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Losartan attenuates progression of osteoarthritis in the synovial temporomandibular and knee joints of a chondrodysplasia mouse model through inhibition of TGF-β1 signaling pathway

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SUMMARY

Objective: Transforming growth factor beta 1 (TGF-β1) is implicated in osteoarthritis (OA). The purpose of this study was to explore the ability of Losartan to inhibit the inflammatory signaling pathway of TGF-β1 observed during osteoarthritic progression in the temporomandibular joint (TMJ) and knee joint using a genetic mouse model.

Methods: A murine OA model displaying the heterozygous chondrodysplasia gene (*cho/+*), a *col11a1* mutation, was used to test this hypothesis. Following a 7-month treatment period with Losartan, the synovial joints were analyzed for histopathological improvement comparing two experimental groups. Tissues were fixed in paraformaldehyde, processed to paraffin section, and stained with Safranin O and Fast Green to visualize proteoglycans and collagen proteins in cartilage. Using the Modified Mankin scoring system, the degree of staining and OA progression were evaluated.

Results: Results show heterozygous animals receiving Losartan having diminished degeneration of TMJ condylar and knee joint articular cartilage. This was confirmed in the TMJ and knee by a statistically significant decrease in the Mankin histopathology score. Decreased expression of HtrA1, a key regulator to the TGF-β1 signaling pathway, was demonstrated *in vitro* as well as *in vivo*, via Losartan inhibition.

Conclusion: Using a genetic mouse model of OA, this study demonstrated the utility of Losartan to improve treatment of human OA in the TMJ and knee joint through inhibition of the TGF-β1 signaling cascade. We further demonstrated inhibition of HtrA1, the lowering of Mankin scores to wild type control levels, and the limiting of OA progressive damage with treatment of Losartan.

Introduction

Recognized as the eleventh highest contributor to global disability, the increasing prevalence of osteoarthritis (OA) poses a major health challenge directly affecting quality of life[1]. There are multiple risk factors for this disease, including increased age, obesity, joint injuries and lifestyle[2]. The increasing presence of these risk factors and the detrimental results of OA recognized as both disabling and with significant financial burden, promote the need for further investigation into contributory molecular mechanisms with anticipation of developing effective therapeutic intervention. Currently, there are no effective disease-modifying treatments for either the prevention or treatment of OA.

Pain relievers, muscle relaxants, mouth guards, physical therapy, injections, and surgery are presently used to treat the progression of osteoarthritis (OA) in the temporomandibular joint (TMJ). While these options have been successful for some, in others TMJ OA continues to be problematic, with prominent recurrence of moderate to severe joint pain[3].

Joint replacement can effectively alleviate pain and restore function of OA; however the procedure is often associated with high risk and does not necessarily prolong life. The potential benefits of joint replacement must be weighed against the risk of

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surgical mortality and morbidity and the discomfort and inconvenience associated with recovery[4]. Although joint replacement has had major impact on the management of OA, outcomes can be accompanied by significant limitations. Advances have also been made in the replacement of the TMJ, however patients still often require multiple surgeries in an effort to obtain relief alongside a multitude of affecting variables[5–7]. For non-replaceable joints, including the TMJ, drug treatments are preferable, and sometimes essential[3].

OA of the synovial joint is characterized by degeneration of the articular cartilage and dysfunction of joints. Histologically, OA is characterized by progressive destruction and loss of the articular and condylar cartilage. Catalysis of existing collagen matrix via the upregulation of matrix metalloproteinases (MMPs) is recognized as a contributing mechanism, with MMP-13 accepted as a viable target[8].

HtrA1 is a highly conserved secreted protease that degrades numerous extracellular matrix (ECM) proteins. HtrA1 has previously been identified as being upregulated in osteoarthritic patients and as having the potential to regulate MMP activity[9–11].

There is a continual search for an effective drug therapy to inhibit the progression of OA of the TMJ and knee joint. TGF-β1, a transforming growth factor, is known to play a critical role during OA development[12–15]. In a recent study we assessed the expression of TGF- β1 in two different OA mouse models[16]. Using immunofluorescence, selected tissue sections of each genotype were stained for the presence of TGF- β1. The immunofluorescence results indicated increased expression of the molecular marker TGF-β1 in the two mutants relative to wild-type controls. Chen *et al.*, showed that TGF-β1 is one of the key initiators of the OA molecular pathway and therefore is, in part, responsible for the initiation of articular cartilage breakdown[17]. Losartan, an angiotensin II receptor antagonist, is a generic drug for the treatment of hypertension. Because this drug also blocks the TGF-β1 signaling pathway, it is therefore a candidate drug for treatment of OA in a genetic mouse model of this disease[18–20].

The *cho* mutation in the C57BL strain of mice is the result of a single nucleotide deletion in the *col11a1* gene, resulting in a frameshift mutation and subsequent early stop codon[21]. Early termination of the translation process affects the availability of Type XI collagen in the ECM, which influences the development and structural integrity of articular cartilage[9]. Likewise, there are genetic factors contributing to the initiation of OA in humans, such as the gene(s) involved with Stickler Syndrome[22–25].

In the present study we evaluated the effectiveness of Losartan in attenuating the progression of OA in heterozygous chondrodysplasia (*cho*/+) mice carrying the mutation in the type XI collagen gene. The *cho* mutation is genetically recessive with regard to chondrodystrophic dwarfism; however, in the heterozygous condition in which case the skeleton is normal, the mutation has been shown to induce early-onset OA in the knee and TMJ, at the same time showing early over-expression of TGF-β1 and HtrA1 in the joint cartilage and associated tissues. By targeting TGF-β1 we report that Losartan therapy is effective in attenuating the progression of OA in the TMJ and knee joint in heterozygous chondrodysplasia (*cho*/+) mice.

Methods

Facilities, experimental animals, and genotyping

Mouse genotyping was performed as previously described[21]. Heterozygous *cho*/+ mice and their wild-type littermates were separated and maintained under a 12-h lighting schedule (12 h with light, 12 h without light).

The C57BL strain of mice carrying the *cho* mutation, a *col11α1* mutation of Type XI collagen, was maintained as a semi-inbred colony in the Brigham Young University's Specific Pathogen Free facility under the Institutional Animal Care and Use Committee (IACUC) protocol number 150901. The mice were genotyped by deoxyribonucleic acid (DNA) sequence analysis from tail samples obtained at 6 weeks of age, then divided into three groups, viz., wild type control mice ($n = 6$; +/+), untreated mutant mice ($n = 6$; *cho*/+) and drug-treated mutant mice ($n = 6$; *cho*/+). The total number of animals used in this study was 18, 12 of which had the desired *cho*/+ genotype and 6 of which were +/+ genotype that served as wild type controls.

The number of animals used allowed appropriate statistical analysis at an inferential uncertainty significance level of $P < 0.05$ with a power of 0.8 using a standard deviation of 0.6, and a minimum detectable difference in the Modified Mankin score of 1 point.

The mice were separated into 6 cages, each cage containing 1 +/+ and 2 *cho*/+ mice. We isolated the cages from the facility's water supply and measured water consumption using volumetric bottles. Three of the cages were given 300 ml of H_2O containing 18 mg of Losartan (0.6 g/L)[17]. The remaining three cages were given 300 ml of H_2O without the drug. We measured water consumption and changed the water supply with or without drug weekly to ensure proper dosage per cage. We also performed daily checks to ensure the health and safety of each animal. Body weight data were gathered monthly to further ensure the health of all animals. The drinking water was replaced weekly, and all animals were monitored for weight gain and water consumption throughout study, i.e., until 9 months of age.

