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The role of tetraspanin CD9 in osteoarthritis using three different mouse models

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ABSTRACT

Although osteoarthritis (OA) is the most prevalent aging-related joint disease, the understanding of mechanisms of OA pathogenesis remains limited. Key features include the progressive degradation of articular cartilage, synovial hyperplasia, and angiogenesis in joint tissues. CD9, a member of the tetraspanin family, is localized in the cell membranes and partly in the endosomes of all mammalian cell types. CD9 is associated with inflammation and angiogenesis through cell adhesion, migration, and signal transduction. This study examined the role of CD9 in OA development in three different mouse models: an aging model, a surgical model and antigen-induced arthritis (AIA) model, using CD9 deficient mice. Our study showed that CD9 deficiency reduced the severity of hallmarks of OA including cartilage degradation and soft tissue inflammation in aged mice. In the AIA model, cartilage damage and inflammation were also reduced in $CD9^{-/-}$ mice. This was in contrast to the surgical OA model where disease severity was similar in wild-type and $CD9^{-/-}$ mice. *Col2a1* and *Aggrecan* expression was increased in chondrocytes of $CD9^{-/-}$ mice compared with those of wild-type mice. Our results indicate that the suppression of cartilage degradation in $CD9^{-/-}$ could be in part related to an increase in the expression of the two main cartilage extracellular matrix proteins aggrecan and type II collagen.

Key pathological features of all forms of arthritis include joint inflammation with infiltration of leukocytes in the synovium, causing joint tissue remodeling and destruction. Osteoarthritis (OA) is the most prevalent joint disease. It is characterized by degradation of articular cartilage and alterations in other joint tissues such as subchondral bone and the synovium. The critical risk factors are aging, trauma and inflammation, and these factors impair the homeostasis of joint tissues through dysregulation of intracellular signaling. The understanding of the mechanisms of OA pathogenesis remains limited (22).

CD9 is localized in the cell membranes and partly in the endosomes of all types of mammalian cells. It is a four membrane-spanning protein and a member of the tetraspanin family, which also includes CD63, CD81, CD82, and CD151. However, CD9 has unique functions in angiogenesis through cell adhesion, migration, and signal transduction, and it is involved in cell fusion processes linked to fertilization, osteoclastogenesis and myogenesis (2, 7, 9, 12, 15, 20,

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21, 24, 30). Furthermore, it was recently reported that CD9$^{-/-}$ mice developed both enhanced macrophage infiltration and TNF-α production in the lungs after intranasal administration of lipopolysaccharide (LPS) (23), while the up-regulation of CD9 decreased lung inflammation in mice (10). Regarding arthritis, previous reports have shown that CD9 is abundantly expressed in the OA synovium (14), in activated osteoclasts in osteoporosis, and in the sites of bone erosion in arthritic lesions of collagen-induced arthritis (8). However, the role of tetraspanins in OA pathogenesis remains unclear. The purpose of this study is to examine the OA development in CD9$^{-/-}$ mice using three different mouse models of arthritis: an aging model of primary OA, a surgical model of posttraumatic OA, and antigen-induced arthritis (AIA) as a transient inflammation model.

MATERIALS AND METHODS

Mice and arthritis models. CD9$^{-/-}$ mice on a C57BL/6J background have been described previously (16). All animal experiments were performed according to protocols approved by the Hiroshima University Animal Care and Use Committee. Male mice were used in the joint analysis in the aging model. Knee joints were evaluated histologically at 6 months (wild-type: $n = 9$, CD9$^{-/-}$: $n = 10$) and at 22 months (wild-type: $n = 11$, CD9$^{-/-}$: $n = 8$), in order to monitor spontaneous age-related OA. Experimental OA was induced by transecting the medial meniscotibial ligament and the medial collateral ligament in the right knees of wild-type mice ($n = 8$) and CD9$^{-/-}$ mice ($n = 8$) (3, 11). Female mice were used in the AIA model. AIA was induced in the knee joints of 10-week old wild-type mice ($n = 19$) and CD9$^{-/-}$ mice ($n = 19$) by intra-articular injection of methylated bovine serum albumin (mBSA) (Sigma-Aldrich, St. Louis, MO, USA) (17).

Histological assessment of the knee joints. The knee joints were embedded in paraffin after fixation and decalcification. Sagittal sections of the knee joints of wild-type mice and CD9$^{-/-}$ mice were stained with Safranin O/Fast Green (Sigma) for histopathological analysis. The severity of cartilage degeneration in aging and surgical models was evaluated using the OARSI scoring system (4). The severity of inflammation in all models was evaluated using five parameters: synovitis, joint space exudate, soft tissue inflammation, cartilage degradation, and bone damage. For each parameter, a score of 0 indicated none, 1 indicated mild, 2 indicated moderate, and 3 indicated severe change (29).

