Osteoarthritis and Cartilage

BMP signaling: A significant player and therapeutic target for osteoarthritis

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Abstract:	Objective: To explore the significance of BMP signaling in osteoarthritis (OA) etiology, and thereafter propose a disease-modifying therapy for OA. Methods: To examine the role of the BMP signaling in pathogenesis of osteoarthritis, an ACLT surgery was performed to incite OA in C57BL6/J mouse line at postnatal day 120 (P120). Thereafter, to investigate whether activation of BMP signaling is necessary and sufficient to induce osteoarthritis, we have used conditional gain- and loss-of-function mouse lines in which BMP signaling can be activated or depleted, respectively, upon intra-peritoneal injection of tamoxifen. Finally, we locally inhibited BMP signaling through intra-articular injection of LDN-193189 pre- and post-onset surgically induced OA. The majority of the investigation has been conducted using micro-CT, histological staining, and immune-histochemistry to assess the disease etiology. Results: Upon induction of OA, depletion of SMURF1—an intra-cellular BMP signaling inhibitor in articular cartilage—coincided with the activation of BMP signaling, as measured by pSMAD1/5/9 expression. In mouse articular cartilage, the BMP gain-of-function mutation is sufficient to induce OA even without surgery. Further, genetic, or pharmacological BMP signaling suppression also prevented pathogenesis of OA. Interestingly, inflammatory indicators were also significantly reduced upon LDN-193189 intra-articular injection which inhibited BMP signaling and slowed OA progression post-onset. Conclusion – Our findings showed that BMP signaling is crucial to the etiology of OA and inhibiting BMP signaling locally can be a potent strategy for alleviating OA.

Title

BMP signalling: A significant player and therapeutic target for osteoarthritis

Running title

Targeting BMP signaling for osteoarthritis therapy

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ABSTRACT:

Objective: To explore the significance of BMP signaling in osteoarthritis (OA) etiology, and thereafter propose a disease-modifying therapy for OA.

Methods: To examine the role of the BMP signaling in pathogenesis of osteoarthritis, an ACLT surgery was performed to incite OA in C57BL/6J mouse line at postnatal day 120 (P120). Thereafter, to investigate whether activation of BMP signaling is necessary and sufficient to induce osteoarthritis, we have used conditional gain- and loss-of-function mouse lines in which BMP signaling can be activated or depleted, respectively, upon intra-peritoneal injection of tamoxifen. Finally, we locally inhibited BMP signaling through intra-articular injection of LDN-193189 pre- and post-onset surgically induced OA. The majority of the investigation has been conducted using micro-CT, histological staining, and immune-histochemistry to assess the disease etiology.

Results: Upon induction of OA, depletion of SMURF1—an intra-cellular BMP signaling inhibitor in articular cartilage coincided with the activation of BMP signaling, as measured by pSMAD1/5/9 expression. In mouse articular cartilage, the BMP gain-of-function mutation is sufficient to induce OA even without surgery. Further, genetic, or pharmacological BMP signaling suppression also prevented pathogenesis of OA. Interestingly, inflammatory indicators were also significantly reduced upon LDN-193189 intra-articular injection which inhibited BMP signaling and slowed OA progression post-onset.

Conclusion – Our findings showed that BMP signaling is crucial to the etiology of OA and inhibiting BMP signaling locally can be a potent strategy for alleviating OA.

Introduction

Osteoarthritis (OA) is a painful, debilitating musculoskeletal disorder with a profound socioeconomic burden and is the primary cause of locomotive disability affecting millions of people worldwide[1–3]. The alarmingly increasing prevalence of OA is exacerbated further as no 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 and aneural 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 formed 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 as 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*. discovered a zone of Col2a1-expressing bipotential proliferating cells known as Distal Proliferative Zone (DPZ) within a developing appendicular skeletal element. The DPZ cells under the influence of BMP signaling undergo transient cartilage differentiation, whereas when exposed to Wnt signaling they 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(p1),11–16].

Previous literature suggests that ectopic activation of BMP signaling in developing cartilage or presumptive joint sites, 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. A surge in BMP2 and BMP4 ligands was reported in human articular cartilage having a moderate to severe form of osteoarthritis[19,20].BMP9 also induces hypertrophic like phenotype in primary chondrocyte which can be rescued by TGF-β1[21,22]. Blocking BMP signaling inhibits chondrocyte hypertrophy and regulates terminal differentiation of BMSCs[23]. Additionally, Noggin administration in an ACLT induced OA model inhibits OA progression by inhibiting IL-1β and BMP-2[24]. A recently published in-vitro study indicates reduction of chondrocyte hypertrophy after BMP receptors were inhibited using LDN-193189[25].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[26]. Recently, it was also demonstrated that pharmacological inhibition of BMP signaling promotes articular cartilage differentiation in hMSC derived chondrocytes and allows the cells to maintain an articular chondrocyte phenotype for a longer duration of time upon implantation in mice[2], suggesting that an embryonic paradigm of spatial restriction of BMP signaling is needed for differentiation and maintenance of the articular cartilage phenotype. However, few studies indicate BMPs have an anabolic effect on articular cartilage integrity[27].

Taken together, we hypothesised that BMP signaling-induced transient cartilage differentiation within the 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 the mouse 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.

Materials and Methods:

Additional information is found in supplementary material.

Animal Study Protocols

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; IITK/IAEC/2013/1040 and IITK/IAEC/2022/1166. Mouse related experiment are performed as per ARRIVE Guidelines (supplementary table 1)

Micro-Computed Tomography (µCT)

Images were reconstructed and analysed using NRecon v1.6 and CTAn 1.16.8.0, respectively. Fixed tissues were taken in 5ml microfuge tube in hydrated condition and imaged using high resolution µCT (Skyscan 1172).

