# Title: Targeting BMP signaling mitigates osteoarthritis

**One Sentence Summary- Pharmacological inhibition of BMP signaling in osteoarthritis, attenuates disease progression and inflammation**

**AUTHORS:**

Akrit Pran Jaswal[1, #], Bhupendra Kumar[1, #], Anke J. Roelofs[3,] Sayeda Fauzia Iqbal[1], Amaresh Kumar Singh[1,4], Anna H.K. Riemen[3], Hui Wang[3], Sadaf Ashraf[3], Sanap Vaibhav Nanasaheb[1], Nitin Agnihotri[1], Cosimo De Bari[3], Amitabha Bandyopadhyay[1, 2 *]

**AFFILIATIONS:**
[1] Department of Biological Sciences and Bioengineering, Indian Institute of Technology Kanpur, Kanpur-208016, Uttar Pradesh, India and
[2] The Mehta Family Centre for Engineering in Medicine, Indian Institute of Technology Kanpur, Kanpur, Uttar Pradesh, India.
[3] Arthritis and Regenerative Medicine Laboratory, Aberdeen Centre for Arthritis and Musculoskeletal Health, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, UK
[4] Department of Zoology, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India

# These two authors contributed equally

*Corresponding Author: Amitabha Bandyopadhyay

Email address: abandopa@iitk.ac.in

**KEYWORDS:** BMP, Osteoarthritis, articular cartilage, local inhibition, LDN-193189, post onset

.

**ABSTRACT:**

Osteoarthritis (OA) is a prominent musculoskeletal disorder characterized by the loss of articular cartilage, ectopic bone formation, and inflammation, causing joint pain and disability. However, the molecular mechanism(s) of articular cartilage maintenance and pathogenesis of OA is poorly understood. Currently, there is no means to revert/retard the progression, and only symptomatic treatments are available to manage OA. In our study, with surgically induced OA in mice, we found an upregulation of BMP signaling, marked by pSMAD1/5/9. We also found upregulation of BMP signaling in the articular cartilage of human osteoarthritic specimens, which corroborated our findings in mice. Further, we established that gain of BMP signaling alone is sufficient to induce OA like phenotype in adult mice articular cartilage. We also demonstrated that loss of BMP2/4 ligands in adult articular cartilage mitigates surgically induced OA in mice. Concomitantly, even after the onset of the disease, local inhibition of BMP signaling by intra-articular injection of the BMP signaling inhibitor, LDN-193189, resulted in reduced severity of OA with reduced expression of inflammatory markers. Therefore, we propose that pharmacological inhibition of BMP signaling blocks OA progression and can be a potential disease-modifying therapy.

## Introduction

Osteoarthritis (OA) is a painful, debilitating musculoskeletal disorder with profound socioeconomic burden and the primary cause of locomotive disability affecting millions of people worldwide(*1–3*). The alarmingly increasing prevalence of OA is exacerbated further as no disease modifying therapy exists to manage OA except for symptomatic treatment with anti-inflammatory drugs or surgical intervention in late stage disease(*4*). It is imperative therefore, to discern the molecular basis of pathogenesis of OA to develop a disease modifying therapy.

Articular cartilage, the tissue affected in OA, is a lubricated, avascular, alymphatic, aneural tissue that lines the ends of the bones at the joints. During OA, the joint surface undergoes a slew of changes characterised by loss of cartilage proteoglycans, hypertrophy of chondrocytes, angiogenesis, osteophyte formation, and ultimately failure of joint function(*4*). The cellular and molecular changes of

the joint cartilage during the onset and progression of OA closely resemble the steps of endochondral ossification, the developmental process by which long bones form within cartilage anlagen(*5*, *6*).

During endochondral ossification, most of the initial cartilage mass in an appendicular skeletal element is replaced by newly forming bone except for the cartilage at the termini. The cartilage that is replaced by bone is referred to as the transient cartilage while the cartilage at the terminal ends is referred to as the joint or articular or permanent cartilage(*7*). During transient cartilage differentiation, type II collagen (Col2a1) expressing cartilage cells undergo a series of changes. These cells undergo pre-hypertrophic differentiation wherein they express Indian hedgehog (IHH), subsequently the transition from pre-hypertrophy to hypertrophy is marked by the expression of type X collagen (ColX). The hypertrophic cells are infiltrated by blood vessels. This is followed by matrix remodelling, where enzymes viz. MMP-13 and ADAMTS-5, degrade the existing collagen matrix and a new matrix, rich in type I collagen (ColI), is synthesised and bone formation is accomplished(*8*, *9*). Ray et.al. demonstrated that within a developing appendicular skeletal element there is a zone of Col2a1 expressing bipotential proliferating cell population referred to as the Distal Proliferative Zone (DPZ). The DPZ cells under the influence of BMP signaling undergo transient cartilage differentiation whereas when exposed to Wnt signaling undergo joint cartilage differentiation(*1*). Some of the molecules involved in transient cartilage differentiation viz. MMP-13, ADAMTS-5, and VEGF-A are reported to be associated and/or necessary for the pathogenesis of OA (*10*–*16*).

Previous literature suggests that ectopic activation of BMP signaling in developing cartilage or presumptive joint site, either by overexpression of BMP ligands(*1*, *17*) or misexpression of constitutively active BMP receptors(*18*), results in transient cartilage differentiation at the expense of joint cartilage. Surge in BMP2 and BMP4 ligands were reported in human articular cartilage having moderate to severe form of osteoarthritis(*19*). Blocking BMP signaling inhibits hypertrophic and mineralization indicates it is important player in terminal differentiation of BMSs (*20*). Additionally, Noggin administration in ACLT induced OA model inhibits OA progression by inhibiting IL-1β and BMP-2(*21*). A recently published in-vitro study indicates reduction of chondrocyte hypertrophy after BMP receptors were inhibited using LDN-193189(*22*).Immobilisation of developing embryonic limbs leads to ectopic differentiation of transient cartilage at the cost of articular cartilage. Moreover, it was shown that immobilization induced OA, leads to ectopic upregulation of BMP signaling within the sub-articular cartilage domain where cartilage precursors are normally exposed only to Wnt signaling (*23*). Recently, it was also

demonstrated that pharmacological inhibition of BMP signalling promotes articular cartilage differentiation in hMSC derived chondrocytes and allowed the cells to maintain articular chondrocyte phenotype for longer duration of time upon implantation in mice(*2*), suggesting that embryonic paradigm of spatial restriction of BMP signalling is needed for differentiation and maintenance of articular cartilage phenotype. However, few studies indicate BMPs have anabolic effect on articular cartilage integrity(*24*). Taken together, we hypothesize that BMP signaling induced transient cartilage differentiation within adult articular cartilage domain is the molecular basis of the pathogenesis of OA. In this study, we tested this hypothesis with conditional gain- and loss-of-function mouse mutants of BMP signaling in conjunction with a surgically induced model of OA. Our findings in mice model are further supported by data obtained from osteoarthritic human cartilage specimens, wherein we found evidence of active BMP signaling in the joint cartilage. Moreover, our data indicates that pharmacological inhibition of BMP signaling in the synovial joint may serve as an effective disease modifying therapy for OA.

**RESULTS:**

**1. Overexpression of BMP signaling in adult joint cartilage is sufficient to induce the development of OA-like phenotype in mice**

To examine whether over expression of BMP signaling in the articular cartilage is sufficient to induce osteoarthritic changes in adult mouse we generated *pMes-caBmpr1a; Col2a1-Cre-ERT2* mouse strain, where BMP signaling can be ectopically activated in adult articular cartilage upon administration of tamoxifen (Fig.1A). We activated BMP signaling in postnatal cartilage at P70 by injecting tamoxifen intraperitoneally (*referred to as induction from here on*) to *pMes-caBmpr1a; Col2a1-Cre-ERT2* mice. However, within seven days of over-expression of *caBmpr1a* in adult mouse articular cartilage, ectopic activation of canonical BMP signaling, as assessed by immunoreactivity towards phosphorylated SMAD1/5/9, is observed and it peaks after two weeks (Fig.1C′-C′′′′). Subsequently, markers of transient cartilage differentiation were also observed in the articular cartilage domain in the same sequence as observed during endochondral ossification. Expression of IHH, which marks pre-hypertrophic state of

cartilage, was observed within 7 days of induction and concurrent with the first detection of pSMAD1/5/9 immunoreactivity. IHH expression is downregulated by the 14$^{th}$ day after induction (Fig.1D-D″). Expression pattern of ColII gets depleted at 14$^{th}$ day of the post-induction and it was minimum at post-induction day 56 (Fig.1E-E‴). ColX expression, which marks cartilage hypertrophy, was observed 14 days post induction and the maximum extent of hypertrophy was at 56 days post induction (Fig.1F-F‴). Embryonic (*23*, *25*), as well as adult articular cartilage cells (*2*) are proliferation deficient while transient cartilage cells are proliferative(*1*). In our experiments we observed that along with other markers of transient cartilage differentiation, proliferation was also stimulated in the adult mice articular cartilage after activation of BMP signaling. Increased BrdU uptake was observed in the adult joint cartilage 7 days after induction which was more apparent on the 14$^{th}$ day post induction (Fig.1G-G″). Loss of proteoglycan staining as indicated by Safranin O /Fast Green in multiple zones was also observed along with vertical cleft in articular cartilage (Fig. 1H-1H′). OARSI scoring for integrity of articular cartilage indicates the severity of loss of articular cartilage in TAM injected versus control samples (Fig.1I). Quantification of ColII expression in control versus TAM injected samples indicate drastic reduction with post BMP signaling activation days (Fig.1J). Similarly, chondrocyte hypertrophic marker ColX shows considerable increase in BMP signaling activated samples versus control (Fig.1K).

Besides the molecular signatures, typical feature of OA is the formation of osteophytes in the joint, which is otherwise devoid of such features. Micro CT imaging of hind limbs revealed extensive osteophyte (indicated by black arrow) formation upon ectopic activation of *Bmpr1a* in articular cartilage (Fig.1B). Taken together, these observations indicate that ectopic activation of BMP signaling is sufficient to induce the development of an OA like phenotype in adult mice.

