrated that intracellular gal-3 protected chondrocytes from cell death.

In vivo role of galectin-3 in the joint

The above results led us to investigate gal-3 effects on cartilage and subchondral bone by comparing WT and gal-3 KO mice during aging or after induction of OA with MIA.

Effects of gal-3 during aging.

Histology and histomorphometric analysis were carried out on both cartilage and subchondral bone in 4-month-old WT and gal-3 KO mice. Data showed that the global (cartilage and subchondral bone together) histologic score was 7.0 [2.0-10.0] (median [range]) and 10.0 [9.0-15.0] for WT and gal-3 KO mice, respectively. A statistically significant difference was obtained for the cartilage parameters (structure/surface, cellularity, and toluidine blue staining) between these two groups ($p=0.023$, Figures 3A and 3B). Indeed a score of 4.0 [2.0-5.0] was obtained for WT and 7.0 [7.0-8.0] for gal-3 KO mice. No differences in the cartilage score were noticed between 6-week-old and 4-month-old WT mice. In contrast, there was a significant increase in the cartilage score in gal-3 KO mice when the 6-week-olds were compared to the 4-month-olds ($p=0.05$).

Data showed that the subchondral bone surface of the 6-week-old WT mice was slightly higher (0.07 [0.04-0.09] μm^2) than that of the age-matched gal-3 KO group (0.06 [0.05-0.07] μm^2). However, when the mice reached 4 months of age, the subchondral bone surface for the WT and

gal-3 KO groups was nearly identical, with 0.09 [0.07-0.10] μm^2 and 0.09 [0.06-0.09] μm^2 respectively (data not illustrated).

The data suggests that gal-3 is essential for the cartilage homeostasis during aging since its absence induced OA-like lesions. In contrast, the absence of gal-3 did not seem to play a crucial role in the subchondral bone during the aging process.

Effects of gal-3 in an OA mouse model

MIA induced structural cartilage changes classically observed in OA, including cartilage surface irregularities and clefts; changes in cellularity (hypocellularity and clustering); loss of extracellular matrix integrity (decrease in toluidine blue staining); narrowing in the deep zone of calcified cartilage; and remodelling of the subchondral bone (Figures 4A and 4B).

The cartilage histologic score in the WT group increased after MIA injection and reached its maximum at day 14 (7.5 [5.0-10.0] p=0.003 vs control; Figure 5A), where the surface irregularity and structural changes were maximal (2.0 [1.0-5.0]) and the cell clustering the most abundant (2.0 [1.0-3.0]). The score tended to decrease at day 21. This was mainly due to a hypercellularity (1.0 [1.0-3.0]) and a reduction in clustering. Conversely, the score for the gal-3 KO group did not show statistical significance during the days following treatment (Figure 5B). With respect to subchondral bone surface, only a minimal decrease was seen in the WT group after MIA injection (Figure 5C). Interestingly, data showed a statistically significant difference in the subchondral bone surface in gal-3 KO mice at days 7 (p=0.008) and 21 (p=0.01) compared to the corresponding conditions in WT mice (median in μm^2: 0.065 vs 0.081 and 0.059 vs 0.074, respectively; Figure 5D).

DISCUSSION

In this study, we demonstrated that intracellular gal-3 plays a key protective role in chondrocytes which, when translated into in vivo mice, protects cartilage structure, as evidenced by an increased cartilage score in the gal-3 KO mice compared to the WT. In fact, cartilage deterioration in 4-month-old gal-3 KO mice was as severe as in WT mice injected with MIA, a treatment known to induce OA-like tissue damage. Moreover, gal-3 also appeared to be important for subchondral bone physiology as in gal-3 KO mice, there was a higher level of remodelling compared to WT mice during OA induction.

It is well known that the aging process modifies the extracellular matrix, thus prompting some changes in chondrocyte metabolism such as an increase in oxidative stress [39-41]. These changes could be compared to the process that chondrocytes are subjected to during endochondral ossification, where an elevated metabolic state is necessary. Therefore, in the absence of gal-3, the capacity of chondrocytes to sustain a high metabolic state, necessary to maintain cartilage of good quality for an extended period, could be weakened. This hypothesis is reinforced as it was shown that gal-3 prevents mitochondrial damage and cytochrome c release as well as the generation of reactive oxygen species [42,43]. Of interest, there is strong evidence to support the mitochondrial impairment of chondrocytes as a mediator of the establishment and progression of OA [44]. As gal-3 expression was lower in normal cartilage than in OA cartilage [7], one may assume that the increase of gal-3 in OA cartilage could be interpreted as a warning signal allowing some preservation of cartilage.