RESULTS:

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

To examine whether overexpression of BMP signaling in the articular cartilage is sufficient to induce osteoarthritis like changes in adult mice, we activated BMP signaling in postnatal cartilage at P70 by injecting tamoxifen in the intraperitoneal cavity of *pMes-caBmpr1a; TgCol2a1-Cre-ERT2* mouse (Fig. 1A) (*Referred as induction*). Seven days of over-expression of constitutively activated BMP receptor

(*caBmpr1a*) in adult mouse articular cartilage resulted in, ectopic activation of canonical BMP signaling, as assessed by immunoreactivity towards phosphorylated SMAD1/5/9, which peaked after two weeks (Fig. 1C′-C′′′′; n=5/5). Expression of IHH, which marks a pre-hypertrophic state of cartilage, was observed within 7 days of induction and by 14th day after induction, IHH expression has been reduced (Fig.1D-D′′; n=5/5). ColII expression pattern got depleted on the 14th post-induction day and reached a nadir on the 56th post-induction day (Fig. 1E-E′′′′; n=5/5). The ColX expression, indicative of cartilage hypertrophy, was observed 14 days after induction, with the largest extent of hypertrophy occurring 56 days later (Fig. 1F-F′′′′; n=5/5). Embryonic[26,28], as well as adult articular cartilage cells[2], are proliferation deficient while transient cartilage cells are proliferative[1]. In our experiments, we observed cell proliferation along with other markers of transient cartilage differentiation markers in the adult mouse articular cartilage after activation of BMP signaling. BrdU uptake increased in joint cartilage 7 days after induction reaching a peak on 14th day of induction (Fig. 1G-G′′). Safranin O/Fast Green staining revealed a loss of proteoglycan staining in multiple zones with vertical clefts in the articular cartilage (Fig. 1H-1H′). OARSI scoring for integrity of articular cartilage indicated the severity of loss of articular cartilage in TAM injected versus control samples (Fig. 1I). A similar trend to transient cartilage differentiation, is indicated by quantification of ColII and ColX expression in control tissues vs samples injected with TAM (Fig. 1J and Fig. 1K).

Besides the molecular signatures, Micro CT imaging of hind limbs revealed extensive osteophyte formation upon ectopic activation of *Bmpr1a* in the 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.

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 the 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[19,29]. We performed Anterior Cruciate Ligament Transection (ACLT) to induce OA in mice and examined BMP signaling readout pSMAD1/5/9 in knee articular cartilage every week following ACLT[30]. In comparison

to sham operated knees (Fig. S1A, S1A′, S1A″ and S1A‴) or 7 days post ACLT (Fig. S1B), we found increased pSMAD1/5/9 immunoreactivity 14 days after ACLT (Fig. S1B′), which lasted until 56 days after ACLT (Fig. S1B″, Fig. S1B‴, and Fig. 2B′). Similar to ectopic BMP signaling activation, we also found increased BrdU uptake in the articular cartilage of mice following ACLT (Fig. S1C and S1D-D‴). In order to prevent activation of BMP signaling post ACLT, we used a previously described *Bmp2/4* double conditional knockout mice strain[31].*Bmp2$^{c/c}$; Bmp4$^{c/c}$; TgCol2a1-Cre-ERT2,* injected tamoxifen intraperitoneally at P70 and thereafter performed ACLT at P84 (Fig. S2A and Fig. 2A).

As expected, after ACLT, pSMAD1/5/9 immunoreactivity was minimal in articular cartilage of Bmp2/4-depleted animals. (Fig. S2B″ and Fig. 2B″). Distribution and abundance of ColII was significantly preserved in *Bmp2/4* depleted animals even after 56 days of ACLT (Fig. S2C-C″ and Fig. 2C-C″). Chondrocyte 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 remarkably elevated after 56 days of ACLT (Fig. 2D′ and Fig. 2E′). However, the depletion of Bmp2/4 rescued the ACLT mediated upregulation of ColX. (Fig. 2D″) and MMP-13 (Fig. 2E″) and maintained at almost comparable level to that of sham control (Fig. 2D and Fig. 2E) Articular cartilage loss was observed in ACLT specimens as measured by Safranin O/Fast green staining, these changes were minimal in BMP ligand depletion specimen (Fig. 2F-F″). Micro-computed tomography (μCT) structural examination revealed that the ACLT + Vehicle group had extensive damage to articular surfaces (roughness) as well as osteophyte formation (marked by red arrows) (Fig. 2G′). However, the severity and extent of these changes were minimal in ACLT+BMP ligand depleted group (Fig. 2G″), and comparable to sham operated group (Fig. 2G), indicating that cartilage protection was provided. Quantification of ColII and ColX in the ACLT+BMP depleted group revealed significant similarity with the Sham control (Fig. 2J & 2K). OARSI scoring indicated significant protection of articular cartilage integrity in the ACLT + BMP depleted group compared to the ACLT+vehicle group (Fig. 2L)

To ascertain the clinical relevance of these findings, we examined both osteoarthritic and non-osteoarthritic human articular cartilage. pSMAD1/5/9 immunoreactivity was found in all zones of osteoarthritic cartilage from patients who had arthroplasty (Fig. 2H″, 2H‴), whereas human cartilage from a donor with no known history of OA showed

no detectable pSMAD1/5/9 immunoreactivity (Fig. 2I″, 2I‴). There was no pSMAD1/5/9 immunoreactivity in phosphatase-treated osteoarthritic cartilage (Fig. 2H′, 2I′)

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

In order to determine if local inhibition of BMP signalling after ACLT would slow the progression of osteoarthritis in mice, LDN-193189, a well-known dorsomorphin derivative and BMP signalling inhibitor, was administered in the joint cavity[32–34]. LDN-193189 activity was assayed using the BRITER (BMP Responsive Immortalized Reporter) cell line[35]. LDN-193189 inhibited BMP signaling in the BRITER cell line at concentrations as low as 100 nM (Fig. S3).

Considering possible dilution and volume loss of LDN-193189 during the injection, we used 6µl of 10 µM LDN-193189 (in 3% w/v 2-hydroxypropyl- β-cyclodextrin in PBS) for intra-articular injection to inhibit BMP signaling following ACLT. Seven consecutive doses of LDN-193189 was given starting from 14th to 21st day post-surgery and tissue were harvested at 28 days post-surgery (Fig. 3A).

We found local inhibition of BMP signaling significantly abrogated OA like changes following ACL transection in mice. The pSMAD1/5/9 positive cells were found in articular cartilage of vehicle administered ACLT group (Fig. 3B′) while lesser immunoreactivity to pSMAD1/5/9 was observed in articular cartilage of LDN-193189 treated ACLT group (Fig. 3B″) and the sham operated group (Fig. 3B).The immunoreactivity against ColII in LDN-193189 treated group and sham operated group (Fig. 3C and 3C″) was similar while it was depleted in ACLT+vehicle group (Fig. 3C′) suggesting protection of ColII in LDN-193189 treatment group. The hypertrophy of cartilage cells was found to be limited to the calcified zones, with minimal ColX immunostaining in the articular cartilage of LDN-193189 treated ACLT induced OA mice (Fig. 3D″), similar to the sham group (Fig. 3D), whereas vehicle injected ACLT group showed extensive hypertrophy throughout the cartilage matrix (Fig. 3D′). Similarly, MMP-13 levels in articular cartilage were found to be significantly reduced after intra-articular administration of LDN-193189 (Fig. 3E″), whereas a robust upregulation of MMP-13 was observed in vehicle-injected knee joints (Fig. 3E′). Proteoglycan depletion and cartilage damage were found to be minimal in the tibial

surface of ACLT+LDN-193189 injected group (Fig. 3F″) when compared to the ACLT+vehicle injected group (Fig. 3F′), and cartilage integrity was found to be comparable to sham operated knees (Fig. 3F). ACLT+LDN-193189 injected samples had ColII quantification data 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 comparable to sham operated samples and significantly higher in ACLT+vehicle injected group (Fig. 3H). Moreover, OARSI scoring of cartilage revealed a significantly attenuated osteoarthritic-like phenotype in the LDN-193189 treated group as compared to the vehicle-treated ACLT group, and it was similar to the 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 the development and progression of OA in a surgically induced osteoarthritic mouse model.

