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(54) Title: INHIBITION OF WNT/β-CATENIN SIGNALING IN THE TREATMENT OF OSTEOARTHRITIS

(57) Abstract: The disclosure provides compositions and methods relating to the treatment of osteoarthritis. In particular, Wnt/β-catenin inhibitors are shown to be effective at treating certain aspects of osteoarthritis.

DESCRIPTION

INHIBITION OF WNT/β-CATENIN SIGNALING IN THE TREATMENT OF OSTEOARTHRITIS

PRIORITY INFORMATION

This application claims benefit of priority to U.S. Provisional Application Serial No. 62/626,784, filed February 6, 2018, the entire contents of which are hereby incorporated by reference.

BACKGROUND

I. Field

The present invention relates to the fields of molecular biology, pathology and medicine. More particularly, it relates to the treatment of osteoarthritis with inhibitors of the Wnt/β-catenin pathway.

II. Related Art

Osteoarthritis (OA) is a degenerative joint disease typically characterized by articular cartilage degeneration, bone remodeling, and osteophytosis as well as fibrosis and hyperplasia of the synovial membrane (Chen *et al.*, 2017). Although the synovium is an important player in OA pathology, synovial contribution toward OA development and progression is less well understood (Scanzello *et al.*, 2012). While OA is highly prevalent and associated with significant morbidity, there remains a lack of effective pharmacologic treatment (Bitton *et al.*, 2009; Holt *et al.*, 2011). Targeting detrimental signaling pathways within cartilage and synovium may holistically decelerate total joint destruction at earlier stages of OA to retard disease progression.

Wnt family proteins are a class of morphogens associated with embryonic skeletal formation, tissue repair, fibrosis, and joint homeostasis (Clevers *et al.*, 2012; Day *et al.*, 2005). Wnts regulate multiple signaling cascades, including the β-catenin–dependent (canonical) pathway (Clevers *et al.*, 2012; Komiya *et al.*, 2008; Nusse, 2005; Miller *et al.*, 1999). The Wnt/β-catenin pathway, which is typically quiescent in many adult organs, is activated in response to injury (Kurimoto *et al.*, 2015; Xiao *et al.*, 2016; Surendran *et al.* 2005). Its role in tissue repair and regeneration is complex and incompletely understood, although an increasing body of data suggests that its activation augments fibrotic repair (Tan *et al.*, 2011). The inventor recently published studies demonstrating that brief therapeutic Wnt inhibition following both

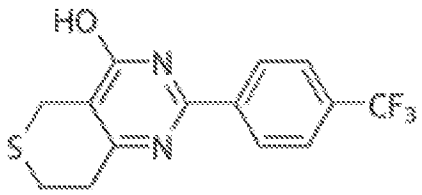
full thickness cutaneous or ischemic cardiac injury resulted in improved regenerative repair with less fibrosis (Bastakoty *et al.*, 2015; 2016).

In OA pathogenesis, activation of canonical Wnt signaling is observed in both articular cartilage and synovium following injury, with increased expression of both Wnt ligands and target genes (Dell'Accio *et al.*, 2006; 2008). Induced overexpression of β-catenin within mature chondrocytes has been shown to exacerbate cartilage degeneration, chondrocyte hypertrophy, and expression of matrix proteases (Day *et al.*, 2005; Yuasa *et al.*, 2008; Zhu *et al.*, 2009; Corr, 2008). However, significant or complete ablation of β-catenin in chondrocytes also results in the deleterious effect of chondrocyte apoptosis (Zhu *et al.*, 2008). Moreover, canonical Wnt expression in the synovium mediated by adenoviral transduction with Wnt ligands resulted in strong induction of cartilage pathology (van den Bosch *et al.*, 2015). Less is known about the effect of Wnt inhibition, with contradictory findings using overexpression or inhibition of *Dkk1* (Oh *et al.*, 2012; Funck-Bretano *et al.*, 2014). Although the sum of the published data using genetic modulation of Wnt suggests deleterious effects of Wnt on OA pathogenesis, there is little published on the cellular target(s) of Wnt modulation and specific cellular phenotypes induced by Wnt modulation, particularly within the synovium (Yuasa *et al.*, 2008; Blom *et al.*, 2010). There is also little known about the therapeutic effect of inhibiting the Wnt pathway within the context of disease.

SUMMARY

Thus, in accordance with the present disclosure, there is provided a method of treating a subject having osteoarthritis (OA) comprising administering to said subject an inhibitor of the Wnt/β-catenin pathway. The inhibitor may be an inhibitor of Porcupine or Tankyrase1/2, may antagonize protein interaction such as Dishevelled with Frizzled, and β-catenin with LEF-1, may inhibit Tcf, CBP, DKK-1, and SFRP-1, may stabilize negative regulators of Wnt signaling, may inhibit the interaction between β-catenin and Tcf4, may inhibit of Wnt processing and secretion, may be COL1A1 inhibitor, may be a Wnt-1 inhibitor, may be an Axin2 inhibitor, may be an SFRP1 inhibitor, may be aTNIK inhibitor, and may be an inhibitor of beta-catenin recruitment with its coactivator CBP.

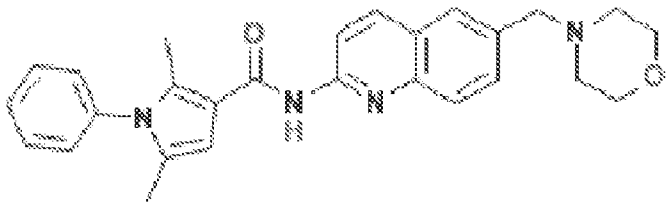
The inhibitor may be administered systemically, or administered local or regional to an OA disease site. The inhibitor may be administered more than once, such as daily, every other day, every third day, twice a week, weekly, monthly and/or chronically. The inhibitor may have the structure:



.

XAV-939

or



C113.

Treating may comprise reducing OA severity, reducing cartilage degeneration and/or synovitis, reducing proliferation of synovial fibroblasts and/or type I collagen synthesis by synovial fibroblasts, and/or increasing *COL2A1* and *PRG4* transcription. Treating may not comprise modulation of OA-driven chondrocyte proliferation.

The method may further comprise treating said subject with a second OA therapy, such as an anti-inflammatory or an immune inhibitor. The may be subject is a human, or a non-human mammal.

It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one."

It is contemplated that any embodiment discussed in this specification can be implemented with respect to any method or composition of the invention, and *vice versa*. Furthermore, compositions and kits of the invention can be used to achieve methods of the invention.

Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIGS. 1A-D. Wnt signaling is upregulated in the joint after injury. (FIG. 1A) Ten-week-old male mice were subjected to destabilization of the medial meniscus (DMM) surgery. Three weeks after injury, they were injected intra-articularly with saline or Wnt inhibitor every 10 days for a total of 5 times, and knee joints were collected 10 weeks after surgery. (FIGS. 1B-C) Synovial tissue (indicated by boxed areas of joint in H&E-stained images was stained by immunofluorescence for β-catenin (red) and periostin (green), a marker of fibroblasts. DAPI (blue) was used as a counterstain, and yellow indicates costaining of Wnt activation in fibroblasts. (FIG. 1B) Uninjured joints and joints after DMM surgery show upregulation of Wnt/β-catenin signaling after injury and increased periostin-positive cells in the synovium. (FIG. 1C) Saline-treated controls, compared with XAV-939-treated joints, indicate that treatment abrogated Wnt/β-catenin signaling in synovial fibroblasts. (FIG. 1D) Additional sections from the same joint from after DMM surgery were used as the isotype control. Images from the same joint region were used, although they are from separate sections. The H&E image is therefore reused from FIG. B. The representative isotype control shows no significant staining. Original magnification, ×40 (top rows); ×80 (bottom rows).

FIGS. 2A-E. Synovial fibroblasts are responsive to Wnt inhibition treatment. (FIG. 2B) Synovial fibroblasts isolated from patients with osteoarthritis (OA) were positive for expression of type I collagen (red) but not type II collagen (green). Nuclei were stained with DAPI (blue). Isotype controls were negative. Original magnification, ×20. (FIG. 2B) Synovial fibroblasts were compared with unaffected synovial fibroblasts (control) by real-time RT-PCR of *AXIN2*, a downstream marker of Wnt signaling. Expression was higher in synovial fibroblasts isolated from patients with OA than those from unaffected controls. Statistical analysis was performed using a *t* test, $***P < 0.001$, $n = 6$. (FIG. 2C) Schematic of the Wnt/β-catenin pathway showing target molecules inhibited by the Wnt inhibitors (C113 and XAV-939) used in the study. (FIG. 2D) Furthermore, synovial fibroblasts were treated with recombinant WNT3A with and without Wnt inhibitors (C113 and XAV-939) and analyzed by real-time RT-PCR of *AXIN2*. Wnt signaling was ameliorated by both inhibitors in unaffected

and OA synovial fibroblasts. One-way ANOVA with Tukey's post-hoc test was used to compare treatment groups, ***$P < 0.001$, $n = 6$. (FIG. 2E) Immunoblotting of β-catenin in OA synovial fibroblast cell lysates after treatment with recombinant WNT3A with or without inhibitors. Densitometry of immunoblots was performed to quantify the reduction of Wnt signaling after inhibitor treatment. One-way ANOVA with Tukey's post-hoc test was used to compare treatment groups, *$P < 0.05$, **$P < 0.01$, $n = 8$. Alteration of β-catenin signaling in the nucleus was confirmed by immunoblotting of synovial fibroblast nuclear lysates compared with lamin B1 loading control. (FIGS. 2B, 2D and 2E) For data presented as box-and-whiskers plots, horizontal lines indicate the medians, cross marks indicate the means, boxes indicate the 25th to 75th percentiles, and whiskers indicate the minimum and maximum values of the data set.

FIGS. 3A-E. Wnt inhibition reduced cartilage degeneration and synovitis in DMM model. (FIG. 3A) Following destabilization of the medial meniscus (DMM) surgery and Wnt inhibitor or saline control treatment, Safranin O/Fast Green staining performed on knee joints showed a reduction in the degree of cartilage degeneration with treatment. Original magnification, ×4 (top row); ×10 (bottom row). (FIG. 3B) The medial femoral condyle and medial tibial plateau were scored using the Osteoarthritis Research Society International (OARSI) system by two independent blinded readers, and both regions had significantly lower scores with Wnt inhibition compared w

ith saline group. (FIG. 3C) Chondrocyte cellularity was significantly reduced in saline-treated group compared with Wnt inhibitor–treated group. (FIGS. 3D-E) Masson's trichrome staining further showed reduced significant reduction in the degree of synovitis in Wnt inhibitor–treated group compared with saline-treated group. Original magnification, ×10 (left); ×4 (right). (FIGS. 3B, 3C and 3E) Statistical analysis was performed using a t test, *$P < 0.05$, $n = 8$. For data presented as box-and-whiskers plots, horizontal lines indicate the medians, cross marks indicate the means, boxes indicate the 25th to 75th percentiles, and whiskers indicate the minimum and maximum values of the data set.

FIGS. 4A-C. Proliferation of synovial fibroblasts is elevated in OA and can be moderated through Wnt inhibition. (FIG. 4A) Immunohistochemistry of Ki-67 on knee synovia from saline control and Wnt inhibitor mice 10 weeks after surgery showed a reduction in proliferation of synovial fibroblasts after treatment with Wnt inhibitor. Original magnification, ×20 (top); ×40 (bottom). (FIG. 4B) Knee synovia were stained by immunofluorescence with periostin (green) and PCNA (red), a proliferating cell marker, and

counterstained with nuclear DAPI (blue). The stained region is represented by the box in the H&E stain of the joint Synovia from saline-treated control mice demonstrated increased levels of periostin- and PCNA-positive cells, when compared with XAV-939–treated synovia. Additional sections from the same joint from the XAV-939–treated mouse were used as the isotype control. Images from the same joint region were used, although they are from separate sections. The H&E image is therefore reused. The representative isotype control shows minimal staining. Original magnification, ×10 (top row); ×80 (bottom 4 rows). (FIG. 4C) Proliferation was assessed by BrdU assay on synovial fibroblasts isolated from unaffected and osteoarthritis (OA) patients. Basal proliferation levels were significantly upregulated in OA patient fibroblasts compared with controls. Furthermore, WNT3A treatment of unaffected fibroblasts also significantly upregulated proliferation. Wnt inhibition in OA fibroblasts decreased the proliferation rate. Two-way ANOVA with Tukey's post-hoc test was performed, $*P < 0.05$, $**P < 0.01$, $n = 6$. For data presented as box-and-whiskers plots, horizontal lines indicate the medians, cross marks indicate the means, boxes indicate the 25th to 75th percentiles, and whiskers indicate the minimum and maximum values of the data set.

