Experimental Evaluation of Discoid Domain Receptor 2 as an Ideal Target for Development of Disease-Modifying Osteoarthritis Drugs

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Experimental Evaluation of Discoidin Domain Receptor 2 as an Ideal Target for Development of Disease-Modifying Osteoarthritis Drugs

A Thesis Presented by

Lauren Brooke Manning, DDS

to

The Faculty of Medicine

In partial fulfillment of the requirements for the degree of

Doctor of Medical Sciences

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We, the undersigned, have read and approved the thesis of Lauren Brooke Manning submitted in partial fulfillment of requirements for the degree of a Doctorate of Medical Sciences at Harvard School of Dental Medicine.

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Abstract

Osteoarthritis (OA) affects 250 million people worldwide. Currently, no targets for disease-modifying osteoarthritis drugs exist. Matrix metalloproteinase-13 (MMP-13) would make it an ideal target; however, its broad biological effects restrict its application as a target enzyme of inhibitory drugs in the treatment of OA. The expression and activation of discoidin domain receptor 2 (DDR2) is increased in human OA tissues and mouse models of OA and was co-localized with elevated expression of MMP-13 in degenerative articular cartilages. In healthy articular cartilage, DDR2 is kept inactivated by the pericellular matrix, which separates the receptor from its ligand, type II collagen. Once enzymes capable of degrading the pericellular molecules expose chondrocytes to type II collagen, DDR2 is activated and induces expression of MMP-13 leading to degradation of type II collagen and proteoglycans resulting in joint destruction and OA.

We tested the hypothesis that complete removal of Ddr2 from the knee joint of mouse adult articular cartilage can delay progression of osteoarthritis prior to or after initiation of articular cartilage degeneration.

To accomplish this goal, conditional knock out techniques were used with Aggrecan-CreERT2 mice and floxed Ddr2 mice, Ddr2 was removed from articular cartilage of knee joints in mice at 8 weeks of age via intraperitoneal Tamoxifen injection (2mg/10g body weight) for 5 consecutive days (Group A). Mice were subjected to destabilization of the medial meniscus (DMM) or sham surgery at 10 weeks of age. An additional experimental group was subjected to DMM or sham surgery at 10 weeks of
age and then DDR2 was removed by intraperitoneal Tamoxifen injection 8 weeks later (Group B). Knee joints from mice in Group A and their corresponding controls were harvested at 8 weeks or 16 weeks post-surgery and mice from Group B and their controls were harvested at 16 weeks post surgery. Histology was performed and the OARSI Modified Mankin Score was used to evaluate articular cartilage degeneration. Statistically significant differences were determined via T-test.

We found the average modified score for Group A 8 week control was 1.64 (n=7) whereas with Ddr2 removed was 0.64 (n=7) [P<0.05]. 2) The average modified score for Group A 16 week control was 4.67 (n=7) and with Ddr2 removed was 1.27 (n=9) [P<0.05]. 3) The average modified score for Group B was 1.1 (n=5).

In conclusion, conditional removal of Ddr2 in articular cartilage attenuated articular cartilage degeneration in mature knee joints of mouse models of OA.
Hypothesis

The hypothesis of this study is that the complete removal of Ddr2 or inhibition of Ddr2 kinase activity at early stages of articular cartilage degeneration can prevent or delay joint destruction in mouse models of osteoarthritis (OA).

Specific Aims

Specific Aim 1:

The first aim of this study is to determine the maximum chondro-protective effect of complete removal of Ddr2 prior to initiation of the articular cartilage degeneration process on the knee joint of a mouse model of OA. By removing Ddr2 before inducing the degenerative process in articular cartilage, the maximum benefit of inhibition of Ddr2 activity can be evaluated. The question posed being can OA be delayed or prevented entirely if the receptor is completely removed before the disease process begins. Observation at two different time points, 8 weeks and 16 weeks post-surgical initiation, gives valuable information on the short term and long term prognosis and outcome of disease prevention.

Specific Aim 2:

The second aim of this study is to determine the maximum delayed chondro-protective effect of complete removal of Ddr2 at an early and delayed stage of the articular cartilage degeneration process on the knee joint of a mouse model of OA. This model more similarly represents the natural progression of the disease and enables evaluation of intervention at two different time points, after 4 weeks and 8 weeks of
articular cartilage destruction. By allowing the disease to progress for a period of time prior to removal of Ddr2, if progression of the disease is arrested or attenuated after the receptor is removed, Ddr2 may be a useful target in pharmacological therapy of OA.
Background

Osteoarthritis (OA), the most common form of arthritis, affects more than 27 million people in the United States alone and 630 million people around the world (1). It has been defined as “a progressive disease of synovial joints that represents failed repair of joint damage that results from stresses that may be initiated by an abnormality in any of the synovial joint tissues.” This eventually leads to development of pain, stiffness and functional disability resulting from breakdown of cartilage and bone (2).