Quantitative real-time polymerase chain reaction. Articular chondrocytes were isolated from 1-month old wild-type mice and CD9$^{-/-}$ mice (25). They were seeded at 5×10^4 cells/well on 24 well plates, and with 1 ng/mL interleukin-1β (IL-1β) (WAKO, Osaka, Japan) for an additional 24 h. Total RNA was extracted from chondrocytes using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized using 500 ng of total RNA with the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific). TaqMan Gene Expression Assay probes (Life Technologies) were used to detect *Col2a1* (Mm01309565_m1), *Acan* (Mm00545807), *Mmp-13* (Mm01168713), *Adamts-5* (Mm01344182_m1). *Gapdh* (Mm99999915_g1) was used as the internal control to normalize the sample differences. The ΔΔCt method was used to analyze the real-time PCR data.

Proteoglycan release assay. Femoral heads were harvested from 4-week old wild type mice and incubated at 37°C for 72 h in 48 well plates. Each well contained 500 μL of DMEM with 10% FBS and 1% penicillin/streptomycin. The cartilage samples were washed 3 times, and cultured at 37°C for an additional 72 h in 500 μL of serum-free DMEM with IL-1β (1 ng/mL) or without IL-1β. The assay was performed on least 3 independent experiments with duplicate wells. The concentration of the released glycosaminoglycan in the conditioned cartilage medium was measured using the Blyscan Glycosaminoglycan assay kit (Biocolor; County Antrim, UK) per the manufacturer's protocol.

Immunohistochemical analysis. Knee joint sections were immunostained with anti-alpha-smooth muscle Actin antibody (Abcam, Austin, TX, USA, ab5694; 1 : 200) and anti-ADAMTS-5 antibody (Abcam, ab52947; 1 : 75), using Vectastain ABC-AP alkaline phosphatase (Vector Laboratories, Burlingame, CA, USA) as described previously (1), and with anti-MMP-13 antibody (Abgent, AP1801a; 1 : 100), using 3,3'-diaminobenzidine (DAB) substrate as described previously (25).

Statistical analysis. The data were analyzed by the Mann-Whitney U test, Steel or Steel-Dwass. Differences were considered statistically significant at * = $P < 0.05$ and ** = $P < 0.01$.