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 and Fig. S4). In contrast to the vehicle-treated knee joints (Fig. 4B′), ColII positive cells were found throughout the articular cartilage in the LDN-193189-treated group (Fig. 4B″), which is very similar to the sham-operated group (Fig. 4B). The vehicle-treated group had significantly higher ColX and MMP13 immunoreactivity than the LDN-193189-injected and sham-operated groups (Fig. 4C-4C″ and Fig. 4D-4D″ respectively). Articular cartilage integrity, as determined by Safranin O staining, was preserved in LDN-193189 treated knee joints and was comparable to sham operated knees (Fig. 4E and 4E″), whereas vertical cleft and articular cartilage loss were observed in vehicle treated ACLT knee joints (Fig. 4E.The µCT imaging revealed that cartilage surface erosion was reduced in the LDN-193189-treated knees compared to the vehicle-

injected knees. (Fig. 4F-4F″; red arrow marks osteophytes).We also analysed synovial membrane of sham control (Fig. S4B and Fig. S4C), Vehicle treated group (Fig. S4B′ and Fig. S4C′) with LDN-193189 treated group (Fig. S4B″ and Fig. S4C″). Massive synovial hyperplasia with loss of membranous structure have been found in the vehicle-treated group while native phenotypes were largely preserved in LDN-193189 treated group and it was similar to the sham control group. Moreover, chondrocyte hypertrophy in meniscus of Vehicle treated group (Fig. S4D′) were increase significantly which was rescued in LDN-193189 treated group (FigS4D″) and it was comparable to sham control group (Fig. S4D) The quantification of ColII expression in articular cartilage was significantly higher in the case of ACLT+LDN-193189 injected samples than vehicle injected control and it was close to sham operated samples (Fig. 4G). Similarly, quantified data for ColX immunoreactivity in articular cartilage was higher in vehicle injected samples while it was significantly reduced in LDN-193189 injected samples and was comparable to a sham operated control (Fig. 4H). The OARSI scores of articular cartilage in the LDN-193189-treated group were significantly lower than those in the 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 molecules as disease modifying agents. 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 the existing literature[36] 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. ColII expression (compare Fig. 5B with Fig. 5B‴) and cartilage specific proteoglycan content (compare Fig. 5D with Fig. 5D‴) were largely preserved in the LDN-193189 injected specimen when compared to the vehicle control. In addition, ColX immunoreactivity was significantly

lower in LDN-193189-treated knee joints compared to vehicle-injected knee joints (compare Figs. 5C and 5C‴). This set of data suggests that even after the onset of surgically induced OA, blocking the BMP signaling pathway locally can offer protection for at least 56 days in mice.

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[26]. Further, this study also demonstrated that this ectopic activation is associated with a concurrent down-regulation of SMURF1, an intracellular inhibitor of the BMP signaling pathway[26].We noticed that SMURF1 expression was lower in mouse articular cartilage 28 and 56 days after ACLT (Fig. 5E-E″). SMURF1 quantification data showed a significant decrease in SMURF1 expression at post-ACLT Days 28 and 56 (Fig. 5F) when compared to the control group. This suggests that the molecular mechanism of articular cartilage maintenance via mechanical regulation is conserved between embryonic and postnatal stages and is likely involved in pathologies such as OA.

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 such as IL1β, NF-κB and TNF-α, which are known to be involved in the development of osteoarthritis (Fig. 6A)[37,38]. We found immunoreactivity against IL1β in vehicle treated group (Fig. 6B′) were significantly higher than LDN-193189 treated group (Fig. 6B″) which as similar to sham control group (Fig. 6B).The NF-κB immunoreactivity in the articular cartilage of the vehicle-treated ACLT group was highly increased (Fig. 6C′), but it was minimal in the LDN-193189-treated or sham-operated groups (Figs. 6C″ and 6C, respectively). We also looked at TNF--α immunoreactivity in osteoarthritic cartilage after LDN-193189 treatment and found that it was significantly higher in the ACLT group injected group with only vehicle group (Fig. 6D′), while the LDN-193189 treated group showed minimal immunoreactivity (Fig. 6D″), and it was similar in sham-operated mice where TNF--α -could be detected minimally (Fig. 6D). Quantitative analysis indicated that TNF-α and NF-κB were significantly lower in LDN-193189-

treated samples compared to vehicle controls (Fig. 6E & 6F, respectively). Therefore, inhibition of BMP signaling not only inhibits OA markers in articular cartilage but also reduces associated inflammation.

Discussion:

This study suggests molecular similarities between osteoarthritis etiology and endochondral ossification. The expression of molecular markers in ACLT-induced OA follows a timeline reminiscent of that of transient cartilage differentiation, also known as endochondral bone formation. Our data and existing literature evince a crucial hint that blocking transient cartilage differentiation is a viable strategy to manage osteoarthritic changes in the articular cartilage. Blocking IHH signaling inhibits transient cartilage differentiation and reduces post-ACLT osteoarthritis severity[39–41]. Though, no IHH signaling inhibitor has been approved so far for clinical use but supressing transient cartilage differentiation appears to be a possible way to inhibit OA pathogenesis. . This hypothesis is in line with what has been suggested earlier in the literature[42–44].

BMP signaling is known to play a critical role in cartilage differentiation. Normal articular cartilage cells express an intracellular BMP inhibitor, SMURF1. Upon ACLT, SMURF1 level goes down and BMP signaling level goes up in the articular cartilage. Thus, it appears that a low level of BMP signaling is maintained by SMURF1 and deviation from it is detrimental to cartilage health. Upregulation of BMP signaling upon ACLT results in hypertrophic differentiation and concomitant down regulation of ColII expression. However, pharmacological, or genetic inhibition of BMP signaling following ACLT does not allow the hypertrophic differentiation to proceed and thus ColII expression is maintained.