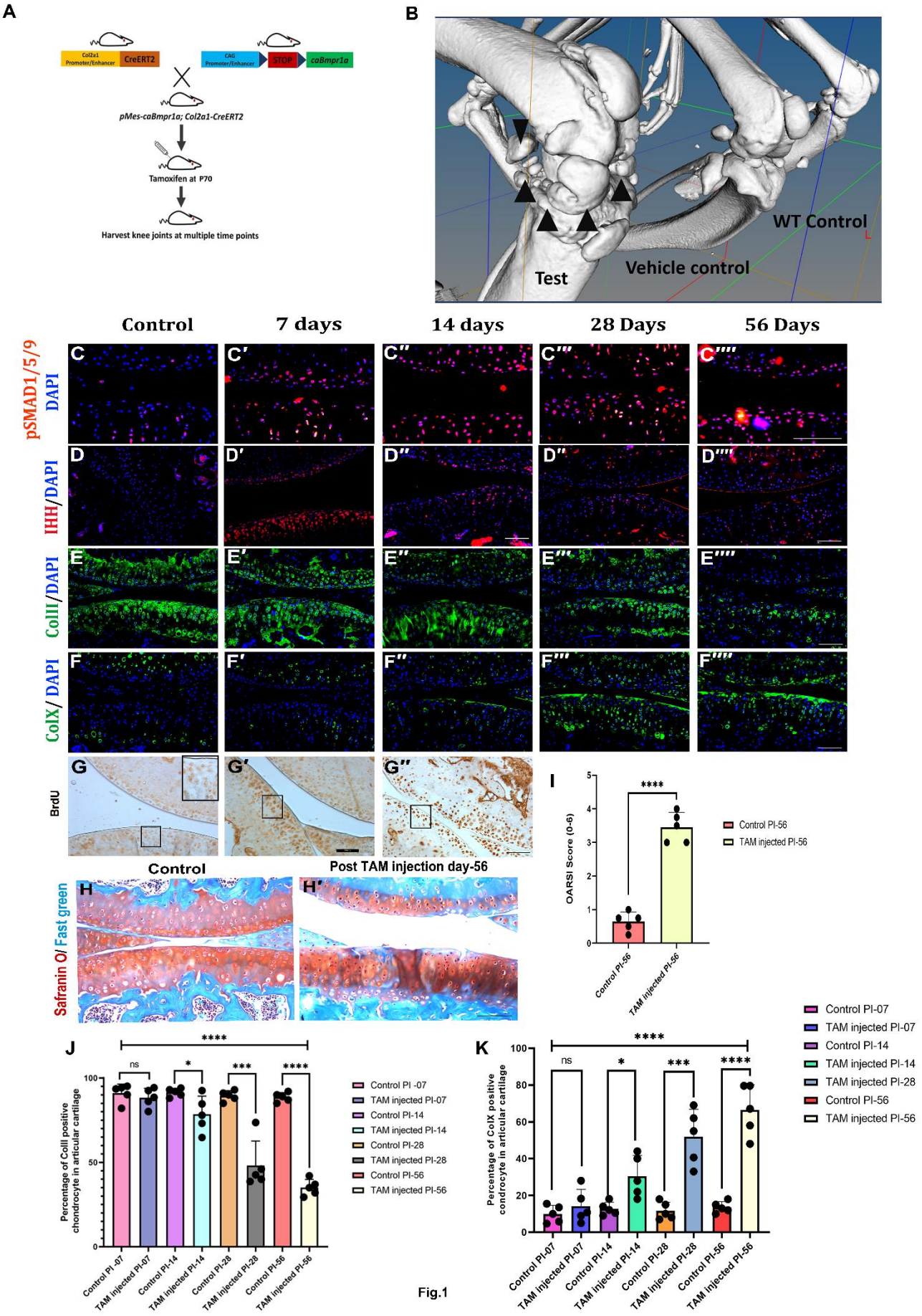
A
Col2a1 Promoter/Enhancer
CreERT2
CAG Promoter/Enhancer
STOP
caBmpr1a
pMes-caBmpr1a; Col2a1-CreERT2
Tamoxifen at P70
Harvest knee joints at multiple time points
B
WT Control
Test
Vehicle control
Control
7 days
14 days
28 Days
56 Days
pSMAD1/5/9 DAPI
IHH/DAPI
CoIII/DAPI
ColX/ DAPI
BrdU
Control
Post TAM injection day-56
Safranin O/ Fast green
OARSI Score (0-6)
Control PI-56
TAM injected PI-56
Percentage of CoIII positive chondrocyte in articular cartilage
Percentage of ColX positive condrocyte in articular cartilage
Control PI -07
TAM injected PI-07
Control PI-14
TAM injected PI-14
Control PI-28
TAM injected PI-28
Control PI-56
TAM injected PI-56
ns
*
***
****

Fig.1

**Fig. 1. Overexpression of BMP signaling in adult joint cartilage is sufficient to induce OA development.**

**(A)** Schematic for generation of *pMes-caBmpr1a; Col2a1-Cre-ERT2* mice and mis-expression of constitutively active *Bmpr1a* in the adult cartilage by injecting tamoxifen (TAM) intraperitoneally at P56. **(B)** 3-D rendering of µCT scan at 40µm resolution in wildtype (WT) control, vehicle control and TAM injected knee joint at 180 days post induction, black arrows show osteophytes **(C-F′′′′)** Longitudinal sections through the adult knee joints of vehicle control (C-H) and mice 7 days (C′-G′), 14 days (C′′-G′′), 28 days (C′′′, F′′′), 56 days (C′′′′-F′′′′) post induction by TAM injection. Immunoreactivity for pSMAD1/5/9 (C-C′′′′), IHH (D-D′′′′), ColII (E-E′′′′) and ColX (F-F′′′′). (**G-G′′)** BrdU incorporation after 7 days (G′) and 14 days (G′′) of TAM injection. (**G**) Vehicle control. (**H-H′)** Safranin O staining in vehicle control (H) and TAM injected knee joints at 180 days (H′) post induction. (**I)** OARSI score at post TAM injection day 56 with control, Unpaired t-test ****=p<0.0001 **(J)** Quantified data for ColII**,** Unpaired t- test, ns = p<0.4573**,** * p<0.00301, *** p<0.0003, ****=p<0.0001. ANOVA -**** p<0.0001 **(K)** Quantification data of ColX. ns=p=0.3731, *p=0.0101, ***=p=0.0004, ****=p<0.0001, ANOVA-**** p<0.0001, n=5 per group. Scale bar = 100µm

**2. BMP signaling induced transient cartilage differentiation is necessary for the pathogenesis of OA**

Next, we investigated the necessity of BMP signaling in the development of osteoarthritic phenotype. It has been previously reported that levels of BMP-2 ligands are elevated in synovial fluid from OA patients and BMP receptor localisation is associated with OA severity (*19*, *26*). To induce OA in mice, we used Anterior Cruciate Ligament Transection (ACLT), an established model for surgical induction of experimental OA (*27*, *28*). We chose to analyse the knee articular cartilage every week after ACLT for the appearance of active BMP signaling. As opposed to sham operated knees (Fig. S1A) or 7 days post ACLT (Fig. S1B) we observed increased pSMAD1/5/9 immunoreactivity in specimens from 14 days post ACLT (Fig. S1B′) which persisted till 56 days post-ACLT (Fig. S1B′′, Fig. S1B′′′ and Fig. 2B′). In keeping with our observation in the context of ectopic activation of BMP signaling, we observed

increased uptake of BrdU in the articular cartilage of mice post ACLT (Fig. S1D-D‴) in contrast to the sham-operated group (Fig. S1C). In order to prevent activation of BMP signaling post ACLT, we used a previously described *Bmp2/4* double conditional knockout strain (*29*). We crossed this strain with *Col2a1-Cre-ERT2* to create *Bmp2*$^{c/c}$*; Bmp4*$^{c/c}$*; Col2a1-Cre-ERT2* strain. This allowed us to deplete BMP ligands in the articular cartilage in a temporally controlled manner through tamoxifen injection. We injected tamoxifen intraperitoneally in P70 mice and ACLT was performed at P84 (Fig. S2A and Fig. 2A).

As expected, BMP signaling was minimally detected in the articular cartilage of *Bmp2/4* depleted animals when subjected to ACLT (Fig. S2B″ and Fig. 2B″). Distribution and abundance of ColII was found to be normal in *Bmp2/4* depleted animals even after 56 days of ACLT (Fig. S2C-C″ and Fig. 2C-C″). Cartilage hypertrophy, as assessed by ColX immunoreactivity (Fig. 2D-D″) as well as expression of MMP-13 (Fig. 2E-E″), a key matrix remodelling enzyme, were significantly elevated after 56 days of ACLT (Fig. 2D′ and Fig. 2E′). However, depletion of *Bmp*2/*4* protected against extensive upregulation and allowed the maintenance of a level of ColX (Fig. 2D″) and MMP-13 (Fig. 2E″) almost comparable to that of sham (Fig. 2D and Fig. 2E). We also noted that while cartilage loss as assessed by Safranin O /Fast green staining was observed in ACLT specimens, these changes were minimum post depletion of BMP ligands (Fig. 2F-F″). Structural examination using micro-computed tomography (µCT) revealed that in “ACLT + Vehicle” group, extensive damage to articular surfaces (roughness) as well as osteophyte (marked by red arrows) formation was observed (Fig.2G′). However, upon depletion of BMP ligands, the severity and extent of these changes were significantly attenuated (Fig. 2G″), and cartilage quality (smoothness) in this group appeared comparable to that in sham operated group (Fig.2G) suggesting cartilage protection was afforded. Quantification of ColII and ColX in ACLT+BMP depleted group reveals significant similarity with Sham control (Fig. 2J & 2K). OARSI scoring indicates significant protection of articular cartilage integrity in BMP depleted +ACLT group than ACLT+Vehicle group (Fig. 2L).

To ascertain the clinical relevance of these findings we examined both osteoarthritic and non-osteoarthritic human articular cartilage. Immunoreactivity of pSMAD1/5/9 was observed in all zones of osteoarthritic cartilage from patients who underwent arthroplasty (Fig. 2H″, 2H‴), while human cartilage

from a donor without known history of OA showed minimal detectable pSMAD1/5/9 immunoreactivity (Fig. 2I″, 2I‴). There was no pSMAD1/5/9 immunoreactivity in osteoarthritic cartilage treated with phosphatase (Fig. 2H′, 2I′).

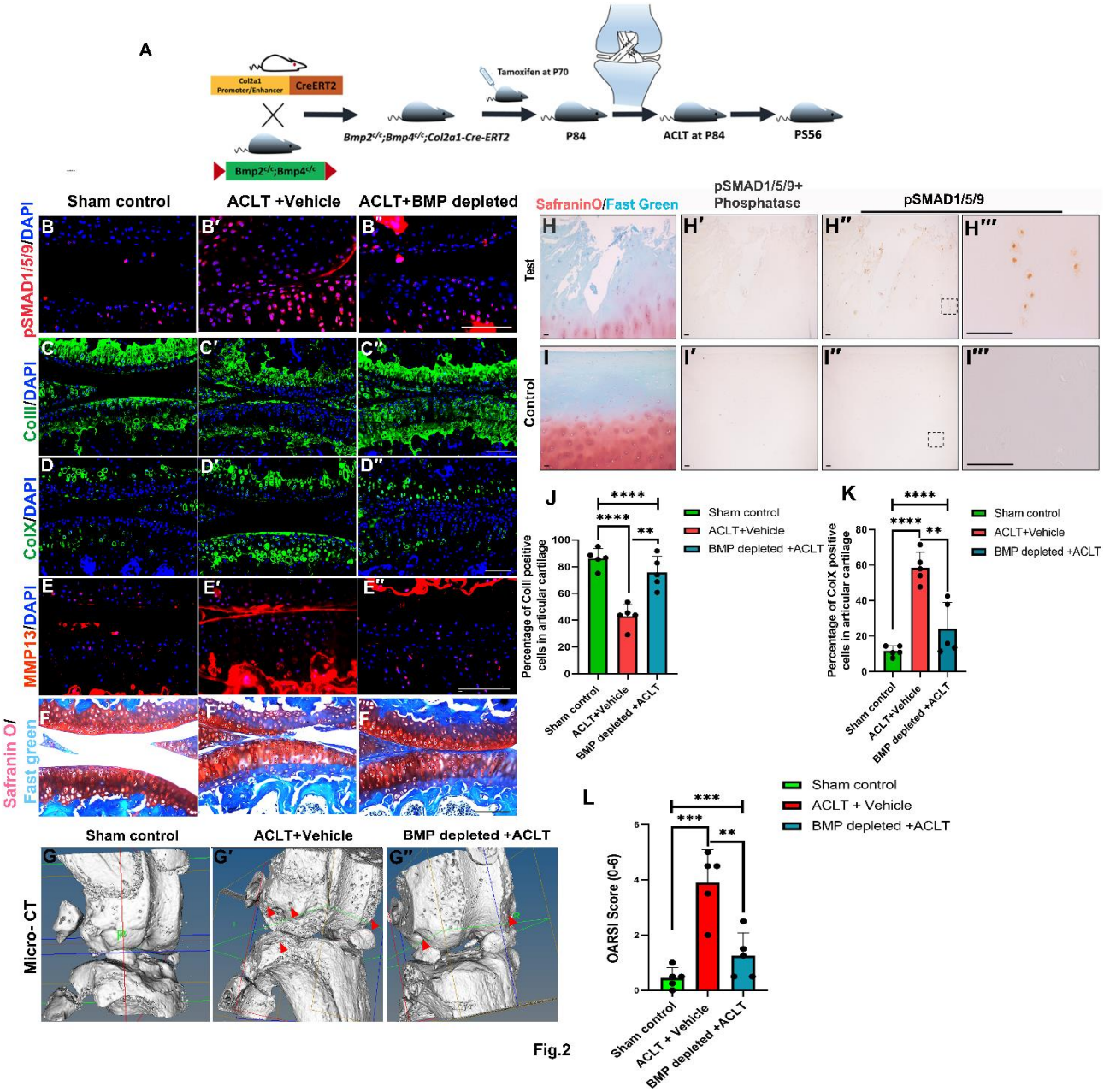


Fig.2

**Fig. 2. BMP signaling induced transient cartilage differentiation is necessary for the pathogenesis of OA.**

**(A)** Schematic representation depicting the generation of *Bmp2$^{c/c}$; Bmp4$^{c/c}$; Col2a1-Cre-ERT2* and the regimen for depletion of BMP signaling by administration of tamoxifen followed by ACLT. **(B-F″)** Longitudinal sections through the knee joints of sham (B-F), "ACLT + vehicle" control (B′-F′) and "BMP depletion + ACLT" (B″-F″) mice at 56 days post-surgery (PS56). Immunoreactivity for pSMAD1/5/9 (B-B″), ColII (C-C″), ColX (D-D″), MMP-13 (E-E″). (F-F″) Safranin O staining. (**G-G″**) 3-D rendering of µCT at PS56. Red arrowheads indicate osteophytes, surface roughness, and damage. n=5 per time point per group. Scale bar = 100µm. **(H-I‴)** Histological sections of knee articular cartilage from OA patients (n=6) (J-J‴), and a patient without known history of knee OA (n=1) (I-I‴). (H, I) Safranin O/Fast Green staining of OA (H) and normal (I) cartilage. Immunoreactivity for pSMAD1/5/9 with (H′, I′) or without phosphatase pre-treatment to verify antibody specificity (H″, H‴, I″, I‴), of OA (H′-H‴) and normal (I′-I‴) cartilage. (H‴, I‴) Higher magnification view of the marked regions in H″ and I″. **(J)** Quantification data for ColII, Unpaired t-test ****p<0.0001, **p<0.0013, ANOVA ****p<0.0001. **(K)** Quantification data of ColX. ****p<0.0001, **p=0.0021. ANOVA=****p<0.0001 **(L)** OARSI score**,** Unpaired t-test ***p=0.0003, ** p=0.0035. ANOVA- ***p=0.0001, Scale bar = 100µm.