FIGS. 5A-B. Wnt inhibitor treatment results in decreased collagen type I expression. (FIG. 5A) Real-time RT-PCR of *COL1A1* on synovial fibroblasts isolated from osteoarthritis (OA) patients shows an increase after recombinant WNT3A treatment and a decrease after Wnt inhibitor treatment. One-way ANOVA with Tukey's post-hoc test was performed, $*P < 0.05$, $**P < 0.01$, $n = 5$. (FIG. 5B) Immunoblotting of type I collagen in OA synovial fibroblasts also demonstrates an increase after activation of Wnt signaling and a decrease in response to C113 or XAV-939 inhibitors. Densitometry was used to quantify COL1A1 protein levels normalized to the β-actin loading control. One-way ANOVA with Tukey's post-hoc test was performed, $*P < 0.05$, $**P < 0.01$, $n = 8$. For data presented as box-and-whiskers plots, horizontal lines indicate the medians, cross marks indicate the means, boxes indicate the 25th to 75th percentiles, and whiskers indicate the minimum and maximum values of the data set.

FIGS. 6A-H. Wnt inhibitor treatment of chondrocytes does not alter proliferation but modifies gene expression. (FIG. 6A) Immunohistochemistry of Ki-67 in the joint space from control and Wnt inhibitor mice 10 weeks after surgery demonstrated no detectable proliferation of chondrocytes after treatment with Wnt inhibitor. Original magnification, ×40. (FIG. 6B) Joints were stained with H&E and also by immunofluorescence with SOX9 (green), a marker for chondrocytes, and PCNA (red), a proliferating cell marker, and counterstained

with DAPI (blue). Additional sections from the same joint from the XAV-939-treated sample were used as the isotype control. Images from the same joint region were used, although they are from separate sections. The H&E image is therefore reused. Isotype controls showed background but no nuclear staining. Original magnification, ×10 (first column); ×80 (rightmost 4 rows). (FIG. 6C) Costained PCNA-positive and SOX9-positive cells were quantified, and no significant difference was detected between control and treated groups. Statistical analysis by *t* test was performed, $P > 0.05$, $n = 3$. (FIG. 6D) Chondrocytes were isolated from patients with osteoarthritis (OA) and stained by immunofluorescence for type II collagen (green) and DAPI (blue) to confirm phenotype. Isotype controls did not show any collagen type II staining. Original magnification, ×20. (FIG. 6E) Proliferation was assessed by BrdU assay and showed no response in proliferation with Wnt treatment or inhibition. (FIG. 6F) Real-time RT-PCR of *AXIN2* demonstrates that chondrocytes are responsive to Wnt activation and inhibition by C113 and XAV-939. (FIG. 6G) Real-time RT-PCR of *COL2A1* demonstrates that type II collagen transcripts are downregulated in response to Wnt expression and increased after inhibition. (FIG. 6H) Real-time RT-PCR of *PRG4* demonstrates that Wnt inhibition in chondrocytes increases *PRG4* transcript levels. (FIGS. 6E-H) Statistical analysis was performed using a 1-way ANOVA with Tukey's post-hoc test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $n = 5$. For data presented as box-and-whiskers plots, horizontal lines indicate the medians, cross marks indicate the means, boxes indicate the 25th to 75th percentiles, and whiskers indicate the minimum and maximum values of the data set.

DETAILED DESCRIPTION

Here, the inventor set out to study Wnt signaling and the effect of local therapeutic Wnt inhibition in a murine model of traumatic osteoarthritis (OA) caused by experimental destabilization of the medial meniscus (DMM). To assess the effect of Wnt inhibition on OA progression, she injected a Wnt inhibitor (XAV-939) into the intra-articular space. She further studied the cell-specific effects of Wnt modulation *in vitro* using primary human synovial fibroblasts and chondrocytes in order to understand the cellular and phenotypic basis for the disease-modifying effects.

Wnt/β-catenin signaling was highly activated in murine synovial fibroblasts as well as in OA-derived human synovial fibroblasts. XAV-939 ameliorated OA severity associated with reduced cartilage degeneration and synovitis *in vivo*. Wnt inhibition using mechanistically distinct small molecule inhibitors, XAV-939 and C113, attenuated the proliferation and type I collagen synthesis in synovial fibroblasts *in vitro*, but did not affect human OA-derived chondrocyte proliferation. However, Wnt modulation increased *COL2A1* and *PRG4* transcripts, which are down-regulated in chondrocytes in OA. In conclusion, therapeutic Wnt inhibition reduced disease severity in a model of traumatic OA via promoting anticatabolic effects on chondrocytes and antifibrotic effects on synovial fibroblasts and may be a promising class of drugs for the treatment of OA.

These and other aspects of the disclosure are set out in detail below.

A. Osteoarthritis

Osteoarthritis (OA) is a type of joint disease that results from breakdown of joint cartilage and underlying bone. The most common symptoms are joint pain and stiffness. Initially, symptoms may occur only following exercise, but over time may become constant. Other symptoms may include joint swelling, decreased range of motion, and when the back is affected weakness or numbness of the arms and legs. The most commonly involved joints are those near the ends of the fingers, at the base of the thumb, neck, lower back, knee, and hips. Joints on one side of the body are often more affected than those on the other. Usually the symptoms come on over years. It can affect work and normal daily activities. Unlike other types of arthritis, only the joints are typically affected.

Causes include previous joint injury, abnormal joint or limb development, and inherited factors. Risk is greater in those who are overweight, have one leg of a different length, and have jobs that result in high levels of joint stress. Osteoarthritis is believed to be caused by

mechanical stress on the joint and low grade inflammatory processes. It develops as cartilage is lost and the underlying bone becomes affected. As pain may make it difficult to exercise, muscle loss may occur. Diagnosis is typically based on signs and symptoms, with medical imaging and other tests occasionally used to either support or rule out other problems. In contrast to rheumatoid arthritis, which is primarily an inflammatory condition, in osteoarthritis, the joints do not typically become hot or red.

Treatment includes exercise, efforts to decrease joint stress, support groups, and pain medications. Efforts to decrease joint stress include resting and the use of a cane. Weight loss may help in those who are overweight. Pain medications may include paracetamol (acetaminophen) as well as NSAIDs such as naproxen or ibuprofen. Long-term opioid use is generally discouraged due to lack of information on benefits as well as risks of addiction and other side effects. If pain interferes with normal life despite other treatments, joint replacement surgery may help. An artificial joint typically lasts 10 to 15 years.

Osteoarthritis is the most common form of arthritis affecting about 237 million (3.3%) of the population. Among those over 60 years old, about 10% of males and 18% of females are affected. It is the cause of about 2% of years lived with disability. In Australia, about 1.9 million people are affected, and in the United States, 30 to 52.5 million people are affected. It becomes more common in both sexes as people become older.

The main symptom is pain, causing loss of ability and often stiffness. The pain is typically made worse by prolonged activity and relieved by rest. Stiffness is most common in the morning, and typically lasts less than thirty minutes after beginning daily activities, but may return after periods of inactivity. Osteoarthritis can cause a crackling noise (called "crepitus") when the affected joint is moved, especially shoulder and knee joint. A person may also complain of joint locking and joint instability. These symptoms would affect their daily activities due to pain and stiffness. Some people report increased pain associated with cold temperature, high humidity, or a drop in barometric pressure, but studies have had mixed results.

Osteoarthritis commonly affects the hands, feet, spine, and the large weight-bearing joints, such as the hips and knees, although in theory, any joint in the body can be affected. As osteoarthritis progresses, movement patterns (such as gait), are typically affected. Osteoarthritis is the most common cause of a joint effusion of the knee.

In smaller joints, such as at the fingers, hard bony enlargements, called Heberden's nodes (on the distal interphalangeal joints) or Bouchard's nodes (on the proximal

interphalangeal joints), may form, and though they are not necessarily painful, they do limit the movement of the fingers significantly. Osteoarthritis of the toes may be a factor causing formation of bunions, rendering them red or swollen.

Damage from mechanical stress with insufficient self-repair by joints is believed to be the primary cause of osteoarthritis. Sources of this stress may include misalignments of bones caused by congenital or pathogenic causes; mechanical injury; excess body weight; loss of strength in the muscles supporting a joint; and impairment of peripheral nerves, leading to sudden or uncoordinated movements. However exercise, including running in the absence of injury, has not been found to increase the risk. Nor has cracking one's knuckles been found to play a role.

A number of studies have shown that there is a greater prevalence of the disease among siblings and especially identical twins, indicating a hereditary basis. Although a single factor is not generally sufficient to cause the disease, about half of the variation in susceptibility has been assigned to genetic factors.

As early human ancestors evolved into bipeds, changes occurred in the pelvis, hip joint and spine which increased the risk of osteoarthritis. Additionally genetic variations that increase the risk were likely not selected against because usually problems only occur after reproductive success.

The development of osteoarthritis is correlated with a history of previous joint injury and with obesity, especially with respect to knees. Since the correlation with obesity has been observed not only for knees but also for non-weight bearing joints and the loss of body fat is more closely related to symptom relief than the loss of body weight, it has been suggested that there may be a metabolic link to body fat as opposed to just mechanical loading.

Changes in sex hormone levels may play a role in the development of osteoarthritis as it is more prevalent among post-menopausal women than among men of the same age. A study of mice found natural female hormones to be protective while injections of the male hormone dihydrotestosterone reduced protection. Increased risk of developing knee and hip osteoarthritis was found in those who work with manual handling (*e.g.*, lifting), have physically demanding work, walk at work or have climbing tasks at work (*e.g.*, climb stairs or ladders). Increased risk of developing hip osteoarthritis over time was found among those who work in bent or twisted positions. Increased risk of knee osteoarthritis was found in those who work in a kneeling or squatting position, experience heavy lifting in combination with a kneeling or squatting posture or work standing up.

Secondary factors of osteoarthritis are is caused by other factors but the resulting pathology is the same as for primary osteoarthritis, and include alkaptonuria, congenital disorders of joints, diabetes doubles the risk of having a joint replacement due to osteoarthritis and people with diabetes have joint replacements at a younger age than those without diabetes, Ehlers-Danlos Syndrome, hemochromatosis and Wilson's disease, inflammatory diseases (such as Perthes' disease; Lyme disease), all chronic forms of arthritis (*e.g.*, costochondritis, gout, and rheumatoid arthritis), gout, where uric acid crystals cause the cartilage to degenerate at a faster pace, injury to joints or ligaments (such as the ACL), as a result of an accident or orthopedic operations, ligamentous deterioration or instability may be a factor, Marfan syndrome, obesity, and joint infection.

While osteoarthritis is a degenerative joint disease that may cause gross cartilage loss and morphological damage to other joint tissues, more subtle biochemical changes occur in the earliest stages of osteoarthritis progression. The water content of healthy cartilage is finely balanced by compressive force driving water out and hydrostatic and osmotic pressure drawing water in. Collagen fibres exert the compressive force, whereas the Gibbs–Donnan effect and cartilage proteoglycans create osmotic pressure which tends to draw water in.

However, during onset of osteoarthritis, the collagen matrix becomes more disorganized and there is a decrease in proteoglycan content within cartilage. The breakdown of collagen fibers results in a net increase in water content. This increase occurs because whilst there is an overall loss of proteoglycans (and thus a decreased osmotic pull), it is outweighed by a loss of collagen. Without the protective effects of the proteoglycans, the collagen fibers of the cartilage can become susceptible to degradation and thus exacerbate the degeneration. Inflammation of the synovium (joint cavity lining) and the surrounding joint capsule can also occur, though often mild (compared to the synovial inflammation that occurs in rheumatoid arthritis). This can happen as breakdown products from the cartilage are released into the synovial space, and the cells lining the joint attempt to remove them.

Other structures within the joint can also be affected. The ligaments within the joint become thickened and fibrotic and the menisci can become damaged and wear away. Menisci can be completely absent by the time a person undergoes a joint replacement. New bone outgrowths, called "spurs" or osteophytes, can form on the margins of the joints, possibly in an attempt to improve the congruence of the articular cartilage surfaces in the absence of the menisci. The subchondral bone volume increases and becomes less mineralized

(hypomineralization). All these changes can cause problems functioning. The pain in an osteoarthritic joint has been related to thickened synovium and subchondral bone lesions.

B. Wnt Inhibitors, Pharmaceutical Compositions and Routes of Administration

1. Wnt Signaling

The Wnt signaling pathways are a group of signal transduction pathways made of proteins that pass signals into a cell through cell surface receptors. Three Wnt signaling pathways have been characterized: the canonical Wnt pathway, the noncanonical planar cell polarity pathway, and the noncanonical Wnt/calcium pathway. All three pathways are activated by binding a Wnt-protein ligand to a Frizzled family receptor, which passes the biological signal to the Dishevelled protein inside the cell. The canonical Wnt pathway leads to regulation of gene transcription, and is thought to be negatively regulated in part by the SPATS1 gene. The noncanonical planar cell polarity pathway regulates the cytoskeleton that is responsible for the shape of the cell. The noncanonical Wnt/calcium pathway regulates calcium inside the cell. Wnt signaling pathways use either nearby cell-cell communication (paracrine) or same-cell communication (autocrine). They are highly evolutionarily conserved in animals, which means they are similar across animal species from fruit flies to humans.