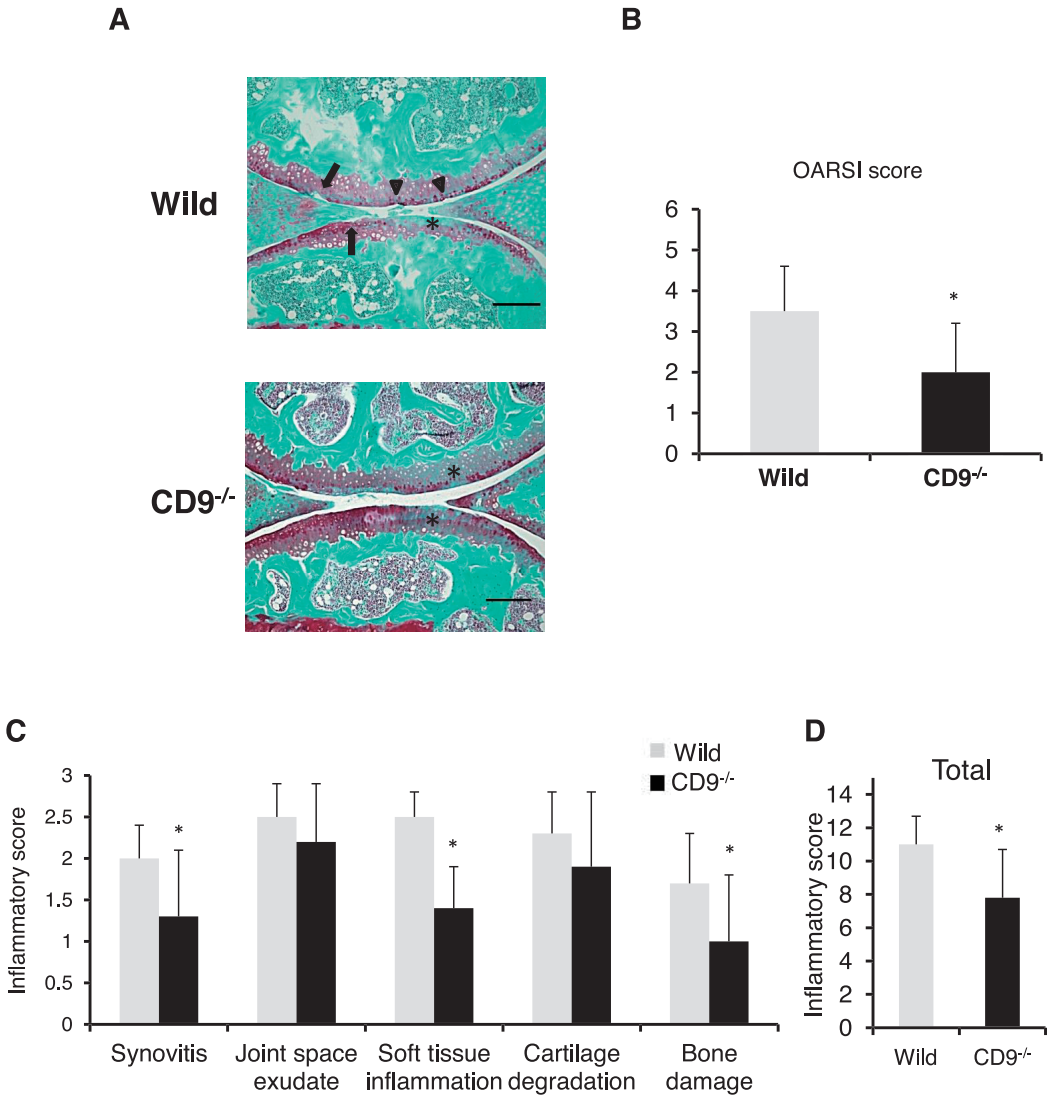

Fig. 1 CD9$^{-/-}$ mice exhibit reduced severity of OA in aged 22-month old mice. **A**. Knee joints from 22-month old wild-type and CD9$^{-/-}$ mice were collected, and the sections were assessed by Safranin O/Fast Green staining. Scale Bars = 200 μm. These samples exhibited thickness defect of cartilage (arrow), cartilage fibrillation (arrowhead), loss of Safranin O (asterisk). These changes were less severe in CD9$^{-/-}$ mice. **B**. Histological assessment using OARSI scoring system of spontaneous cartilage degeneration exacerbated with aging in wild-type mice and CD9$^{-/-}$ mice. **C**. Histological assessment using inflammatory scoring system in mice at 22 months old, including 5 parameters: synovitis, joint space exudate, soft tissue inflammation, cartilage degradation, and bone damage. **D**. Total score of 5 inflammatory parameters. Values are the mean ± SD. Statistical analysis was performed with the Mann-Whitney U test. * = $P < 0.05$ versus wild-type mice.

RESULTS

Age-related OA in CD9$^{-/-}$ mice

To reveal whether CD9 is involved in the development of OA with aging, we examined the knee joints of wild-type and CD9$^{-/-}$ mice at 6- and 22-months. CD9$^{-/-}$ mice exhibited normal skeletal development. From 1 to 6 months of age, both strains of mice showed intact articular cartilage and similar proteoglycan staining (data not shown). At 22 months of age, OA severity in wild-type mice ranged from minimal changes to cartilage fibrillation or partial and full thickness defects with exposure of subchondral bone (Fig. 1A). These changes were less severe in CD9$^{-/-}$ mice (Fig. 1A), and the OARSI score indicated significantly decreased severity of OA in the CD9$^{-/-}$ mice compared with that in the wild-type mice (Fig. 1B). In the inflammation scores, synovitis, soft tissue inflammation and bone damage in the knee joint were less severe in the CD9$^{-/-}$ mice, and