The panels where *Bmp2/4* depleted animals were subjected to ACLT are marked as "BMP depletion + ACLT". Vehicle injected animals were used as genotype controls ("ACLT + Vehicle". "Sham" refers to *Bmp2$^{c/c}$; Bmp4$^{c/c}$; Col2a1-Cre-ERT2* animals which underwent sham surgery without ACLT.

**3. Local pharmacological inhibition of BMP signaling halts the progression of osteoarthritic changes**

We next investigated whether inhibiting BMP signaling locally in articular cartilage can retard the progression of osteoarthritic changes after surgical induction of OA. For this purpose, we used a well

characterized BMP signaling inhibitor, LDN-193189, a derivative of dorsomorphin (*30–32*). LDN-193189 activity was assayed using BRITER (BMP Responsive Immortalized Reporter) cell line (*33*) (please see Materials and Methods). As low as 100nM concentration of LDN-193189 significantly inhibited BMP signaling in BRITER cell line (Fig. S3).

Considering possible dilution and volume loss of LDN-193189 during the injection, we used 10 µM (in 3% w/v 2-hydroxypropyl- β-cyclodextrin in PBS) of LDN-193189 for intra-articular injection to locally inhibit BMP signaling. We devised a regime where we performed ACLT on wild type mice at P120 and injected LDN-193189 into the knee joint intra-articular space for seven consecutive days starting on the 14th day post-surgery and harvested 28 days post-surgery (Fig. 3A).

We observed that local inhibition of BMP signaling severely abrogates OA-associated changes in articular cartilage post transection of ACL. Lesser immunoreactivity to pSMAD1/5/9 was observed in articular cartilage of LDN-193189 treated set post ACLT (Fig. 3B″) and the sham operated knee cartilage (Fig.3B), while pSMAD1/5/9 positive cells were found in zones of articular cartilage in vehicle administered ACLT knee joints (Fig.3B′). The immunoreactivity against ColII in LDN-193189 treated knee joints was like sham operated (Fig. 3C and 3C″) as compared to ACLT+vehicle group (Fig.3C′) suggesting reduced depletion of ColII post-surgery and protection of cartilage. Hypertrophy of cartilage cells was found to be restricted to the calcified zones and minimal ColX immunostaining was observed in the articular cartilage of LDN-193189 treated ACLT induced OA mice (Fig. 3D″) similar to sham set (Fig.3D), while vehicle injected animals showed extensive hypertrophy throughout the cartilage matrix (Fig.3D′). Similarly, MMP-13 levels were found to be significantly reduced in articular cartilage upon intra-articular administration of LDN-193189 (Fig.3E″) whereas in vehicle injected knee joints, a global upregulation of MMP-13 was observed throughout multiple zones of articular cartilage (Fig.3E′). Proteoglycan depletion and cartilage damage was observed to be minimal in tibial surface of LDN-193189 injected (Fig.3F″) as compared to vehicle injected controls (Fig. 3F′) and cartilage integrity was found to be comparable to sham operated knees (Fig. 3F). ColII quantification data of ACLT+LDN-193189 injected samples were similar to sham operated controls. However, it was significantly lower in ACLT+Vehicle injected samples (Fig.3G). Similarly, quantitative data for ColX expression in ACLT+LDN-193189 injected samples was similar to sham operated samples and significantly lower than ACLT+Vehicle injected samples (Fig.3H). Moreover, OARSI scoring of cartilage revealed a

significantly attenuated osteoarthritic-like phenotype in LDN-193189 treated group as compared to the vehicle-treated ACLT group, and it was similar to sham-operated group (Fig. 3I).

Taken together, these findings suggest that *in situ* inhibition of BMP signaling in articular cartilage is sufficient to prevent the phenotypic and molecular changes associated with development and progression of OA in a surgically induced osteoarthritic mouse model.

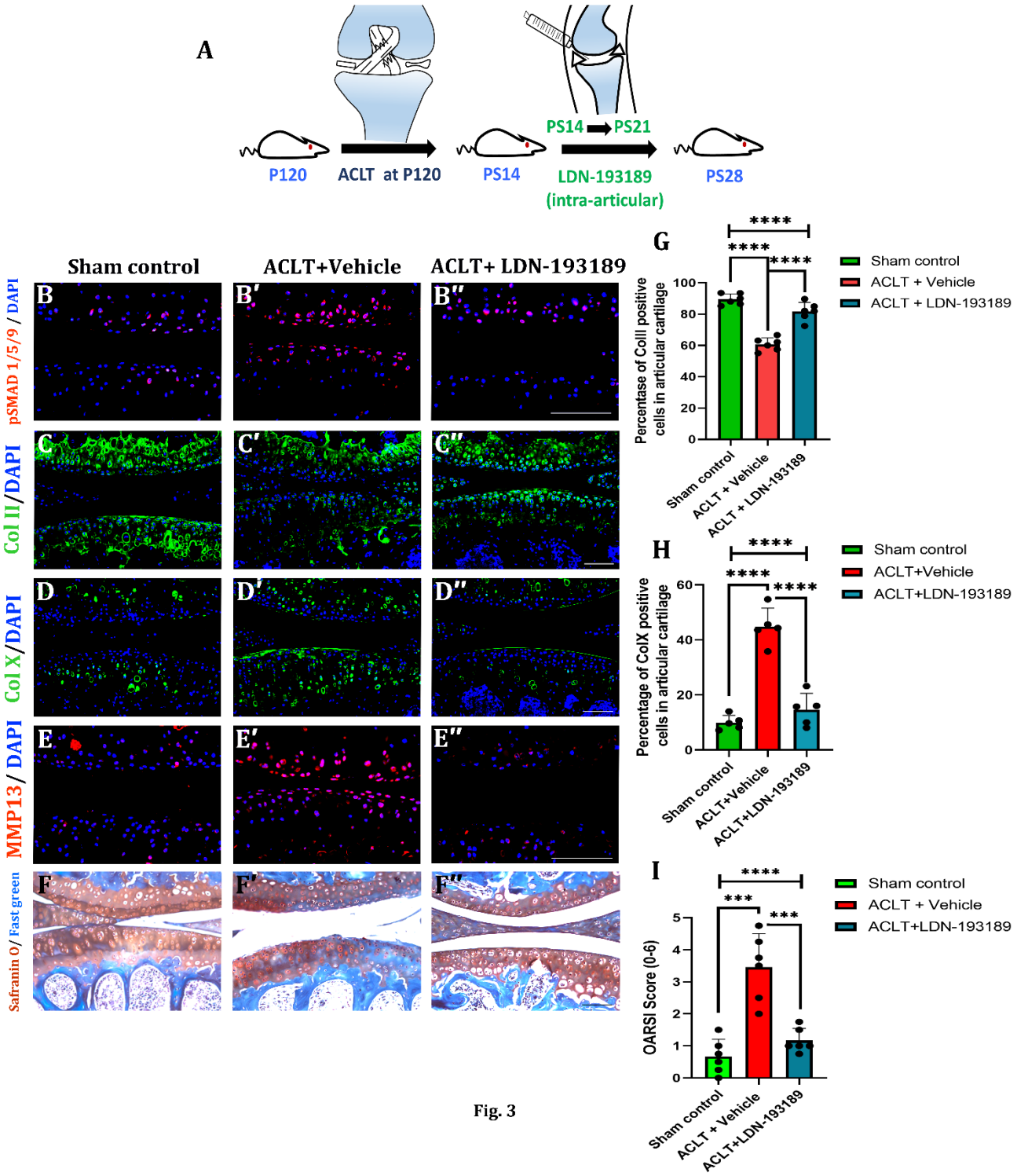
A
P120
ACLT at P120
PS14
PS14 → PS21
LDN-193189
(intra-articular)
PS28
Sham control
ACLT+Vehicle
ACLT+ LDN-193189
pSMAD 1/5/9 / DAPI
Col II/DAPI
Col X/DAPI
MMP13/ DAPI
Safranin O/ Fast green
B
B′
B″
C
C′
C″
D
D′
D″
E
E′
E″
F
F′
F″
G
Percentase of ColII positive cells in articular cartilage
Sham control
ACLT + Vehicle
ACLT + LDN-193189
H
Percentage of ColX positive cells in articular cartilage
ACLT+Vehicle
ACLT+LDN-193189
I
OARSI Score (0-6)
****
***

Fig. 3

**Fig. 3. Local pharmacological inhibition of BMP signaling halts the progression of osteoarthritic changes.**

**(A)** Schematic for local inhibition of BMP signaling using LDN-193189 in surgically induced OA in wildtype mice. **(B-F″)** Longitudinal sections through the knee joints of sham (B-F), "ACLT + vehicle" control (B′-F′) and "ACLT + LDN-193189" (B″-F″) mice at 28 days post-surgery (PS28). Immunoreactivity for pSMAD1/5/9 (B-B″), ColII (C-C″), ColX (D-D″), MMP-13 (E-E″). (F-F″) Safranin O staining. **(G)** Quantification data for ColII, Unpaired t-test ****p<0.0001, ANOVA-****p<0.0001. **(H)** Quantification of ColX, Unpaired t-test- ****p<0. 0001.ANOVA-****p<0.0001. **(I)** OARSI severity score for "ACLT + vehicle" vs "ACLT + LDN-193189" and "ACLT + vehicle" vs Sham, ***p=0.0002, ***p=0. 0005.ANOVA-****p<0.0001. Scale bar = 100µm, n=5 per group.

**4. Inhibition of BMP signaling post onset of OA attenuates disease severity**

*In situ* inhibition of BMP signaling before the onset of OA following ACL transection in mice retards the progression of OA. However, in a clinical setting, patients report to the clinic after the disease has set in. We therefore investigated if local inhibition of BMP signaling can mitigate the severity of osteoarthritic changes even after the disease has set in. For this purpose, seven consecutive intra-articular LDN-193189 injections were administered starting on post-surgery day 35 and finishing on post-surgery day 42. The knees were harvested at post-surgery day 56 (Fig.4A).

In contrast to vehicle treated knee joints (Fig. 4B′), ColII positive cells could be observed throughout articular cartilage in LDN-193189 treated samples (Fig. 4B″), which are very similar to sham operated group (Fig. 4B). The ColX immunoreactivity in vehicle treated group was significantly higher than LDN-193189 injected group which was similar to sham operated group (Fig.4C- 4C″). The level of MMP-13 was also observed to be markedly less in the articular cartilage of LDN-193189 administered knees (Fig. 4D″), as compared to the vehicle control ACLT group (Fig. 4D′) where a high level of MMP-13 expression was observed throughout. Articular cartilage integrity as indicated by Safranin O staining was maintained in case of LDN-193189 treated knee joints and it was similar to sham operated knees (Fig.4Eand 4E″) while Vertical cleft and articular cartilage loss were observed in vehicle treated ACLT knee joints (Fig. 4E′). Evaluation of the structural parameters using µCT imaging revealed that cartilage surface erosion was reduced in LDN-193189 treated set as compared to vehicle injected knees (Fig. 4F-4F″, red arrow marks osteophytes). Quantification of ColII expression was significantly higher in

case of ACLT+LDN-193189 injected samples than vehicle injected control and it was near to sham operated samples (Fig. 4G). Similarly, Quantified data for ColX immunoreactivity was higher in vehicle injected samples while it was significantly reduced in LDN-193189 injected samples and it was like sham operated control (Fig.4H). The OARSI scores in LDN-193189-treated group were significantly lower than that for ACLT group, even though administration of LDN-193189 was performed after the onset of disease. It should be noted though that less protection of cartilage was afforded, as judged by the OARSI severity scores, to the knee joints treated with LDN-193189 post-onset of OA compared to when knee joints were treated with LDN-193189 pre-onset of OA (compare Fig. 3I and Fig. 4I).