Wnt signaling was first identified for its role in carcinogenesis, then for its function in embryonic development. The embryonic processes it controls include body axis patterning, cell fate specification, cell proliferation and cell migration. These processes are necessary for proper formation of important tissues including bone, heart and muscle. Its role in embryonic development was discovered when genetic mutations in Wnt pathway proteins produced abnormal fruit fly embryos. Wnt signaling also controls tissue regeneration in adult bone marrow, skin and intestine. Later research found that the genes responsible for these abnormalities also influenced breast cancer development in mice.

This pathway's clinical importance was demonstrated by mutations that lead to various diseases, including breast and prostate cancer, glioblastoma, type II diabetes and others. Encouragingly, in recent years researchers reported first successful use of Wnt pathway inhibitors in mouse models of disease.

The three best characterized Wnt signaling pathways are the canonical Wnt pathway, the noncanonical planar cell polarity pathway, and the noncanonical Wnt/calcium pathway. As their names suggest, these pathways belong to one of two categories: canonical or

noncanonical. The difference between the categories is that a canonical pathway involves the protein β-catenin while a noncanonical pathway operates independently of it.

In order to ensure proper functioning, Wnt signaling is constantly regulated at several points along its signaling pathways. For example, Wnt proteins are palmitoylated. The protein *porcupine* mediates this process, which means that it helps regulate when the Wnt ligand is secreted by determining when it is fully formed. Secretion is further controlled with proteins such as wntless and evenness interrupted and complexes such as the retromer complex. Upon secretion, the ligand can be prevented from reaching its receptor through the binding of proteins such as the stabilizers Dally and glypican 3, which inhibit diffusion. At the Fz receptor, the binding of proteins other than Wnt can antagonize signaling. Specific antagonists include Dickkopf (Dkk), Wnt inhibitory factor 1 (WIF-1), secreted Frizzled-related proteins (SFRP), Cerberus, Frzb, Wise, SOST, and Naked cuticle. These constitute inhibitors of Wnt signaling. However, other molecules also act as activators. Norrin and R-Spondin2 activate Wnt signaling in the absence of Wnt ligand. Interactions between Wnt signaling pathways also regulate Wnt signaling. As previously mentioned, the Wnt/calcium pathway can inhibit TCF/β-catenin, preventing canonical Wnt pathway signaling. Prostaglandin E2 is an essential activator of the canonical Wnt signaling pathway. Interaction of PGE2 with its receptors E2/E4 stabilizes β-catenin through cAMP/PKA mediated phosphorylation. The synthesis of PGE2 is necessary for Wnt signaling mediated processes such as tissue regeneration and control of stem cell population in zebrafish and mouse. Intriguingly, the unstructured regions of several oversized intrinsically disordered proteins play crucial roles in regulating Wnt signaling.

2. Wnt Pathway Inhibitors

Wnt inhibitors targeting various target (*e.g.*, non-ligand dependent targets) within the Wnt pathway cam be evaluated for reduction in fibrosis markers in human fibroblast-like synoviocytes (hFLS) *ex vivo*, anti-catabolic effects, and effects of Wnt inhibitors on human synovial and cartilage tissues *ex vivo*. The tests may include looking for inhibition of canonical Wnt signaling by different mechanisms including modulating Wnt signaling enzymes, such as porcupine and tankyrase, antagonizing protein interaction such as: Dishevelled with Frizzled and β-catenin with LEF-1, Tcf, CBP, DKK-1, SFRP-1, and stabilizing negative regulators of Wnt signaling such as Axin. They may specifically include porcupine inhibitors, inhibitors of the interaction between β-cat and Tcf4, inhibitors of Wnt processing and secretion, tankyrase

1/2 inhibitors, SFRP1 inhibitors, TNIK inhibitors, and inhibitors of beta-catenin recruitment with its coactivator CBP.

Porcupine inhibitors contemplated for use in accordance with the present disclosure include LGK974, IWP-L6, Wnt-C59, ETC-159, IWP-2, C59, IWP 12, Wnt-C59 and GNF6231. Tankyrase inhibitors include XAV939, AZ6102, G007-LK, E7449, IWR-1, WIKI4, JW55, JW74, G244-LM and NCB-0846. Other inhibitors include those that antagonizing protein interaction of Dishevelled with Frizzled (NSC 668036, 3289-8625, J01-017a, J01-015, J01-007, TMEM88, KY-02061, KY-02327, BMD4702, Niclosamide, DK-520, Sulindac, OTSA101 mAb, Vantictumab, UM206, 322338), those antagonizing protein interaction of β-catenin with Tcf/LEF-1 (PNU-74654, ICG-001/PRI-724, Windorphen, iCRT3, iCRT5, iCRT14, ethacrynic acid, BC21, Genistein, Kaempferol, Isorhamnentin, Baicalein, Quercetin, PKF115-584, PKF118-310, PKF118-744, PKF222-815, CGP049090, CCT036477 and NC043), CK 1a stabilizers (C113, pyrvinium), CREB-binding protein (ICG-001/PRI-724), DKK-1 inhibitors (Case *et al.*, WO 2014144817 A2), SFRP-1 inhibitors, (WAY316606; U.S. Patent 7563813 B2), TNIK inhibitors (NCB-0846), inhibitors of LPR6 phosphorylation (salinomycin), Sam68 binders (CWP232291, CWP232204), Omp-18r5 mAbs.

3. Pharmaceutic Compositions/Route of Administration

Pharmaceutical compositions of the present disclosure comprise an effective amount of an agent dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of a pharmaceutical composition that contains at least one active agent, and optionally an additional active ingredient, will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference. Moreover, for animal (*e.g.*, human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (*e.g.*, antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents,

flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, 1289-1329, incorporated herein by reference). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the pharmaceutical compositions is contemplated.

The agent may be admixed with different types of carriers depending on whether it is to be administered orally or by injection. The agent can be administered buccally, intravenously, intradermally, transdermally, intrathecally, intraarterially, intraperitoneally, intranasally, intravaginally, intrarectally, topically, intramuscularly, intra-articularly, subcutaneously, mucosally, orally, topically, locally, inhalation (*e.g.*, aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in cremes, in lipid compositions (*e.g.*, nanoparticles, liposomes), or by other method or any combination of the forgoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference).

The agent may be formulated into a composition in a free base, neutral or salt form or ester. It may also be synthesized/formulated in a prodrug form. Pharmaceutically acceptable salts, include the acid addition salts, *e.g.*, those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, fumaric, or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

Further in accordance with the present disclosure, the agent suitable for administration is provided in a pharmaceutically acceptable carrier with or without an inert diluent. The carrier should be assimilable and includes liquid, semi-solid, *i.e.*, pastes, or solid carriers. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of the composition contained therein, its use in administrable composition for use in practicing the methods of the present invention is appropriate. Examples of carriers or diluents include fats, oils, water, saline solutions, lipids, liposomes, resins, binders, fillers and the like, or combinations thereof. The composition may also comprise

various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited to parabens (*e.g.*, methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

In a specific embodiment, the composition is combined or mixed thoroughly with a semi-solid or solid carrier. The mixing can be carried out in any convenient manner such as grinding. Stabilizing agents can be also added in the mixing process in order to protect the composition from loss of therapeutic activity, *i.e.*, denaturation in the stomach. Examples of stabilizers for use in the composition include buffers, amino acids such as glycine and lysine, carbohydrates such as dextrose, mannose, galactose, fructose, lactose, sucrose, maltose, sorbitol, mannitol, *etc.*

In further embodiments, the present disclosure may concern the use of a pharmaceutical lipid vehicle compositions that include agents, one or more lipids, and an aqueous solvent. As used herein, the term "lipid" will be defined to include any of a broad range of substances that is characteristically insoluble in water and extractable with an organic solvent. This broad class of compounds are well known to those of skill in the art, and as the term "lipid" is used herein, it is not limited to any particular structure. Examples include compounds which contain long-chain aliphatic hydrocarbons and their derivatives. A lipid may be naturally-occurring or synthetic (*i.e.*, designed or produced by man). Lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof.

One of ordinary skill in the art would be familiar with the range of techniques that can be employed for dispersing a composition in a lipid vehicle. For example, the agent may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid, contained or complexed with a micelle or liposome, or otherwise associated with a lipid or lipid structure by any means known to those of ordinary skill in the art. The dispersion may or may not result in the formation of liposomes.

The actual dosage amount of an agent administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of

the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount may vary according to the response of the subject. The practitioner responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of the agent, about 0.5% of the agent, or about 1.0% of the agent. In other embodiments, the agent may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of the antagonist in each therapeutically useful composition may be prepared is such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

In other non-limiting examples, a dose of agent may also comprise from about 0.1 microgram/kg/body weight, about 0.2 microgram/kg/body weight, about 0.5 microgram/kg/body weight, about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, *etc.*, can be administered, based on the numbers described above.

In particular embodiments of the present invention, the agent is formulated to be administered via an alimentary route. Alimentary routes include all possible routes of administration in which the composition is in direct contact with the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered orally,

buccally, rectally, or sublingually. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

In certain embodiments, the active compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (Mathiowitz *et al.*, 1997; U.S. Patents 5,641,515, 5,580,579 and 5,792,451, each specifically incorporated herein by reference in its entirety). The tablets, troches, pills, capsules and the like may also contain the following: a binder, such as, for example, gum tragacanth, acacia, cornstarch, gelatin or combinations thereof; an excipient, such as, for example, dicalcium phosphate, mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate or combinations thereof; a disintegrating agent, such as, for example, corn starch, potato starch, alginic acid or combinations thereof; a lubricant, such as, for example, magnesium stearate; a sweetening agent, such as, for example, sucrose, lactose, saccharin or combinations thereof; a flavoring agent, such as, for example peppermint, oil of wintergreen, cherry flavoring, orange flavoring, *etc.* When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. When the dosage form is a capsule, it may contain, in addition to materials of the above type, carriers such as a liquid carrier. Gelatin capsules, tablets, or pills may be enterically coated. Enteric coatings prevent denaturation of the composition in the stomach or upper bowel where the pH is acidic. See, *e.g.*, U.S. Patent 5,629,001. Upon reaching the small intestines, the basic pH therein dissolves the coating and permits the composition to be released and absorbed by specialized cells, *e.g.*, epithelial enterocytes and Peyer's patch M cells. A syrup of elixir may contain the active compound sucrose as a sweetening agent methyl and propylparabens as preservatives, a dye and flavoring, such as cherry or orange flavor. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

In further embodiments, the agent may be administered via a parenteral route. As used herein, the term "parenteral" includes routes that bypass the alimentary tract. Specifically, the pharmaceutical compositions disclosed herein may be administered for example, but not

limited to intravenously, intradermally, intramuscularly, intraarterially, intrathecally, subcutaneous, or intraperitoneally U.S. Patents 6,537,514, 6,613,308, 5,466,468, 5,543,158; 5,641,515; and 5,399,363 (each specifically incorporated herein by reference in its entirety). Solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions (U.S. Patent 5,466,468, specifically incorporated herein by reference in its entirety). In all cases the form must be sterile and must be fluid to the extent that easy injectability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (*i.e.*, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it may be desirable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous, and intraperitoneal administration. In this connection, sterile aqueous media that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the

condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. A powdered composition is combined with a liquid carrier such as, *e.g.*, water or a saline solution, with or without a stabilizing agent.

C. Combination Treatments

As discussed, the present disclosure provides for the treatment of osteoarthritis. Other agents may be used in combination with the agents of the present disclosure for the same therapeutic purpose. This may achieve a greater therapeutic benefit to the patient, and/or may reduce side effects by permitting a lower doses of one or the other agent, or both. More specifically, these agents would be provided in a combined amount (along with the agent) to produce any of the effects that either agent might produce on their own. This process may involve contacting the subject with both agents at the same time, such as by contacting the subject with a single composition or pharmacological formulation that includes both agents, or by contacting the subject with two distinct compositions or formulations at the same time.

Alternatively, one agent may precede or follow the other by intervals ranging from minutes to weeks. In embodiments where the agents are applied separately to the subject, one would generally ensure that a significant period of time did not expire between the each delivery, such that the agents would still be able to exert an advantageously combined effect on the subject. In such instances, it is contemplated that one may contact the subject with both modalities within about 12-24 h of each other and, more preferably, within about 6-12 h of each other. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

Various combinations may be employed, the agent of the present disclosure is "A" and the other agent is "B":

A/B/A	B/A/B	B/B/A	A/A/B	A/B/B	B/A/A	A/B/B/B	B/A/B/B
B/B/B/A	B/B/A/B	A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A		
B/A/B/A	B/A/A/B	A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A		

Administration protocols and formulation of such agents will generally follow those of standard pharmaceutical drugs. A particular agent to be used in combination with the agents of the present disclosure are anti-inflammatories, such as NSAIDs, and immune inhibitory agents known for the treatment of osteoarthritis.

D. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

Example 1 – Materials and Methods

Animal model. All mice were obtained from The Jackson Laboratory. Ten-week-old male C57BL/6J mice were subjected to DMM surgery to induce OA as described previously (Vashegnhani *et al.*, 2015; Zhang *et al.*, 2015). Three weeks after surgery, five intra-articular injections of XAV-939 (0.4 mM, Seleck Chemicals, S1180) or saline were administered at 10-day intervals, with a total volume of 5 µl. Knee joints from control (n = 8) and Wnt inhibitor mice (n = 8) were collected at 10 weeks after surgery.

Histology. Joints were fixed overnight in TissuFix (Chaptec), decalcified for 1.5 hours in RDO Rapid Decalcifier (Apex Engineering), and further fixed in TissuFix overnight, followed by embedding in paraffin and sectioning. Sections were deparaffinized in xylene, followed by a graded series of alcohol washes. To determine the extent of cartilage deterioration, sections were stained with Safranin O/Fast Green (Millipore-Sigma), and the histological scoring method issued by OARSI was used for analysis as described previously

(Glasson *et al.*, 2010; Vasheghani *et al.*, 2015; Zhang *et al.*, 2015). To determine degree of synovitis, joint sections were stained with Masson's trichrome stain and scored on a scale of 0–3 (0, no synovitis; 1, mild synovitis; 2, moderate synovitis; and 3, severe synovitis) (Glasson *et al.*, 2010). Subchondral bone plate histomorphometry was performed on Safranin O/Fast Green-stained sections of knee joints of control and Wnt inhibitor-treated groups. Blinded histomorphometric assessment was performed on the medial compartment as described previously (Valverde-Franco *et al.*, 2012). Immunohistochemical staining for Ki-67 (Cell Signaling, 12202S), F480 (Novus Biologicals, NB600-404), CD68 (Leica, PA0191), and αSMA (ThermoFisher Scientific, RB-9010-P1) was performed by the Vanderbilt Translational Pathology Shared Resource. Briefly, the Leica Bond Max IHC stainer was used for deparaffinization, heat-induced antigen retrieval was performed with Epitope Retrieval 2 solution (Leica), blocking was performed with Protein Block (DAKO, 0909), and primary antibody incubation and visualization were performed with the Bond Refine Polymer detection system Slides were then dehydrated, cleared, and mounted. Two independent readers evaluated slides in a blinded fashion.

Plasma sample processing. Plasma was isolated from the saphenous vein of 10-week-old male C57BL/6J mice before injection with saline or XAV-939, 3 and 24 hours after injection. Plasma was stored at –80°C in 2-ml polypropylene tubes and allowed to thaw on ice before processing. Aliquots of thawed plasma (25 ml) were transferred to clean microcentrifuge tubes and spiked with the internal standard carbamazepine (2.5 ml). Calibration standards (25 ml) were spiked with internal standard and the appropriate working stocks of XAV-939 (2.5 ml). Spiked samples were lightly vortexed, allowed to stand at room temperature for 15–20 minutes, and deproteinized with 75 ml cold acetonitrile. Precipitated proteins were removed by centrifugation (18,000 *g*, 30 minutes, 5 °C). The clear supernatant (~75 ml) of each sample was transferred to a clean Eppendorf tube and evaporated under a gentle stream of N2 gas. The residue was reconstituted in 75 ml H_2O/CH_3OH (3:1) containing 0.15% acetic acid, vigorously vortexed, centrifuged to remove particulates (18,000 *g*, 30 minutes, 5 °C), and transferred to autosampler vials equipped with 200 ml polypropylene inserts and Teflon-lined bonded rubber septa.

Nanoflow liquid chromatography/mass spectrometry analysis. XAV-939 was diluted in wild-type mouse plasma to generate a standard curve. Quantification of XAV-939 was based on single reaction monitoring (SRM) detection using an Orbitrap mass spectrometer (ThermoFisher Scientific). The mass spectrometer was operated in positive ion mode using the

following optimized SRM transitions: XAV-939, *m/z* 313.1 → 267; CID CE 27%, isolation width 2.0 *m/z*; carbamazepine, *m/z* 237.1 → 194; CID CE 27%, isolation width 2.0 *m/z*. The following optimized parameters were used for the detection of analyte and internal standard: spray voltage, 2.3 kV; capillary temperature, 200 °C; capillary offset, 3 V; tube lens offset, 80 V; 3 microscans, AGC maximum inject time, 50 ms. The instrument was calibrated weekly over a mass range of *m/z* 195–1,722 (caffeine, MRFA, and Ultramark 1621) using the manufacturer's calibration procedure. All chromatographic separations were performed using an Easy-nLC 1000 (ThermoFisher Scientific) nanoflow LC system. Mobile phases were made up of 0.1% HCOOH in (A) H_2O and in (B) CH_3CN. Gradient conditions were as follows: 0–2 minutes, B = 15%; 2–27 minutes, B = 15–100%; 27–32 minutes, B = 100%; 32–33.5 minutes, B = 100–15%; 33.5–46.5 minutes, B = 15%. The flow rate was maintained at 500 nl/min. The autosampler injection valve and syringe needle were flushed sequentially with mobile phase B (2 × 20 ml) and mobile phase A (2 × 20 ml) between sample injections. The autosampler tray temperature was maintained at 10 °C. The analytical column was preequilibrated with 4 ml mobile phase A at a constant back pressure of 200 bar prior to each injection cycle. Plasma extracts (1 ml) were loaded at a constant back pressure of 200 bar (6 ml loading volume) onto a polyimide-coated, fused-silica capillary column (360 mm OD × 100 mm ID, Polymicro Technologies) packed with 15 cm Aqua-C18 bulk packing material (Phenomenex). Emitter tips (2 mm) were pulled using a P-2000 Laser Based Micropipette Puller (Sutter Instrument). The Aqua C18 material was suspended in methanol, and columns were slurry packed to a desired capacity on a PicoView pressure loader (New Objective, Scientific Instruments Services) under 1,000 psi of ultra-high purity helium gas.

Data acquisition and processing. Data acquisition and quantitative spectral analysis were performed using Thermo-Finnigan Xcalibur version 2.0.7 and Thermo-Finnigan LCQuan version 2.5.6, respectively. Calibration curves were constructed by plotting the peak area ratio (XAV-939/carbamazepine) against the concentration of XAV-939 for a series of 6 plasma standards (20 pM–100 nM). A weighting factor of 1/C2 was applied in the linear least-squares regression analysis to maintain homogeneity of variance across the concentration range. The lower limit of quantitation was 100 pM, defined as the lowest standard on the calibration curve, where %RE ≤ 20%. The lower limit of detection was approximately 20 pM.

Cell culture. Human OA synovial tissues were obtained from patients that underwent knee joint replacement surgery under informed written consent. OA was diagnosed in all patients according to the American College of Rheumatology Diagnostic Criteria for OA

(Altman *et al.*, 1986). Synovial fibroblasts were prepared as previously described (Nakamachi *et al.*, 2003). Human chondrocytes were released from articular cartilage isolated from the femoral condyles by sequential enzymatic digestion as previously described (Martel-Pelletier *et al.*, 1999). To preserve the chondrocyte phenotype, only first-passage cultured chondrocytes were used for cell culture experiments. Chondrocytes and fibroblasts were serum starved in DMEM + 0% FBS for 4 hours and treated with WNT3A (50 ng/μl) (Vanderbilt Antibody and Protein Resource) in DMEM + 2% FBS for 24 hours; then, they were subjected to no treatment, C113 (2 μM), or XAV-939 (10 μM) for 24 hours and collected for protein or RNA extraction. The small-molecule Wnt inhibitor C113 was a gift from Ethan Lee, Department of Cell and Developmental Biology, Vanderbilt University (Nashville, Tennessee, USA) and was synthesized by the Vanderbilt Institute of Chemical Biology's medicinal chemistry core (Thorne *et al.*, 2010). XAV-939 was purchased from Selleck Chemicals (S1180) (Huang *et al.*, 2009). For conditioned media experiments, synovial fibroblasts were treated as above with WNT3A and inhibitors. After treatment, media were changed to serum-free DMEM for 24 hours and then collected. These media were either used for protein arrays (RayBiotech, Cytokine Array C5) or to treat chondrocytes for 24 hours. To measure the effect of Wnt inhibition with XAV-939 and C113, NIH3T3 cells that overexpress *Wnt3A* were seeded overnight and then treated with XAV-939 and C113 over 24 hours. At timed intervals, the RNA levels of *Axin2* were assessed by qRT-PCR. The media were then changed and cells were collected every 24 hours to assess reexpression of *Axin2*. For apoptosis of chondrocytes, cells were treated with 80 mM ethanol in conditioned medium for 24 hours in 8-well chamber slides and fixed with 4% paraformaldehyde.

Real-time RT-PCR. Quantitative real-time RT-PCR was used to quantify mRNA expression of *AXIN2*, *COL1A1*, *COL2A1*, *PRG4*, and *MMP13* (data not shown). Total RNA was isolated using TRIzol (Invitrogen). cDNA was generated using the iScript cDNA Synthesis kit (Bio-Rad), and RT-PCR was performed as described previously (Segedy *et al.*, 2014).

Immunoblotting. Total cell lysates were collected by suspension of cell pellets in RIPA buffer. Cell fractionation was performed with the NE-PER nuclear and cytoplasmic extraction kit per manufacturer's directions (ThermoFisher Scientific, catalog 78833). Protein was denatured and resolved by SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane (PerkinElmer). Blots were blocked in 5% milk with TBST and then probed with COL1A1 (MDBioproducts, 203002), β-catenin (BD Transduction Laboratories, 610153), or β-actin (MilliporeSigma, A5441). Secondary antibodies were conjugated to HRP

(goat anti-mouse (SouthernBiotech, 1010-05) or goat anti-rabbit (SouthernBiotech, 4050-05), and bands were visualized by chemiluminescence.

Immunofluorescence. Cells were seeded overnight in chamber slides and then fixed with acetone. Tissue sections were deparaffinized in xylene, followed by a graded series of alcohol washes. Antigen retrieval was performed by boiling in citrate buffer. Tissue sections were blocked with 10% goat serum; incubated with antibodies against COL1A1 (MDBioproducts, 203002), COL2A1 (Millipore, MAB8887), Vimentin (ThermoFisher Scientific, PA1-10003), active Jnk (Promega, V7931), Periostin (Santa Cruz, sc-67233), β-catenin (BD Transduction Laboratories, 610153), PCNA (Santa Cruz, sc-56), or SOX9 (Millipore, AB5535); incubated with secondary antibody (goat anti-mouse FITC (Leinco Technologies, M359) or goat anti-rabbit Cy3 (Jackson ImmunoResearch 111-165-144); and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Isotype controls to rabbit (ThermoFisher Scientific, 02-6102) or mouse IgG (BioLegend, 400223) were included as appropriate with the same conditions and secondary antibodies. For TUNEL staining, a 1:10 mix of enzyme/label was added to the samples (*In Situ* Cell Death Detection Kit TMR Red, Roche) and then mounted with ProLong Gold Antifade reagent with DAPI (Invitrogen). Slides were viewed with a confocal laser scanning microscope (Zeiss LSM510).

Proliferation assay. Proliferation was assessed using the BrdU Cell Proliferation Assay kit (Calbiochem, EMD Millipore Corporation). Briefly, cells were seeded with or without WNT3A (50 ng/ml) for 24 hours. A subset of cells were treated with either C113 (1 mM) or XAV-939 (2 mM) for an additional 24 hours. The amount of BrdU incorporated into cultured cells was quantified calorimetrically using a spectrophotometer.

Statistics. The statistical significance was determined by 1-way or 2-way ANOVA with Tukey correction for multiple comparisons using multiple groups, as indicated in the figure legends. For comparison between two groups of data, unpaired 2-tailed *t* test was used for normally distributed data sets with Welch's correction. GraphPad Prism software was used for all statistical analyses. $P < 0.05$ was considered statistically significant.

Study approval. All animal studies were approved by the Animal Resources Centre, University Health Network. This study was approved by the University Health Network Institutional Ethics Committee Board (REB: 14-7529AE) and the Vanderbilt Institutional Review Board in order to obtain human-derived synovial fibroblasts and chondrocytes (IRB 131308).