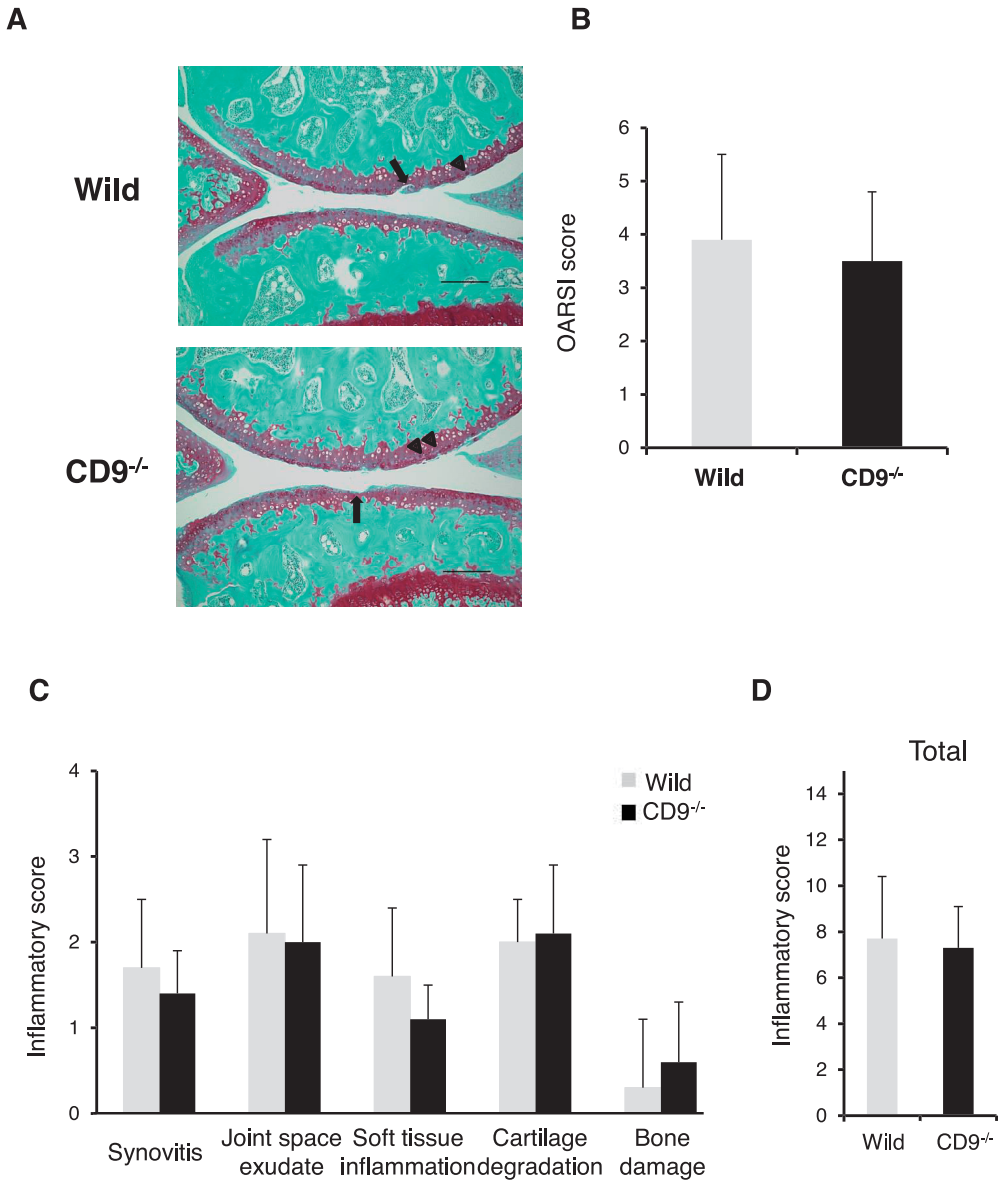

Fig. 2 Histological assessment in knee joints of mice with surgical OA. **A**. Knee joints from wild-type and CD9$^{-/-}$ mice with surgical OA were collected and the sections were assessed by Safranin O/Fast Green staining. Scale Bars = 200 μm. **B**. Histological assessment using OARSI scoring system of spontaneous cartilage degeneration in wild-type mice and CD9$^{-/-}$ mice. **C**. Histological assessment using inflammatory scoring system in 10-week old mice, including 5 parameters: synovitis, joint space exudate, soft tissue inflammation, cartilage degradation and bone damage. **D**. Total score of 5 inflammatory parameters. Values are the mean ± SD. Statistical analysis was performed using the Mann-Whitney U test.

the total inflammation score was also decreased in the CD9$^{-/-}$ mice (Fig. 1C, D).

Surgical posttraumatic OA model

A surgical model of posttraumatic OA was generated in 10-week-old wild-type and CD9$^{-/-}$ mice by transecting the medial meniscotibial ligament and the medial collateral ligament. In this model, OA-like changes such as loss of safranin-O or cartilage fibrillation, vertical clefts and erosion with sclerosis of subchondral bone were observed in similar patterns in both wild-type and CD9$^{-/-}$ mice at 12 weeks after surgery (Fig. 2A). However, the OARSI score (Fig. 2B) and inflammation score (synovitis, joint space exudate, soft tissue inflammation, cartilage degradation, and bone damage) were not significantly different between the wild-type and CD9$^{-/-}$ mice (Fig. 2C, D).