The precise mechanism by which OA is initiated is unknown. However, the general consensus is that the etiology of OA is multifactorial and includes both genetic and environmental factors (1). Genetic components constitute mutations in genes resulting in defects in articular cartilage matrix and chondrocyte metabolism. When defects in the articular cartilage are present, age-dependent OA may develop from normal mechanical stresses on a defective joint (3). Environmental factors include, but are not limited to, obesity, over-loading on joints, repetitive injuries that can involve ligaments and menisci, a decrease in muscle strength and malalignment of the joint (4). Any of these conditions can lead to abnormal mechanical stresses on a normal joint inevitably resulting in the development of OA. The initiation of OA cannot only be cause by genetic or environmental factor, or a combination of both, but can also involve multiple joint tissues. It is not surprising that the understanding the detailed pathogenesis in all of these conditions continues to be challenging. Still, despite the lack of minute understanding of the etiology of OA, the development of OA due to breakdown of
articul cartilage follows a consistent pathological pattern. (5).

Studies have shown using both human OA tissues as well as animal modes of OA that the earliest indication of articular cartilage degeneration is chondrocyte clustering and an up-regulation in synthetic activity of chondrocytes (6). Chondrocytes then synthesize and release extracellular matrix (ECM)-degrading enzymes, including matrix metalloproteinase-13 (MMP-13). The loss of proteoglycans on the surface of articular cartilage occurs simultaneously with type II collagen degradation. The articular surface subsequently develops cracks, producing this histological image termed fibrillation. Eventually, late in the degenerative process, fibrocartilage and osteophytes are formed.

Early in the degeneration process, there is a degradation of the pericellular network surrounding the chondrocyte. A likely culprit involved is high temperature requirement A1 (HTRA1). HTRA1, a serine protease, is highly expressed in these tissues and whose substrates include fibromodulin, aggrecans, fibronectin, decorin and biglycan, all of which constitutes the pericellular components of chondrocytes in articular cartilage (6). HTRA1 is the most abundant protease in human OA cartilage and its protein level and activity were increased in knee and temporomandibular joints of OA mouse models (7), including models where OA is surgically induced. These findings suggest that altered mechanical stress, such as that in surgical models, can directly or indirectly induce HTRA1 in chondrocytes and this elevated expression has been associated with disruption of the pericellular matrix in OA mouse models. Additionally, Li et al found that expression of HTRA1 increased prior to up-regulation of Ddr2 and Mmp-13 in these
mouse models indicating HTRA1 may contribute to the development of OA by degrading the pericellular network.

It should be noted that loss of the entire pericellular network, not only aggrecans, is required. Two recent studies report an surprising finding that the disappearance of aggrecans in articular cartilage of knee joints in adult mice, secondary to genetic inactivation of a transcription factor, sex-determining region Y box 9 (Sox9) or the conditional up-regulated expression of bone morphogenic protein 2 in articular cartilage, does not initiate or accelerate progression of cartilage degeneration (8,9). A possible explanation for this finding is that the loss of proteoglycans alone may not be sufficient to initiate or accelerate articular cartilage degeneration, but the degradation of both proteoglycans and type II collagen may be required.

Matrix metalloproteinase 13 (MMP-13), also known as collagenase-3 has been found to degrade both proteoglycans and type II collagen (10,11). In normal mature articular cartilage MMP-13 expression is hardly detected; however, its activity and expression is increased in both human OA cartilage and the joints of mouse models of OA (12-15). Other studies have demonstrated that degradation of articular cartilage is irreversible after MMP-mediated degradation of aggrecan and type II collagen is induced (16). It is only logical that inhibition of the activity and expression of MMP-13 in articular cartilage would be a possible method of intervening in the development of OA and multiple pharmacological companies have attempted to develop disease modifying osteoarthritis drugs (DMOADs) targeting this enzyme. However, the broad expression
and activity of MMP-13 limits the possibility for developing inhibitory drugs in the treatment of OA, as it is not tissue specific (17).

Collagen in humans has a half-life of 117 years (18) indicating the slow turnover of collagen in mature articular cartilage. This suggests the need for continual production of type II collagen may not exist and chondrocytes may have limited potential to replenish type II collagen once it has been degraded. TGF-β1 can stimulate chondrocytes to synthesize and release extracellular matrix molecules including type II collagen and proteoglycans (19-22) consistent with results from animal models, which indicate a lack of Tgf-β1 signaling in germ line cells results in OA. However, other studies indicate that increased TGF-β1 signaling may not only initiate but also accelerate articular cartilage degeneration. Results from experiments execute in the Li Lab have demonstrated that DDR2 may play a significant role in how a normal joint develops osteoarthritis. It is equally important given findings from multiple studies, to take into account that molecules, such as HTRA1 and TGF-β1, may eventually lead to activation of DDR2 and consider their importance in the development of OA.