In order to investigate whether gal-3 was a key molecule for chondrocyte survival, chondrocytes were treated with SNP, which has been shown to induce cell death [45-50]. Our results agree with those of Colnot et al, which suggested that gal-3 could be a regulator of chondrocyte survival [25]. Our data showed that in human OA chondrocytes gal-3 levels were more dramatically decreased in SNP-treated chondrocytes than Bcl-2 levels in these cells under similar conditions [51]. This

indicates that, although there was some redundancy in the cell death preventing genes, some may have primordial roles depending on the cell type. Moreover, additional experiments performed with non transfected ATDC5 (i.e. cells with non gal-3 expression) showed that NOC-12 could not reverse SNP-induced death (data not shown). Therefore, the transfection of ATDC5 cells with a gal-3 expressing vector indicated that gal-3 is involved in the NOC-12 reversibility of SNP-induced death. The effect of gal-3 appears to be related to its phosphorylation state, which is in accordance with previous results [38].

When OA was induced in 4-month-old WT mice, the cartilage histological score was significantly increased and values were similar to those of control gal-3 KO mice. However, gal-3 KO mice did not respond to MIA treatment, which might be attributed to a decrease in the inflammatory component. Indeed, gal-3 KO mice have consistently been shown to develop less inflammation than WT mice [52,53]. However, inflammation is a part of the OA process, mainly by the release of pro-inflammatory cytokines from the synovial membrane [54].

The trend in decrease of subchondral bone surface induced by MIA in WT mice was further accentuated in gal-3 KO mice, suggesting that gal-3 may be essential in subchondral bone remodelling, as was recently demonstrated for carminerin [55], another protein expressed during endochondral ossification.

In summary, this study demonstrated a definite protective role for intracellular gal-3, suggesting that any downregulation of its expression during the OA process may induce structural changes in the joint.

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FIGURE LEGENDS

Figure 1. Intracellular gal-3 level in chondrocytes under cell death stimulation **(A)** or under recovering conditions with NOC-12 **(B)** and with NF-κB inhibitors **(C)**. Ten µg of cellular proteins were blotted and immunodetection was performed with a rabbit polyclonal anti-galectin-3 antibody. **(A)** Cells were treated with SNP for the indicated periods of time and then replaced in fresh medium for an 18h total incubation. **(B)** Cells were treated with SNP for 2h and then replaced in fresh medium containing NOC for an 18h incubation. **(C)** Cells were treated as indicated in **(B)** but NOC was replaced by NF-κB inhibitors (MG132; Lactacystin). Immunoblottings are representative of three independent experiments. **(D)** Cells were treated as indicated in **(B)** and LDH activity was assessed in the conditioned medium (n = 4).

Figure 2. (A) Levels of total and phosphorylated gal-3 under SNP treatment in human OA chondrocytes. Cells were incubated with 1 mM SNP for the indicated periods of time, then harvested and sonicated in cold PBS. Total and phosphorylated gal-3 as well as actin levels were then determined by immunoblotting as described in Materials and Methods. This is a representative of 3 independent experiments. **(B, C)** ATDC5 cell survival after transfection with a gal-3-expressing vector**.** ATDC-5 cells were transfected with either an empty vector or a gal-3-expressing vector. Immunodetection of three independent experiments done in duplicate was shown. **(C)** Cell survival was assessed measuring LDH activity in the conditioned medium. The luciferase activity was used to normalize the transfection efficacy (n = 4).

Figure 3. Cartilage histologic score for the WT and gal-3 KO mice. **(A)** Data are expressed as median and [range] and are presented in a box plot, where the boxes represent the 1st and 3rd quartiles, the line within the box represents the median, and the lines outside the box represent the spread of the values (n = 4 to 6 animals per group). P values <0.05 were considered significant. *: p vs 4-month-old WT group; #: p vs 6-week-old gal-3 KO group. **(B)** Representative histological sections of specimens from WT and gal-3 KO mice from the 6-week-old and 4 month-old stained with safranin O (upper panel) or toluidine blue (lower panel). Magnification x 100.