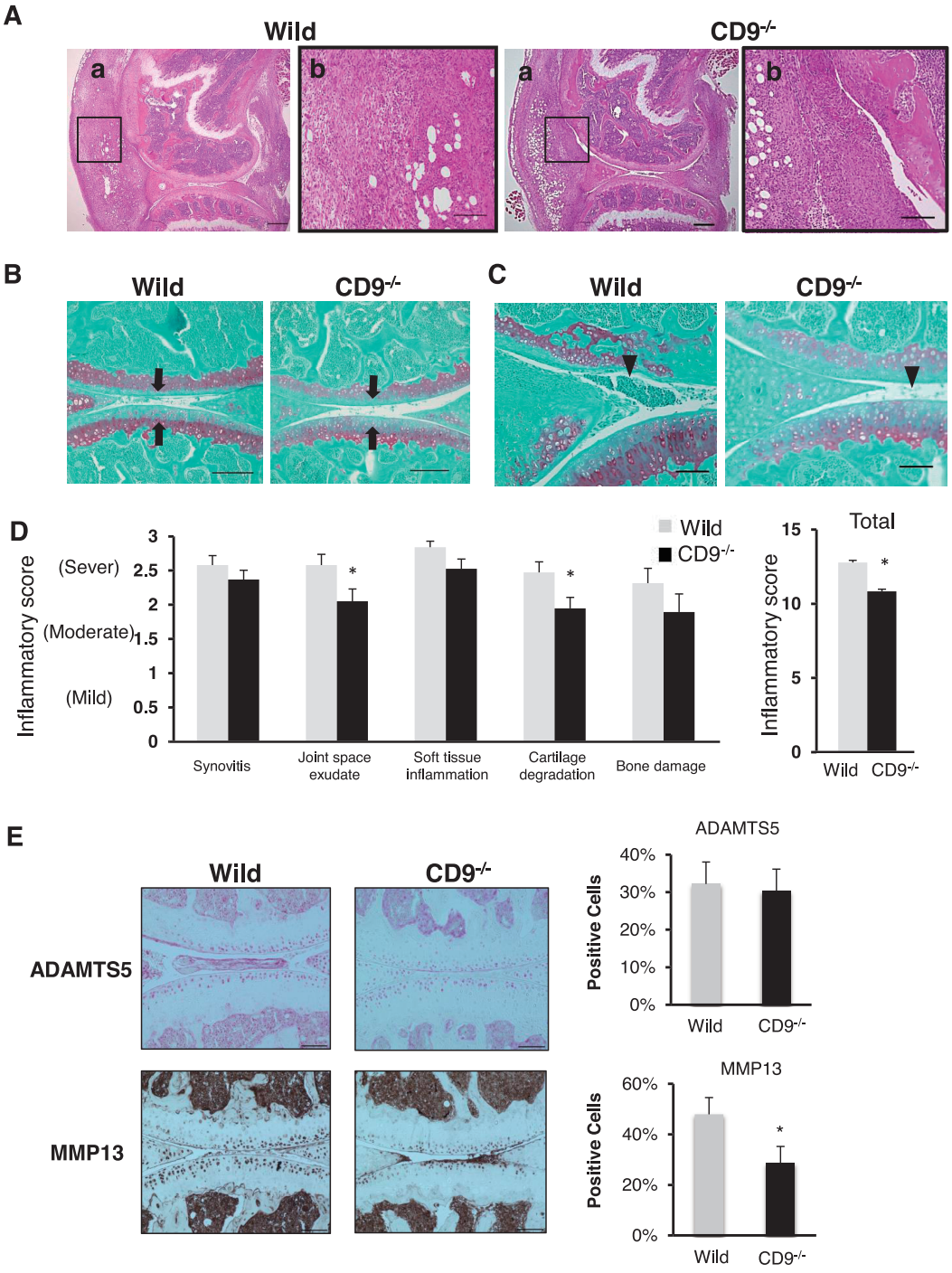

Fig. 3 Histological assessment of knee joints in the AIA model. **A**. Knee joints from wild-type and CD9$^{-/-}$ mice of AIA model were collected, and the sections were assessed by H-E staining. In both wild-type and CD9$^{-/-}$ mice, synovial hyperplasia was exhibited. (a) Scale Bars = 300 μm. (b) Scale Bars = 200 μm **B**. Photographs of a knee from AIA mouse model focused on the difference in degree of cartilage degradation (arrow) between the wild-type and CD9$^{-/-}$ mouse. Scale Bars = 200 μm. **C**. Photographs of a knee from AIA mouse model focused on the difference in degree of joint space exudate (arrowhead) between the wild-type and CD9$^{-/-}$ mouse. Scale Bars = 100 μm. **D**. Histological assessment using the inflammatory scoring system in 10-week old mice, including 5 parameters. Values are the mean ± SD. Statistical analysis was performed using the Mann-Whitney U test. * = $P < 0.05$ versus wild-type mice. **E**. Knee joints from wild-type mice and CD9$^{-/-}$ mice were analyzed by immunohistochemistry for ADAMTS-5 and MMP-13. Total numbers of ADAMTS-5 and MMP-13 positive cells in six fields were counted, and the percentage of positive cells was calculated. Values are the mean ± SD. Statistical analysis was performed with the Mann-Whitney U test. * = $P < 0.05$ versus wild-type mice. Scale Bars = 200 μm.

Inflammatory arthritis model

We further examined the role of CD9 in the AIA model. Inflammatory arthritis signs such as synovial hyperplasia (Fig. 3A), and proteoglycan loss (Fig. 3B) were exhibited in both the wild-type and CD9$^{-/-}$ mice. The scores for synovitis, soft tissue inflammation and bone damage were not significantly different between the wild-type and CD9$^{-/-}$ mice (Fig. 3D). However, the cartilage degeneration (Fig. 3B), joint space exudate of leukocytes (Fig. 3C), and the total score for AIA were significantly lower in the CD9$^{-/-}$ mice than in the wild-type mice (Fig. 3D). The expression of ADAMTS-5 and MMP-13, main cartilage degradation enzymes, was characterized by immunohistochemistry in the articular cartilage of mice with AIA. Although ADAMTS-5 was not significantly different, the number of MMP-13 positive chondrocytes was significantly lower in the CD9$^{-/-}$ mice than in the wild-type mice (Fig. 3E).

Effect of CD9 deficiency on articular chondrocytes