Tissue processing

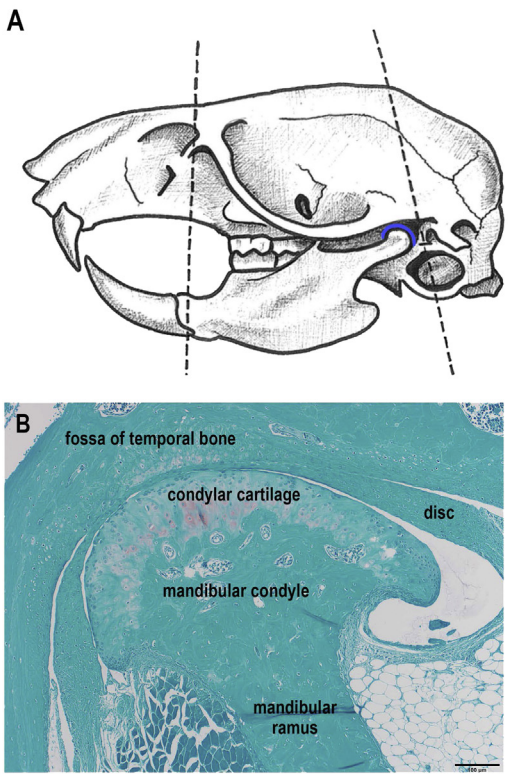
Following the 7-month treatment period, mice from each genotype were weighed, before being euthanized, with the right and left TMJs collected. The knee joints were also excised and similarly processed as follows. Tissues were fixed in 4% paraformaldehyde, decalcified, embedded in paraffin, sectioned, and stained with Safranin-O and Fast Green. The TMJs were processed into coronal sections at a thickness of 6 μm obtained posterior to anterior [Fig. 1(A) and (B)]. Coronal sections of the knee joint were prepared similarly for histological examination as previously described[26].

Tissue evaluation and histopathological scoring via Modified Mankin procedure

Once stained with Safranin-O and Fast Green, tissues were scored for histopathology according to the Modified Mankin scoring system[26,27(p1)]. The extent of OA histopathology was quantified by the sum of scores of the following four criteria: surface fissuring (0–3), pericellular matrix staining (0–2), spatial arrangement of chondrocytes (0–3), and interterritorial matrix staining (0–3) [Fig. 1(C)]. The scores of each genotype were averaged and compared for statistical significance; the higher the score implies more advanced OA histopathology. Both TMJ and knee joint tissues were also analyzed using the Osteoarthritis Research Society International (OARSI) procedure[16,28]. The statistical analysis in the present study is based on a two-way analysis of variance (ANOVA)' and was used to compare the means of each treatment group for statistically significant differences at the $P < 0.05$ level[26].

Immunohistochemical analysis

Immunohistochemistry using antibodies against HtrA1was performed on the TMJ sections from *cho*/+ Losartan treated and untreated mice. Each slide was deparaffinized and then blocked



C

Modified Mankin Scoring Procedure

Surface Fissuring	
Smooth noneroded cartilage	0
Rough noneroded cartilage	1
Superficial fibrillation	2
Separation of uncalcified from calcified cartilage	3
Pericellular Matrix Staining	
Normal intensity	0
Focal points of enhanced intensity	1
More than 40% of cartilage with enhanced staining	2
Spatial Arrangements of Chondrocytes	
Normal	0
Diffuse hypercellularity	1
Focal points of hypocellularity	2
Diffuse hypocellularity	3
Interterritorial Matrix Staining	
Normal staining	0
Reduced staining	1
Focal points without any staining	2
More than 40% of cartilage without any staining	3

Fig. 1. (**A, B) Diagram of the mouse skull and a representative histological section of the TMJ.** The vertical coronal sections allowed removal of the anterior and posterior regions of the skull prior to paraffin embedment and tissue sectioning. The condylar cartilage is indicated in blue (A). Serial microtome sections 6 μ thick and collected from posterior to anterior (rostral) and stained with Safranin O and Fast Green show the condylar cartilage (B) that was analyzed via the Mankin procedure. The bar in the lower right corner of the image represents 100 μm. **(C) Modified Mankin scoring system used to quantify the extent of OA.** These criteria, considered to be the hallmark in the progression of OA, can be seen in the Safranin-O and Fast Green stained sections of wild-type cartilage in comparison to mutant cartilage with and without Losartan treatment. Surface fissuring represents the extent of cartilage erosion of the condyle. Pericellular matrix staining refers to the staining around each chondrocyte, determined by Safranin-O staining the proteoglycan. With regard to spatial arrangement of cells, typically the chondrocytes are arranged in a columnar fashion, but as the progression of OA continues the chondrocytes group together forming clusters, leaving areas of hypocellularity. Interterritorial matrix staining refers to staining of the extracellular matrix (ECM) by Safranin-O.

with donkey serum for 1 h. Antibodies were diluted 1:200, applied to specimens, and incubated overnight at 4°C. On the following day, samples were rinsed with phosphate buffered saline (PBS) and incubated with avid/biotin complex (ABC) mix. The samples were then rinsed again with PBS before incubation with biotinylated secondary rabbit antibody. After a final rinse, a color reaction was initiated using a peroxidase substrate (Vector Labs, NovaRED). Differences in staining intensity between the *cho*/+ Losartan treated and the *cho*/+ untreated tissue sections were then compared by visual inspection under the light microscope. Stained and unstained cells were quantified within a defined area, providing an approximate percentage of cells rescued from upregulation of HtrA1 protein.

Cell culture

Immortalized mouse articular chondrocytes (iMAC) purchased from abm (Vancouver, BC) were grown in PriCoat™ flasks, PriGrow III™ media with 10% fetal bovine serum (FBS) and penicillin/streptomycin and kept at 37°C in a humidified air chamber with 5% CO_2. Cells were seeded at 3×10^5 cells/flask for the experiments and then grown to confluency. Cells between 3 and 4 passages were used for all experiments.

Losartan (Sigma–Aldrich) was dissolved in dimethylsulfoxide (DMSO) to obtain a 1 mM stock solution. Cell cultures were simultaneously induced with TGF-β1 (10 ng/ml) alone or TGF-β1 and Losartan (10 μM). The cells were allowed to continue growing in the incubator for 24 h.

Protein extraction

Cells were washed with cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, 1% Sodium deoxycholate, 1% Triton X-100% and 0.1% sodium dodecyl sulfate (SDS)). Cells were collected with a cell scraper and centrifuged at 14,000 rpm for 30 min. Supernatant was collected and assayed for protein concentration. Samples were mixed with 4× SDS Loading buffer (40% Glycerol, 8% SDS, 200 mM Tris–HCl, 400 mM Dithiothreitol, 0.005% bromophenol blue) for further analysis.

Gel electrophoresis

A 12% Sodium dodecyl sulfate (SDS)-polyacrylamide solution was prepared by standard methods and samples were electrophoresed for 1 h at 150 V.

Western blotting

Proteins from gels were transferred to 0.45 μm Nitrocellulose paper (Bio-Rad) and then blocked with ProteinSimple Blocking Buffer (PBS) (ProteinSimple). Primary antibodies were diluted in blocking solution containing 0.1% Tween and incubated overnight at 4°C with monoclonal antibody to HtrA1 (Abcam). Blots were normalized via membrane probes with Histone H3 (Cell Signaling Technology, Beverly, MA).

Secondary antibody was incubation performed in blocking solution with the MultiFluor kit (ProteinSimple). The proteins were

detected and visualized by fluorescence using a FluorChem M system (ProteinSimple, San Jose, CA). Densitometry analysis of specific bands was completed using AlphaView software. Statistical analysis, including 1-way ANOVA with Dunnett's Multiple Comparison Test, was performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

Results

Health, body weight and water consumption unaffected by Losartan treatment

Baseline data for health, animal weight, and water consumption was consistent across all treatment groups. Average Losartan dose was 1.09 ± 0.09 mg/day/10 g. No marked increase or decrease in animal weight or water consumption was noted during administration of Losartan. At the termination of the experiment, animal weights of the mutant control group averaged 34.15 g ± 4.03, as compared to the Losartan treated group of 35.78 g ± 6.06.