Our data strongly suggest that BMP signaling is necessary and sufficient in pathogenesis of OA. The necessity of BMP signaling in the onset of osteoarthritis-like changes in articular cartilage has been shown genetically and pharmacologically, while sufficiency has been shown genetically. Moreover, patient sample analysis also suggests that BMP signaling activation in articular cartilage cells is linked to osteoarthritis.

We cannot rule out the possibility that BMP signaling has also been activated in the growth plate cartilage of adult mice and that the molecular and cellular changes

observed are partially attributable to activated BMP signaling in the growth plate cartilage since we used TgCol2a1-Cre-ERT2 mediated recombination (Fig. S5). All our experiments were conducted after the mice had reached adulthood , so changes in the growth plate chondrocyte contribute minimally to the observed phenotype. Moreover, the changes were first seen in articular cartilage, suggesting they were due to ectopic 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. Despite having a low regeneration potential and proliferative capacity of articular cartilage cells, our data suggests that articular cartilage cells display a regenerative response upon ACLT or upregulation of BMP signaling. However, altered tissue microenvironment due to activated BMP signaling post ACLT , promotes transient cartilage differentiation over articular cartilage. Consequently, instead of healing, regeneration exacerbate disease condition.

LDN-193189, a BMP signaling inhibitor, has been found to reverse the phenotype of Fibrodysplasia ossificans progressive (FOP), a disorder characterized by progressive heterotopic ossification of muscle upon injury, caused by the constitutive activation of BMP signaling[32]. The study demonstrates following surgical induction of osteoarthritic in mice, prophylactic in situ blockade of BMP signaling with LDN-193189 reduced its severity. 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. A recent study by Liu et *al*, also suggests BMP inhibition can target osteoarthritis by Intra-peritoneal administration of the inhibitor, however, it has global effects on the body and is therefore not an option for patients.

Finally, while transient cartilage differentiation may play a role in the onset of OA, it is the inflammation that ultimately determines the severity and course of the disease. Despite a large body of literature, the hierarchy between inflammation and cartilage differentiation is unclear. BMP signaling also modulates endothelial inflammation following cardiac ischemia[45].Our study also signifies that pharmacologically blocking BMP signaling in surgically induced OA also prevents inflammatory response

activation. However, whether BMP signaling directly regulates inflammatory pathway or it induces chondrocyte hypertrophy causing inflammation due to altered joint mechanics, further needs to be investigated.

Since, we used only male mice in all our experiments, it remains to be seen if the observations made in this study hold true in females as well. However, based on the observations reported in , it is likely that the conclusions derived using the male mice will be applicable to females as well[46]. Also, since we have not assessed the levels of BMP signaling in mice of different ages, our conclusions cannot be extrapolated beyond Post-Traumatic OA. Nonetheless our study demonstrates that *in situ* inhibition of BMP signaling, and consequently transient cartilage differentiation, can be a potent means of disease-modifying therapy for osteoarthritis.

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Author's contribution:

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.

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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**.

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5. Dr. Manohar Mahato - Institute for Stem Cell Biology and Regenerative Medicine, India.

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Figures legend:

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

(A) Schematic for generation of *pMes-caBmpr1a; TgCol2a1-Cre-ERT2* mice and mis-expression of constitutively active *Bmpr1a* in the adult cartilage by injecting tamoxifen (TAM) intraperitoneally at P70. **(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 7 days (G′) and 14 days (G″) after TAM injection. (**G**) Vehicle control. (**H-H′)** Safranin O staining in vehicle control (H) and TAM injected knee joints at 56 days (H′) post induction. Black arrow indicates vertical cleft at the articular cartilage surface. (**I)** Statistical analysis by Unpaired Mann-Whitney t-test of OARSI scores at post TAM injection day 56 with control, p=0.0079 (**).**(J)** Quantification data for ColII**,** Unpaired t- test was performed to compare the means of stage matched control vs post injected (PI) TAM test animals at different time points, Control vs Test-PI day 7, p=0.4573 (ns)**,** Control vs Test, PI day 14, p=0.0301(*), Control vs Test, PI day 28, p=0.0003 (***), Control vs Test, PI day 56, p<0.0001(****). **(K)** Quantification data for ColX**,** Unpaired t- test was performed to compare the means of stage matched control vs post injected (PI) TAM test animals at different time points, Control vs Test-PI day 7, p=0.3731 (ns)**,** Control vs Test, PI day 14, p=0.0101 (*), Control vs Test, PI day 28, p=0.0004 (***), Control vs Test, PI day 56, p<0.0001 (****). n=5 per group. Scale bar = 100µm

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$^{c/c}$; TgCol2a1-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, one way ANOVA was performed along the three sets and p<0.0001(****). We compared the means of sham control vs ACLT+vehicle; p<0.0001 (****), sham control vs BMP depleted+ACLT; p=0.2319 (ns) and ACLT+vehicle vs. BMP depleted+ ACLT p=0.0005(***) **(K)** Quantification data of ColX., one way ANOVA was performed along the three sets and p<0.0001(****) the means of sham control vs ACLT+vehicle; p<0.0001 (****), sham control vs BMP depleted+ACLT; p=0.1595 (ns) and ACLT+vehicle vs. BMP depleted+ ACLT p=0.0004(***) **(L)** OARSI score**,** Brown - Forsythe and Welch ANOVA was performed, p=0.0003(***), the means of sham control vs ACLT+vehicle; p=0.0015 (**), sham control vs BMP depleted+ACLT; p=0.2065 (ns) and ACLT+vehicle vs. BMP depleted+ ACLT p=0.00114 (*) 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$^{c/c}$; TgCol2a1-Cre-ERT2* animals which underwent sham surgery without ACLT.

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, one way ANOVA was performed along the three sets and p<0.0001(****). The comparison of Sham control vs ACLT+vehicle; p<0.0001 (****), Sham control vs ACLT+ LDN-193189; p=0.0263 (*) and ACLT+vehicle vs. ACLT+ LDN-193189 p<0.0001(****). **(H)** Quantification data for ColX, one way ANOVA was performed

along the three sets and p<0.0001(****). The comparison of Sham control vs ACLT+vehicle; p<0.0001 (****), Sham control vs ACLT+ LDN-193189; p=0.3897 (ns) and ACLT+vehicle vs. ACLT+ LDN-193189 p<0.0001(****). **(I)** OARSI score**,** Brown - Forsythe and Welch ANOVA was performed, p<0.0001(****), the comparison of means of Sham control vs ACLT+vehicle; p=0.0001 (***), Sham control vs ACLT+LDN-193189; p=0.2058(ns) and ACLT+vehicle vs. ACLT+LDN-193189 p= 0.0007(***); Scale bar = 100μm, n=5 per group.