IL-1β is one of the critical mediators driving the inflammatory process which leads to OA pathogenesis. To determine whether CD9 in cartilage is important for the degradation of the cartilage-related matrix, we quantified proteoglycan release from mouse femoral head cartilage explants. Cartilage explants from wild-type and CD9$^{-/-}$ mice treated with IL-1β showed significantly increased proteoglycan release. However, it was not significantly different between the wild-type and CD9$^{-/-}$ mice with or without IL-1β (Fig. 4A). *Adamts-5* mRNA levels with or without IL-1 treatment were also not significantly different between wild-type and CD9$^{-/-}$ chondrocytes (Fig. 4B). Although there was a trend towards reduced *Mmp-13* in IL-1β treated CD9$^{-/-}$ chondrocytes, this difference was not statistically significant from wild type chondrocytes (Fig. 4B). The levels of cartilage matrix genes *Col2a1* and *Aggrecan* in control were significantly higher in CD9$^{-/-}$ chondrocytes than in wild-type chondrocytes. In response to IL-1β stimulation, the levels of *Col2a1* and *Aggrecan* were significantly reduced in wild-type and CD9$^{-/-}$ chondrocytes but even under IL-1 treatment remained higher in CD9$^{-/-}$ chondrocytes (Fig. 4B).

DISCUSSION

This is the first study to demonstrate a role of CD9 in OA development in three different animal models. CD9 deficiency did not lead to apparent abnormalities in joint development and joints of CD9$^{-/-}$ mice were completely normal on histological analysis at 6 months. However, intra-articular inflammation and cartilage degeneration during aging was attenuated in the CD9$^{-/-}$ mice compared with the wild-type mice. Our study also showed CD9 deficiency had certain protective effects in the AIA model where it reduced the severity of cartilage degradation, some inflammation markers and the total AIA score. By contrast, the severity of the surgical OA model was not significantly different between wild-type and CD9$^{-/-}$ mice. Thus CD9 deficiency has certain protective effect in the aging and AIA models but not in the surgical model. This difference among the three models could be due to the fact that the surgical model is much more severe than the two other models. The profound joint instability caused by transection of the meniscotibial and the medial collateral ligaments may override potential protective effects of CD9 deletion in this model.