We have observed that intra-articular administration of LDN-193189 provides protection against OA like changes at least for 14 days post injection (Fig.4). Next, we wanted to investigate the potential for clinical translatability of LDN-193189 or similar molecule as a disease modifying agent. We examined whether LDN-193189 can confer longer term protection against surgically induced OA by emulating a clinic-like regimen of minimum dosage and maximum efficacy over extended durations of time. Our data (Fig. S2) as well as existing literature (*34*) suggest that molecular changes associated with OA are apparent within 28 days of ACLT. Hence, we conducted ACLT at P120, injected LDN-193189 intra-articularly on PS28, PS30 and PS32 and harvested the knee joint 56 days later at PS84. As compared to vehicle control, in the LDN-193189 injected specimen, ColII expression (compare Fig. 5B with Fig. 5B‴) as well as cartilage specific proteoglycan content, as assessed by Safranin O staining (compare Fig. 5D with Fig. 5D‴), were largely preserved. Also, ColX immunoreactivity was significantly lower in LDN-193189 treated specimen in compared to Vehicle injected knee joints (compare Fig. 5C with Fig. 5C‴). This set of data suggests that even after onset of surgically induced OA, blocking BMP signaling pathway locally can offer protection for at least 56 days in mice.

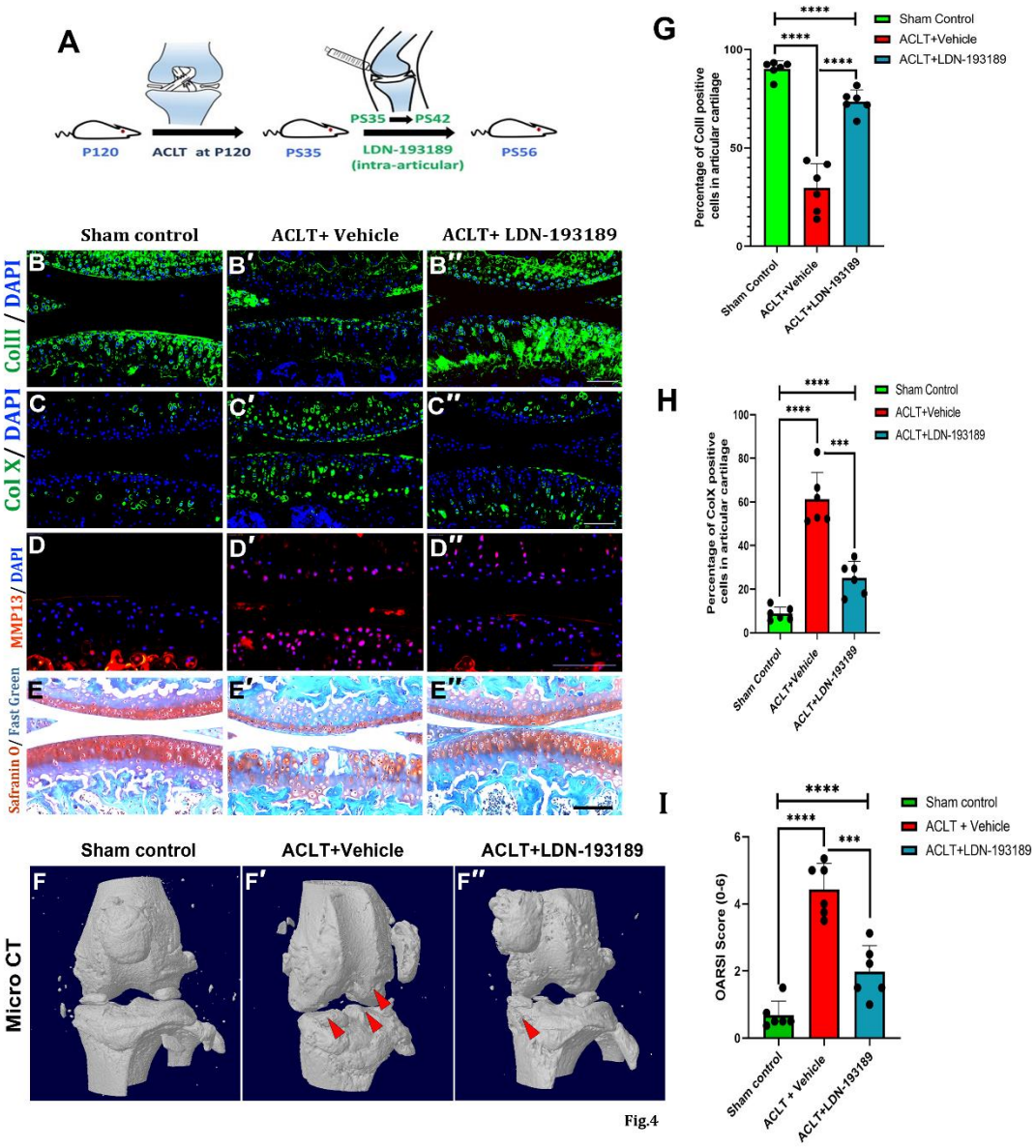


**Fig. 4. Inhibition of BMP signaling post onset of OA attenuates disease severity.**

**(A)** Schematic for local inhibition of BMP signaling, using of LDN-193189, in the knee joint of wildtype mouse post-surgical onset of OA. **(B-E″)** Longitudinal sections through the knee joints of sham (B-E), "ACLT + vehicle" control (B′-E′) and "ACLT + LDN-193189" (B″-E″) mice at 56 days post-surgery (PS56). Immunoreactivity for CoIII (B-B″), ColX(C-C″), MMP13 (D-D″). (E-E″) Safranin O staining. (**F-F″**) 3-D rendering of μCT scan at resolution of 5.86 μm per pixel in sham, "ACLT + vehicle" control and "ACLT + LDN-193189" injected knee joint at PS56 (Red arrows mark osteophytes)**. (G)** Quantification data for

ColII, Unpaired t-test-****p<0.0001, ANOVA-****p<0.0001. **(H)** Quantification data for ColX. Unpaired t-test ****p<0.0001, ***p=0.0001, ANOVA-****p<0.0001 **(I)** OARSI severity score for “ACLT + vehicle” vs “ACLT + LDN-193189”, “ACLT + vehicle” vs Sham, Unpaired t-test- ****p<0.0001, ***p= 0.0003, ANOVA-****p < 0.0001. Scale bar = 100μm, n=6 per group,

**5. Mechanistic insight into the pathogenesis of OA from a developmental biology perspective**

Recently, Singh *et al.*, demonstrated that immobilisation of chick or mouse embryos results in transient cartilage differentiation at the expense of articular cartilage differentiation which is associated with ectopic activation of BMP signaling (*23*). Further, this study also demonstrated that this ectopic activation is associated with a concurrent downregulation of expression of SMURF1, an intracellular inhibitor of BMP signaling pathway (*23*). We detected a downregulation of SMURF1 (Fig. 5E-E″) expression in the mouse articular cartilage 28- and 56-days post ACLT. Quantified data of SMURF1 suggest drastic reduction in SMURF1 expression at post ACLT Day 28 and 56 (Fig. 5F). This suggests that the molecular mechanism of articular cartilage maintenance via mechanical regulation is conserved between embryonic and postnatal stages and likely involved in pathologies such as OA.

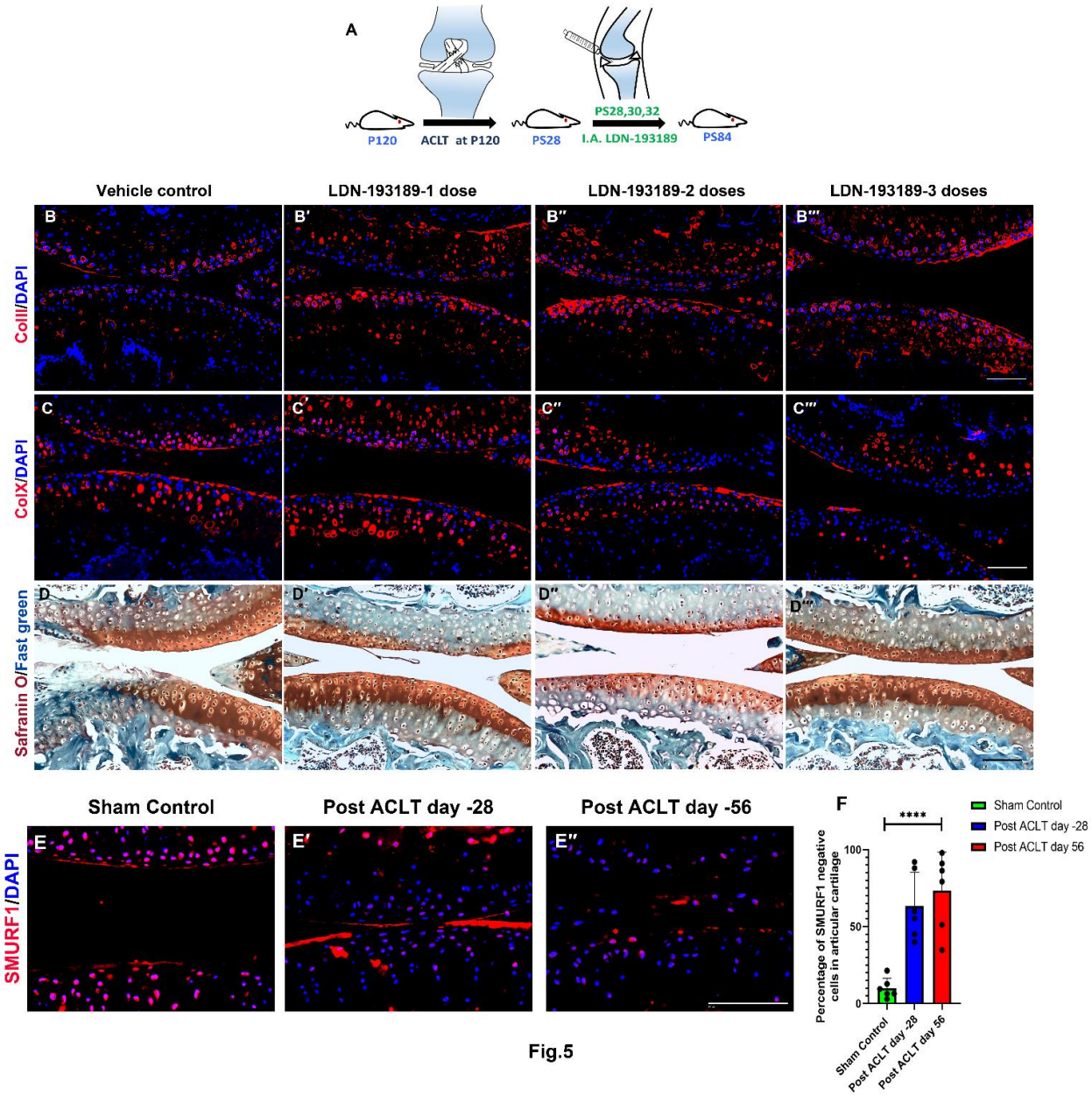


**Fig. 5. Local inhibition of BMP signaling post-onset of surgically induced OA attenuates the severity of OA associated changes for longer duration.**

**(A)** Schematic for local inhibition of BMP signaling, using of LDN-193189, in the knee joint of wildtype mouse post-surgical onset of OA. Longitudinal sections through the knee joints of ACLT induced OA mice (**B-D‴**). “ACLT + vehicle” control (B, C and D), “ACLT + LDN-193189 one dose” (B′, C′ and D′), “ACLT + LDN-193189 two doses” (B″, C″ and D″), “ACLT + LDN-193189 three doses” (B‴, C‴ and D‴) mice at 84 days post ACLT. Immunoreactivity for ColII (B- B‴), ColX (C- C‴) and Safranin O staining (D- D‴). **(E-E″)** Immunoreactivity for SMURF1 in sham control (E), post ACLT 28 days (E′) and 56 days (E″). **(F)** Quantification of SMURF1 negative cells in articular cartilage of sham control post ACLT Day28 and 56, Unpaired t-test-***p=0.0002, ns=0.4741, ANOVA-****p<0.0001. Scale bar = 100µm, n=6 per group.