Example 2 - Results

Wnt signaling is upregulated in the DMM model of OA and human OA synovial fibroblasts. Previous studies have demonstrated upregulation of Wnt signaling in response to injury in a range of tissue types (Chen *et al.*, 2004; 2007; Fernadez –Martos *et al.*, 2001; Konigshoff *et al.*, 2009). Several studies have linked upregulation of Wnt signaling to cartilage degradation in OA and rheumatoid arthritis (Lories *et al.*, 2013; Ma *et al.* 2012; Landman *et al.*, 2013; Takamatsu *et al.*, 2014; Shi *et al.*, 2016). A frequently used model for traumatic and chronic OA is the DMM model in mice that is generated by the transection of the medial meniscotibial ligament, leading to altered stability of the knee joint and subsequent cartilage damage (Glasson *et al.*, 2007). In order to assess whether ablation of Wnt activity can ameliorate the severity of OA in the knee joint, 10-week-old male mice underwent DMM surgery. Three weeks following surgery, the mice were treated with intraarticular injections of either saline control or small-molecule Wnt inhibitor, XAV-939, every 10 days. Knee joints were collected 10 weeks after surgery, 10 days following the last injection (FIG. 1A). The inventor assessed the temporal and spatial changes in canonical Wnt/β-catenin signaling by performing immunofluorescence of joint sections using an antibody to β-catenin, a marker of Wnt signaling, and costained with periostin, a stromal/fibroblast marker. Prior to injury, minimal staining was noted; however, after injury, β-catenin was strongly upregulated in the knee joint, particularly in the synovium (FIG. 1B), and was attenuated after XAV-939 treatment (FIG. 1C). These data demonstrated a striking and dynamic increase in canonical Wnt signaling in the synovium, and the enhanced staining was distinctly colocalized with periostin.

In order to determine how long Wnt inhibition using a single dose of inhibitor would persist, NIH3T3 fibroblast cells that overexpress Wnt3a were treated with both inhibitors. C113 treatment reduced Axin2 expression levels to 6.4% of their baseline level within 1 hour; XAV-939 treatment reduced Axin2 expression to 3.7% within 1 hour. Levels of Axin2 for both inhibitors were below 1% of the starting value after 2 hours and undetectable within 24 hours of treatment. Axin2 was restored to baseline within 72 hours for both inhibitors (data not shown). To determine if intraarticular injections of XAV-939 led to circulating drug, peripheral blood was isolated 3 and 24 hours after intraarticular injection, and XAV-939 was not detectable in plasma, with a detection limit of 0.100 nM (data not shown). This finding is similar to those in previous studies in which topical administration resulted in nondetectable

levels of pyrvinium, C113, or XAV-939 in the plasma (Bastakoty *et al.*, 2015). Macrophages were very rare in both control and treated joints after injury, indicating an overall minor inflammatory component (data not shown). The inventor did not perform a longitudinal analysis.

To compare basal Wnt signaling as well as capacity for activation in response to Wnt ligands in diseased versus control synovial fibroblasts, the inventor isolated primary human synovial fibroblasts derived from patients with or without OA (unaffected). As expected, these cells expressed collagen type I, but not collagen type II, by immunofluorescent staining (FIG. 2A). Quantitative real-time RT-PCR was performed on cDNA collected from these fibroblasts to assess levels of AXIN2 transcripts, a direct target of the Wnt signaling pathway that is often used as a marker to assess the intensity and duration of canonical Wnt activation (Jho *et al.*, 2002) (FIG. 2B). Synovial fibroblasts isolated from OA patients had higher basal levels of Wnt activity. These cells were then stimulated with WNT3A recombinant protein to stimulate Wnt pathway activity in the absence or presence of two small-molecule Wnt inhibitors, C113 and XAV-939, which inhibit Wnt activity through two distinct mechanisms (Huang *et al.*, 2009; Thorne *et al.*, 2010) (FIG. 2C). In cells from both unaffected (normal, healthy controls) and OA patients, Wnt pathway activation was significantly attenuated with both inhibitors (FIG. 2D). The inventor further confirmed these data by analyzing levels of β-catenin in the total cell lysates and nuclear fraction by immunoblotting (FIG. 2E).

To assess the contribution of noncanonical Wnt signaling, the inventor stained murine synovium, human synovial fibroblasts, murine cartilage, and human chondrocytes with an antibody to active Jnk by immunofluorescence. Relatively low levels of staining were observed, with no discernible changes between treatment groups in murine tissue or human cells (data not shown).

Inhibition of Wnt signaling *in vivo* ameliorates OA severity. To visualize the severity of cartilage damage in the injured knees, Safranin O/Fast Green staining was utilized (FIG. 3A), and quantification of articular cartilage damage was determined using Osteoarthritis Research Society International (OARSI) scoring (Glasson *et al.*, 2010) by two blinded observers (FIG. 3B). Results showed marked protection in the severity of cartilage degeneration and significant reduction in the OARSI scores in both medial tibial plateau and medial femoral condyle in the Wnt inhibitor-treated group compared with saline-treated controls. Chondrocyte cellularity analysis further showed greater chondrocyte loss in the saline-treated group compared with the Wnt inhibitor-treated group (FIG. 3C). To assess

synovitis, Masson's trichrome staining was performed, and the degree of synovitis was blindly scored as described previously (Krenn *et al.*, 2002) (FIGS. 3D-E). Results showed a marked reduction in the degree of synovitis in the Wnt inhibitor–treated group compared with the saline-treated group. The inventor also performed subchondral bone plate histomorphometry in the Wnt inhibitor-treated group compared with the saline-treated group as previously described (Valverde-Franco *et al.*, 2012). Results showed no significant differences in the subchondral bone plate thickness in the Wnt inhibitor-treated group compared with the saline-treated group (data not shown).

Wnt inhibition reduces both proliferation and type I collagen levels of synovial fibroblasts. The inventor sought to assess the cellular effects of Wnt inhibitors on synovial fibroblasts to clarify the basis for the observed decrease in synovitis. Increased proliferation of synovial fibroblasts has been shown in the context of OA and rheumatoid arthritis (Bhattaram *et al.*, 2017; Lacey *et al.*, 2003; Qu *et al.*, 1994). She identified that treatment with XAV-939 reduced the numbers of proliferating fibroblasts in the synovium, as assessed by immunostaining with Ki-67 and PCNA on sections from control- or XAV-939–treated mice after DMM injury (FIGS. 4A-B). To determine if Wnt directly modulated synovial fibroblast proliferation, the inventor assessed the proliferation response of isolated human synovial fibroblasts by BrdU incorporation assay (FIG. 4C). Synovial fibroblasts from OA patients showed significantly higher basal levels of proliferation than fibroblasts from healthy controls. While both unaffected and OA synovial fibroblasts demonstrated an increase in proliferation with the addition of WNT3A, the effect was only significant in synovial fibroblasts from unaffected patients. The elevated basal proliferation rate of OA-derived synovial fibroblasts may be an explanation for the lack of response in the presence of WNT3A. Importantly, both small-molecule Wnt inhibitors resulted in a statistically significant decrease in proliferation of OA-derived synovial fibroblasts to below basal levels. The antiproliferative effect of XAV-939 and C113 on unaffected synovial fibroblasts demonstrated a decreased trend but did not achieve statistical significance.

The primary constituent of synovial fibrotic matrix is collagen type I, which is produced by synovial fibroblasts (Remst *et al.*, 2014). Since there was a striking effect of XAV-939 treatment on synovitis, the inventor further assessed the effect of Wnt modulation on type I collagen synthesis of synovial fibroblasts *in vitro*. Treatment of cultured synovial fibroblasts with WNT3A ligand resulted in a significant increase in COL1A1 transcript levels, and this increase was not only abrogated with the addition of either Wnt inhibitor, but levels were

reduced to below baseline (C113 = 43.77%, XAV-939 = 83.09%) (FIG. 5A). As further confirmation, the inventor demonstrated the same trends in collagen type I protein expression (FIG. 5B). Taken together, Wnt inhibitors addressed two key aspects of synovial fibrosis by reducing both proliferation of synovial fibroblasts and their synthesis of type I collagen.

Effect of Wnt modulation on OA-derived human articular chondrocytes. The inventor sought to evaluate Wnt-mediated effects on cartilage/chondrocytes. To determine whether XAV-939 treatment was inducing proliferation of chondrocytes in the articular cartilage, the inventor performed immunostaining of both Ki-67 and PCNA on control or treated murine joints (FIGS. 6A-C). Staining of the joint space showed no significant difference in the proliferative activity of the chondrocytes. To further confirm that Wnt inhibition did not induce proliferation, chondrocytes were isolated from the articular cartilage of patients with OA and were verified by expression of the chondrocyte-specific marker, collagen type II (FIG. 6D). The inventors utilized a BrdU incorporation assay and noted no significant differences in the proliferative activity of the cells with Wnt pathway activation or inhibition, supporting the inventor's finding that the cartilage-sparing effects observed with XAV-939 treatment were not due to promoting chondrocyte regeneration (FIG. 6E).

The inventor next determined whether chondrocytes were responsive to Wnt signaling by evaluating AXIN2 transcript levels by real-time RT-PCR (FIG. 6F). AXIN2 expression was activated >22-fold 24 hours after activation with WNT3A, and addition of either XAV-939 or C113 significantly reduced Wnt signaling activation, as gauged by AXIN2 transcript levels. She next assessed if the cartilage-sparing effects of Wnt inhibitors may be modulating expression of type II collagen matrix or the expression of lubricin, encoded by PRG4, a proteoglycan secreted by chondrocytes that has been shown to play a positive role in OA in animal models (Alquraini *et al.*, 2015; Waller *et al.*, 2017; Heathfield *et al.*, 2001; Jia *et al.*, 2016). These data showed a significant decrease in COL2A1 transcript levels after the addition of WNT3A, while Wnt inhibition rescued the mRNA expression (FIG. 6G). Transcript levels of PRG4 were also significantly increased by either C113 or XAV-939 treatment, suggesting a positive effect on expression (C113 = 11.693-fold increase, XAV-939 = 2.448-fold increase) (FIG. 6H). Collectively, the cartilage-sparing effects of XAV-939 and C113 were not likely due to spurring chondrocyte/cartilage regeneration. However, enhancing type II collagen matrix and specific proteoglycan secretion may contribute to the disease-ameliorating phenotype.

The inventor further sought to determine if the secretome of synovial fibroblasts had apparent effects on the chondrocyte phenotype. Synovial fibroblasts were treated with WNT3A, WNT3A plus C113, or WNT3A plus XAV-939. Conditioned media were collected for 24 hours following treatment and then transferred to human chondrocytes. No changes were observed in apoptosis, hypertrophy (indicated by qRT-PCR of COLX), or proteinase levels (indicated by qRT-PCR of MMP13). Additionally, conditioned media were analyzed by cytokine array, with few notable significant changes between treatment groups, indicating minimal significant secretome modulation by Wnt signaling in synovial fibroblasts (data not shown).

Example 3 - Discussion

This study demonstrated that traumatic joint injury through DMM surgery induced robust activation of canonical Wnt signaling, most striking in the synovium, and this upregulation was downregulated with intermittent (every 10 days), local (intra-articular) treatment using a small-molecule Wnt inhibitor, resulting in amelioration of both synovitis and cartilage loss. An important advantage of identifying therapeutic benefits with local administration is avoiding systemic effects of Wnt inhibition on Wnt-dependent tissues, such as intestinal stem cells/intestinal turnover, hematopoiesis, and bone density (Kahn *et al.*, 2014). The use of small-molecule Wnt inhibitors bypasses the restrictions of genetic approaches by targeting the entire injury milieu, rather than a particular cell type, as is the case in genetic models (Bastakoty *et al.*, 2016). Additionally, since different cell types exhibit different levels of Wnt activation and sensitivity to Wnt inhibition, one can also fine-tune the degree and timing of Wnt inhibition using dosing strategies that are calibrated. This is exemplified in the cutaneous wound model, in which the dose of the inhibitor utilized primarily reduced Wnt signaling in the dermis without significant reduction in the epidermis (Bastakoty *et al.*, 2015). The inventor's findings indicated that there was continued Wnt signaling elevation 10 weeks after DMM surgery within the synovium, suggesting that Wnt activation following injury did not return to basal levels after the initial damage response. This was further confirmed by the inventor's analysis of human OA patient–derived synovial fibroblasts, which demonstrated elevated basal Wnt signaling levels as compared with unaffected synovial fibroblasts, suggesting that Wnt signaling is chronically elevated in the synovial cells of OA patients. There have not been reports identifying Wnt effectors activated and sustained following injury, although specific Wnt effectors, such as *Wnt16*, a weak canonical activator, have been shown

to increase following injury but return completely to basal levels in DMM-operated knees after 8 weeks (Nalesso *et al.*, 2017). Sustained Wnt activation following injury, as observed in the synovium after DMM injury, is in contrast to cutaneous injury and ischemic myocardial injury models in which the activation was transient (Bastakoty *et al.*, 2015; 2016) and may reflect the progressive nature of the disease. Although published studies using mice suggest that Wnt target gene expression is elevated after DMM in chondrocytes/cartilage (van den Bosch *et al.*, 2012; Nalesso *et al.*, 2017), the inventor did not observe a notable upregulation of Wnt signaling in the cartilage by immunofluorescent staining for β-catenin, likely due to reduced sensitivity of immunofluorescence. These data highlight that changes in Wnt signaling following DMM were more dynamic and robust in the synovium as compared with cartilage.