Previous research in macrophages suggested anti-inflammatory properties of CD9. Statins exert anti-inflammatory effects by up-regulating CD9 in macrophages, and the up-regulation decreases lung inflammation in mice (10). However, CD9 expression is up-regulated in the synovium of OA (14), and we also demonstrated an increase in CD9 expression by synovial fibroblasts with IL-1β stimulation, while CD9 expression was significantly decreased in chondrocytes with IL-1β (Supplemental Figure). Two research groups analyzed the effects of statins on OA using the STR/Ort spontaneous OA mouse model (28, 31), but observed divergent results.

In the present study, cartilage degradation was significantly reduced in CD9$^{-/-}$ mice in aging and in the AIA model. Thus we compared chondrocytes from CD9$^{-/-}$ and wild-type mice. *Col2a1* and *Aggrecan* expression was increased in chondrocytes of CD9$^{-/-}$ mice compared with wild-type mice. IL-1β-induced proteoglycan release from cartilage explants, and *Adamts-5* and *Mmp-13* expression were not significantly different between CD9$^{-/-}$ and wild-type chondrocytes. Thus, these results suggest that the suppression of cartilage degradation in CD9$^{-/-}$ could be in part related to an increase in the expression of the two main cartilage extracellular matrix proteins aggrecan and type II collagen.

Synovial fibroblasts proliferate, and macrophages migrate into OA synoviums. These cells are associated with cartilage degradation through their release of inflammatory cytokines and cartilage degradation enzymes (5, 32). CD9 is well known to be associated with formation of functional complexes between integrin and other family. It is also a characteristic marker of and membranous components of microves-