**6. Effect of local inhibition of BMP signaling on inflammatory responses in a surgically induced osteoarthritic mouse model**

We performed an analysis for candidate inflammatory response molecules which are known to be involved in the development of osteoarthritis (*35*, *36*). Immunoreactivity for NFkB was detected in the articular cartilage of the vehicle treated ACLT group (Fig. 6B′), while it was minimum observed in either LDN-193189 treated group or in the sham operated group (Fig. 6B″ and 6B, respectively). We also investigated TNF-α immunoreactivity in osteoarthritic cartilage upon treatment with LDN-193189 and found that TNF-α immunoreactivity was significantly high in the ACLT group injected with only vehicle (Fig. 6C′, white arrow), while the LDN-193189 treated group displayed minimal immunoreactivity (Fig. 6C″), and it was like that in sham-operated mice where TNF-α could be detected in subchondral bone (Fig.6C). Quantification of NFkB and TNF-α suggest significant reduction in case of LDN-193189 treated samples than vehicle control (Fig.6D & 6E respectively).

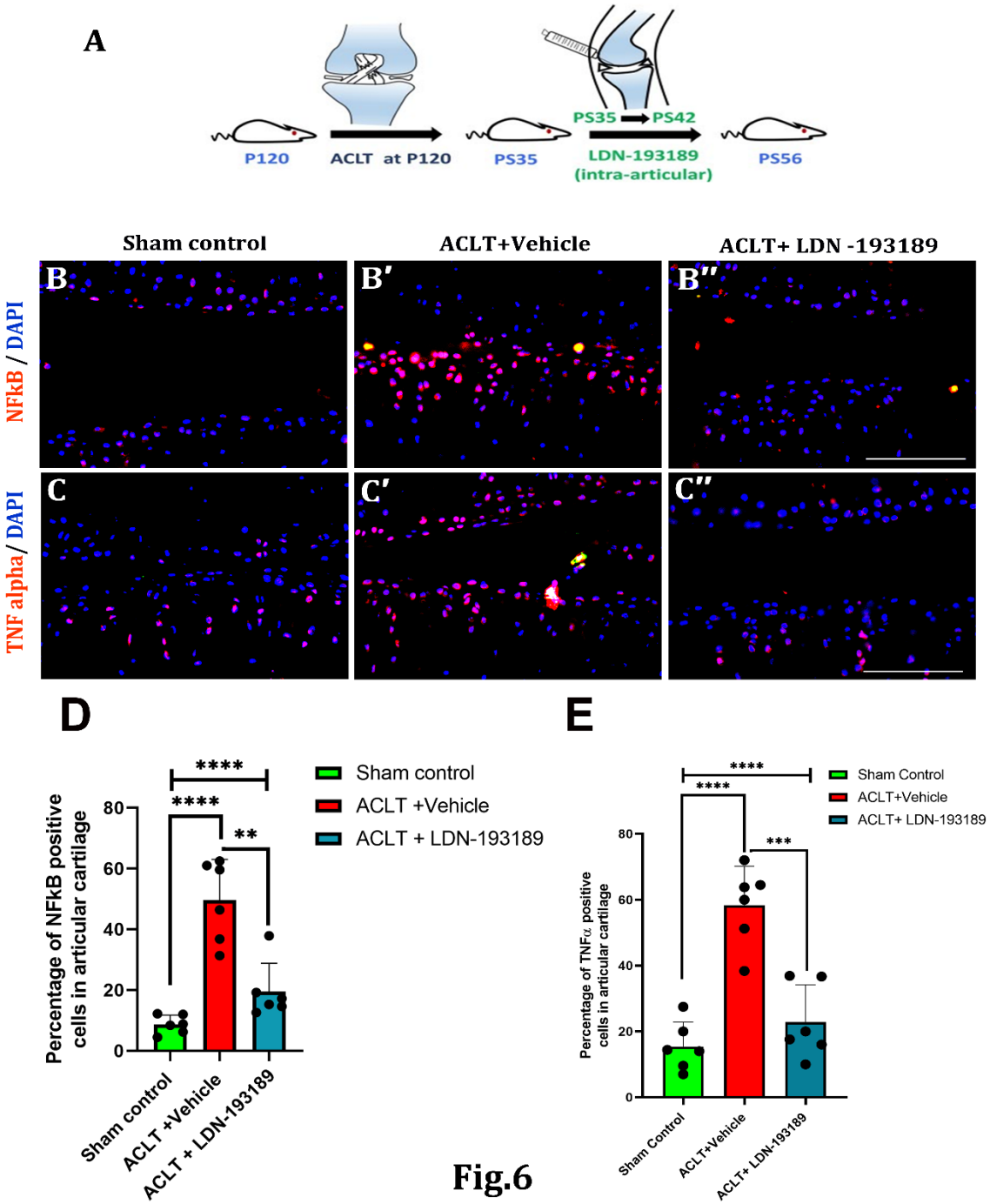


**Fig. 6. Effect of local inhibition of BMP signaling on inflammatory responses in a surgically induced osteoarthritic mouse model.**

**(A)** Schematic for local inhibition of BMP signaling, using of LDN-193189, in the knee joint of wildtype mouse post-surgical onset of OA. **(B-C″)** Longitudinal sections through the knee joints of sham (B-C),

“ACLT + vehicle” control (B′-C′) and “ACLT + LDN-193189” (B″-C″) mice at 56 days post-surgery (PS56). Immunoreactivity for NF-κB (B-B″) and TNF-α (C-C″) levels. White arrows indicate TNF-α positive cells. **(D)** Quantification data for NF-κB, Unpaired t-test-****p<0.0001, **p=0.0011, ANOVA-****p<0.0001. **(E)** Quantification data for TNF- α., Unpaired t-test- ****p<0.0001, ***p=0.0003, ANOVA-****p<0.0001. Scale bar = 100μm, n=6 per group.

**Discussion:**

Our findings suggest existence of striking resemblance between the molecular changes associated with pathogenesis of osteoarthritis and endochondral ossification. Moreover, the temporal order of the expression of molecular markers appear in ACLT induced OA was found to be similar with transient cartilage differentiation i.e., endochondral bone formation. Inhibition of transient cartilage differentiation via blocking IHH signaling has been reported to inhibit and attenuate the severity of osteoarthritic phenotype post ACLT (*37–39*). However, so far, no IHH signaling inhibitor has been approved for clinical use. This could be because Ihh loss-of-function, during embryonic development, results only in a temporary delay in hypertrophic differentiation – an essential step of endochondral ossification, which eventually gets restored and even accelerated at postnatal stages in mice (*39*, *40*). Taken together, these studies give a crucial hint that blocking transient cartilage differentiation is a viable strategy to manage osteoarthritic changes in the articular cartilage. This hypothesis is in line with what has been suggested earlier in the literature (*41–43*).

BMP signaling is known to play a critical role in transient cartilage differentiation. Previously, intraperitoneal administration of a BMP signaling inhibitor, LDN-193189, has been shown to reverse the phenotype associated with *Fibrodysplasia ossificans progressiva,* a condition where progressive heterotopic ossification of muscle is observed upon injury, due to constitutive activation of BMP signaling (*30*). In this study, we found that activation of BMP signaling is both necessary and sufficient for the pathogenesis of OA in mice. The necessity of BMP signaling in the onset of osteoarthritis like changes in the articular cartilage has been demonstrated using both genetic and pharmacological means whereas sufficiency has been demonstrated using genetic means. Further, analysis of patient samples suggests an association between osteoarthritis and activation of BMP signaling in the articular cartilage cells. Since we have used *Col2a1-Cre-ERT2* mediated recombination as the means to activate

expression of caBMPRIA we cannot rule out the possibility that BMP signaling has also been activated in the growth plate cartilage of adult mice and the molecular and cellular changes observed is partly due to activated BMP signaling in the growth plate cartilage. However, all our experiments have been done after skeletal maturity of mice so there are least contribution of observed phenotype due to change in the growth plate chondrocyte (Fig. 1). Moreover, the changes were first observed in the superficial layers of articular cartilage suggesting that the changes observed were primarily due to ectopic activation of BMP signaling in the articular cartilage.

Interestingly, we also observed proliferation in articular cartilage cells, as assessed by enhanced BrdU uptake, post ACLT or activation of BMP signaling. Our data suggests that articular cartilage cells, originally having low regenerative potential and proliferative capacity, display a regenerative response upon ACLT or upregulation of BMP signaling. However, this leads to an altered tissue microenvironment that promotes transient cartilage differentiation at the expense of articular cartilage. Thus, instead of healing by regeneration it further promotes the disease condition. Prophylactically blocking BMP signaling *in situ* using LDN-193189 led to an attenuation in the severity of osteoarthritic phenotype following surgical induction in mice. Further, our investigation suggests that administration of LDN-193189 after the onset of OA not only halts the progression of OA but also an intense Safranin O-stained cartilage tissue appears which is negative for transient cartilage markers, suggesting that new cartilage formation takes place.

Finally, it has to be acknowledged that while transient cartilage differentiation could be involved in the initiation of the disease, the inflammation associated with OA can determine the severity and course of disease progression (*44*, *45*). However, despite a large body of existing literature exploring the role of inflammation in the pathology of OA, there is no clear demonstration of the hierarchy between onset of inflammation and transient cartilage differentiation. This study suggests that pharmacologically blocking BMP signaling in surgically induced OA knees prevents activation of inflammatory response in pathogenesis of OA (Fig. 6). Thus, in *situ* inhibition of BMP signaling, and consequently transient cartilage differentiation, may be a potent means of disease modifying therapy for osteoarthritis.

**Acknowledgements**

We are immensely grateful to Prof. YiPing Chen at Tulane University, USA for the gift of mouse strains. We thank Prof. Frank Beier of Western University, Ontario, Canada for teaching APJ the method of ACL transection. We sincerely thank Shuchi Arora and Ankita Jena for their critical comments on the manuscript. We are highly grateful to Niveda Udaykumar and Saahiba Thaleshwari for their help in blind OARSI scoring. We thank Mr. Naresh Gupta for assistance with mouse experiments.

 **Funding**

This work was supported by grants from the Department of Biotechnology, India (DBT) BT/PR17362/MED/30/1648/2017 and BT/IN/DENMARK/08/JD/2016 to A.B.; Versus Arthritis Grants 19667 and 21156 to CDB and AJR, Fellowships to APJ, BK, and SFI are supported by fellowships from the Ministry of Education, Govt. of India. Fellowship to AKS was supported by Science and Engineering Research Board, Govt. of India. APJ travelled to Western University Canada with Shastri Research Student Fellowship (SRSF, 2015-'16). A.H.K.R. was supported by the Wellcome Trust through the Scottish Translational Medicine and Therapeutics Initiative (Grant No. WT 085664).

**AUTHOR CONTRIBUTIONS:** A.B., A.P.J. and B.K. designed the experiments and A.P.J., B.K., A.K.S. S.V.N. and S.F.I. conducted experiments, collected, and analysed data. A.P.J., B.K. and S.F.I. prepared the manuscript; N.A. conducted the cell-based LDN-193189 assay. A.B., C.D.B., A.J.R. edited the manuscript along with A.P.J.; B.K. and A.K.S. provided the data for inflammation response studies and mechanistic data including Smurf expression analysis; A.J.R. and A.H.K.R. collected and analysed human cartilage samples; H.W. and S.A. performed the scoring for osteoarthritis

**Competing Interests:**

The authors declare the following competing interests:

The use of BMP inhibitors as locally administered agents using sustained drug delivery vehicle(s) has been submitted for patent via Indian patent application number – **201911044840**.

The inventor(s) are:


1. Dr. Amitabha Bandyopadhyay–IIT Kanpur, India,
2. Mr. Akrit Pran Jaswal -IIT Kanpur, India.
3. Mr. Bhupendra Kumar-IIT Kanpur,India.
4. Dr. Praveen Vemula – Institute for Stem Cell Biology and Regenerative Medicine, India.
5. Dr. Manohar Mahato - Institute for Stem Cell Biology and Regenerative Medicine, India.