One of the earliest signs of articular cartilage degeneration is overproduction of proteoglycans in mouse models of OA (3,5). Results from Van den Berg et al show overexpression of active TGF-β1 in mature mouse knee joints is associated with hyperplasia of synovium, increased production of proteoglycans articular cartilage, and chondro-osteophyte formation (23). Two studies indicate increased TGF-β1 levels are
present in human osteoarthritic tissues when compared to healthy articular cartilages (24,25) concurring with the human genetic study reporting an alteration in SMAD-3 that demonstrated higher levels of TGF-β1 activity and TGF-β1 signaling pathway in two families associated with early-onset OA (89). Two investigations to determine if Tgf-β1 induced HtrA1 in two mouse models demonstrated increases in expression of Tgf-β1, p-Smad2/3 and HtrA1 in articular chondrocytes. The expression of p-Smad2/3 and HtrA1 were co-localized, and TGF-β1-induced expression of HTRA1 was inhibited by an ALK-5 inhibitor in human and mouse chondrocytes. These findings imply that Tgf-β1 canonical signaling could be activated to induce HtrA1 (6,27).

In recent investigations to see if inhibiting Tgf-β1 signaling would prevent degeneration of mature knee joints in mouse models of OA (28), three different scenarios including a Tgfbr2 conditional knockout, intra-articular injection with TGF-β1 neutralizing antibody in a genetic model of OA (type XI collagen haploinsufficiency [Col11a1+/−]), and Losartan induced inhibition of TGF-β1 signaling in a surgical (DMM) model of OA, lead to the conclusion that inhibition of TGF-β1 signaling attenuates articular cartilage degeneration (28). Findings from these experiments indicate that complete removal of Tgf-β1 signaling could delay progression of degeneration but not prevent it entirely suggesting induction of HRTA1 in chondrocytes may be the result of factors other than TGF-β1, including but not limited to Wnt/β-catenin and LPS/TLR4 (toll-like receptor) (29,30). Once activated, either by TGF-β1 or other factors, HTRA1
leads to loss of the pericellular matrix surrounding the chondrocyte and initiation of the pathway the leads to activation of MMP-13 and articular cartilage degradation. However, native type II collagen cannot enter chondrocytes to induce MMP-13. Therefore, collagen has to interact with a cell surface molecule instead and results from recent investigations implicate DDR2 as such a molecule.

Originally cloned as a cell surface receptor tyrosine kinase (RTKs), mRNA transcripts of DDR2 are detected in several human and mouse tissues, primarily in cartilage, skeletal muscle, skin and adipose tissue (31-34). Two research groups described native fibrillar collagens as the ligands for DDR2 (35,36). The specific amino acid sequences on DDR2 and type II collagen (Figure 1) critical for the interaction of the receptor with its ligand was identified by two additional research groups and a change in any of these amino acids affect the affinity of DDR2 for type II collagen drastically.

![Figure 1. Binding amino acid sequence of type II collagen to DDR2](image)

When expression profiles of DDR2 were analyzed, it was discovered that the protein was hardly detectable in normal, healthy articular cartilage. However, DDR2 expression was increased in human osteoarthritic tissues and mouse models of OA and this expression was co-localized with increased activity and expression of MMP-13 (37-42). The results from these experiments showed 4 things: 1) When chondrocytes are
cultured on type II collagen expression of MMP-13 was elevated as was that of DDR2 indicating chondrocytes exposed to native type II collagen induced expression of MMP-13 and the receptor itself. 2) When cultured on denatured type II collagen, the expression of MMP-13 and DDR2 was not induced in chondrocytes. 3) Overexpression of full-length DDR2 cDNA resulted in higher levels of MMP-13 expression, while overexpression of a truncated DDR2 cDNA, one lacking the protein tyrosine kinase, inhibited increased expression of MMP-13. Also, when the DDR2 receptor lacks the portion responsible for binding type II collagen (discoidin domain), there is no effect on the expression of MMP-13 or the receptor itself. This confirms that an increase in expression of MMP-13 in chondrocytes, the interaction of DDR2 with type II collagen is required. 4) Ras/Raf/MED/ERK and p38 signaling pathways played a role in the increased expression of MMP-13 by type II collagen-DDR2 interaction.

Additional in vivo experiments looking at if reduction of Ddr2 expression in heterozygous Ddr2^{+/−} mice could delay progression of articular cartilage degeneration were carried out in two mouse models of OA, genetic collagen type XI deficient mice and surgical mice via destabilization of the medial meniscus. It was reported that decreased expression of Ddr2 did attenuate articular cartilage degeneration (43) and this result was confirmed by 3 independent research groups (44-46).

It has been determined that the basic structural and functional unit of articular cartilage is a chondron, composed of a chondrocyte, its surrounding pericellular matrix and a capsule surrounding the pericellular matrix (47-53). The capsule is composed of
type VI and IX collagen and proteoglycans. The extracellular matrix components comprising the pericellular matrix include laminin, fibronectin, biglycan, decorin, fibromodulin, matrillin 3, and cartilage oligo matrix protein (COMP). Under normal healthy circumstances, the pericellular matrix and capsule separate chondrocytes from type II collagen. However, once this construction system is disrupted, as with activation of HTRA1 in which many of the pericellular matrix components are substrates of, chondrocytes are exposed to type II collagen. When DDR2 binds type II collagen, MMP-13 expression is induced leading to destruction of articular cartilage.