Losartan ameliorates TMJ histopathology in mice predisposed to OA

Histologically, the wild type (+/+) control TMJ condylar cartilage showed an intact superficial border, normal columnar cellular organization and typical staining of the cartilage ECM and the pericellular matrix immediately surrounding the chondrocyte [Fig. 2(A), (D) and (G)]. The untreated *cho*/+ group showed marked histopathological changes including surface fissuring, cell clustering, areas of hypocellularity and deficient staining of the ECM [Fig. 2(B), (E) and (H)]. The Losartan-treated *cho*/+ mice displayed normal chondrocyte placement including lack of cell clustering and clear columnarization as observed in the wild type (+/+) group. In addition, staining of the matrix in the Losartan treated group was similar to that observed in the wild type condylar cartilage, unlike the altered staining observed in the untreated *cho*/+ TMJ [Fig. 2(C), (F), and (I)].

There was a statistically significant difference ($P < 0.05$) in the Mankin score of the TMJ comparing wild type (3.8 ± 0.8) and untreated mutant mice (8.1 ± 1.5), but not between the wild type and Losartan treated mutant mice (3.7 ± 1.2). Mankin scoring thus confirms statistically that which was observed by visual inspection (Fig. 3).

Losartan ameliorates knee joint histopathology in mice predisposed to OA

Histologically, the wild type (+/+) control knee joint articular cartilage for both femur and tibia showed an intact superficial border, typical cellular organization superior and inferior to the tide mark, and typical staining of cartilage (ECM) and the pericellular

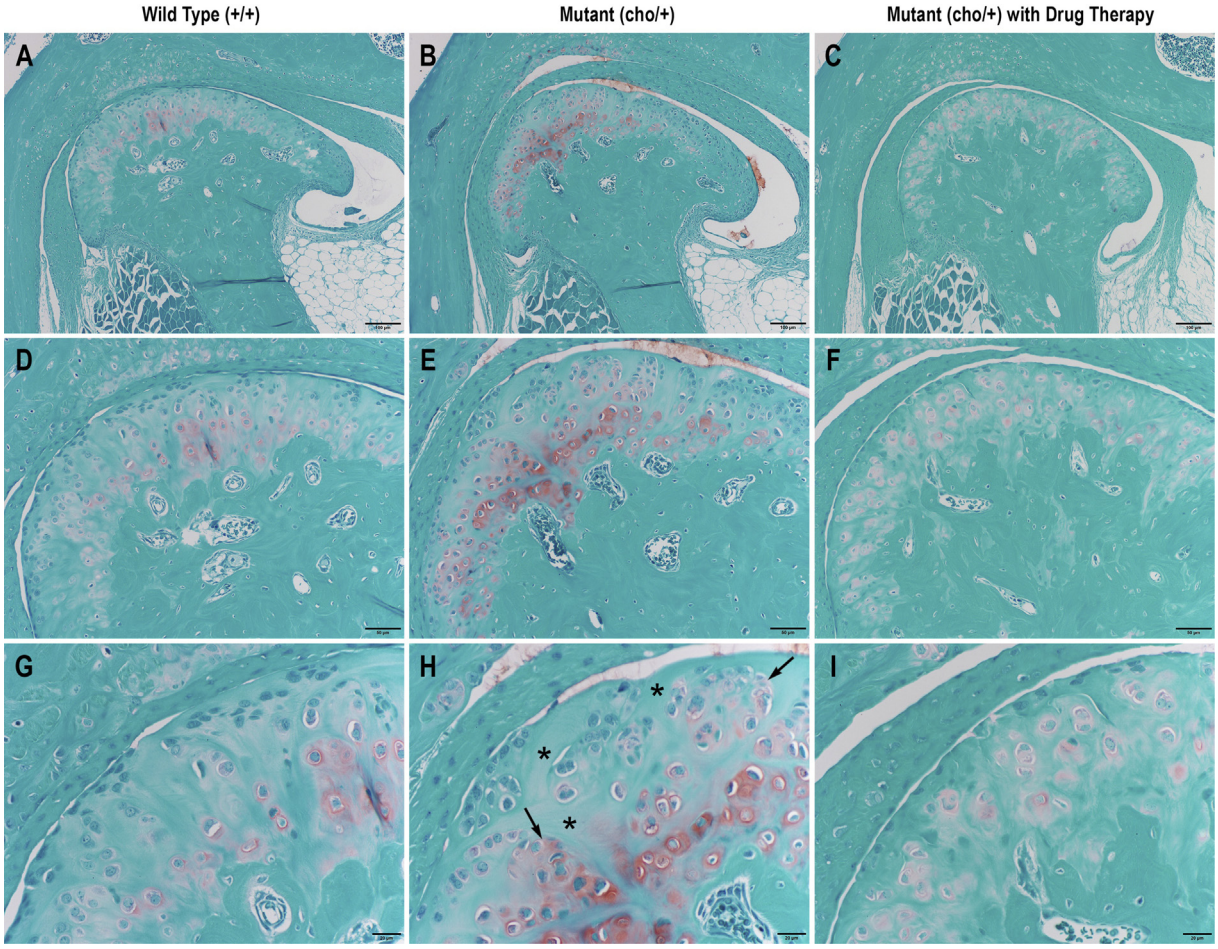


Fig. 2. Histological comparison of wild type, untreated mutant and drug-treated mutant TMJ after staining with Safranin-O/Fast Green. Comparing wild type (A, D, G) with mutant untreated condylar cartilage (B, E, H), the untreated mutant mice show signs of OA, i.e., breakdown of condylar cartilage including hypocellularity, (asterisk) fissuring, and chondrocyte clustering (arrow) accompanied by increased staining of the pericellular space. Mutant treated with Losartan displayed normal structural morphology and proteoglycan staining of the condylar cartilage (C, F, I). The bar in the lower right corner of photographs A-C, D-F, and G-I represents 100, 50 and 20 μm respectively.

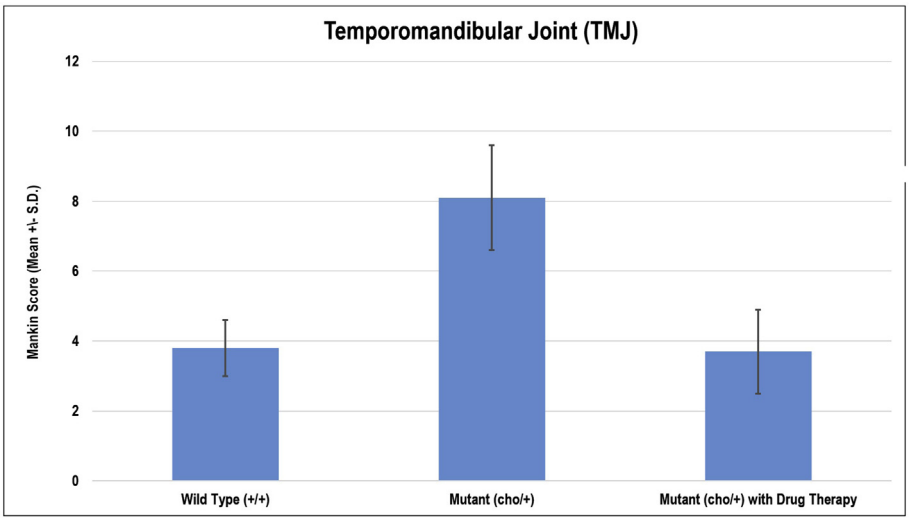


Fig. 3. Mankin scoring confirms the improvement in OA histopathology of the TMJ following drug therapy. The wild type had a mean Mankin score of 3.8 ± 0.8, the untreated mutant had a mean Mankin score of 8.1 ± 1.5, and the mutant treated with Losartan had a score of 3.7 ± 1.2. By comparing the Losartan treatment group with the mutant untreated group, Losartan is shown to significantly reduce the average Mankin score ($P < 0.05$, $n = 6$).