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 ColII (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, one way ANOVA was performed along the three sets and p<0.0001(****). The comparison of
Sham control vs ACLT+vehicle; p<0.0001 (****), Sham control vs ACLT+ LDN-193189; p=0.0088 (**) and ACLT+vehicle vs. ACLT+ LDN-193189 p<0.0001(****). **(H)** Quantification data for ColX, one way ANOVA was performed along the three sets and p<0.0001(****). The comparison of Sham control vs ACLT+vehicle; p<0.0001 (****), Sham control vs ACLT+ LDN-193189; p=0.0111 (*) and ACLT+vehicle vs. ACLT+ LDN-193189 p<0.0001(****). **(I)** OARSI score**,** Brown -Forsythe and Welch ANOVA was performed, p<0.0001(****), the comparison of means of Sham control vs ACLT+vehicle; p<0.0001 (****), Sham control vs ACLT+LDN-193189; p=0.0195 (*) and ACLT+vehicle vs. ACLT+LDN-193189 p=0.0032(**). Scale bar = 100μm, n=6 per group.

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, one way ANOVA was performed along the three sets with $p<0.0001$. The comparison of Sham control vs. post ACLT Day28 $p=0.0007$ (***), Sham control vs. Post ACLT Day 56 $p=0.0001$(***) and Post ACLT Day28 vs. post ACLT Day 56 $p=0.6523$ (ns). Scale bar = 100μm, n=6 per group.

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-D″)** Longitudinal sections through the knee joints of sham (B-D), “ACLT + vehicle” control (B′-D′) and “ACLT + LDN-193189” (B″-D″) mice at 56 days post-surgery (PS56). Immunoreactivity for IL1-β(B-B″), NF-κB(C-C″) and TNF-α (D-D″) levels. **(E)** Quantification data for NF-κB, one way ANOVA was performed along the three sets and $p<0.0001$(****). The comparison of Sham control vs ACLT+vehicle; $p<0.0001$ (****), Sham control vs ACLT+ LDN-193189; $p=0.1601$ (ns) and ACLT+vehicle vs. ACLT+ LDN-193189 $p=0.0002$(***). **(F)** Quantification data for TNF- α, one way ANOVA was performed along the three sets and $p<0.0001$(****). The comparison of Sham control vs ACLT+vehicle; $p<0.0001$ (****), Sham control vs ACLT+ LDN-193189; $p=0.4479$ (ns) and ACLT+vehicle vs. ACLT+ LDN-193189 $p<0.0001$(****). Scale bar = 100μm, n=6 per group.

Figure.1

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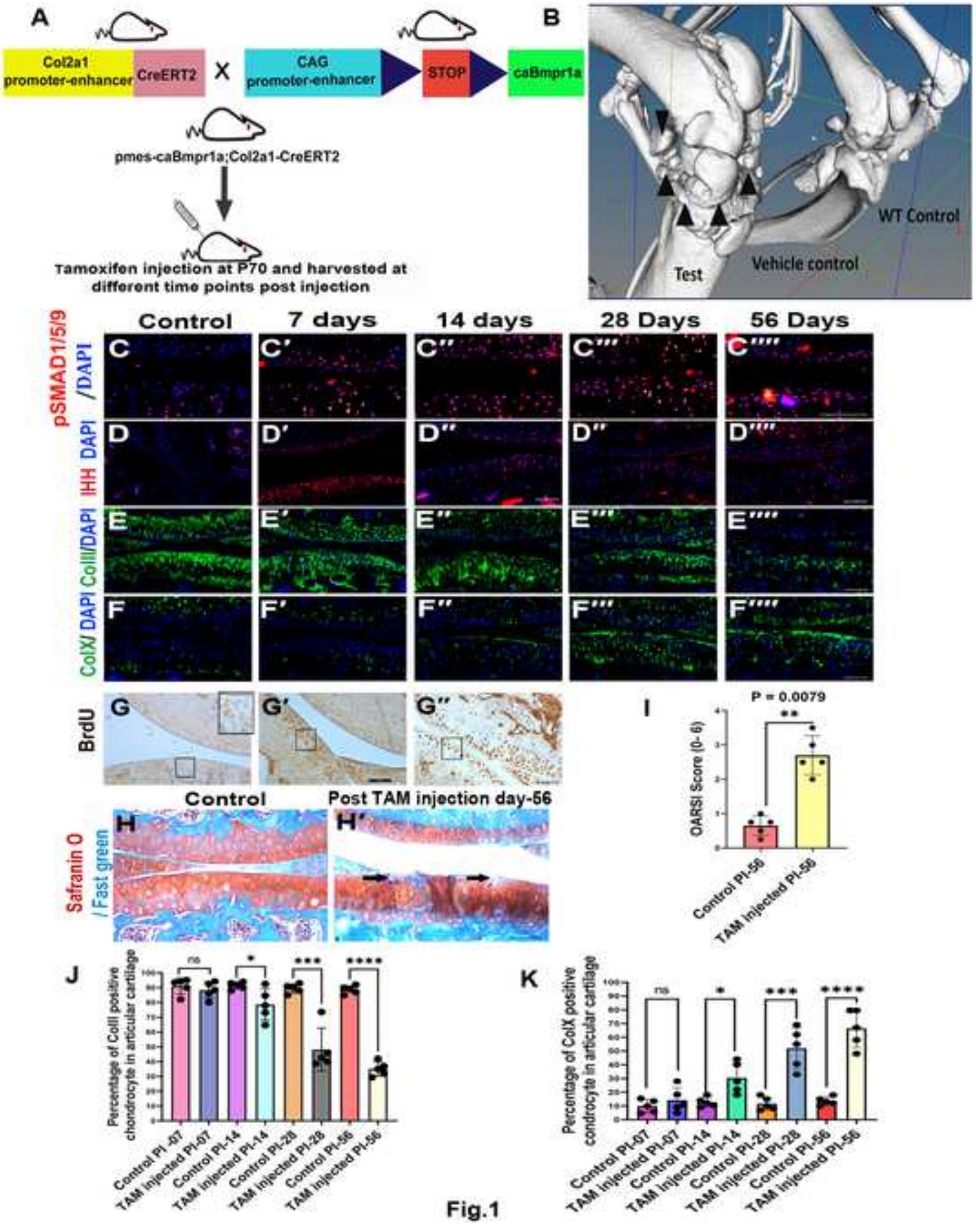