## References


1. A. Ray, P. N. P. Singh, M. L. Sohaskey, R. M. Harland, A. Bandyopadhyay, Precise spatial restriction of BMP signaling is essential for articular cartilage differentiation. *Development*. **142**, 1169–1179 (2015).

2. P. Occhetta, S. Pigeot, M. Rasponi, B. Dasen, A. Mehrkens, T. Ullrich, I. Kramer, S. Guth-Gundel, A. Barbero, I. Martin, Developmentally inspired programming of adult human mesenchymal stromal cells toward stable chondrogenesis. *Proc Natl Acad Sci U S A*. **115**, 4625–4630 (2018).

3. D. Symmons, C. Mathers, B. Pfleger, Global burden of osteoarthritis in the year 2000. *WHO global burden of disease* (2003).

4. D. Chen, J. Shen, W. Zhao, T. Wang, L. Han, J. L. Hamilton, H.-J. Im, Osteoarthritis: toward a comprehensive understanding of pathological mechanism. *Bone Res*. **5**, 16044 (2017).

5. V. Rosen, Mechanisms of endochondral ossification in the pathogenesis of OA. *Osteoarthritis and Cartilage*. **21**, S1 (2013).

6. L. Pulsatelli, O. Addimanda, V. Brusi, B. Pavloska, R. Meliconi, New findings in osteoarthritis pathogenesis: therapeutic implications. *Ther Adv Chronic Dis*. **4**, 23–43 (2013).

7. E. Kozhemyakina, A. B. Lassar, E. Zelzer, A pathway to bone: signaling molecules and transcription factors involved in chondrocyte development and maturation. *Development*. **142**, 817–831 (2015).

8. N. Ortega, D. J. Behonick, Z. Werb, Matrix remodeling during endochondral ossification. *Trends Cell Biol*. **14**, 86–93 (2004).

9. F. Long, D. M. Ornitz, Development of the endochondral skeleton. *Cold Spring Harb Perspect Biol*. **5**, a008334 (2013).

10. L. Dahlberg, R. C. Billinghurst, P. Manner, F. Nelson, G. Webb, M. Ionescu, A. Reiner, M. Tanzer, D. Zukor, J. Chen, H. E. van Wart, A. R. Poole, Selective enhancement of collagenase-mediated cleavage of resident type II collagen in cultured osteoarthritic cartilage and arrest with a synthetic inhibitor that spares collagenase 1 (matrix metalloproteinase 1). *Arthritis Rheum*. **43**, 673–682 (2000).

11. L. A. Neuhold, L. Killar, W. Zhao, M. L. Sung, L. Warner, J. Kulik, J. Turner, W. Wu, C. Billinghurst, T. Meijers, A. R. Poole, P. Babij, L. J. DeGennaro, Postnatal expression in hyaline cartilage of constitutively active human collagenase-3 (MMP-13) induces osteoarthritis in mice. *J Clin Invest*. **107**, 35–44 (2001).

12. H. Enomoto, I. Inoki, K. Komiya, T. Shiomi, E. Ikeda, K. Obata, H. Matsumoto, Y. Toyama, Y. Okada, Vascular endothelial growth factor isoforms and their receptors are expressed in human osteoarthritic cartilage. *Am J Pathol*. **162**, 171–181 (2003).

13. J.-Y. Ko, M. S. Lee, W.-S. Lian, W.-T. Weng, Y.-C. Sun, Y.-S. Chen, F.-S. Wang, MicroRNA-29a Counteracts Synovitis in Knee Osteoarthritis Pathogenesis by Targeting VEGF. *Sci Rep*. **7**, 3584 (2017).

14. M. W. Lark, E. K. Bayne, J. Flanagan, C. F. Harper, L. A. Hoerrner, N. I. Hutchinson, I. I. Singer, S. A. Donatelli, J. R. Weidner, H. R. Williams, R. A. Mumford, L. S. Lohmander, Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. *J Clin Invest*. **100**, 93–106 (1997).

15. S. S. Glasson, R. Askew, B. Sheppard, B. Carito, T. Blanchet, H.-L. Ma, C. R. Flannery, D. Peluso, K. Kanki, Z. Yang, M. K. Majumdar, E. A. Morris, Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature*. **434**, 644–648 (2005).

16. Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5, doi:10.1002/art.22334.

17. D. Duprez, E. Bell, M. Richardson, C. Archer, L. Wolpert, P. Brickell, P. Francis-West, Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mechanisms of Development* (1996), doi:10.1016/0925-4773(96)00540-0.

18. H. Zou, R. Wieser, J. Massagué, L. Niswander, Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev*. **11**, 2191–2203 (1997).

19. T. Nakase, T. Miyaji, T. Tomita, M. Kaneko, K. Kuriyama, A. Myoui, K. Sugamoto, T. Ochi, H. Yoshikawa, Localization of bone morphogenetic protein-2 in human osteoarthritic cartilage and osteophyte. *Osteoarthritis and Cartilage*. **11**, 278–284 (2003).

20. C. A. Hellingman, E. N. B. Davidson, W. Koevoet, E. L. Vitters, W. B. van den Berg, G. J. V. M. van Osch, P. M. van der Kraan, Smad signaling determines chondrogenic differentiation of bone-marrow-derived mesenchymal stem cells: inhibition of Smad1/5/8P prevents terminal differentiation and calcification. *Tissue Eng Part A*. **17**, 1157–1167 (2011).

21. S.-Y. Chien, C.-H. Tsai, S.-C. Liu, C.-C. Huang, T.-H. Lin, Y.-Z. Yang, C.-H. Tang, Noggin Inhibits IL-1β and BMP-2 Expression, and Attenuates Cartilage Degeneration and Subchondral Bone Destruction in Experimental Osteoarthritis. *Cells*. **9**, 927 (2020).

22. S. Chawla, M. H. M. Berkelaar, B. Dasen, C. Halleux, S. Guth-Gundel, I. Kramer, S. Ghosh, I. Martin, A. Barbero, P. Occhetta, Blockage of bone morphogenetic protein signalling counteracts hypertrophy in a human osteoarthritic micro-cartilage model. *J Cell Sci*. **133**, jcs249094 (2020).

23. P. N. P. Singh, C. A. Shea, S. K. Sonker, R. A. Rolfe, A. Ray, S. Kumar, P. Gupta, P. Murphy, A. Bandyopadhyay, Precise spatial restriction of BMP signaling in developing joints is perturbed upon loss of embryo movement. *Development*. **145**, dev153460 (2018).

24. E. Blaney Davidson, E. Vitters, P. Lent, F. Loo, W. Berg, P. Kraan, Elevated extracellular matrix production and degradation upon bone morphogenetic protein-2 (BMP-2) stimulation point toward a role for BMP-2 in cartilage repair and remodeling. *Arthritis research & therapy*. **9**, R102 (2007).

25. M. P. Khan, K. Khan, P. S. Yadav, A. K. Singh, A. Nag, P. Prasahar, M. Mittal, S. P. China, M. C. Tewari, G. K. Nagar, D. Tewari, A. K. Trivedi, S. Sanyal, A. Bandyopadhyay, N. Chattopadhyay, BMP signaling is required for adult skeletal homeostasis and mediates bone anabolic action of parathyroid hormone. *Bone*. **92**, 132–144 (2016).

26. H. Schmal, I. H. Pilz, A. T. Mehlhorn, D. Dovi-Akue, C. Kirchhoff, N. P. Südkamp, U. Gerlach, P. Niemeyer, Expression of BMP-receptor type 1A correlates with progress of osteoarthritis in human knee joints with focal cartilage lesions. *Cytotherapy*. **14**, 868–876 (2012).

27. T. Hayami, M. Pickarski, Y. Zhuo, G. A. Wesolowski, G. A. Rodan, L. T. Duong, Characterization of articular cartilage and subchondral bone changes in the rat anterior cruciate ligament transection and meniscectomized models of osteoarthritis. *Bone*. **38**, 234–243 (2006).

28. S. S. Glasson, T. J. Blanchet, E. A. Morris, The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartilage*. **15**, 1061–1069 (2007).

29. A. Bandyopadhyay, K. Tsuji, K. Cox, B. D. Harfe, V. Rosen, C. J. Tabin, Genetic Analysis of the Roles of BMP2, BMP4, and BMP7 in Limb Patterning and Skeletogenesis. *PLOS Genetics*. **2**, e216 (2006).

30. P. B. Yu, D. Y. Deng, C. S. Lai, C. C. Hong, G. D. Cuny, M. L. Bouxsein, D. W. Hong, P. M. McManus, T. Katagiri, C. Sachidanandan, N. Kamiya, T. Fukuda, Y. Mishina, R. T. Peterson, K. D. Bloch, BMP type I receptor inhibition reduces heterotopic [corrected] ossification. *Nat Med*. **14**, 1363–1369 (2008).

31. J. Hao, J. N. Ho, J. A. Lewis, K. A. Karim, R. N. Daniels, P. R. Gentry, C. R. Hopkins, C. W. Lindsley, C. C. Hong, In vivo structure-activity relationship study of dorsomorphin analogues identifies selective VEGF and BMP inhibitors. *ACS Chem Biol*. **5**, 245–253 (2010).

32. C. E. Sanvitale, G. Kerr, A. Chaikuad, M.-C. Ramel, A. H. Mohedas, S. Reichert, Y. Wang, J. T. Triffitt, G. D. Cuny, P. B. Yu, C. S. Hill, A. N. Bullock, A new class of small molecule inhibitor of BMP signaling. *PLoS One*. **8**, e62721 (2013).

33. P. S. Yadav, P. Prashar, A. Bandyopadhyay, BRITER: a BMP responsive osteoblast reporter cell line. *PLoS One*. **7**, e37134 (2012).

34. S. Kamekura, K. Hoshi, T. Shimoaka, U. Chung, H. Chikuda, T. Yamada, M. Uchida, N. Ogata, A. Seichi, K. Nakamura, H. Kawaguchi, Osteoarthritis development in novel experimental mouse models induced by knee joint instability. *Osteoarthritis Cartilage*. **13**, 632–641 (2005).

35. M. Kobayashi, G. R. Squires, A. Mousa, M. Tanzer, D. J. Zukor, J. Antoniou, U. Feige, A. R. Poole, Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. *Arthritis Rheum*. **52**, 128–135 (2005).

36. L. C. Tetlow, D. J. Adlam, D. E. Woolley, Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. *Arthritis Rheum*. **44**, 585–594 (2001).

37. A. C. Lin, B. L. Seeto, J. M. Bartoszko, M. A. Khoury, H. Whetstone, L. Ho, C. Hsu, S. A. Ali, A. S. Ali, B. A. Alman, Modulating hedgehog signaling can attenuate the severity of osteoarthritis. *Nat Med*. **15**, 1421–1425 (2009).

38. J. Zhou, Q. Chen, B. Lanske, B. C. Fleming, R. Terek, X. Wei, G. Zhang, S. Wang, K. Li, L. Wei, Disrupting the Indian hedgehog signaling pathway in vivo attenuates surgically induced osteoarthritis progression in Col2a1-CreERT2; Ihhfl/fl mice. *Arthritis Res Ther*. **16**, R11 (2014).

39. A. Vortkamp, K. Lee, B. Lanske, G. V. Segre, H. M. Kronenberg, C. J. Tabin, Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science*. **273**, 613–622 (1996).

40. Indian hedgehog signals independently of PTHrP to promote chondrocyte hypertrophy - PMC, (available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7188307/).

41. K. Gelse, A. B. Ekici, F. Cipa, B. Swoboda, H. D. Carl, A. Olk, F. F. Hennig, P. Klinger, Molecular differentiation between osteophytic and articular cartilage--clues for a transient and permanent chondrocyte phenotype. *Osteoarthritis Cartilage*. **20**, 162–171 (2012).

42. A. A. Pitsillides, F. Beier, Cartilage biology in osteoarthritis--lessons from developmental biology. *Nat Rev Rheumatol*. **7**, 654–663 (2011).

43. L. J. Sandell, T. Aigner, Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. *Arthritis Res*. **3**, 107–113 (2001).

44. J. Sokolove, C. M. Lepus, Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Ther Adv Musculoskelet Dis*. **5**, 77–94 (2013).

45. M. B. Goldring, M. Otero, Inflammation in osteoarthritis. *Curr Opin Rheumatol*. **23**, 471–478 (2011).

46. E. Nakamura, M.-T. Nguyen, S. Mackem, Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER(T) to assay temporal activity windows along the proximodistal limb skeleton. *Dev Dyn*. **235**, 2603–2612 (2006).