Extensive investigation of the Wnt/β-catenin pathway using transgenic mouse models suggests that Wnt/β-catenin signaling needs to be carefully modulated for cartilage development as well as maintenance of normal articular cartilage function (Zhu *et al.*, 2008; Usami *et al.*, 2016). Both excessive Wnt signaling and Wnt suppression lead to chondrocyte pathologies and cartilage loss in mice as well as humans (Zhu *et al.*, 2009; Zhu *et al.*, 2008; Loughlin *et al.*, 2004; Diarra *et al.*, 2007; van den Bosch *et al.*, 2017). Studies that have examined activation of Wnt signaling in chondrocytes and synovium *in vivo* have noted stimulation of MMP3 and MMP13 and a catabolic profile in chondrocytes, though the precise genes altered differ based on the Wnt ligand and/or receptor (Yuasa *et al.*, 2008; Zhu *et al.*, 2009; van den Bosch *et al.*, 2017; Chen *et al.*, 2016). Overexpression of *Dkk1*, a negative regulator of the Wnt signaling pathway, in cartilage via the *Col2a1* promoter protected against cartilage destruction. Furthermore, in an ongoing phase II clinical trial, SM05690 (Samumed), a small-molecule inhibitor of Wnt signaling, is applied intra-articularly in moderate-to-severe knee OA (identification no. NCT03122860). Preclinical studies reported improved outcome through both reduced cartilage degeneration and reduction in inflammatory cytokine and protease production, and no toxicity was noted (Hood *et al.*, 2016). The cartilage-sparing effects *in vivo* using XAV-939 and the *in vitro* data from isolated human chondrocytes exposed to XAV-939 and C113 were consistent with these genetic studies in that the inventor observed that Wnt inhibitors acted to restore a chondrocyte-protective profile and reverse the catabolic profile following Wnt activation. For example, lubricin (*PRG4*) and type II collagen are essential for joint homeostasis, and their disruption results in cartilage breakdown (Tchetina *et al.*, 2005; Musumeci *et al.*, 2014; Loeser *et al.*, 2013). Expression of *PRG4* and *COL2A1* increased with Wnt inhibition, supporting the prior studies linking Wnt activation to a catabolic

phenotype in chondrocytes, which can be reversed with Wnt inhibition. *PRG4* expression has been shown to be protective in OA and, furthermore, has been shown to upregulate hypoxia-inducible factor 3α, which inhibits transcriptional programs that promote cartilage catabolism and hypertrophy (Waller *et al.*, 2017; Ruan *et al.*, 2013). The inventor did not observe changes in *MMP13*, as previously reported with Wnt modulation of chondrocytes, and this may be due to dosing and other differences in the experimental context. Such differences may also be attributed to sustained versus acute inhibition of this pathway. While Wnt inhibition significantly reduced the proliferation of OA synovial fibroblasts, it did not appear to alter proliferation of OA chondrocytes. This study suggests that Wnt inhibition spared cartilage loss through mechanisms other than increased chondrocyte proliferation and true regeneration.

Excessive synovial fibroblast proliferation and deposition of type I collagen are hallmarks of synovitis (Benito *et al.*, 2005). Intra-articular injection of XAV-939 was shown to result in significantly reduced synovitis. Concurrently, type I collagen deposition was vastly reduced. Consistent with these findings, primary human synovial fibroblasts demonstrated heightened proliferation and type I collagen synthesis in response to WNT3A. Both proliferation and collagen synthesis were significantly reduced with both XAV-939 and C113, suggesting that these are direct targets of Wnt signaling. To the inventor's knowledge, this is the first report to identify synovial fibroblasts as key targets of Wnt modulation and to identify their proliferation and matrix synthesis as direct Wnt-modulated cellular effects. Although earlier studies in which *Dkk1* was overexpressed under the *Col1a1* promoter (which would target expression in matrix-synthesizing synoviocytes) demonstrated reduced cartilage lesions and osteophytes as well as reduced overall OA score, specific evaluation of the effect of Wnt inhibition in cellular phenotype of synovial fibroblasts was not reported (Funck-Bretano *et al.*, 2014). The inventor's findings *in vivo* were further supported using mechanistically distinct Wnt inhibitors to assess the cellular responsiveness to Wnt inhibition on human synovial fibroblasts in culture. The inventor demonstrated that basal Wnt signaling as well as the basal proliferation rate was higher in synovial fibroblasts derived from OA patients. This elevation in Wnt signaling may be responsible for the higher baseline proliferation of OA-derived synovial fibroblasts, as their proliferation rate was only minimally responsive to WNT3A, in contrast to unaffected synovial fibroblasts. Both XAV-939 and C113 mediated decreased proliferation and decreased expression of type I collagen. Interestingly, despite synovial hyperplasia in the DMM model of OA, there was minimal evidence of both myofibroblasts and cellular inflammation. The inventor also did not observe statistically significant Wnt-

dependent modulation of *MMP13* or *MMP1* in human synovial fibroblasts (data not shown). The inventor observed that WNT3A increased *MMP13* transcripts slightly (~1.3-fold, albeit statistically significantly) in human chondrocytes; however, the inventor did not observe a statistically significant abrogation of the increased transcript levels with XAV-939 or C113 (data not shown). Hence, the inventor cannot extrapolate the effect of therapeutic Wnt inhibition on disease severity on OA models that exhibit a significant inflammatory component.

These data support a role for therapeutic Wnt inhibition in the treatment of traumatic OA and suggest that the disease-mitigating effects of Wnt inhibition may be due to effects on both synovial fibroblasts and chondrocytes, although it is difficult to discern if the cartilage-sparing effects are primarily due to direct effects on articular cartilage or secondary to effects on synovial tissues. The inventor's findings particularly underscore the antifibrotic effects of therapeutic Wnt inhibition following injury observed in other models of therapeutic Wnt downregulation (Tan *et al.*, 2014; Bastakoty *et al.*, 2015; 2016; Saraswati *et al.*, 2010).

* * * * * * * * * * * * * * * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

E. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

U.S. Patent 6,537,514

U.S. Patent 6,613,308

U.S. Patent 5,629,000

U.S. Patent 5,466,468

U.S. Patent 5,543,158

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CLAIMS

1. A method of treating a subject having osteoarthritis (OA) comprising administering to said subject an inhibitor of the Wnt/β-catenin pathway.

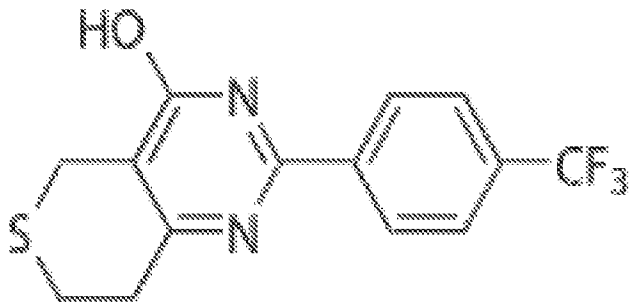
2. The method of claim 1, wherein the inhibitor is an inhibitor of Porcupine, an inhibitor Tankyrase1/2, antagonizes protein interactions such as Dishevelled with Frizzled, and β-catenin with LEF-1, an inhibitor of Tcf, CBP, DKK-1, or SFRP-1, stabilizes a negative regulator of Wnt signaling, inhibits the interaction between β-catenin and Tcf4, inhibits of Wnt processing and secretion, a COL1A1 inhibitor, a Wnt-1 inhibitor, an Axin2 inhibitor, an SFRP1 inhibitor, a TNIK inhibitor, or an inhibitor of beta-catenin recruitment with its coactivator CBP.

3. The method of claims 1-2, wherein said inhibitor is administered systemically.

4. The method of claims 1-2, wherein said inhibitor is administered local or regional to an OA disease site.

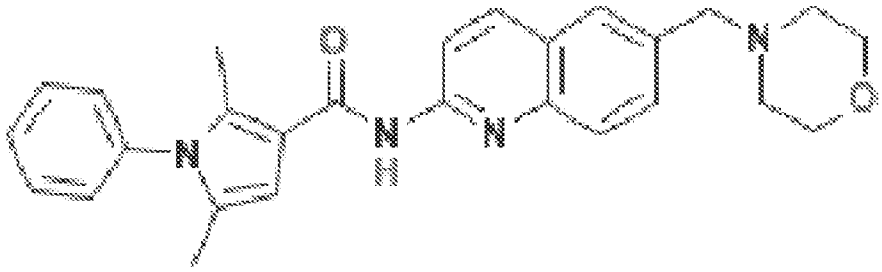
5. The method of claims 1-4, wherein said inhibitor is administered more than once, such as daily, every other day, every third day, twice a week, weekly, monthly and/or chronically.

6. The method of claims 1-5, wherein said inhibitor has the structure:



XAV-939

or



C113

7. The method of claims 1-6, wherein treating comprises reducing OA severity.

8. The method of claims 1-6, wherein treating comprises reducing cartilage degeneration and/or synovitis.

9. The method of claims 1-6, wherein treating comprises reducing proliferation of synovial fibroblasts and/or type I collagen synthesis by synovial fibroblasts.

10. The method of claims 1-6, wherein treating comprises increased *COL2A1* and *PRG4* transcription.

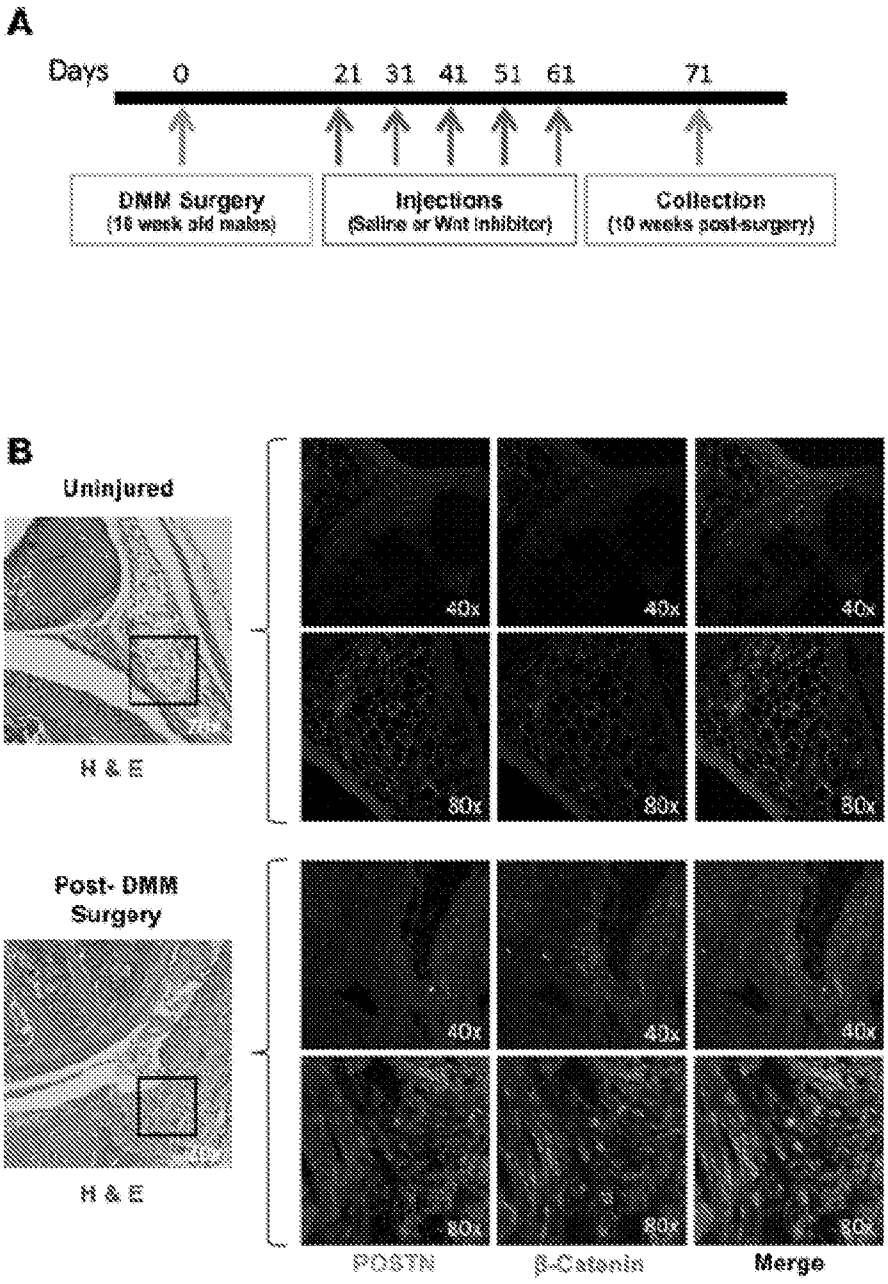
11. The method of claims 1-6, wherein treating does not comprise modulation of OA-driven chondrocyte proliferation.