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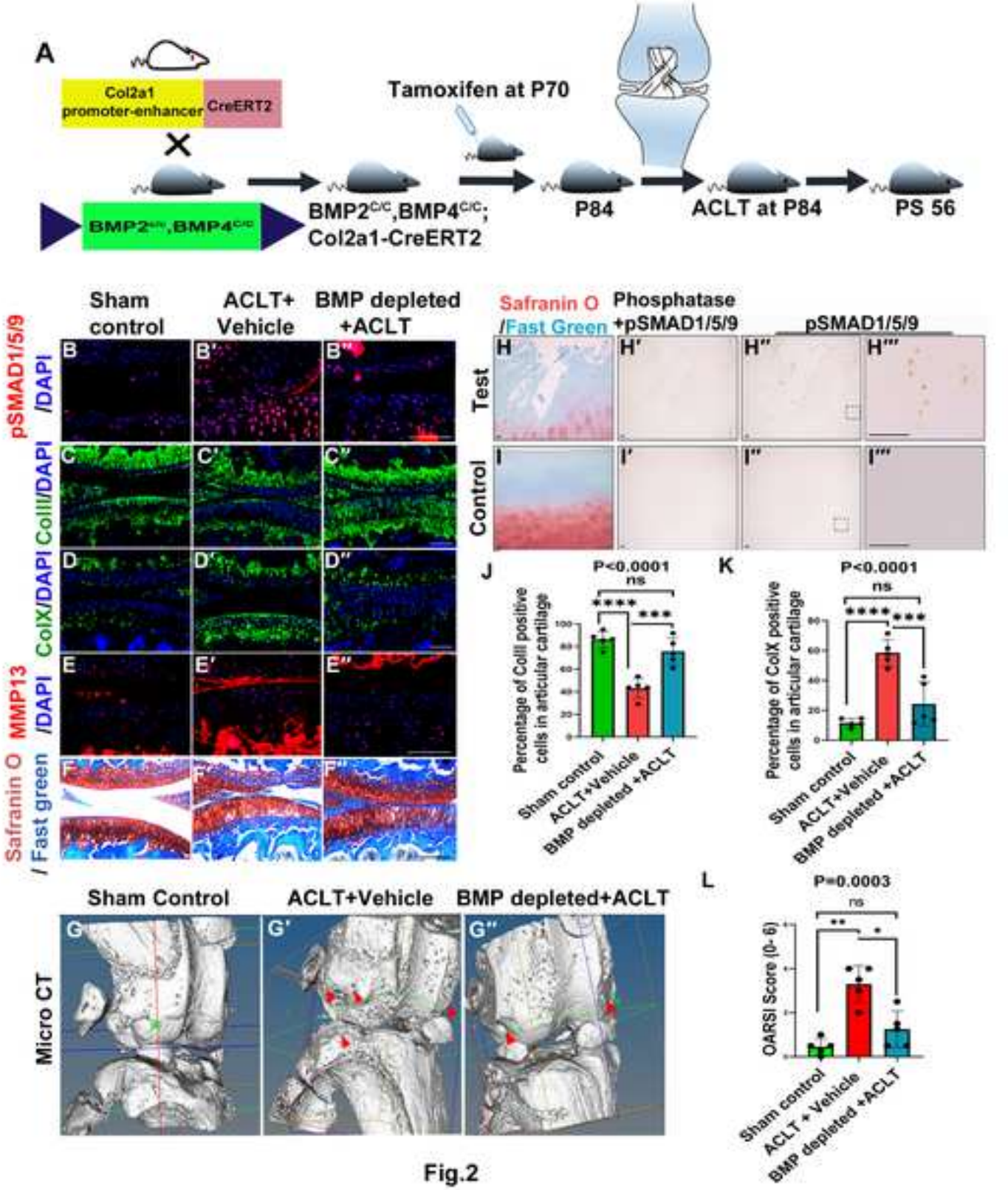

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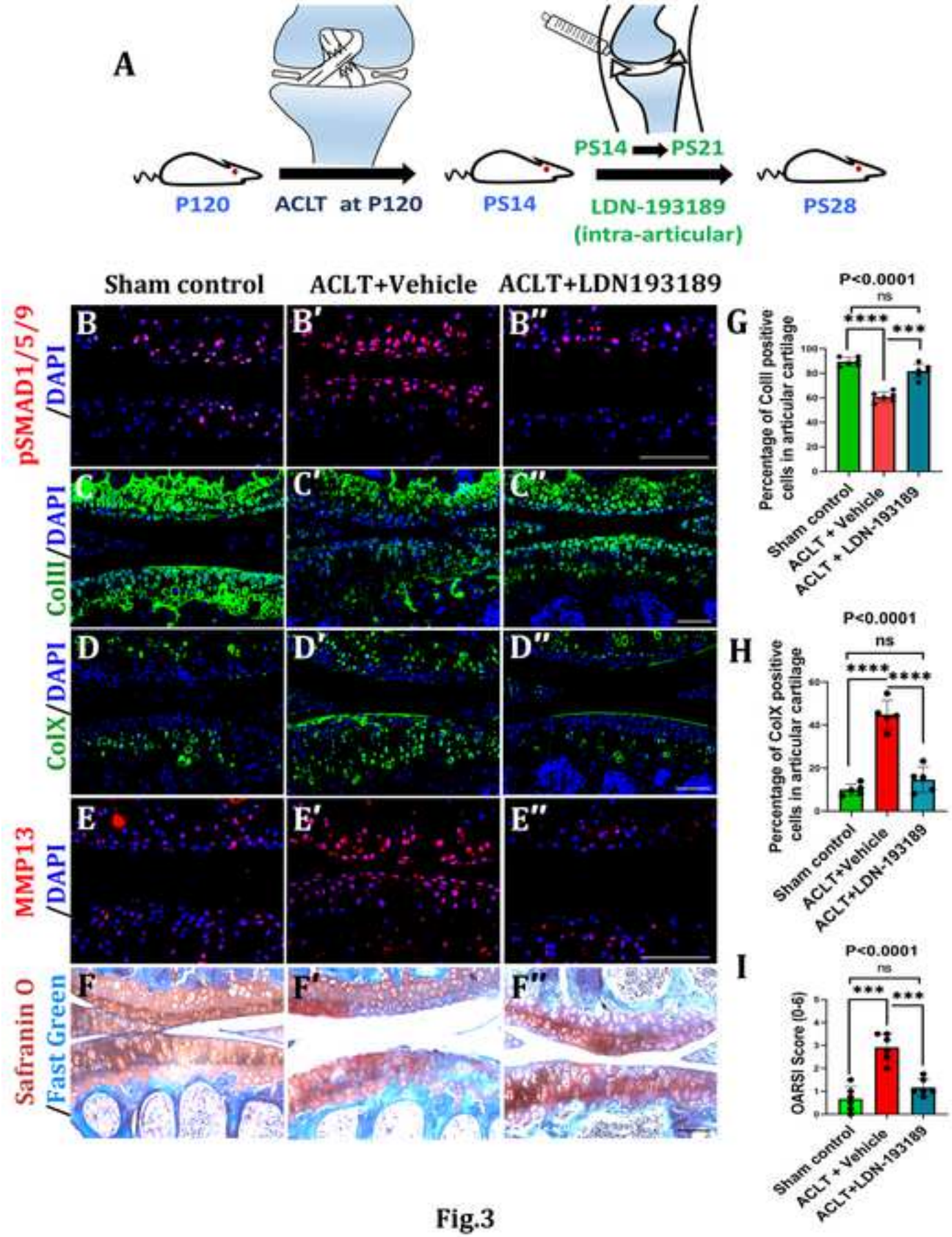