Results from both human and mouse genetic models implicate the pericellular matrix’s role in protecting loss of articular cartilage and the development of OA (38,54-63). In a tetracycline-controlled gene expression system (Tet-Off system), conditionally induced expression of DDR2 did not induced expression of Mmp-13 and no OA-like pathologic changes were seen in the knee joints of the transgenic mice (50). These results indicated that a high-level of inactivated DDR2 does not cause initiation or progression of OA indicating that if up-regulated DDR2 was surrounded by an intact pericellular matrix, DDR2 is prevented from binding type II collagen. However, the loss of the pericellular matrix is required prior to DDR2 activation by binding type II collagen, pericellular matrix degrading enzyme(s) must play a vital role in OA development. One study suggested that proteases, no matrix metalloproteinases may be involved, and many independent research groups have reported HTRA1 as such an enzyme (64).
Based on the data provided by investigations by other groups and the experiments conducted in the Li Lab, a molecular pathway underlying articular cartilage degeneration was developed (Figure 2). Excessive mechanical stress caused by either normal mechanical loading on a defective joint or overloading of a normal joint stimulates the synthesis and release of TGF-β1 into synovial fluid from chondrocytes. Three independent studies support this premise reporting mechanical injury of bovine cartilage explants causing a significant increase in TGF-β1 gene expression, indicating that hydrostatic pressure on human articular chondrocytes increased expression of TGF-β1 in culture, and latent TGF-β1 is then activated by mechanical shearing of synovial fluid (65-67). TGF-β1 then binds to its receptor, TGF-β receptor II, inducing HTRA1 expression in chondrocytes. A negative feedback loop may exist between TGF-β1 and HTRA1 demonstrated by the potential an inhibitory effect of HTRA1 on TGF-β1 signaling. This is based on the structural and functional similarity of the insulin-like growth factor-binding protein domain of HTRA1 with follistatin and the capability to cleave pro-TGF-β1 (68,69). Secondary effects of HTRA1 induction include degradation of the pericellular matrix and exposure of chondrocytes to type II collagen, enabling enhanced signaling through DDR2. Activation of DDR2 then leads to induction of MMP-13 expression and increased expression of DDR2 itself. MMP-13 activity results in type II collagen and aggrecan fragments, which activates signals further increasing production of MMP-13 (70). The evitable result is a feedback amplification loop causes irreversible
articular cartilage degeneration.

Figure 2. A molecular pathway underlying articular cartilage degeneration.
**Innovation**

Current FDA-approved treatments have been mainly be targeted at symptom relief for OA; however, they have not been shown to prevent ongoing structural damage of the joint. Recent discoveries indicate that the activation of DDR2 induces MMP-13 in chondrocytes, *in vitro* and *in vivo*, resulting in development of OA. It was also found that reduction in the expression of DDR2 in heterozygous conventional knockout mice (*Ddr2*+/−) could delay progression of articular cartilage degeneration in knee joints.

This revelation allows the novel utilization of the tamoxifen-inducible system to completely remove *Ddr2* from knee articular cartilage before and after the onset of articular cartilage degeneration in mouse models of OA. Once completed, the time point at which the deletion of *Ddr2* can prevent the joint from being destroyed will be determined. In previous studies, complete deletion of *Ddr2* in knee articular cartilage at a desirable time was not accomplished due to the lack of a floxed *Ddr2* mouse strain. Additionally, homozygous conventional *Ddr2* knockout mice exhibit short stature (dwarfism) and could not be used in OA studies.

This study utilizes the creation of floxed *Ddr2* mice and plans to determine whether sufficiency of inhibiting the kinase activity of Ddr2 against development of OA.
Materials and Methods

Generation of AgcCreERT2+/−;Ddr2floxflox mice

Initially, floxed Ddr2 mice were generated in Dr. Li’s lab, then crossed with FLPer mice to remove the DNA fragment containing several elements, En2SA-IRES-LacZ-Neo (Figure 3). By removing this fragment, any potential effects of the previous elements on the development of mice were eliminated. Currently, the floxed Ddr2 mice have been maintained for further experiments.