Figure 4. Representative histological sections of specimens from WT and gal-3 KO mice from the 4-month-old control and MIA injected mice stained with safranin O **(A)** or toluidine blue **(B)**. Magnification x 100.

Figure 5. Effect of MIA injection on the cartilage and on the subchondral bone in 4-month-old WT **(A,C)** and gal-3 KO **(B,D)** groups. The data presented are the average of 2 measurements per specimen in a 250 x 500 µm box. Data are the median [range] and were analysed using the Mann-Whitney U test (n = 4 to 6 animals per group). P values <0.05 were considered significant. *: p vs control (CTL) group, #: p vs the corresponding conditions in WT mice.

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Figure 1

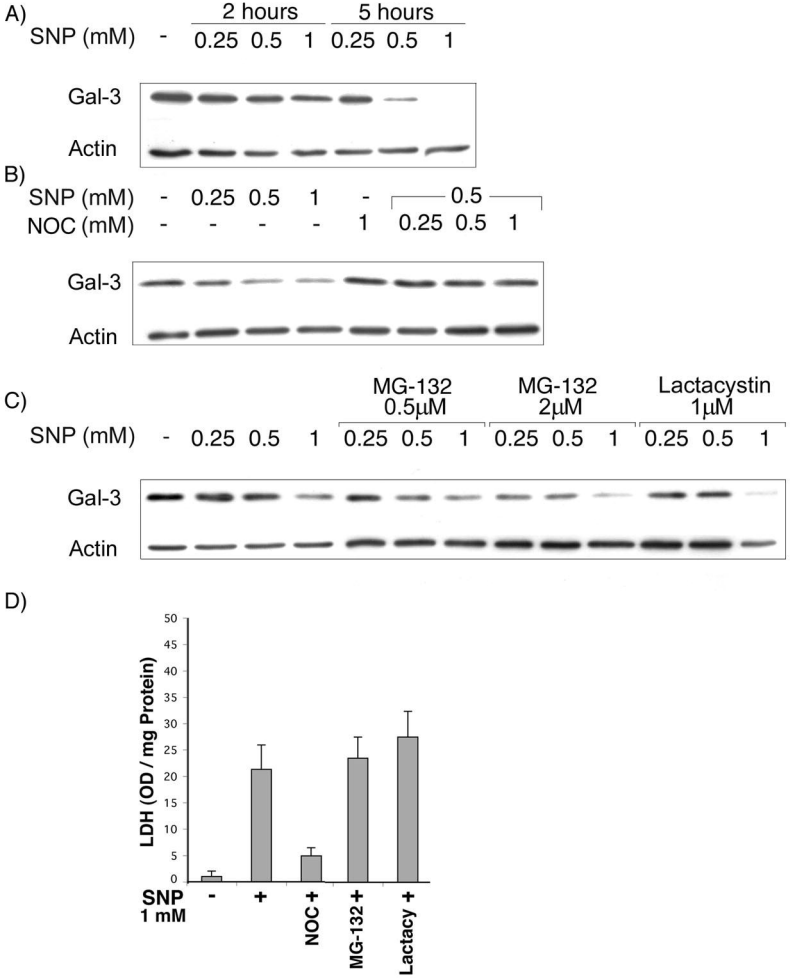
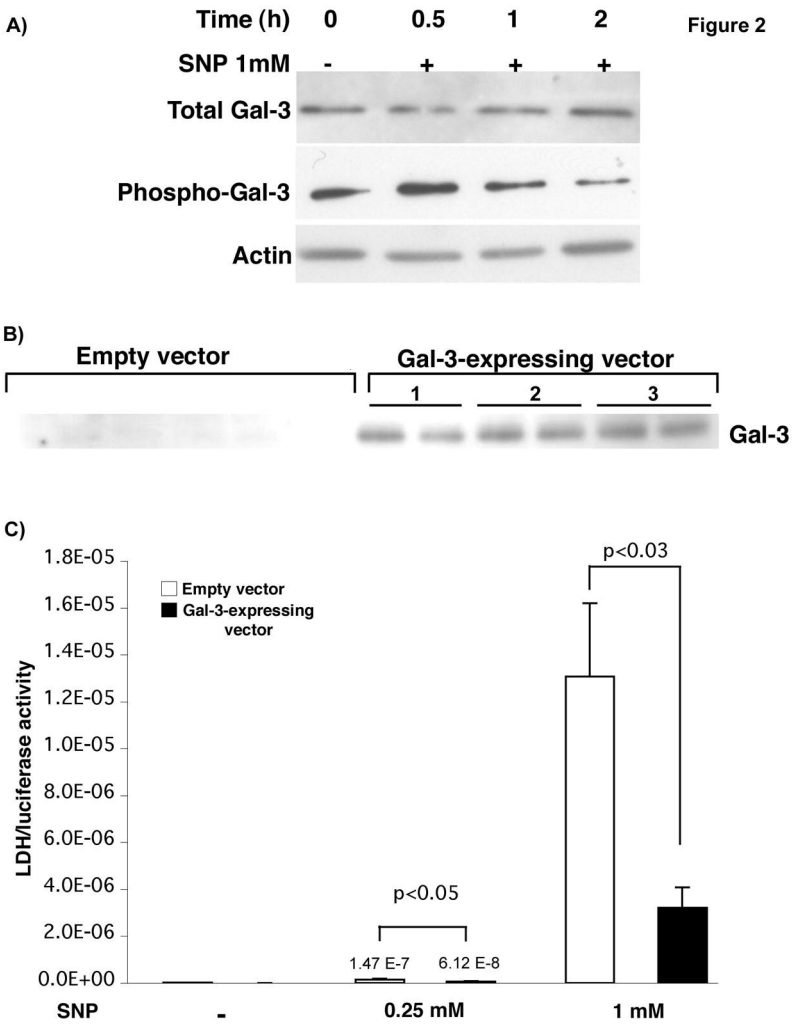
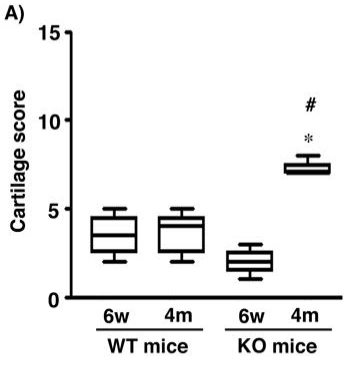