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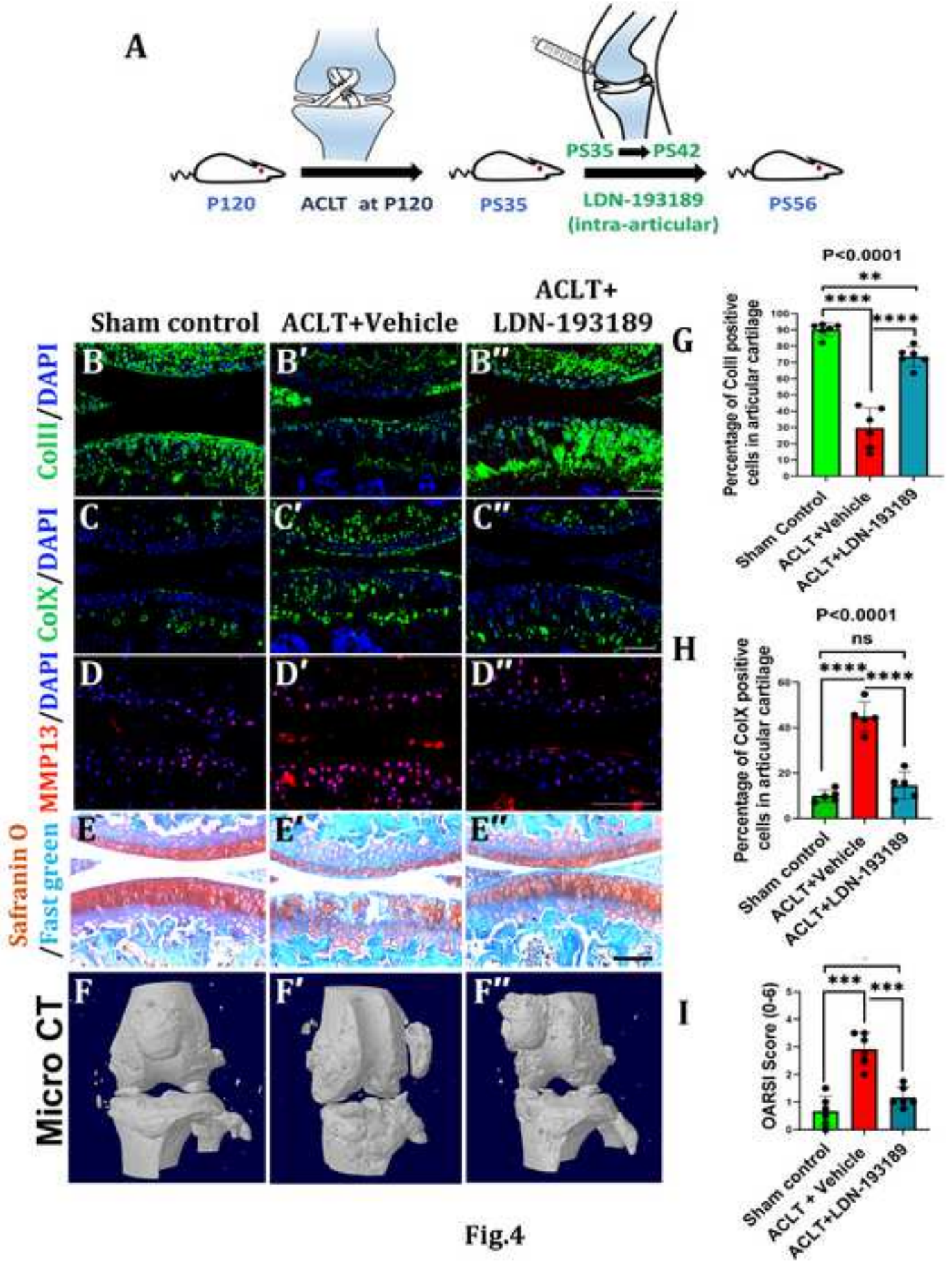