An additional mouse strain, AgcCreET2 (Figure 4), is needed for this experiment and provided by Dr. Henry from M.D. Anderson Cancer Center at Houston, TX. The mouse strain has been maintained in the Li laboratory. This mouse strain expresses a recombinant protein consisting of Cre-recombinase and the modified estrogen receptor (CreERT2), driven by the endogenous aggregan promoter. In this mouse strain, the CreERT2 is expressed in chondrocytes of un-calcified articular cartilage in these mice, but not in the chondrocytes of calcified cartilage in adult mice.
Mouse Genotyping

Mouse genotyping for AggrecanCreERT2 and the floxed Ddr2 gene was performed. Genomic DNA was isolated from the mouse tail. To determine if mice were Aggrecan-CreERT2 positive (AgcCreERT2+/−) forward primer CreERt2-F TAACTACCTGTTTTGGCCGGG(20) and the reverse primer CompCre-R8 GTCTGCCAGTTGGTCAGTAA(21) were used. AgcCreERT2 protocol was set for primary denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and elongation at 72°C for 1 minute, with final elongation for 10 minutes at 72°C. To determine if mice were homozygous for the floxed Ddr2 gene (Ddr2flox/flox) forward primer Ddr2-5’arm AGTAGGTGCTAGCTACCTCCCACC(24) and the reverse primer Ddr2-3’arm CTGCTTCCTCCCAGGTACCTTCCC(24) were used. Ddr2flox protocol was set for primary denaturation at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 30 seconds, with final elongation for 10 minutes and 72°C.

Destabilization of the Medial Meniscus

The experimental surgical procedure was performed following approval from the Harvard Medical School Institutional Animal Care and Use Committee. Male and female mice at the age of 10-12 weeks were anesthetized intra-peritoneally with Ketamine (90mg) and Xylazine (10mg)/kg mouse body weight. The right knees were prepared for
aseptic surgery. The joint capsule immediately medial to the patellar tendon was opened to provide visualization of the medial meniscotibial ligament. The ligament was sectioned and the medial meniscus displaced medially, resulting in the destabilization of the medial meniscus (DMM). The joint capsule was closed with 8-0 tapered Vicryl suture and the subcutaneous later was closed with 7-0 cutting Vicryl. The skin was closed by the application of tissue adhesive. The mice were maintained under a daily schedule of 12 hours with light and 12 hours without light for further experiments. Sham surgery in which the ligament was visualized but not transected in mice was also performed as a control.

**Removal of Ddr2 in the Articular Cartilage of Knee Joints AgcCreERT2+/−;Ddr2^{flox/flox} Mice**

Mice were injected with 2 mg of tamoxifen/10g of body weight intra-peritoneally for 5 consecutive days at three different time points. Phase 1 mice were injected at 8 weeks of age. Phase 2 mice were subjected to DMM surgery at 10 weeks of age and were subsequently placed into two different groups. Group 1 mice were injected at 4 weeks after DMM surgery was completed. Group 2 mice were injected at 8 weeks after DMM surgery was completed. These time points were selected based on the observation that protein expression of Ddr2 was dramatically increased in knee articular cartilage of mice at 8 weeks following DMM surgery.

ERT2, modified human ER, does not bind its natural ligand (17β-estradiol) at
physiologic concentrations, but instead binds synthetic estrogen receptor ligand, tamoxifen. Once bound, CreERT2 is transferred into the nucleus. The Cre-recombinase recognizes a specific DNA sequence, LoxP, and functions as an endonuclease to remove the DNA sequence between the two LoxP sequences, which is the exon 9 of the Ddr2 gene, resulting in the knockout Ddr2 in \textit{AgcCreERT2}^{+/+};\textit{Ddr2}^{floxfloxflox} mice (Figure 5).

\textit{Characterization of Mouse Knee Joints}

Phase 1 \textit{AgcCreERT2}^{+/+};\textit{Ddr2}^{floxfloxflox} mice and corresponding controls were sacrificed at 8 weeks (n=6-7) and 16 weeks (n=6-9) following surgery. Phase 2 \textit{AgcCreERT2}^{+/+};\textit{Ddr2}^{floxfloxflox} mice (n=5), which had Ddr2 removed 8 weeks after surgery, were sacrificed at 16 weeks following surgery. The selected final termination point was 16 weeks after surgery because the knee joints at this time reveal a typical OA joint, including fibrillation, loss of articular cartilage, inflammation and formation of osteophytes.

With regard to the sample size of animals in each experimental group, we performed the power analysis on a pilot study, which is the effect of the removal of Ddr2 on articular cartilages of mouse knee joints. From results of the pilot
experiment, we concluded that a minimal sample size, 5-6, is required to achieve the 95% confidence level with at least 50% reduction of the score in the treatment group.

Histology

Knee joints from AgcCreERT2+/−;Ddr2flox/flox and their corresponding controls were collected and fixed. Before they were processed for paraffin embedding, the samples were decalcified in Morse’s solution. For each knee joint from AgcCreERT2+/−;Ddr2flox/flox mice and the Ddr2flox/flox mice, 6 µm thick serial coronal sections were cut. Every tenth section was collected for Safranin O/Fast green staining. Sections from knee joints were also used for immunofluorescence.