12. The method of claim 1-11, further comprising treating said subject with a second OA therapy.

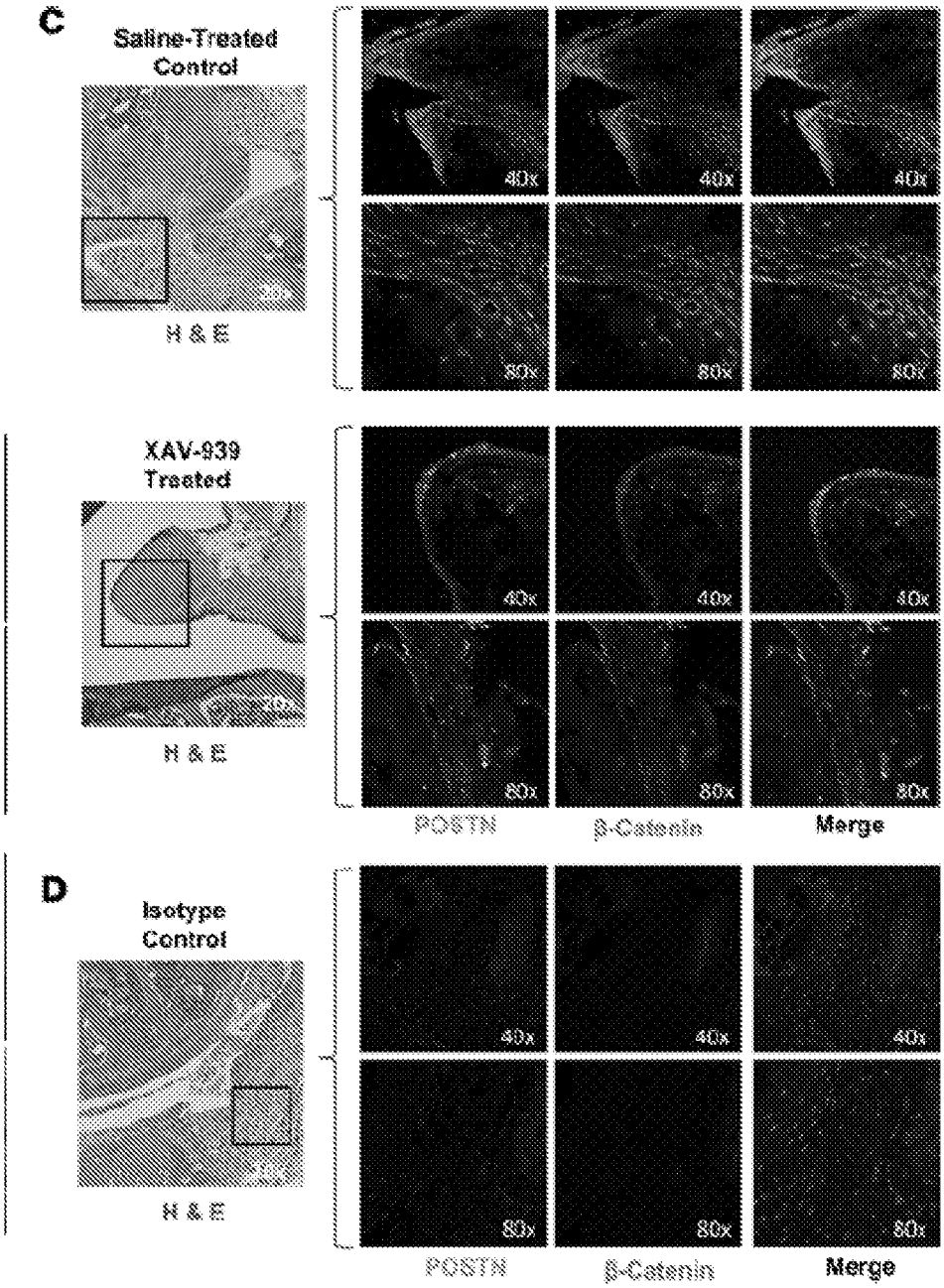
13. The method of claim 12, wherein said second OA therapy is an anti-inflammatory or an immune inhibitor.

14. The method of claim 1-13, wherein said subject is a human.

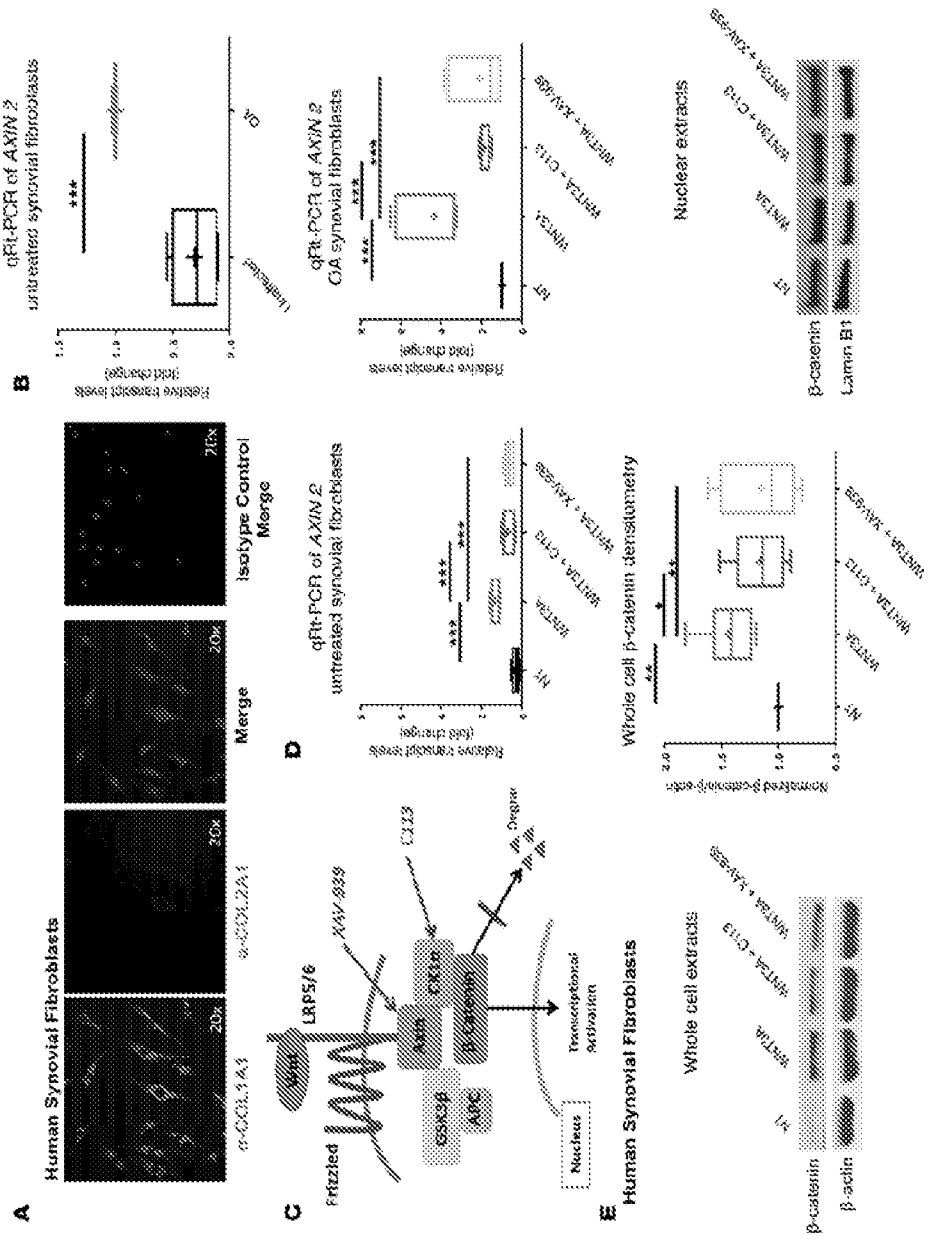
15. The method of claim 1-13, wherein said subject is a non-human mammal.


A
Days
0
21
31
41
51
61
71
DMM Surgery
(16 week old males)
Injections
(Saline or Wnt inhibitor)
Collection
(10 weeks post-surgery)
B
Uninjured
H & E
Post- DMM Surgery
H & E
40x
80x
POSTN
β-Catenin
Merge

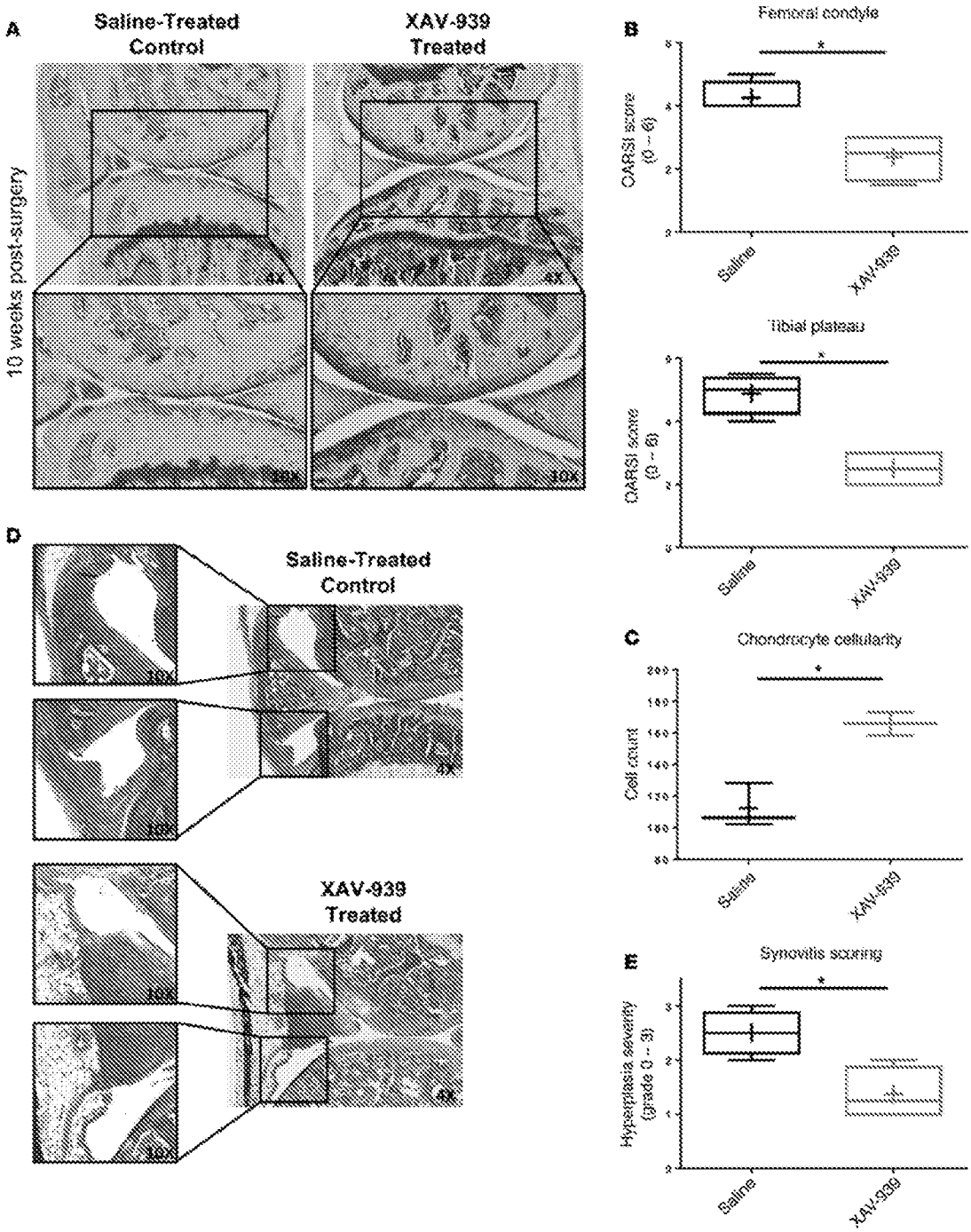
FIGS. 1A-B


C
Saline-Treated Control
H & E
XAV-939 Treated
H & E
D
Isotype Control
H & E
40x
80x
POSTN
β-Catenin
Merge