47. M. D. Muzumdar, B. Tasic, K. Miyamichi, L. Li, L. Luo, A global double-fluorescent Cre reporter mouse. *Genesis*. **45**, 593–605 (2007).

48. A. J. Roelofs, C. De Bari, Immunostaining of Skeletal Tissues. *Methods Mol Biol*. **1914**, 437–450 (2019).

# Supplementary Materials (SM)

## Materials and Methods

### Study design:

*Sample size:* We used statistical power calculations using G*Power3.1 software to generate the sample size from previously conducted studies in mice using Bmp2/4 DCKO mice (24).

*Data Exclusion:* No data was excluded from the end study.

*Rules for stopping data collection:* Data collection was stopped at end points pertinent to different experiments since tissues were harvested at the end of each experiment. The relevant time points were determined for each experiment based on similar experiments reported previously as well as from developmental biology insights and no outliers were present or reported.

*Replication* of data was ensured for all experiments including protocols involving classical histology/ molecular histology by repeating multiple times to ensure reproducibility of data.

*Experimental design:* The study was a controlled laboratory experiment involving animal subject, human samples, and cell culture studies. Primary nature of data collected, recorded, and analyzed was

imaging data, viz. microscopic imaging, micro-CT imaging data and statistical software used accordingly.

*Randomization:* Age matched, gender matched, genotype matched animals were used to minimize randomization in experiments. For all genetic induction experiments 2 groups were used: 1) Vehicle injected and 2) tamoxifen injected. In experiments involving surgical induction with or without genetic induction, 3 groups were used: 1) Sham surgical control 2) ACLT + vehicle 3) ACLT + Tamoxifen / LDN-191389 treated, depending on the type of experiment.

*Blinding:* OARSI scoring was performed by researchers who were not involved in the animal experiments and who were blinded to the treatment groups. Data was then unblinded and analysed by the researchers carrying out the experiments.

Details for animal and human subjects are provided in the sections following this.

**Generation of mice lines**

All animals were housed, bred and maintained in Central Experimental Animal Facility (CEAF) of Indian Institute of Technology Kanpur, India. All experiments were performed in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC) as well as under the aegis of the Centre for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India under protocols IITK/IAEC/2013/1002; IITK/IAEC/2013/1015; and IITK/IAEC/2013/1040. We obtained *B6By/J* wild type mice, *TgCol2a1-Cre-ERT2* (*46*) and *ROSA26 mT/mG* (*47*) strains from Jackson Laboratories, USA; *pMes-caBmpr1a* mice as gift from Prof. YiPing Chen at Tulane University, USA; *Bmp2*$^{c/c}$; *Bmp4*$^{c/c}$ mice from Prof. Clifford Tabin at Harvard Medical School, USA. For BMP signaling gain-of-function, *pMes-caBmpr1a* mice were crossed with *TgCol2a1-Cre-ERT2* mice to generate *pMes-caBmpr1a; TgCol2a1-Cre-ERT2. Bmp2*$^{c/c}$*; Bmp4*$^{c/c}$ (*29*) animals were crossed with *TgCol2a1-Cre-ERT2* to generate *Bmp2*$^{c/c}$*; Bmp4*$^{c/c}$; *TgCol2a1-Cre-ERT2* for BMP loss-of-function mutation. Mice were genotyped for the presence of *caBmpr1a* using PCR with primers 5′- CGAAGATATGCGTGAGGTTGTGT-3′ and 5′-TGGGCGAGAGGGGAAAGAC - 3′ while *TgCol2a1-Cre-ERT2* transgene was genotyped using primer pair 5′- CACTGCGGGCTCTACTTCAT-3′ and 5′- ACCAGCAGCACTTTTGGAAG- 3′. *ROSA26 mT/mG* was genotyped using primers 5′-CTCTGCTGCCTCCTGGCTTCT-3′ and 5′- TCAATG GGCGGGGGTCGTT - 3′. *Bmp2*$^{c/c}$ was genotyped using 5′- GTGTGGTCCACCGCATCAC-3′ and 5′-

GGCAGACATTGTATCTCTAGG - 3′ and *Bmp4*$^{c/c}$ was genotyped using 5′-AGACTCTTTAGTGAGCATTTTCAAC-3′ and 5′- AGCCCAATTTCCACAACTTC - 3′ primer pairs.

**Anterior Cruciate Ligament Transection (ACLT)**

All ACLT surgeries were conducted at P120. Unless specifically mentioned, ACLT was conducted on *B6By/J* wild type male mice. Animals were subjected to ACLT at P120 and harvested at post ACLT day 07, 14, 21, 28, and 56 for molecular and histological analysis. SMURF1 immunoreactivity was analyzed after one and two months of ACLT in wild type mice. All animals were anesthetized using isoflurane during ACLT surgery following standard protocol (28). ACLT was performed in left hind limb of all mice. Patella was dislocated medially to expose femorotibial joint cavity and ACL was transected using a micro-surgical blade under the microscope. After ACL transection, patella was repositioned and quadriceps femoris muscle was sutured to prevent its dislocation. In the sham group, the joint cavity and ACL were exposed but left intact and sutured. Surgical incision was sealed using Vicryl 5-0 Absorbable suture. Povidone-Iodine solution was applied over the sutured skin to prevent microbial infections.

**BMP signaling gain-of-function**

*pMes-caBmpr1a;TgCol2a1-Cre-ERT2* male mice were injected with three consecutive doses of tamoxifen (Sigma-Aldrich, 2.5 mg/25 g body weight) starting from P56 to induce Cre mediated recombination and harvested at post TAM injection day 7,14, 21, 28, 56 and 6 months for molecular and histological analysis.

**BMP signaling loss-of-function**

All animals used for BMP signaling loss-of-function were homozygous males for the conditional alleles of *Bmp2* and *Bmp4*. Three doses of tamoxifen (Sigma-Aldrich, 2.5 mg/25 g body weight) were injected in *Bmp2*$^{c/c}$*; Bmp4*$^{c/c}$; *TgCol2a1-Cre-ERT2* mice intraperitoneally starting from P56 for three consecutive days to activate Cre-mediated recombination to deplete BMP2 and BMP4 in adult cartilage.

pSMAD1/5/9 immunoreactivity was used to assess the status of BMP signaling before/after ACLT and/or with/without TAM-induced recombination.

Local inhibition of BMP signaling was achieved through administration of 6µl solution of 10µM LDN-193189, prepared in 3% 2-(hydroxypropyl)-β-cyclodextrin (w/v in PBS) in the knee joint cavity. Local inhibition of BMP signaling was done at two different time points. In one set, LDN-193189 was injected daily from 14$^{th}$ to 21$^{st}$ day post ACLT. In the other set LDN-193189 was administered daily from 35$^{th}$ to 42$^{nd}$ day post ACLT.

To observe the persistent effect of LDN-193189 over a prolonged period as well as to ascertain the optimum number of doses needed for providing long term protection following ACLT, LDN-193189 was injected on post-surgery day 28 (single dose) or post-surgery days 28 and 30 (two doses) or post-surgery day 28, 30 and 32 (three doses). Tissues were harvested at post ACLT day 84 for further analysis. In all experiments the vehicle control animals were injected with 3% 2-(hydroxypropyl)-β-cyclodextrin (w/v in PBS).

## Determination of the recombination frequency at P70 in mice articular cartilage.

*For determination of recombination frequency under the Col2a1Cre, TgCol2a1::Cre-ERT2;ROSA26::TdTomato-EGFP* compound mice line has been used. Tamoxifen was injected at P70 along with vehicle control group. At post TAM injection day 14, animals were harvested, tissue sectioned and anti GFP antibody was performed. Total no. of GFP positive cells in articular cartilage were calculated in test v control sample. 75.50 % percent of articular cartilage cells were positive for anti GFP antibody which results 75.50% recombination frequency of articular chondrocyte at P70 (Fig. S4).

### Cell proliferation analysis

Cell proliferation status was assessed using BrdU incorporation assay following intraperitoneal delivery of 100mg BrdU/10ml PBS/kg of body weight for three consecutive days prior to the day of harvesting tissues.

**LDN-193189 activity assay**

BRITER (BMP Responsive Immortalized Reporter) cell line(*33*) were used to determine optimum concentration of LDN-193189 required to inhibit BMP signaling.

**Human sample collection**

Osteoarthritic cartilage from 6 patients and non-osteoarthritic cartilage from one patient undergoing knee joint excision for malignancy at a site not involving sampled area, were obtained after informed consent and in accordance with the relevant guidelines and regulations, with approval from the NHS Grampian Biorepository Tissue Bank Committee, UK.

**Processing of tissue, histology, and immunohistochemistry**

Post harvesting, tissues were fixed in 4% paraformaldehyde for 16h at 4°C and then washed in PBS for 2h. The tissues were decalcified in 14% EDTA solution (pH 7.4) for 28 days with regular changes of EDTA solution at least once in a week. Decalcified tissues were embedded in paraffin wax and sectioned at 5 µm thickness.

For immunohistochemistry, sections were treated with xylene and then rehydrated using descending alcohol gradient. Antigen retrieval was done using citrate buffer except for collagens ColII and ColX antigens were retrieved by treatment of acidic solution of 0.05% pepsin at 37ºC for 25 minutes. Tissue sections were washed with PBS and treated with 3% $H_2O_2$ for 15 minutes to block endogenous peroxidase activity. After blocking in 5% HINGS (Heat-inactivated Goat Serum) solution for an hour, tissue sections were incubated with primary antibodies against Type X Collagen (DSHB, Cat.no. X-AC9-s, 1:20), Type II Collagen (DSHB, Cat.no. CIIC1-s, 1:20, SMURF1 (Santa Cruz Biotechnology,Cat.no.SC100616,1:100), NF-κB (CST,Cat.no.D14E12,1:400, MMP-13 (Santa Cruz Biotechnology, Cat.no.SC30073,1:100), pSMAD1/5/9 (CST Cat.no.13820S, 1:100), GFP (Invitrogen, Cat no.A6455, 1:100), BrdU (Sigma, Cat.no. B8434), iNOS (Abcam, Cat. no.3523, 1:50), and TNF-α (Santa Cruz Biotechnology, Cat.no. SC-52746, 1:100) for 16 hours at 4ºC. Tissue sections were washed with PBST and incubated with fluorescence labelled secondary antibodies for overnight at 4ºC. Secondary antibody against BrdU was conjugated with HRP (horseradish peroxidase) and detected by DAB (3,3 Diaminobenzidine).

Histological analysis was performed using Safranin O and Toluidine blue staining following standard protocols. For Safranin O/ Fast green staining, rehydrated tissue sections were treated with

haematoxylin solution for 10 minutes, washed in running tap water, and treated with 0.5% Fast Green solution for 2 minutes. Thereafter, sections were dipped in 1% acetic acid and finally stained with 0.5% Safranin O solution for 2 minutes. Stained sections were dehydrated with 95% and 100% alcohol respectively, cleared with Xylene and mounted in DPX. All images were taken using Leica DM 5000B compound microscope and processed using manufacturer provided software suite(s).

Human cartilage sections were stained by immunohistochemistry for pSmad1/5/9 using standard protocols (*48*). Briefly, antigen retrieval was performed by incubation for 1 hour at 65ºC in antigen unmasking citrate buffer solution (pH 6, Vector Laboratories, UK), after which sections were incubated with phosphatase (CST Lambda Phosphatase kit, New England Biolabs, #P0753) according to manufacturer's instructions, or incubated with buffer only. Sections were then incubated with an antibody against pSmad1/5/9 (Millipore, cat no. ab3848, at 1:50 (10 µg/ml)) followed by biotinylated goat anti-rabbit IgG (Vector Labs, cat no. BA-1000, at 1:200). Stained sections were imaged using Zeiss Axioskop 40 (Zeiss) with Progress XT Core 5 colour digital camera and ProgRes CapturePro 2.9.0.1 software (JenOptik, Germany).

The OARSI scores were calculated using the recommended guidelines for assessment of osteoarthritic severity in small animals (mice). OARSI scoring was performed by researchers who were not involved in the animal experiments and who were blinded to the treatment groups. Data was then unblinded and analysed by the researchers carrying out the experiments.

Quantification of ColX, ColII, NFkB and TNF alpha was done by counting percentage of articular chondrocyte was positive for respective molecules. The quantification of SMURF1 was done by calculating percentage of negative cells for SMURF1 in articular cartilage.