The pathologic condition of the knee joints was evaluated using the Osteoarthritis Research Society International (OARSI) scoring system. The OARSI histopathology initiative recommends scoring the condition of articular cartilage in the following way: the score 0 is for normal mouse articular cartilage; 0.5 for loss of Safranin-O without structural changes; 1 for small fibrillations without loss of cartilage; 2 for vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina; 3 for vertical clefts/erosion to the calcified cartilage extending to <25% of the articular surface; 4 for vertical clefts/erosion to the calcified cartilage extending to 25 to 50% of the articular surface; 5 for vertical clefts/erosion to the calcified cartilage extending to 50 to 75% of the articular surface; and 6, the maximal score, for vertical clefts/erosion to the calcified cartilage extending >75% of the articular surface.
The significance of differences between \textit{AgCreERT2}^{+/−};\textit{Ddr2}^{flx/flx} and \textit{Ddr2}^{flx/flx} mice were evaluated using the Student T test. The meniscus, subchondral bone, and osteophyte formation in knee joints were also examined to evaluate the overall condition of the joint.

\textit{Immunofluorescence}

Expression of MMP-13 is known to be up-regulated in human OA articular cartilage and is believed to be involved in the degradation of matrix proteins including collagens and proteoglycans (71). Immunohistostaining on knee joints from \textit{Ddr2}^{−/−} and their controls at 8 weeks after DMM surgery was completed. Knee joints from mice at 16 weeks post-operatively were too damaged to perform immunostaining. For analysis of the protein expression of MM-13, ten paraffin sections, even distributed throughout each joint, were selected for immunohistostaining. Sections were de-paraffinized and quenched for endogenous peroxidase activity. The sections were incubated with rabbit polyclonal antibody after at 1\(\mu\)g/\(\mu\)L (1:400) against Mmp-13 protein (Cat. No. ab84594, www.abcam.com). After overnight incubation at 4°C, the sections were washed three time with phosphate buffered saline (PBS) and subsequently treated with a biotinylated secondary antibody (1:200) (goat anti-rabbit IgG, Cat. No. BA-1000, VectorLabs, Burlingame, CA) at room temperature for 30 minutes. Color development was performed using peroxidase substrate (NovaRED Substrate, Cat. No. SK-4800, VectorLabs) following treatment of sections of the sections with a mixture of avidin and biotinylated horseradish peroxidase (VECTASTAIN ABC Kit, Cat. No. PK-4000,
VectorLabs). Sections were counterstained with 0.2% Fast Green solution. Staining without primary antibody was performed as negative controls. The sections were then washed three times with PBS and examined under a fluorescence microscope.

*Analysis of Ddr2 DNA in articular chondrocytes by real-time PCR*

DNA was isolated from mouse knee articular cartilages using standard DNA extraction protocol. Taqman Copy Number Assay was used to evaluate the removal efficiency. In this assay, Taqman Copy Number Mouse Ddr2 Assay Probe (Cat. No. 4400291) was labeled with FAM and Taqman Copy Number Mouse Tfrc Reference Assay Probe (Cat. No. 4458366) was labeled with fluorophore-Vic. The amplicon for Ddr2 is 70 bp and that for Tfrc is 91 bp. Duplex real-time PCR was performed using the StepOnePlus Real-Time PCR System (Life Technologies) and the PCR reaction was carried out at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds, 60°C for 60 seconds.

*Statistical Analysis*

For statistical analysis, 9 to 10 paraffin sections from each knee joint were used. The score from the section with the worst condition was selected to represent the joint. Six to eight scores were obtained for each experimental group and an average score for each experimental group was then calculated from the individual scores. A two-sample t-test with a significance level of 0.05 was used to determine if any significant difference
between any two average scores exists.
Results

Generation of Experimental Mice and Genotyping

Removal of Ddr2

Through tamoxifen induced recombination, exon 9 of the Ddr2 gene was removed in articular cartilage resulting in a single band 720bp in length when compared to the wild-type Ddr2\(^{+/+}\) gene at \(~1600bp\). See figure 8.
Histology

Phase I: Ddr2 removed prior to initiation of OA via DMM surgery

At the 8 week time point, a significant disparity in the progressive process of articular cartilage degeneration in knee joints between Ddr2\textsuperscript{−/−} and Ddr2\textsuperscript{+/+} mice. Figure 9 shows progression toward OA was delayed in the Ddr2\textsuperscript{−/−} mice after the surgery was completed. See appendix figures 1-3 for higher magnification.

![Representative images of Phase I mice 8 weeks after DMM surgery](image)

Figure 9. Representative images of Phase I mice 8 weeks after DMM surgery

In particular, we found that the appearance of fibrillation was one of the hallmarks in the superficial layer of articular cartilage of knee joints in Ddr2\textsuperscript{+/+} mice. However, no fibrillation as observed in Ddr2\textsuperscript{−/−} mice. It is of note that proteoglycan degradation was noticed in the superficial layer of articular cartilage of Ddr2\textsuperscript{−/−} mice, suggesting that other factors must be involved in proteoglycan degradation at 8 weeks. However, it is not known whether this degradation plays any significant role in the acceleration of articular cartilage degeneration. There were no abnormal morphological changes in the sham surgery groups. Table 1 shows the conditions of articular cartilage evaluated individually by the scoring system used.
Table 1: OARSI Score reported per animal in Phase I 8 weeks after surgery