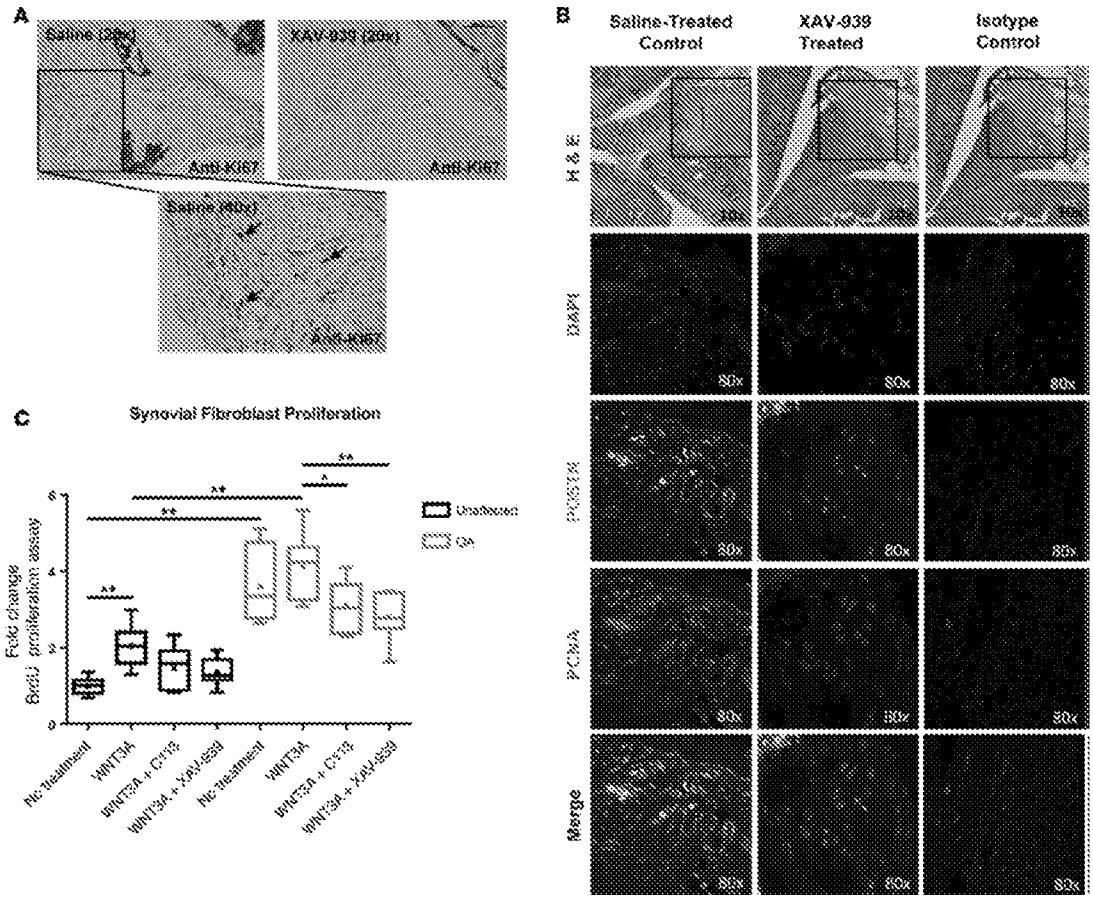
FIGS. 1C-D


A
Human Synovial Fibroblasts
α-COL1A1
α-COL2A1
Merge
Isotype Control
Merge
B
qRt-PCR of AXIN 2
untreated synovial fibroblasts
Relative transcript levels
(fold change)
Isotype
siCTR
siAXIN2
C
Frizzled
LRP5/6
XAV-939
CK1α
GSK3
Axin
β-catenin
APC
Nucleus
Transcriptional Activation
No Degradation
D
qRt-PCR of AXIN 2
OA synovial fibroblasts
NT
siAXIN2
siCTR
siAXIN2 + CK1α
siAXIN2 + XAV-939
E
Human Synovial Fibroblasts
Whole cell β-catenin densitometry
Normalized β-catenin/β-actin
Whole cell extracts
β-catenin
β-actin
Nuclear extracts
β-catenin
Lamin B1

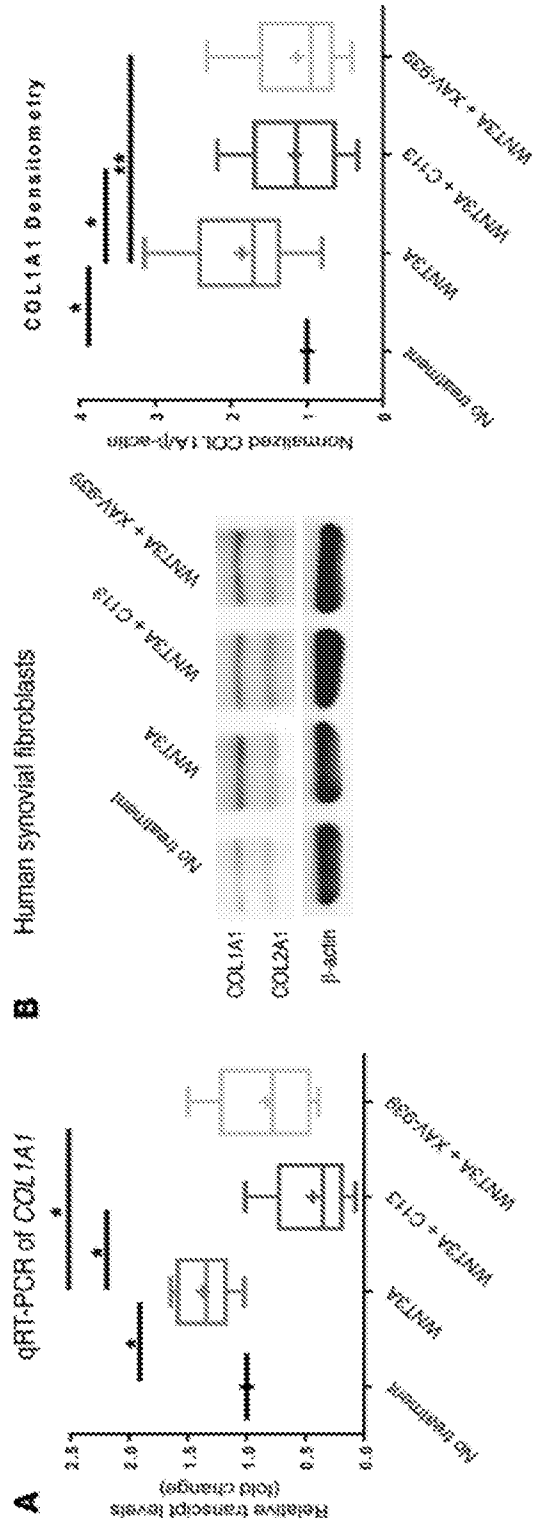
FIGS. 2A-E


A
Saline-Treated Control
XAV-939 Treated
10 weeks post-surgery
4X
10X
B
Femoral condyle
OARSI score (0 – 6)
Saline
XAV-939
Tibial plateau
C
Chondrocyte cellularity
Cell count
D
Saline-Treated Control
XAV-939 Treated
E
Synovitis scoring
Hyperplasia severity (grade 0 – 3)

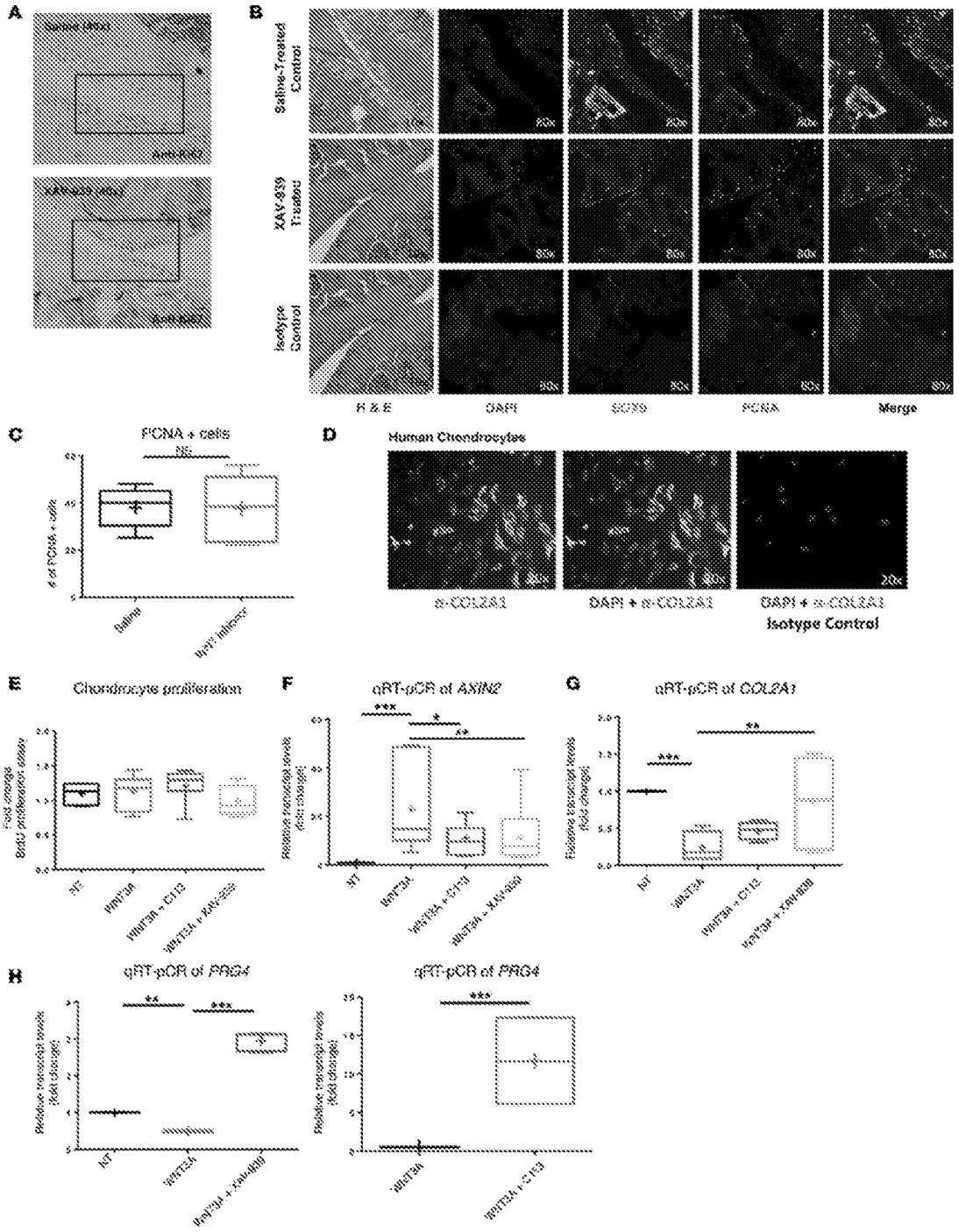
FIGS. 3A-E


A
Saline (28d)
XAV-939 (28d)
Anti-KI67
Saline (48h)
Anti-KI67
B
Saline-Treated Control
XAV-939 Treated
Isotype Control
H & E
DAPI
PCNA
Merge
80x
C
Synovial Fibroblast Proliferation
Fold change BrdU proliferation assay
Unstained
Ctrl
No Treatment
WNT3A
WNT3A + Ctrl
WNT3A + XAV-939

FIGS. 4A-C


A
qRT-PCR of COL1A1
Relative transcript levels (fold change)
2.5
2.0
1.5
1.0
0.5
0.0
No treatment
WNT3A
WNT3A + C113
WNT3A + XAV-939
B
Human synovial fibroblasts
COL1A1
COL2A1
β-actin
No treatment
WNT3A
WNT3A + C113
WNT3A + XAV-939
COL1A1 Densitometry
Normalized COL1A1/β-actin
4
3
2
1
0
No treatment
WNT3A
WNT3A + C113
WNT3A + XAV-939

FIGS. 5A-B


A
B
Saline-Treated Control
XAV-939 Treated
Isotype Control
H & E
DAPI
SOX9
PCNA
Merge
C
PCNA + cells
# of PCNA + cells
Saline
WNT Inhibitor
D
Human Chondrocytes
α-COL2A1
DAPI + α-COL2A1
DAPI + α-COL2A1
Isotype Control
E
Chondrocyte proliferation
Fold change BrdU proliferation assay
NT
WNT3A
WNT3A + C113
WNT3A + XAV-939
F
qRT-pCR of AXIN2
Relative transcript levels (fold change)
NT
WNT3A
WNT3A + C113
WNT3A + XAV-939
G
qRT-pCR of COL2A1
Relative transcript levels (fold change)
NT
WNT3A
WNT3A + C113
WNT3A + XAV-939
H
qRT-pCR of PRG4
Relative transcript levels (fold change)
NT
WNT3A
WNT3A + XAV-939
qRT-pCR of PRG4
Relative transcript levels (fold change)
WNT3A
WNT3A + C113

FIGS. 6A-H