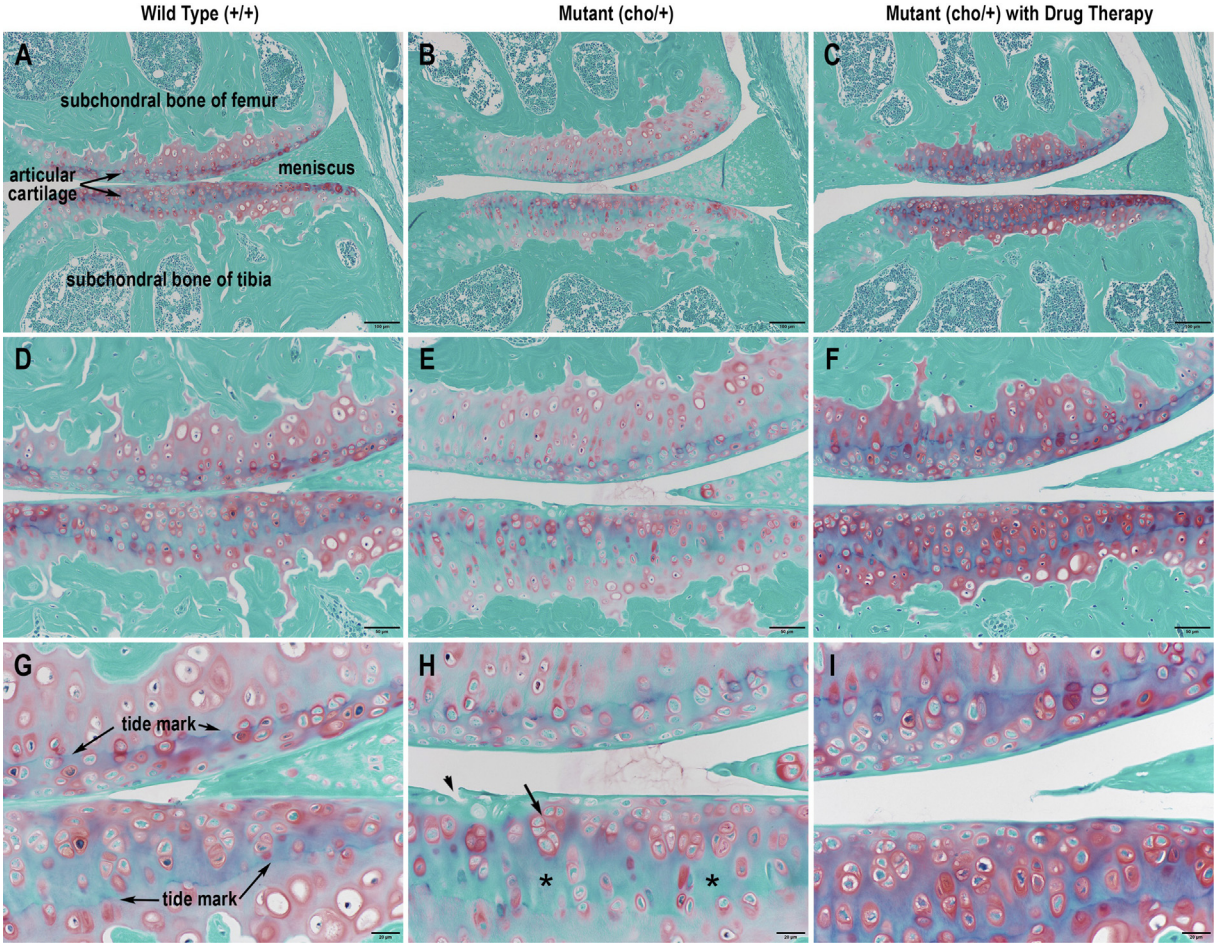


Fig. 4. Light microscopy of the knee joint comparing the three groups of mice shows marked improvement in overall histology following drug therapy. The wild type (+/+) control group, with the exception of mild osteoarthritic expression expected in 9-month old mice, shows an intact articular cartilage surface, columnar organization of chondrocytes superior and inferior to the tide mark, and typical Safranin O staining of the extracellular matrix and matrix immediately surrounding the cells (Figs. A, D, G). The untreated mutant (*cho/+*) shows surface irregularity noted here by fissuring (arrowhead) on the articular surface of the joint. The untreated mutant also shows increased hypocellularity, i.e., extracellular matrix devoid of chondrocytes (asterisk), and cell clustering (arrow), in addition to a decrease in Safranin O staining of the extracellular matrix (Figs. B, E, H). Comparable to that observed in wild type (+/+) control mice, mutant (*cho/+*) mice treated with Losartan show normal histology (Figs. C, F, I). Results shown for each treatment group are representative of all animals in each group. The bar in the lower right corner of photographs A–C, D–F, and G–I represents 100, 50 and 20 μm respectively.

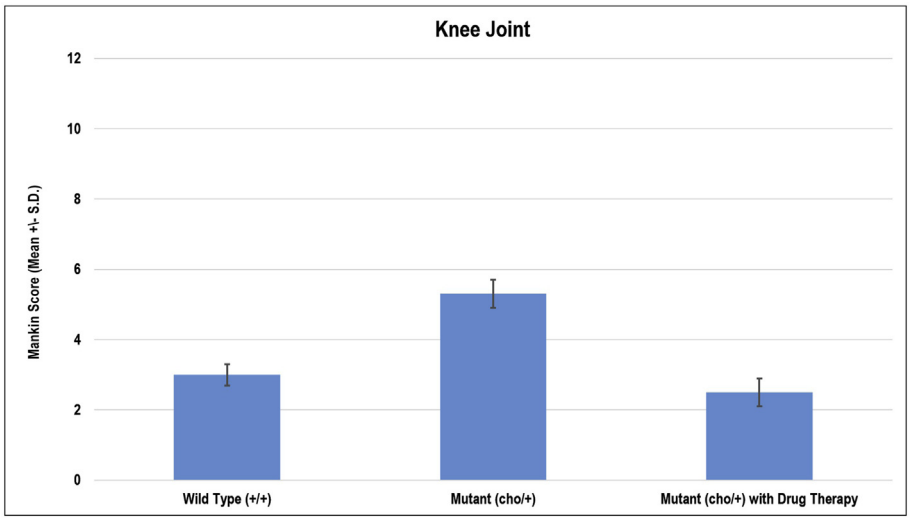


Fig. 5. Average Mankin scores show significant improvement in the mutant knee score following Losartan treatment. The mean Mankin score for the untreated mutant (*cho/+*) group was significantly higher than that of the wild type (+/+) control group ($P < 0.05$, $n = 6$). The Losartan treatment group had a significantly lower average Mankin score in comparison with the untreated mutant group. The mean score for the Losartan-treated mutant group was comparable to that of the wild type (+/+) control mice.

matrix of the space immediately surrounding the chondrocyte [Fig. 4(A), (D) and (G)]. The untreated *cho*/+ group showed marked histopathological changes including surface fissuring, cell clustering, areas of hypocellularity, and deficient staining of the ECM [Fig. 4(B), (E) and (H)]. The Losartan-treated *cho*/+ mice displayed normal chondrocyte placement including lack of cell clustering and clear columnarization as observed in the wild type group. In addition, staining of the ECM was similar to that observed in the wild type articular cartilage, unlike the altered staining observed in the untreated *cho*/+ knee joint [Fig. 4(C), (F) and (I)].