A)
2 hours
5 hours
SNP (mM) - 0.25 0.5 1 0.25 0.5 1
Gal-3
Actin
B)
SNP (mM) - 0.25 0.5 1 - 0.5
NOC (mM) - - - - 1 0.25 0.5 1
Gal-3
Actin
C)
MG-132 0.5μM
MG-132 2μM
Lactacystin 1μM
SNP (mM) - 0.25 0.5 1 0.25 0.5 1 0.25 0.5 1 0.25 0.5 1
Gal-3
Actin
D)
LDH (OD / mg Protein)
50
45
40
35
30
25
20
15
10
5
0
SNP 1 mM
-
+
NOC +
MG-132 +
Lactacy +

Figure 2


A)
Time (h) 0 0.5 1 2
SNP 1mM - + + +
Total Gal-3
Phospho-Gal-3
Actin
B)
Empty vector
Gal-3-expressing vector
1 2 3
Gal-3
C)
1.8E-05
1.6E-05
1.4E-05
1.2E-05
1.0E-05
8.0E-06
6.0E-06
4.0E-06
2.0E-06
0.0E+00
LDH/luciferase activity
Empty vector
Gal-3-expressing vector
p<0.03
p<0.05
1.47 E-7
6.12 E-8
SNP
-
0.25 mM
1 mM