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<td>1.0</td>
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<td>0</td>
<td>0.5</td>
</tr>
<tr>
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<td>2.0</td>
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</tr>
<tr>
<td>Animal 5</td>
<td>2.0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Animal 6</td>
<td>1.0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>Animal 7</td>
<td>2.0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Modified Mankin Score</strong></td>
<td>1.71</td>
<td>0</td>
<td>0.64</td>
</tr>
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</table>

Mice at 8 weeks following sham surgery were used as a normal control (score=0). The average scores for *Ddr<sup>2</sup><sup>+/+</sup>* and *Ddr<sup>2</sup><sup>−/−</sup>* mice were 1.71 and 0.64, respectively. There was a significant difference between the scores of the two groups, p<0.01.

At the 16-week time point, a significant disparity in the progressive process of articular cartilage degeneration in the knee joints between *Ddr<sup>2</sup><sup>+/+</sup>* and *Ddr<sup>2</sup><sup>−/−</sup>* mice was seen. Figure 10 also shows progression towards OA was delayed in the *Ddr<sup>2</sup><sup>−/−</sup>* mice after surgery. See appendix figures 4-6 for higher magnification.

![Representative images of Phase I mice 16 weeks after DMM surgery](image)

**Figure 10.** Representative images of Phase I mice 16 weeks after DMM surgery
In particular, proteoglycan degradation, fibrillation, and missing cartilage were seen in the knee joints in $Ddr2^{+/+}$ mice. Although proteoglycan and mild fibrillation was observed in the $Ddr2^{-/-}$ mice, the superficial layer of articular cartilage had been maintained. Table 2 shows the conditions of articular cartilage evaluated individually by the scoring system used.

Table 2. OARSI Score reported per animal in Phase I 16 weeks after surgery

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>16 week</th>
<th>P</th>
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<tbody>
<tr>
<td></td>
<td>Control (n=7)</td>
<td>$Ddr2^{-/-}$ (n=9)</td>
</tr>
<tr>
<td>$Ddr2^{+/+}$ DMM</td>
<td>0.5</td>
<td>4</td>
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<tr>
<td>$Ddr2^{+/+}$ Sham</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>$Ddr2^{-/-}$ DMM</td>
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<td>5</td>
</tr>
<tr>
<td>$Ddr2^{-/-}$ Sham</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>$Ddr2^{-/-}$ DMM</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>$Ddr2^{-/-}$ Sham</td>
<td>0.5</td>
<td>5</td>
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<tr>
<td>$Ddr2^{-/-}$ DMM</td>
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<td>0.5</td>
</tr>
<tr>
<td>$Ddr2^{-/-}$ Sham</td>
<td>4</td>
<td>1</td>
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<tr>
<td>$Ddr2^{-/-}$ DMM</td>
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<td>0.4</td>
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<td>$Ddr2^{-/-}$ Sham</td>
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<td>1</td>
</tr>
<tr>
<td>Modified Mankin Score</td>
<td>4.6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The average scores for $Ddr2^{+/+}$ and $Ddr2^{-/-}$ mice were 4.6 and 1.27, respectively. There was a significant difference between the scores of the two groups, p<0.01.

The phase II group mice that had $Ddr2$ removed 8 weeks after being subjected to DMM surgery were also found to have delayed progression of articular cartilage degeneration. Figure 11 shows delayed progression of articular cartilage degeneration in 16 week post-operative phase II $Ddr2^{-/-}$ mice in comparison to 16 week post-operative phase I $Ddr2^{+/+}$ mice. See appendix figures 5 and 7 for higher magnification.
Figure 11. Representative images of Phase I mice 16 weeks after DMM surgery (left image) and Phase II mice 16 weeks after DMM surgery (right image)

The average scores for phase II $Ddr2^{-/-}$ mice was 1.1, and here was a significant difference between the scores of this group and that of the 16 week post operative $Ddr2^{+/+}$ mice from phase I groups, $p<0.01$.

**Immunohistochemistry**

We found that the condition of the articular cartilage damage at 8 weeks post-operatively was mild and expression of Mmp-13 was hardly detected in $Ddr2^{-/-}$ mice, when compared with that observed in their wild-type littermates Figure 12. See figures 8-10 in index for higher magnification.
Analysis of Ddr2 Removal Efficiency

In our experiment, we examined the efficiency of the ablation of Ddr2 by AgcCreERT2 in the articular chondrocyte of adult mouse knee joints. After several rounds of crossing AgcCreERT2+/− mice with floxed Ddr2 mice, we obtained compound mutant mice, heterozygous CreERT2 driven by the aggrecan promoter and homozygous floxed Ddr2 (AgcCreERT2+/−; Ddr2lox/lox). We found that the exon 9 genomic DNA of Ddr2 floxed by loxP sites was deleted in 75% articular chondrocytes of adult mouse knee joints, (figure ). The loss of the exon 9 resulted in a pre-mature stop codon immediately after the exon 9 of Ddr2. This result indicated that Ddr2 was deleted in 75% of articular chondrocytes of adult knee joints in AgcCreERT2+/−; Ddr2lox/lox mice.
Discussion