Applying the Mankin scoring procedure to knee articular cartilage revealed that the +/+ control group mice had an average score of 3.0 ± 0.3, the untreated *cho*/+ animals had an average score of 5.3 ± 0.4, and the Losartan-treated *cho*/+ mice had an average score

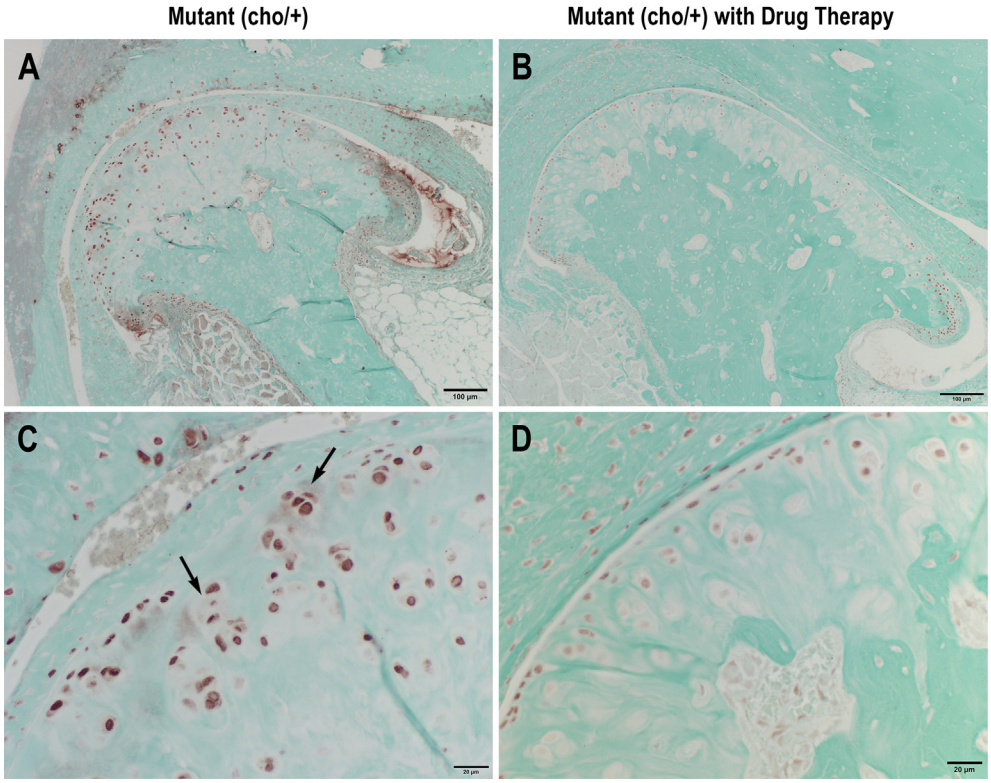


Fig. 6. Immunohistochemical comparison of mutant mouse (*cho/+*) TMJ shows the effectiveness of Losartan treatment in blocking up-regulation of HtrA1, an inflammatory OA marker. Mutant (*cho/+*) without Losartan treatment (A and C) shows clusters of HtrA1 positive chondrocytes (arrows). By comparison, treatment with Losartan (B and D) shows only background antibody staining, indicating a diminished inflammatory response. The bar in the lower right of the image represents 100 μ and 20 μ (A, B and C, D) respectively.

of 2.5 ± 0.4 (Fig. 5). The statistical analysis for the observed differences in knee histopathology showed that there was a significant difference ($P < 0.05$) between Losartan treated and untreated *cho*/+ groups, but no statistical difference between the Losartan-treated *cho*/+ and +/+ control groups.

In summary, the Mankin scores for the TMJ condylar cartilage and knee joint articular cartilage support what we had observed by visual inspection, i.e., the Losartan-treated *cho*/+ group exhibited similar histological features to that of the wild type control group, thereby demonstrating that Losartan therapy had ameliorated the progression of OA in the *cho*/+ animal model.

Losartan inhibits upregulation of HtrA1 as detected by immunohistochemistry

Immunohistochemical analysis of the TMJ in untreated *cho*/+ mice showed intense antibody staining of HtrA1throughout the condylar cartilage [Fig. 6(A) and (C)]. All untreated mice expressed intense antibody staining, with virtually all cells within the chondrocyte clusters being positively stained. By comparison, the Losartan treated *cho*/+ mice showed marked decreases in both frequency and intensity of cellular staining [Fig. 6(B) and (D)].

Losartan inhibits upregulation of HtrA1 within immortalized mouse chondrocytes

As shown in Fig. 7, Western Blot analysis demonstrates exposure of immortalized mouse chondrocytes (iMAC) to TGF- β1 significantly upregulates the presence of HtrA1 serine protease with respect to unexposed iMAC control ($P < 0.0001$, $n = 4$). However, treatment with Losartan in the presence of TGF- β1 inhibits the TGF- β1 induced upregulation of HtrA1 serine protease. We hereby report that in the presence of Losartan, TGF- β1 induced upregulation of HtrA1 is halted within iMAC *in vitro*, as was similarly observed with immunohistochemistry.

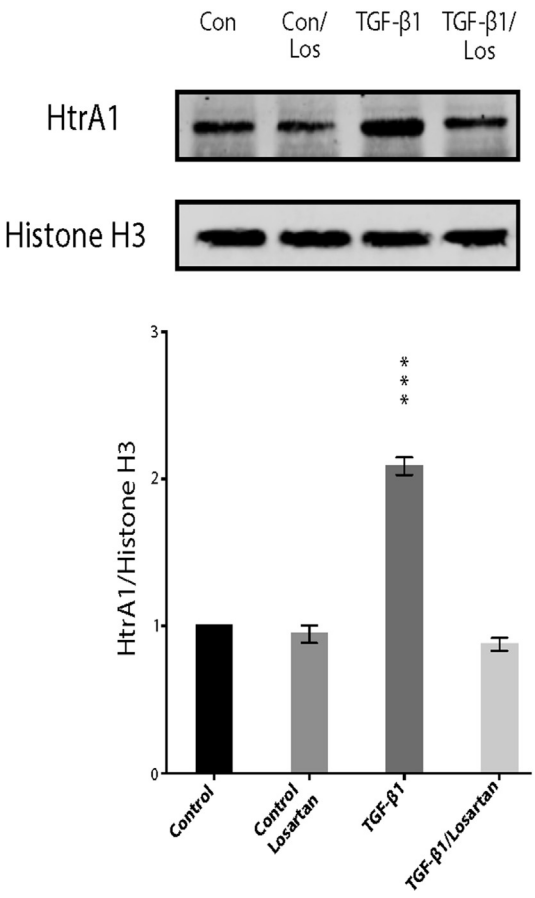


Fig. 7. Losartan prevents up-regulation of HtrA1 in vitro. Immortalized mouse chondrocytes (iMAC cells) were induced with 10 ng/mL of TGF-β and treated with Losartan at 10 μM for 24 h. HtrA1 expression was significantly increased in the induced group (TGF-β1) when compared with control (Con or Con/Los; $P < 0.0001$, $n = 4$) (1-way ANOVA, Dunnett). HtrA1 expression was not significantly different from that of control chondrocytes (Con or Con/Los) when iMAC cells were induced with TGF-β1 and concomitantly treated with Losartan (TGF-β1/Los). Samples were obtained from cytoplasmic protein extraction and normalized to Histone H3.

Discussion

Although TGF- β1 expression is required for normal differentiation of cartilage in the early stages of joint development, after completion of chondrocyte differentiation in adult mice the increased expression of TGF-β1 appears unnecessary[12,13]. As the untreated *cho*/+ mutants exhibited significantly increased Mankin scores compared with the wild type, the overexpression of TGF-β1 observed in the TMJ of *cho*/+ mutants suggests involvement of this inflammatory molecule in the pathogenesis of TMJ OA[16]. Overexpression of TGF-β1 has also been observed in knee OA of *cho*/+ mutant mice at 3 months of age and in a surgical mouse model of knee OA[16,17].

Our findings with 9 month old mice support the hypothesis that normal forces within a *col11a1* genetic defect, as seen with Stickler Syndrome, exacerbates the mechanical load transmitted through the ECM involved with OA. The *col11a1* genetic animal model offers a promising platform for characterization of the progression of OA in human. A common etiology of OA is the upregulation and binding of TGF-β1 with its receptor TGFBRII. Such signaling activates SMAD2/3 proteins that regulate cell proliferation, differentiation and cellular death[12,29–31]. This in turn induces the production of HtrA1, activating degradation of the pericellular matrix and leading to exposure of chondrocytes to type II collagen and signaling of Discoidin domain-containing receptor 2 (DDR2). The consequence of such signaling is that MMP-13 is upregulated and released into the matrix, destroying type II collagen and chondroitin sulfate proteoglycans of the ECM. Regardless of the synovial joint, i.e., TMJ or knee, this degeneration progresses to OA[9,32–36] (Fig. 8).