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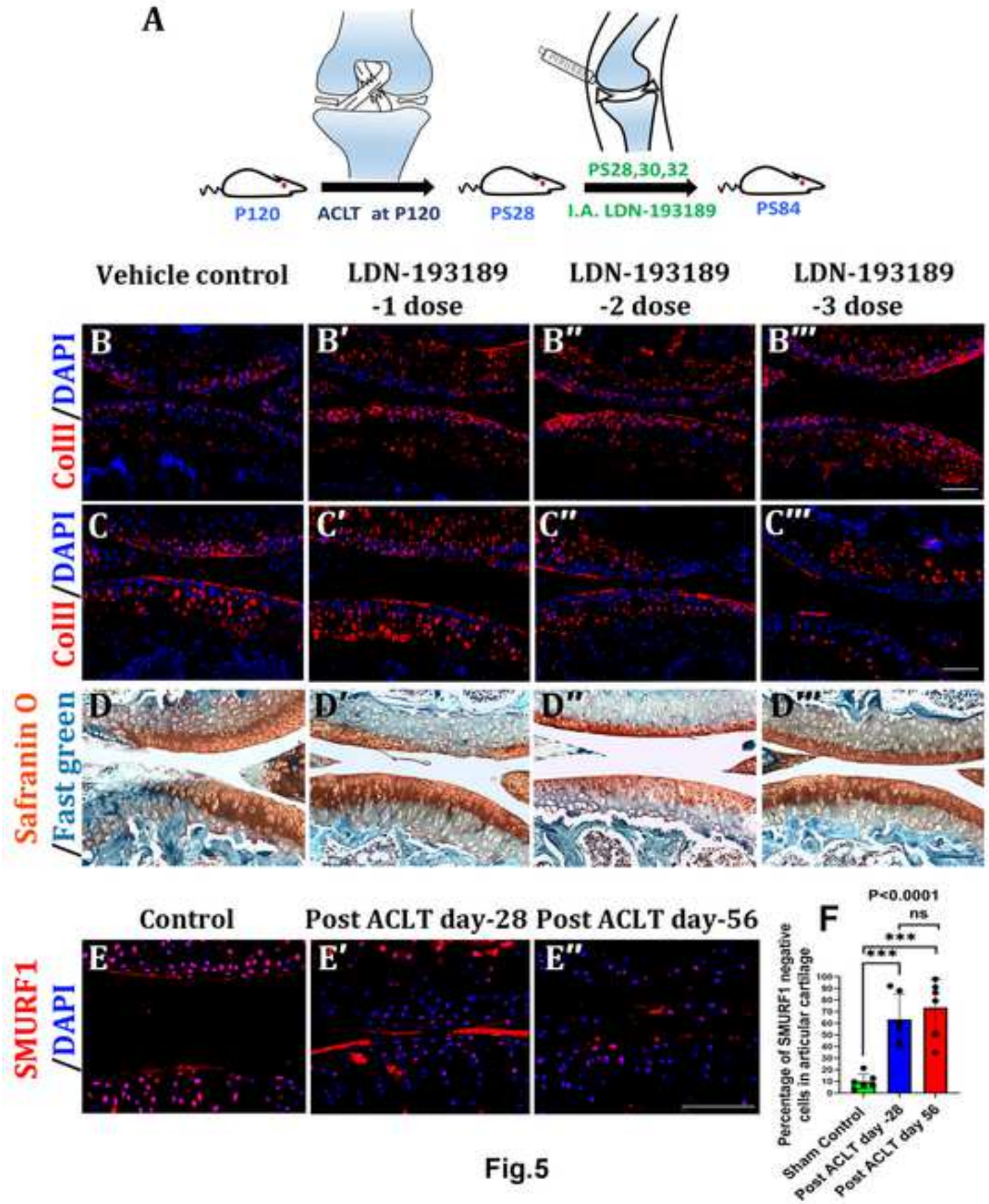

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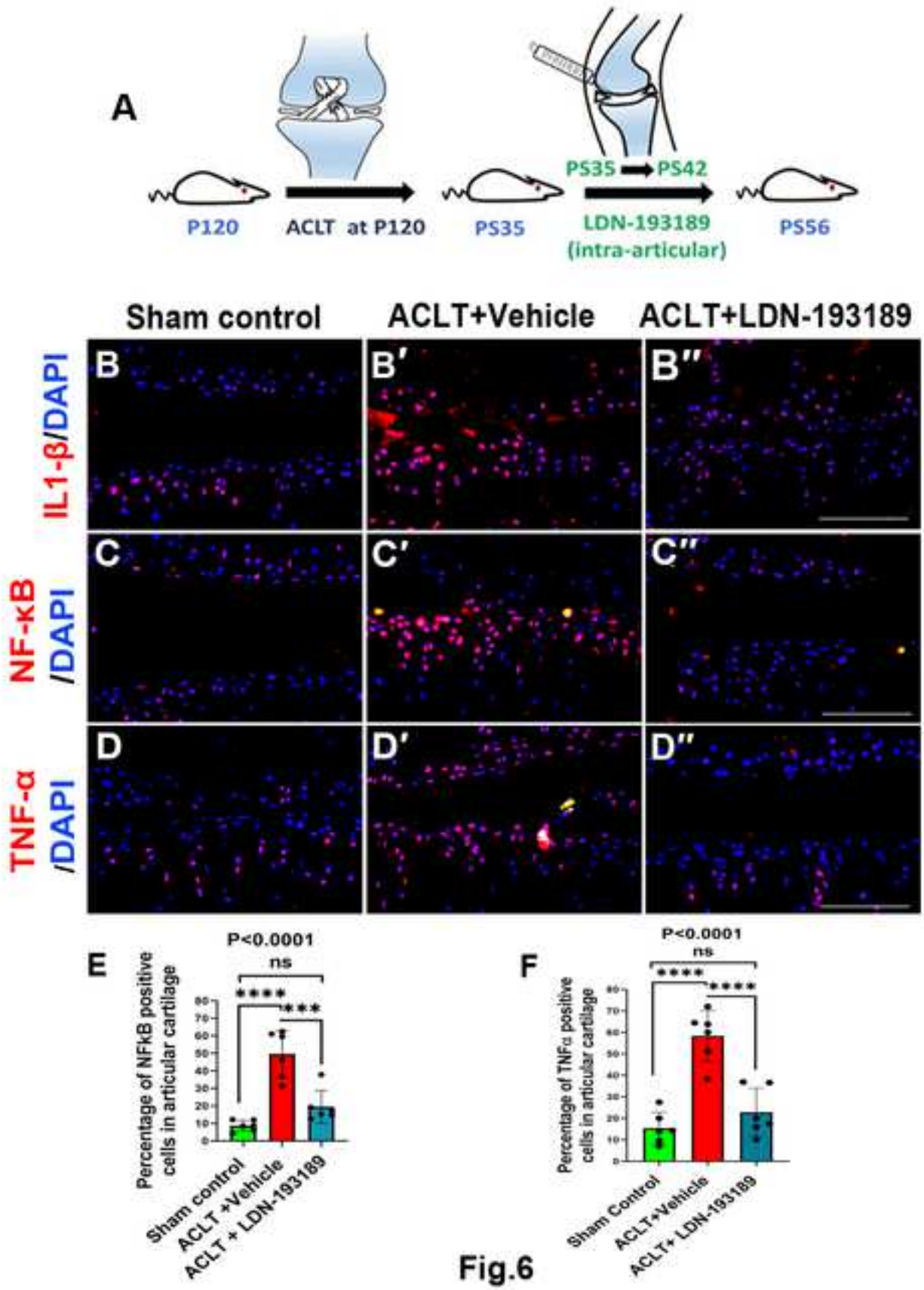

Fig.6