**Micro-Computed Tomography (µCT)**

Fixed tissues were taken in 5ml microfuge tube in hydrated condition and imaged using high resolution µCT (Skyscan 1172). Images were reconstructed and analyzed using NRecon v1.6 and CTAn 1.16.8.0, respectively. 3-D volumetric projections were generated using CTVol 2.0 software. The scanner was set to operate at a voltage of 50 kVp, a current of 200 µA and the resolution of the images was 5.86 µm per pixel. µCT for BMP signaling gain-of-function specimens was performed, processed, and analyzed

at the Institutional facility of Western University, Ontario, Canada using eXplore Locus GE scanner. The resolution of the image is 40 µm per pixel and 3-D isosurface projection was generated using Parallax Innovations MicroView 2.5.0- rc15 (2.5.0-3557).

**Statistics**

Graph Pad Prism 8 software was used to ascertain statistical significance of the OARSI histopathological scoring data. One-way ANOVA with post-hoc analysis (Dunnett's test) was performed to calculate the statistical significance and interdependence between different experimental groups. The results were plotted using Graph Pad Prism 8 software. The error bars represent Standard Deviation (S.D.).

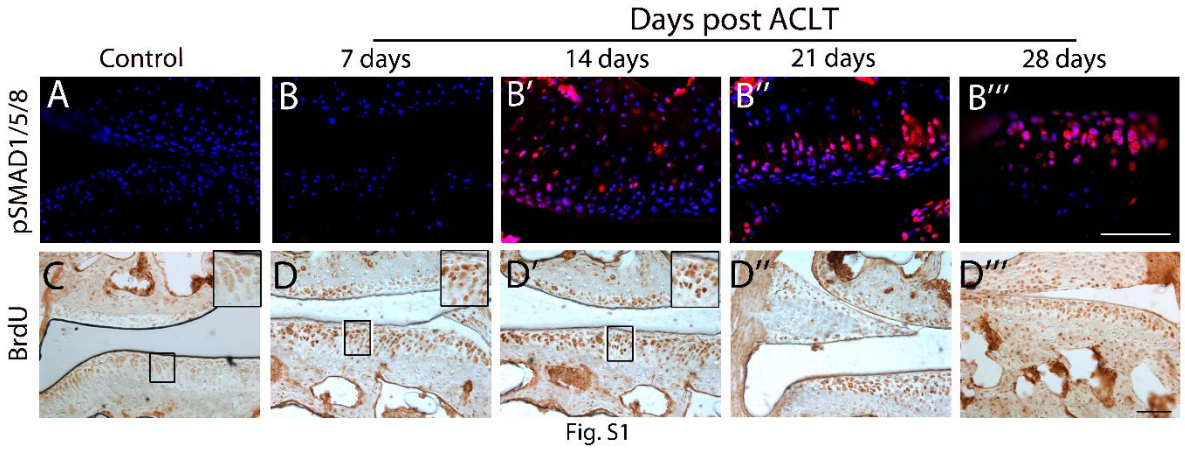


**Fig. S1. Molecular characterization of mouse knee joint post ACLT**

**(A-D‴)** Longitudinal sections through the knee joints of sham (A, C), 7 days (B, D), 14 days (B′, D′), 21 days (B″, D″), 28 days (B‴, D‴) post ACLT. **(A-B‴)** Immunoreactivity for pSMAD1/5/9. **(C-D‴)** BrdU uptake. Insets display the higher magnification view of the marked regions. Scale bar = 100µm, n=5 per group

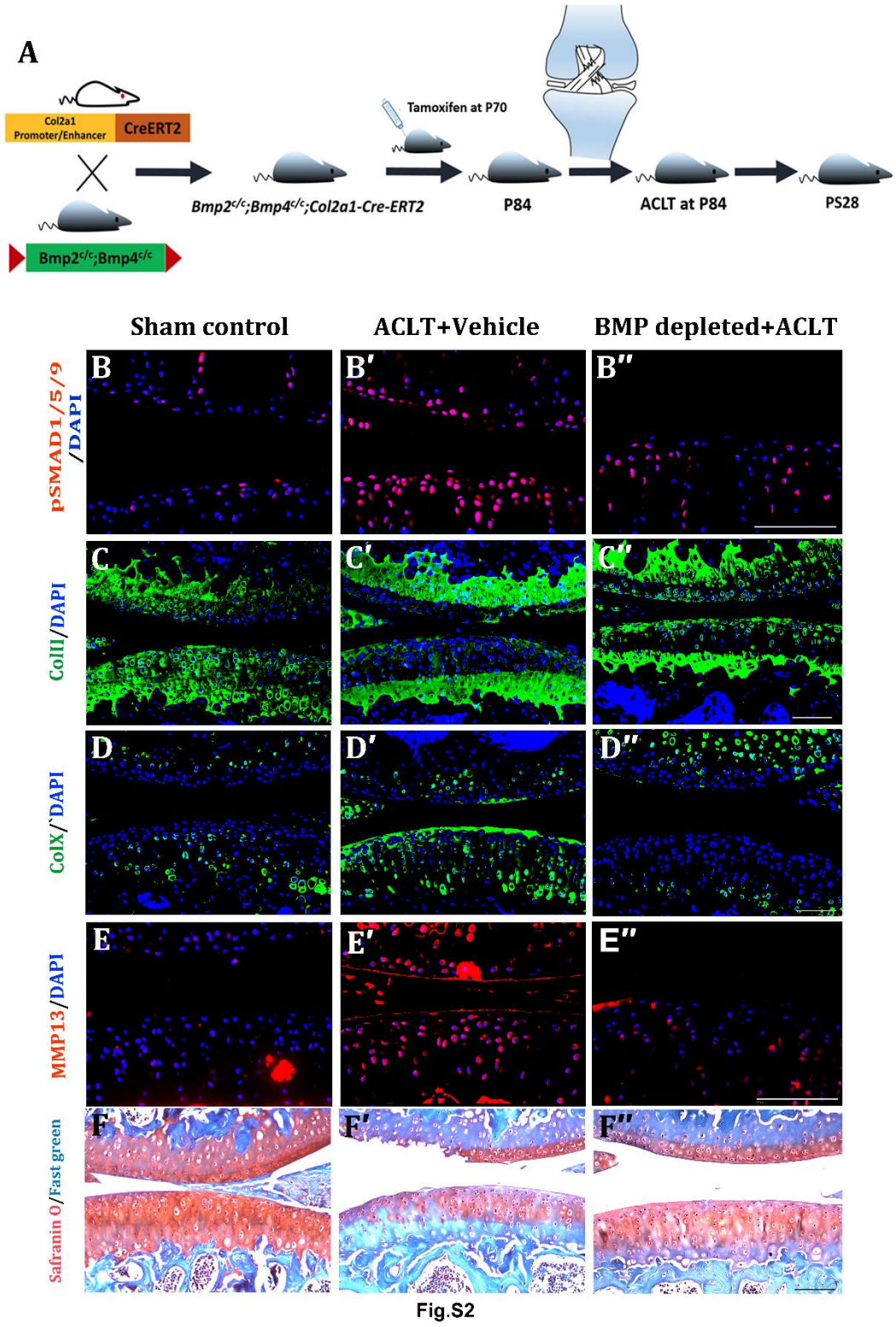


**Fig. S2. BMP signaling induced transient cartilage differentiation is necessary for the pathogenesis of OA.**

**(A)** Schematic representation depicting the generation of *Bmp2*$^{c/c}$*; Bmp4*$^{c/c}$*; Col2a1-Cre-ERT2* and the regimen for depletion of BMP signaling by administration of tamoxifen followed by ACLT. **(B-F″)**

Longitudinal sections through the knee joints of sham (B-F), “ACLT + vehicle control” (B′-F′) and “BMP depletion + ACLT” (B″-F″) mice at 28 days post-surgery (PS28). Immunoreactivity for pSMAD1/5/9 (B-B″), ColII (C-C″), ColX (D-D″), MMP-13 (E-E″). **(F-F″)** Safranin O staining. n=5 per group. Scale bar = 100µm.

The panels where *Bmp2/4* depleted animals were subjected to ACLT are marked as “BMP depletion + ACLT”. Vehicle injected animals were used as genotype controls (“ACLT + Vehicle”. Scale bar = 100µm, n=5 per group

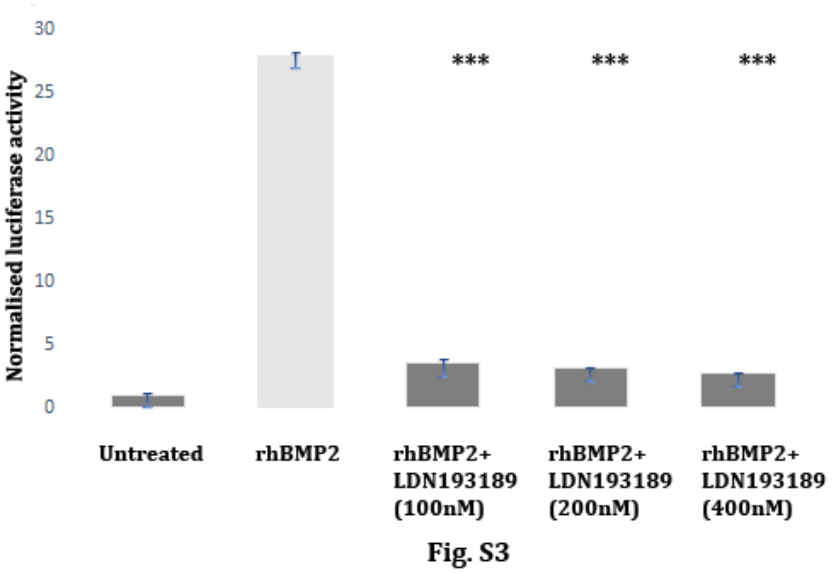


**Fig. S3. Determination of optimum LDN-193189 concentration to inhibit BMP signaling using BRITER (BMP Responsive Immortalized Reporter) cell line.**

Normalized relative luciferase activity after 3h of LDN-193189 treatment with different concentration (100nM, 200nM, 400nM) to inhibit BMP signaling in BRITER cell line (Fig.S3).

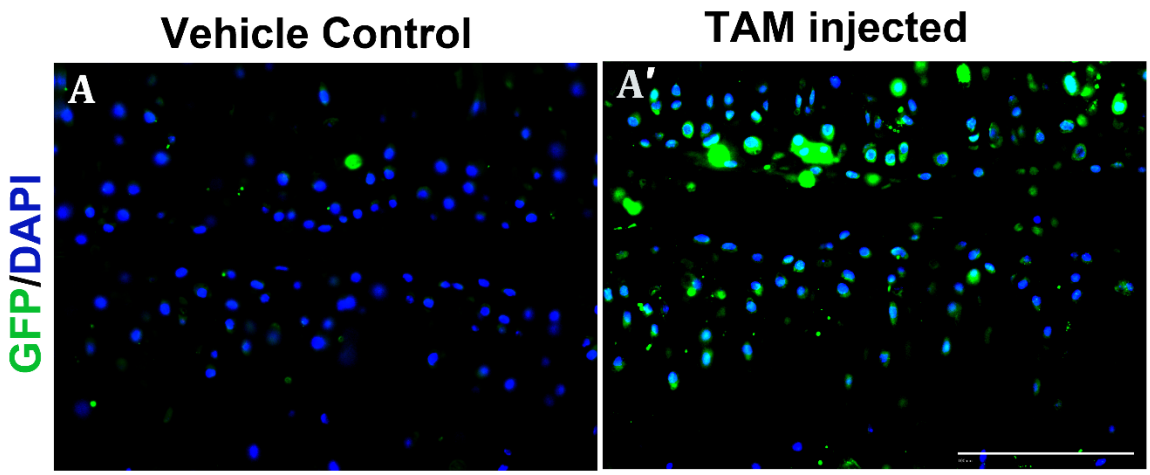


Fig.S4

**Fig. S4. Determination of the recombination frequency at P70 in mice articular cartilage.**

*For determination of recombination frequency under the Col2a1Cre, TgCol2a1::Cre-ERT2;ROSA26::TdTomato-EGFP* compound mice line has been used. Tamoxifen injected *at* P70 along with corresponding vehicle groups. (A) Vehicle control. (A′) Post TAM injected Day 14. Scale bar = 100μm, n=4 per group