The present study demonstrates that Losartan treatment has a significant ameliorating impact on the progression of OA histopathology. Inasmuch as elevated expression and co-localization of TGF-β1, HtrA1 and p-Smad2 have been reported in TMJ condylar cartilage of *cho*/+ mice, inhibition of the TGF-β1 cascade may serve as a potential therapeutic for OA of the TMJ and knee[16,17,37]. Losartan's role as a TGF-β1 antagonist leading to the inhibition of the arthritic pathway would likely be similar in human OA, as seen in Stickler syndrome, as such patients experience early onset OA[24,38].

The reduction of arthritic-associated histopathology following treatment with Losartan in the *cho*/+ TMJ was especially pronounced in regard to fissuring and cell clustering. In the drug treatment group, the lack of clustering and presence of highly organized chondrocyte columns comparable to that observed in wild type animals demonstrate a positive effect of Losartan on the cartilage of the synovial joint of *cho*/+ animals. Also, minimal proteoglycan staining in the pericellular space of the *cho*/+ Losartan treated animals was observed, affirming the evidence of Losartan's beneficial effect. These results are further supported when compared to the untreated *cho*/+ mice, which showed numerous clusters of chondrocytes arranged in a disordered

fashion, multiple fissures on the surface of the cartilage, and increased proteoglycan staining of the pericellular space.

No evidence of osteophytes was observed in any of the untreated cho/+ mice. These observations are in agreement with the OA study of cho/+ mice by Xu *et al*., 2003. They examined for the presence of osteophytes but reported that "signs of advanced OA are not present in cho/+ mice until after 9 months".

The present study ended at 9 months, whereas Xu *et al*., 2003 extended to 15 months.

Confirming results come from the knee joint. In the untreated mutant, both the tibial plateau and femoral condyle show surface irregularities throughout, i.e., multiple fissures. Losartan treatment promoted surface normality, i.e., absence of fissuring and also promoted normal cellularity, thus contributing to the almost 3 point difference between the means of the mutant untreated and mutant Losartan-treated groups. The statistical analysis of the Mankin scores for the three knee groups (wild type control, mutant without treatment, and mutant treated with Losartan) demonstrate a marked decrease in the Mankin scoring of mutants following treated with Losartan.

Observing that treatment with Losartan limits the expression of HtrA1 within TMJ chondrocytes confirms Losartan as an inhibitor of

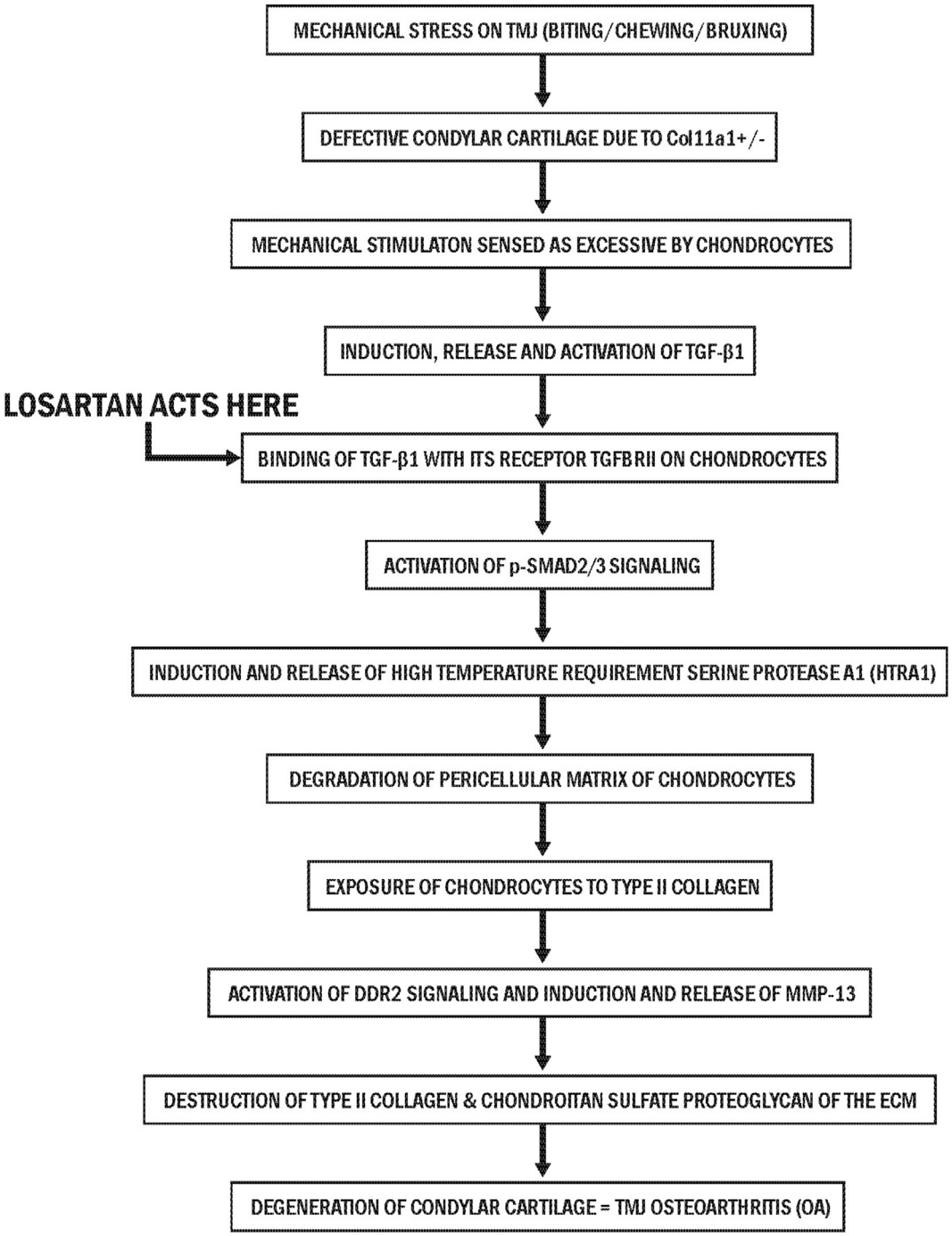


Fig. 8. Flow chart showing initiation of the inflammatory response pathway that leads to OA and point of antagonism by Losartan. This chart indicates the TGF-β1 inflammatory response pathway in *cho*/+ mice and the point at which Losartan blocks the molecular signaling pathway in the TMJ. This pathway and point antagonism would be similar to that postulated for onset and progression of knee OA, thereby ameliorating OA in the synovial joint[17].

TGF-β1 binding to its receptor TGFBRII located on condylar chondrocytes and other cells of this synovial joint. This restriction prevents TGF-β1 from initiating the signaling cascade necessary for the degeneration of the cartilage in the synovial joint. Specifically, the *cho*/+ Losartan treatment group displayed an absence of surface irregularities, histopathological features that would normally appear in the untreated *cho*/+ group. Furthermore, there were no signs of deterioration of the pericellular matrix, and the chondrocytes were organized in a columnar fashion in the Losartan-treated mutant cartilage. Each of these observations is indicative of an advantageous implementation of Losartan.

Our demonstrating that treatment with Losartan in the presence of TGF-β1 limits the expression of HtrA1 within iMAC cell culture supports previous studies showing Losartan as having an inhibitory effect on the TGF-β1 signalling cascade[18,20,39–43]. These data support the histological improvement that we observed *in vivo*, and provide insight into the therapeutic value of Losartan treatment. As inhibition of HtrA1 is critical in mediating the propagation of MMP-13 collagen destruction and disease progression, Losartan acts to halt the detrimental effects seen in OA.