A)
15
10
5
0
Cartilage score
#
*
6w
4m
6w
4m
WT mice
KO mice

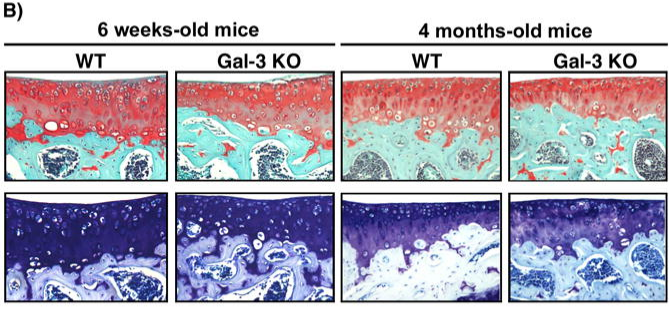

B)
6 weeks-old mice
4 months-old mice
WT
Gal-3 KO
WT
Gal-3 KO

Figure 4

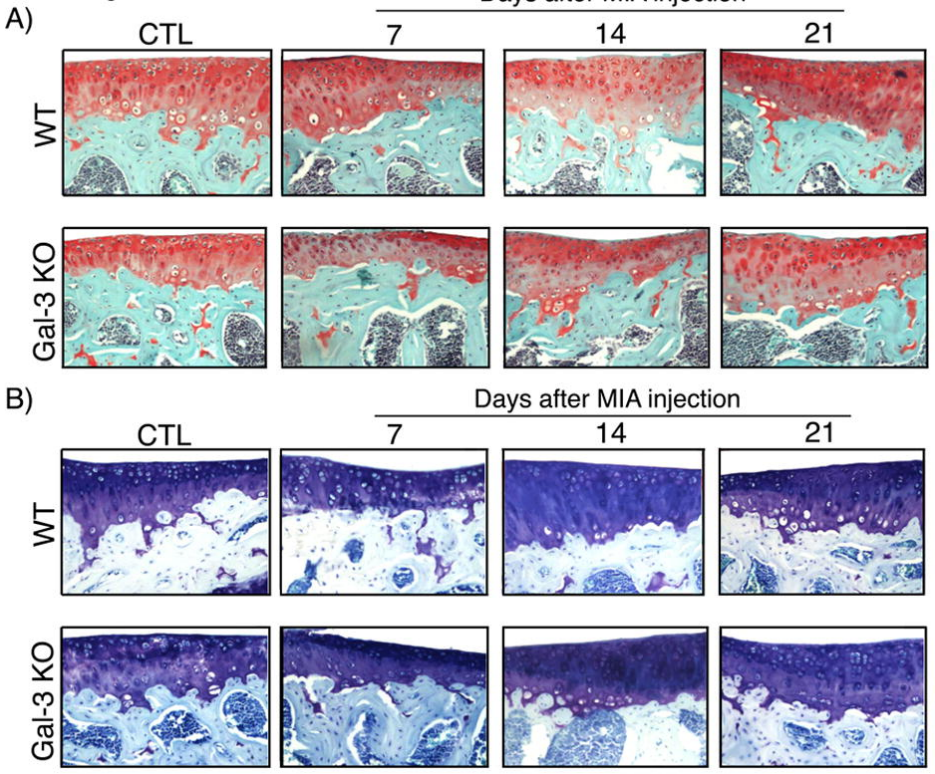
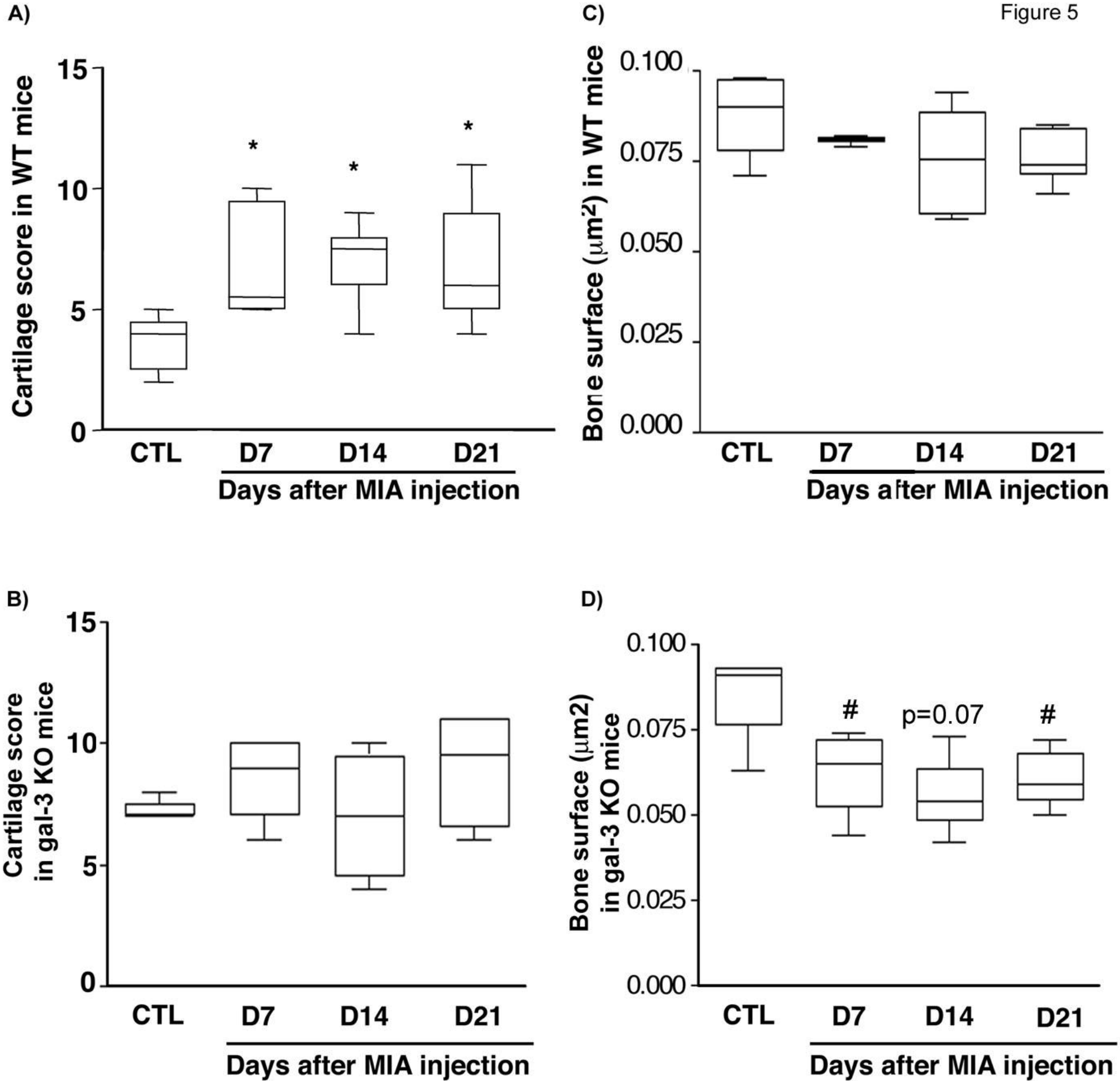
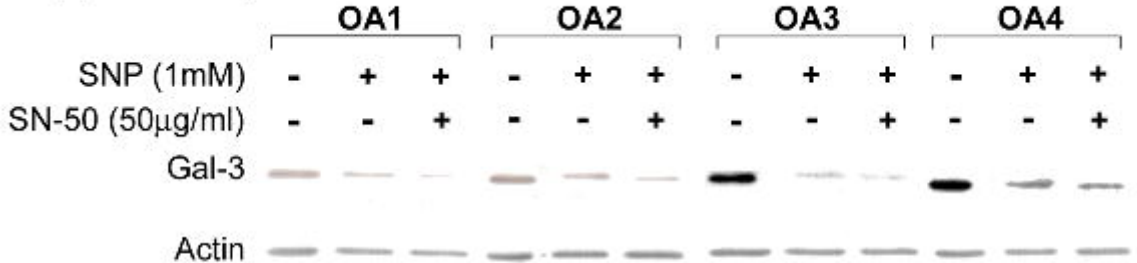

A)
Days after MIA injection
CTL
7
14
21
WT
Gal-3 KO
B)
Days after MIA injection
CTL
7
14
21
WT
Gal-3 KO

Figure 5


A)
Cartilage score in WT mice
15
10
5
0
*
*
*
CTL
D7
D14
D21
Days after MIA injection
C)
Bone surface (μm²) in WT mice
0.100
0.075
0.050
0.025
0.000
CTL
D7
D14
D21
Days after MIA injection
B)
Cartilage score in gal-3 KO mice
15
10
5
0
CTL
D7
D14
D21
Days after MIA injection
D)
Bone surface (μm2) in gal-3 KO mice
0.100
0.075
0.050
0.025
0.000
#
p=0.07
#
CTL
D7
D14
D21
Days after MIA injection

Supplemental figure



Cells were treated with SNP for 2h and then replaced in fresh medium containing SN50 for an 18h incubation. Immunoblottings of gal-3 and actin were performed with cell protein lysates. Four different specimens (OA1 to OA4) were analyzed.

Intracellular localization of Galectin-3 has a protective role

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Notes