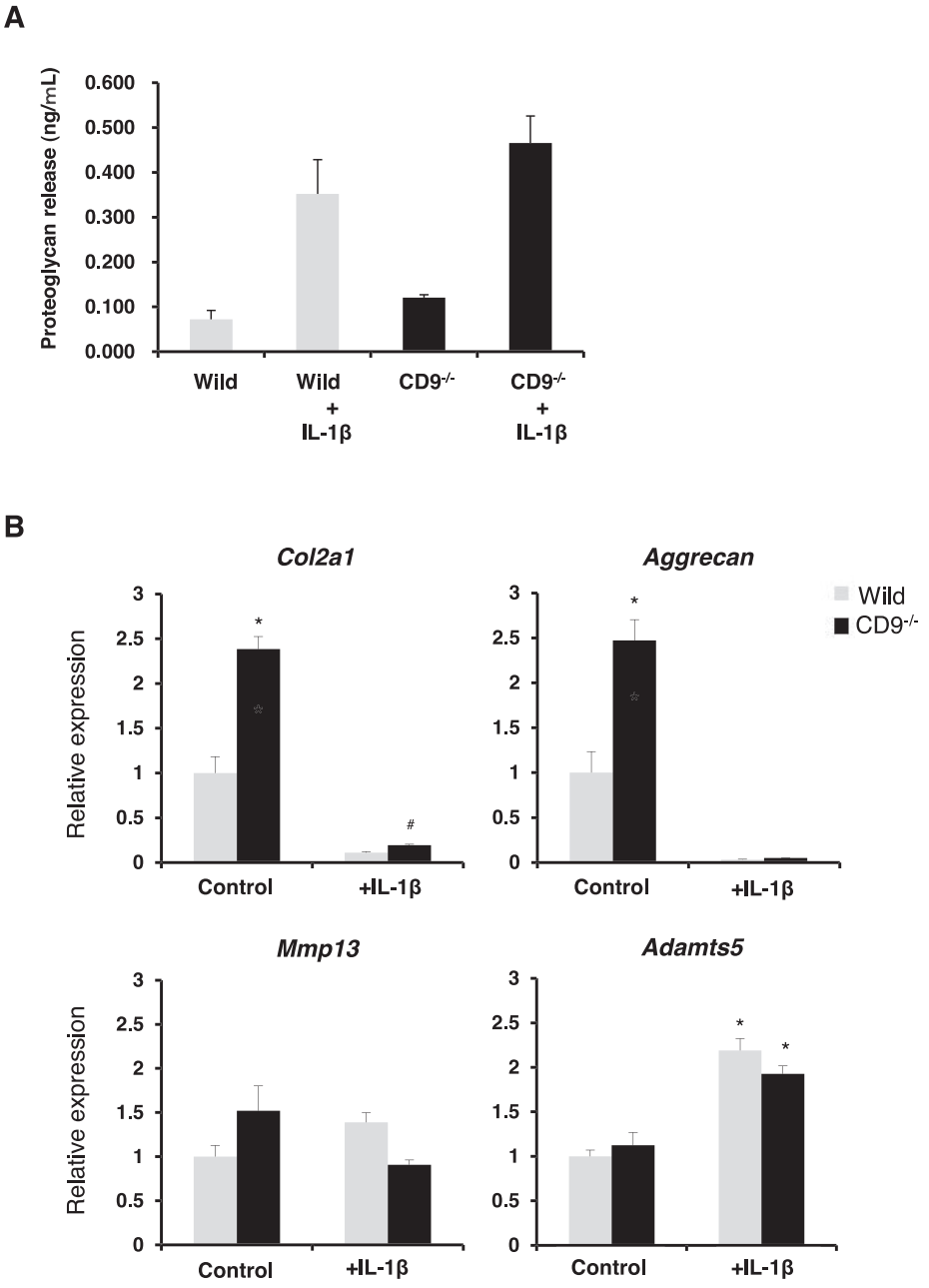

Fig. 4 Effect of CD9 deficiency in articular chondrocytes. **A**. Femoral head cartilage explants from wild-type mice and CD9$^{-/-}$ mice cultured with or without IL-1β. Proteoglycan release into the conditioned medium from cartilage was assayed as concentration of glycosaminoglycan (GAG). Data are the mean ± SEM. Comparison of mean values was performed by the Steel-Dwass test. **B**. Articular chondrocytes from wild-type mice and CD9$^{-/-}$ mice were treated with or without IL-1β. The expression of OA- and cartilage-related genes was analyzed by real-time PCR. Data are the mean ± SEM. Comparison of mean values was performed by the Steel-Dwass test. * = $P < 0.01$ versus chondrocytes without IL-1β from wild-type mice. # = $P < 0.01$ versus chondrocytes with IL-1β from wild-type mice.

icles such as exosomes. We recently reported that exosomes derived from IL-1β-stimulated synovial fibroblasts induce OA-like changes to normal chondrocytes and cartilage (13). These exosomes also induce migration and tube formation in endothelial cells (13). Angiogensis is associated with the progression of arthritis (6, 15, 18, 27), and angiogenesis inhibitor shows potential for use in OA treatment (19). CD9 knockdown or CD9$^{-/-}$ mice previously showed an antiangiogenic effect (9, 12). CD9$^{-/-}$ mice

exhibited the attenuation of cartilage degradation in the aged mice and in the AIA model mice. These results might be at least in part mediated by the anti-angiogenic effect and the dysfunction of exosomes through CD9 deficiency.

The present study showed that CD9 deficiency reduces the severity of OA such as cartilage degradation in aged mice, as well as in AIA transient inflammation model mice. Thus, CD9 might have potential to open new insight for the mechanism and protection of OA.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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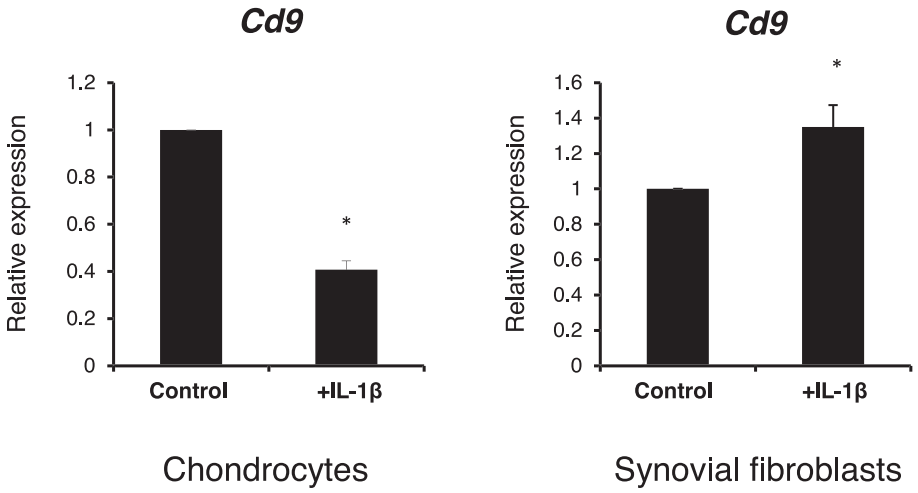

Supplemental Figure Articular chondrocytes and synovial fibroblasts from wild-type mice were treated with or without IL-1β. The expression of *Cd9* was analyzed by real-time PCR. Data are the means ± SEM. Comparison of mean values was performed by the Steel test. $^{*}P < 0.01$ versus control.