Further research into the exact mechanism by which Losartan works is necessary in order to extend experimentation to clinical trials with humans. These trials would be essential to assess the effectiveness of Losartan in treating and preventing OA of most if not all etiological types in human populations. Further characterization of TGF-β1 regulation in genetic mouse models is warranted, along with concurrent data on signaling pathway proteins including SMADs, HtrA1, and MMP-13. Indeed some of these molecules have the potential of serving as OA biomarkers.

Conclusion

Using Losartan drug therapy to block binding of TGF-β1 to its receptor on the TMJ and knee joint chondrocyte has significantly reduced the Mankin scoring and prevented upregulation of HtrA1 of the chondrodysplasia mutant mouse. Furthermore, Losartan in the presence of TGF-β1 limits the expression of HtrA1 *in vitro*. Combined, these findings suggest that the progression of OA in humans can be slowed or stopped with treatment that is antagonistic to TGF-β1.

Our findings are consistent with data from several other recent investigations, indicating that inhibition activity, rather than therapeutic application of TGF-β1, should be considered in treating OA. The present study demonstrates that HtrA1, a serine protease, is one of the down-stream efforts of the increased TGF-β1 activity in the development of OA. We propose that Losartan, an angiotensin receptor II antagonist, may be repurposed for treatment of OA.

Further research into the development of TGF-β1 blockers is anticipated to benefit many who suffer from OA.

Contributions

Michael Thomas: Created photo images of slides, wrote Discussion and Results, compiled initial Mankin Scores, assisted with staining of slides.

Zackary Fronk: Animal curation and treatment, tissue procurement and processing, collection, analysis and interpretation of data, and drafting and critical revision of the manuscript.

Andrew Gross, *PhD*, *DMD*: Corresponding author, analysis, interpretation, and collection of cell culture data, drafting of the manuscript, critical revision, collection and assembly of data, and final approval of the manuscript.

Danielle Willmore: Animal curation and treatment, tissue harvesting and processing, data collection, analysis and interpretation, and drafting and critical revision of the manuscript.

Alejandro Arango: Drew mouse skull drawing, wrote the Abstract, dissected skulls to obtain TMJs, transported specimens for tissue sectioning, assisted with staining slides, and poster creation for science meeting.

Charles Higham: Co-authored the Introduction, wrote figure legends, assisted in initial Mankin scoring, and assisted in staining slides.

Victoria Nguyen: Co-authored the Introduction, assisted in staining and Mankin scoring, and created Google documents.

Hana Lim: Co-authored Methods section, assisted in staining and Mankin scoring.

Vijay Kale, *Bpharm*, *Mpharm*, *PhD*: DNA isolation, purification, quantification, and sequencing, polymerase chain reaction (PCR) genotyping, Preparation of pharmaceuticals, Manuscript review and approval.

Glen McMillan, *DDS*: Scheduled research lab meetings, edited manuscript layout, oversaw all phases of project completed at Roseman University.

Robert Seegmiller, *PhD*: Lead investigator, made all arrangements for animal husbandry, taught students research principles, staining techniques, microscope protocol, scoring methods, and manuscript preparation standards.

Conflict of interests

No author had competing or conflicting interests during the duration of this study.

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Ethical approval

All animal work described in this paper was pre-approved by Brigham Young University under IACUC protocol #150901.

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References

1. Cross M, Smith E, Hoy D, *et al*. The global burden of hip and knee osteoarthritis: estimates from the Global Burden of

Disease 2010 study. Ann Rheum Dis February 2014, https://doi.org/10.1136/annrheumdis-2013-204763. annrheumdis-2013-204763.
2. Musumeci G, Aiello FC, Szychlinska MA, Di Rosa M, Castrogiovanni P, Mobasheri A. Osteoarthritis in the XXIst century: risk factors and behaviours that influence disease onset and progression. Int J Mol Sci 2015;16(3):6093–112, https://doi.org/10.3390/ijms16036093.
3. Ingawalé S, Goswami T. Temporomandibular joint: disorders, treatments, and biomechanics. Ann Biomed Eng 2009;37(5):976–96, https://doi.org/10.1007/s10439-009-9659-4.
4. Hamel MB, Toth M, Legedza A, Rosen MP. Joint replacement surgery in elderly patients with severe osteoarthritis of the hip or knee: decision making, postoperative recovery, and clinical outcomes. Arch Intern Med 2008;168(13):1430–40, https://doi.org/10.1001/archinte.168.13.1430.
5. Morrey BF. Difficult complications after hip joint replacement. Dislocation. Clin Orthop 1997;344:179–87.
6. Basilico FC, Sweeney G, Losina E, Gaydos James, Skoniecki Debra, Wright Elizabeth A, *et al*. Risk factors for cardiovascular complications following total joint replacement surgery. Arthritis Rheum 2008;58(7):1915–20, https://doi.org/10.1002/art.23607.
7. Wagner H. Surface replacement arthroplasty of the hip. Clin Orthop 1978;134:102–30.
8. Rodriguez RR, Seegmiller RE, Stark MR, Bridgewater LC. A type XI collagen mutation leads to increased degradation of type II collagen in articular cartilage. Osteoarthritis Cartilage 2004;12(4):314–20, https://doi.org/10.1016/j.joca.2003.12.002.
9. Xu L, Flahiff CM, Waldman BA, Wu D, Olsen BR, Setton LA, *et al*. Osteoarthritis-like changes and decreased mechanical function of articular cartilage in the joints of mice with the chondrodysplasia gene (cho). Arthritis Rheum 2003;48(9):2509–18, https://doi.org/10.1002/art.11233.
10. Tiaden AN, Bahrenberg G, Mirsaidi A, Glanz S, Blüher M, Richards PJ. Novel function of serine protease HTRA1 in inhibiting adipogenic differentiation of human mesenchymal stem cells via MAP kinase-mediated MMP upregulation. Stem Cells Dayt Ohio 2016;34(6):1601–14, https://doi.org/10.1002/stem.2297.
11. Tiaden AN, Klawitter M, Lux V, Mirsaidi Ali, Bahrenberg Gregor, Glanz Stephan, *et al*. Detrimental role for human high temperature requirement serine protease A1 (HTRA1) in the pathogenesis of intervertebral disc (IVD) degeneration. J Biol Chem 2012;287(25):21335–45, https://doi.org/10.1074/jbc.M112.341032.
12. Blaney Davidson EN, van der Kraan PM, van den Berg WB. TGF-beta and osteoarthritis. Osteoarthritis Cartilage 2007;15(6):597–604, https://doi.org/10.1016/j.joca.2007.02.005.
13. Fang J, Xu L, Li Y, Zhao Z. Roles of TGF-beta 1 signaling in the development of osteoarthritis. Histol Histopathol 2016;31(11):1161–7, https://doi.org/10.14670/HH-11-779.
14. Xie L, Tintani F, Wang X, Li Fengfeng, Zhen Gehua, Qiu Tao, *et al*. Systemic neutralization of TGF-β attenuates osteoarthritis. Ann N Y Acad Sci 2016;1376(1):53–64, https://doi.org/10.1111/nyas.13000.
15. Finnson KW, Chi Y, Bou-Gharios G, Leask A, Philip A. TGF-b signaling in cartilage homeostasis and osteoarthritis. Front Biosci Sch Ed 2012;4:251–68.
16. Long E, Motwani R, Reece D, Pettit N, Hepworth J, Wong P, *et al*. The role of TGF-ß1 in osteoarthritis of the temporomandibular joint in two genetic mouse models. Arch Oral Biol 2016;67:68–73, https://doi.org/10.1016/j.archoralbio.2016.03.004.
17. Chen R, Mian M, Fu M, Zhao Jing Ying, Yang Liang, Li Yefu, *et al*. Attenuation of the progression of articular cartilage degeneration by inhibition of TGF-β1 signaling in a mouse model of osteoarthritis. Am J Pathol 2015;185(11):2875–85, https://doi.org/10.1016/j.ajpath.2015.07.003.
18. Bar-Klein G, Cacheaux LP, Kamintsky L, Prager Ofer, Weissberg Itai, Schoknecht Karl, *et al*. Losartan prevents acquired epilepsy via TGF-β signaling suppression. Ann Neurol 2014;75(6):864–75, https://doi.org/10.1002/ana.24147.
19. Campistol JM, Iñigo P, Jimenez W, Lario Sergio, Clesca Paul H, Oppenheimer Federico, *et al*. Losartan decreases plasma levels of TGF-β1 in transplant patients with chronic allograft nephropathy. Kidney Int 1999;56(2):714–9, https://doi.org/10.1046/j.1523-1755.1999.00597.x.
20. Cohn RD, van Erp C, Habashi JP, Soleimani Arshia A, Klein Erin C, Lisi Matthew T, *et al*. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. Nat Med 2007;13(2):204–10, https://doi.org/10.1038/nm1536.
21. Li Y, Lacerda DA, Warman ML, Yoshioka H, Ninomiya Y, Oxford JT, *et al*. A fibrillar collagen gene, Col11a1, is essential for skeletal morphogenesis. Cell 1995;80(3):423–30, https://doi.org/10.1016/0092-8674(95)90492-1.
22. Robin NH, Moran RT, Ala-Kokko L. Stickler syndrome. In: Adam MP, Ardinger HH, Pagon RA, *et al*, Eds. GeneReviews®. Seattle (WA). Seattle: University of Washington, 1993, http://www.ncbi.nlm.nih.gov/books/NBK1302/. Accessed June 12, 2018.
23. Aury-Landas J, Marcelli C, Leclercq S, Boumédiene K, Baugé C. Genetic determinism of primary early-onset osteoarthritis. Trends Mol Med 2016;22(1):38–52, https://doi.org/10.1016/j.molmed.2015.11.006.
24. Francomano CA, Liberfarb RM, Hirose T, Maumenee IH, Streeten EA, Meyers DA, *et al*. The Stickler syndrome: evidence for close linkage to the structural gene for type II collagen. Genomics 1987;1(4):293–6, https://doi.org/10.1016/0888-7543(87)90027-9.
25. Snead MP, Yates JRW. Clinical and molecular genetics of Stickler syndrome. J Med Genet 1999;36(5):353–9, https://doi.org/10.1136/jmg.36.5.353.
26. Bomsta BD, Bridgewater LC, Seegmiller RE. Premature osteoarthritis in the Disproportionate micromelia (Dmm) mouse. Osteoarthritis Cartilage 2006;14(5):477–85, https://doi.org/10.1016/j.joca.2005.11.011.
27. Ricks ML, Farrell JT, Falk DJ, Holt DW, Rees M, Carr J, *et al*. Osteoarthritis in temporomandibular joint of Col2a1 mutant mice. Arch Oral Biol 2013;58(9):1092–9, https://doi.org/10.1016/j.archoralbio.2013.02.008.
28. Cohen WA, Servais JM, Polur I, Li Y, Xu L. Articular cartilage degeneration in the contralateral non-surgical temporomandibular joint in mice with a unilateral partial discectomy. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol 2014;43(2):162–5, https://doi.org/10.1111/jop.12113.
29. Davidson ENB, Vitters EL, Kraan PM van der, Berg WB van den. Expression of transforming growth factor-β (TGFβ) and the TGFβ signalling molecule SMAD-2P in spontaneous and instability-induced osteoarthritis: role in cartilage degradation, chondrogenesis and osteophyte formation. Ann Rheum Dis 2006;65(11):1414–21, https://doi.org/10.1136/ard.2005.045971.
30. Kraan PM van der, Davidson ENB, Blom A, Berg WB van den. TGF-beta signaling in chondrocyte terminal differentiation and osteoarthritis: modulation and integration of signaling pathways through receptor-Smads. Osteoarthritis Cartilage 2009;17(12):1539–45, https://doi.org/10.1016/j.joca.2009.06.008.
31. Yang X, Chen L, Xu X, Li C, Huang C, Deng C-X. TGF-β/Smad3 signals repress chondrocyte hypertrophic differentiation and