Through the various techniques utilized in this study, we have shown that the presence of DDR2 leads to MMP-13 expression and destruction of articular cartilage. However, the question still remains as to what would happen if we could completely remove DDR2 from mature articular cartilage with regard to the protective effect. A conventional knock out model could not be utilized to due to the differences in loading of the joint that is a result of the hypomorphic (dwarf) phenotype in the homozygous condition. Therefore, employing a conditional knock out and surgical model eliminated any potential developmental problems that may occur due to the lack of Ddr2. The tamoxifen induced recombination system can result it in a wide-range variation. Other studies have looked at the local removal of the gene of interest driven by AgcCreERT2 from articular cartilage in adult mice by counting chondrocytes that did not express the gene of interest on histologic sections. They found the removal efficiency to be between 70-80% (8,72). In this study, we looked at global removal of Ddr2 from articular cartilage of the knee joint from experimental mice by qPCR with DNA from the articular cartilage of the whole joint, not one portion. We discovered Ddr2 was successfully removed from 75% chondrocytes in articular cartilage of the mouse knee joints. Still, 25% of the chondrocytes had Ddr2 expressed, which could contribute to the delay of progression of cartilage degeneration, but not the complete cessation of the degenerative process.

The first phase of this study showed that removal of Ddr2 is an effective treatment
of OA providing a proof of concept. This supports the hypothesis that removal of DDR2 decreases articular cartilage degeneration and results from immunostaining of MMP-13 expression demonstrate if Ddr2 is removed, Mmp-13 expression is markedly reduced, also supporting the hypothesis that activation of DDR2 induces MMP-13 expression. Furthermore, the differences between phase I and phase II of this study allowed the conclusion that prophylactic removal of Ddr2 is not indicated in the proposed treatment of OA. The results from phase II show that removal of Ddr2 even half way through development of an osteoarthritic joint at 8 weeks in the surgical model still delayed progression of the disease process. This implies that high-risk groups, such as athletes participating in contact sports with high injury rates, should not have DDR2 removed prior to injury, because degeneration of articular cartilage could still be delayed after the injury occurs.

The results from the immunohistochemistry demonstrate that if Ddr2 is removed, little, if any, Mmp-13 expression is present, supporting our hypothesis and the findings from various other independent research groups (37-42,50). Less DDR2 activity resulted in less MMP-13 activity inevitably leading to less articular cartilage degeneration. Although MMP-13 was not an effective target for DMOADS due to its board expression throughout the body, DDR2 is an ideal target because not only does reduction in DDR2 expression lead to decreased MMP-13 activity, but also it is also only expressed in the testes, and renal pelvis in wild-type mice. These findings support the conclusion that DDR2 is an ideal target for DMOADS.
The key in future studies will be to show the effectiveness of Ddr2 removal in a more conventional model of OA, such as the Col11A1 haploinsufficient mouse, where the progression of articular cartilage degeneration is not as rapid as in the DMM surgical model. Establishing the timing of Ddr2 inhibition is also very important. Once the joint is considered to be an osteoarthritic joint and little if any articular cartilage remains, this form of intervention therapy may not be indicated. Defining the limit at which DDR2 inhibition therapy would still be beneficial will have very large clinical implications.
Appendix

Appendix Figure 1. Phase I 8 weeks after sham surgery control ($Ddr2^{-/-}$)
Appendix Figure 2. Phase I 8 weeks after DMM surgery ($Ddr^{2+/+}$)
Appendix Figure 3. Phase I 8 weeks after DMM surgery ($Ddr2^{-/-}$) [$Ddr2$ removed prior to DMM surgery]
Appendix Figure 4. Phase I 16 weeks after sham surgery control ($Ddr2^{-/-}$)
Appendix Figure 5. Phase I 16 weeks after DMM surgery ($Ddr2^{+/+}$)
Appendix Figure 6. Phase I 16 weeks after DMM surgery ($Ddr2^{−/−}$) [$Ddr2$ removed prior to DMM surgery]
Appendix Figure 7. Phase II 16 weeks after DMM surgery ($Ddr2^{−/−}$) [$Ddr2$ removed 8 week after DMM surgery completed]
Appendix Figure 8. MMP-13 expression in Phase I mice 8 weeks after sham surgery control ($Ddr2^{-/-}$)
Appendix Figure 9. MMP-13 expression in Phase I mice 8 weeks after DMM surgery control ($Ddr2^{+/+}$)
Appendix Figure 10. MMP-13 expression in Phase I mice 8 weeks after DMM surgery control \((Ddr2^{-/-})\) [\(Ddr2\) removed prior to DMM surgery]
References