are required for maintaining articular cartilage. J Cell Biol 2001;153(1):35–46, https://doi.org/10.1083/jcb.153.1.35.
32. Goldring MB, Otero M. Inflammation in osteoarthritis. Curr Opin Rheumatol 2011;23(5):471–8, https://doi.org/10.1097/BOR.0b013e328349c2b1.
33. Holt DW, Henderson ML, Stockdale CE, Farrell JT, Kooyman DL, Bridgewater LC, *et al*. Osteoarthritis-like changes in the heterozygous sedc mouse associated with the HtrA1-Ddr2-Mmp-13 degradative pathway: a new model of osteoarthritis. Osteoarthritis Cartilage 2012;20(5):430–9, https://doi.org/10.1016/j.joca.2011.11.008.
34. Hu K, Xu L, Cao L, Flahiff CM, Brussiau J, Ho K, *et al*. Pathogenesis of osteoarthritis-like changes in the joints of mice deficient in type IX collagen. Arthritis Rheum 2006;54(9):2891–900, https://doi.org/10.1002/art.22040.
35. Troeberg L, Nagase H. Proteases involved in cartilage matrix degradation in osteoarthritis. Biochim Biophys Acta BBA Protein Proteomics 2012;1824(1):133–45, https://doi.org/10.1016/j.bbapap.2011.06.020.
36. Xu L, Peng H, Glasson S, Lee Peter L, Hu Kenpan, Ijiri Kosei, *et al*. Increased expression of the collagen receptor discoidin domain receptor 2 in articular cartilage as a key event in the pathogenesis of osteoarthritis. Arthritis Rheum 2007;56(8):2663–73, https://doi.org/10.1002/art.22761.
37. Li Y, Xu L. Advances in understanding cartilage remodeling. F1000Res 2015;4(F1000 Faculty Rev), https://doi.org/10.12688/f1000research.6514.1.
38. Li K, Thorne C. Adult presentation of Stickler syndrome type III. Clin Rheumatol 2010;29(7):795–7, https://doi.org/10.1007/s10067-010-1382-x.
39. Lacro RV, Dietz HC, Wruck LM, Bradley Timothy J, Colan Steven D, Devereux Richard B, *et al*. Rationale and design of a randomized clinical trial of β-blocker therapy (atenolol) versus angiotensin II receptor blocker therapy (losartan) in individuals with Marfan syndrome. Am Heart J 2007;154(4):624–31, https://doi.org/10.1016/j.ahj.2007.06.024.
40. Li Y, Chen R, Mian M, Zhao J, Golshirazian I, Asbury B, *et al*. Inhibition of TGF-β1 attenuates articular cartilage degeneration in mature knee joints of mouse models of osteoarthritis. Osteoarthritis Cartilage 2014;22:S17–8, https://doi.org/10.1016/j.joca.2014.02.054.
41. Nyström A, Thriene K, Mittapalli V, Kern Johannes S, Kiritsi Dimitra, Dengjel Jörn, *et al*. Losartan ameliorates dystrophic epidermolysis bullosa and uncovers new disease mechanisms. EMBO Mol Med 2015;7(9):1211–28, https://doi.org/10.15252/emmm.201505061.
42. Wolf G, Schneider A, Wenzel U, Helmchen U, Stahl RA. Regulation of glomerular TGF-beta expression in the contralateral kidney of two-kidney, one-clip hypertensive rats. J Am Soc Nephrol 1998;9(5):763–72.
43. Zhang L, Gao L-G, Zhang M, Zhou X-L. Genotype-phenotype analysis of F-helix mutations at the kinase domain of TGFBR2, including a type 2 Marfan syndrome familial study. Mol Vis 2012;18:55–63.