

RESEARCH ARTICLE

Blockage of bone morphogenetic protein signalling counteracts hypertrophy in a human osteoarthritic micro-cartilage model

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ABSTRACT

Bone morphogenetic protein (BMP) signalling plays a significant role during embryonic cartilage development and has been associated with osteoarthritis (OA) pathogenesis, being in both cases involved in triggering hypertrophy. Inspired by recent findings that BMP inhibition counteracts hypertrophic differentiation of human mesenchymal progenitors, we hypothesized that selective inhibition of BMP signalling would mitigate hypertrophic features in OA cartilage. First, a 3D *in vitro* OA micro-cartilage model was established using minimally expanded OA chondrocytes that was reproducibly able to capture OA-like hypertrophic features. BMP signalling was then restricted by means of two BMP receptor type I inhibitors, resulting in reduction of OA hypertrophic traits while maintaining synthesis of cartilage extracellular matrix. Our findings open potential pharmacological strategies for counteracting cartilage hypertrophy in OA and support the broader perspective that key signalling pathways known from developmental processes can guide the understanding, and possibly the mitigation, of adult pathological features.

KEY WORDS: Osteoarthritis, Hypertrophy, BMP signalling, *In vitro* models

INTRODUCTION

Osteoarthritis (OA) is a highly prevalent disease associated with age, mechanical injury and genetic predisposition. Other than palliative pharmacological treatment (i.e. opioids, non-steroidal anti-inflammatory drugs, etc.) aiming at reducing the pain (Zhang et al., 2016), the only definitive therapeutic option is surgical replacement of the total joint (Zhang et al., 2008). Therefore, increasing attention has been dedicated in the last decade towards the identification of pharmacological or regenerative approaches aimed at reversing key pathological features.

During OA progression, remarkable changes occur in articular joint chondrocytes both at the gene expression level and in their phenotypic behaviour. Articular chondrocytes exit their quiescent state and start to

undergo hypertrophic differentiation. This switch is characterized by upregulation of hypertrophic genes (i.e. *COL10A1* and *IHH*), which eventually leads to increased synthesis of calcified cartilage matrix (Kirsch et al., 2000; Zhang et al., 2008). Although cartilage hypertrophy is an essential transient developmental step during growth plate formation, it has detrimental effects in articular cartilage because it induces a series of events that eventually cause cartilage degeneration and OA (Dreier, 2010). The OA chondrocytes start to exhibit an increased tendency to synthesize cartilage-degrading enzymes (e.g. aggrecanases and matrix metalloproteinases; MMPs) (Van der Kraan and Van den Berg, 2012), thus tilting the homeostatic balance of cartilage extracellular matrix (ECM) towards catabolic events such as degradation and inflammation. Moreover, OA articular cartilage is characterized by an increase in the size and number of chondrocyte clusters, limiting diffusion of nutrients and reducing the mechanical properties of articular cartilage (Kouri et al., 1996; Lee et al., 2000; Lotz et al., 2010).

Among other signalling pathways, bone morphogenetic protein (BMP) signalling has been extensively reported to play a crucial role in OA progression (Jaswal and Bandyopadhyay, 2019). BMPs signal via specific cell-surface receptor complexes, namely type I (BMPRI) and type II (BMPRII) serine/threonine kinase receptors. In detail, BMPs first bind to BMPRI, which subsequently forms a heterodimer with BMPRII. BMPRII in turn phosphorylates BMPRI, triggering its kinase activity, which initiates intracellular signalling (Lin et al., 2016). However, there are also reports of the presence of pre-formed BMPRI and BMPRII hetero- and homo-complexes (Gilboa et al., 2000). In both cases, intracellular SMAD proteins [SMAD1, SMAD5 and SMAD8 (also known as SMAD9)] are phosphorylated and released from the receptors to form a heterodimer complex with SMAD4, which translocates into the nucleus to modulate gene expression. Nevertheless, studies have also reported a SMAD-independent BMP pathway that acts *in vitro* in chondrocytes through activation of p38 MAPK by inducing TGF- β -activated kinase (Retting et al., 2009).

Recent experimental evidence points towards an association of BMP signalling with the onset of OA. Increased BMP-2 protein expression was reported in the proximity of chondrocyte clusters as well as in osteophytes in human OA osteochondral samples, and its localization in cartilage lesions in mouse OA models has been shown (Nakase et al., 2003; van der Kraan et al., 2010). Additionally, BMP pathway activation and downstream SMAD1/5 phosphorylation was demonstrated upon mechanical injury in an *ex vivo* OA model using human articular cartilage (Dell'Accio et al., 2006). BMP signalling is also known to play a crucial role in triggering cartilage hypertrophy during growth plate development *in vivo* (Kobayashi et al., 2005). It has been shown in mice models that, during the development of embryonic joints, BMP signalling primes mesenchymal progenitors towards transient cartilage differentiation followed by expression of hypertrophic markers, ultimately leading to development of bone

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(Kobayashi et al., 2005). Furthermore, immunolocalization of BMPs in hypertrophic chondrocytes during fracture healing has been identified by Yu and colleagues, providing a proof of concept for activation of BMP signalling during hypertrophic differentiation of chondrocytes (Yu et al., 2010). In a recent study, we demonstrated that modulation of BMP signalling helps to control the fate of adult human mesenchymal stromal cells (hMSCs) towards attainment of a stable cartilage phenotype, rather than a hypertrophic transient phenotype (Occhetta et al., 2018).

Based on the similarities between hypertrophy in growth plate development and in OA progression (Ripmeester et al., 2018; Van der Kraan and Van den Berg, 2012), and considering the role of BMP signalling in these events (Meliconi et al., 2013), attempts have been made to modulate BMP signalling as a potential strategy for mitigating or reversing OA traits. Direct BMP signalling inhibition strategies include the use of BMP-2-responsive microRNAs (e.g. miR-199a*), which induces the expression of chondrogenic marker genes in multipotent stem cells (C3H10T1/2) (Lin et al., 2009). Modulation of Col2a1 receptors was also inversely correlated to BMP signalling. Col2a1 loss was observed during an increase in hypertrophic differentiation of chondrocytes via the BMP-SMAD1 pathway. Conversely, interaction between Col2a1 and its receptors (i.e. integrin β 1, ITGB1) was shown to exert a competitive role that prevents BMP receptors from binding to SMAD1, eventually blocking the BMP-SMAD1-mediated triggering of chondrocyte hypertrophy (Lian et al., 2019). The involvement of SMAD1 signalling in various physiological functions beyond chondrogenesis, however, poses a challenge to the use of BMP inhibitors for OA treatment because of general safety concerns. This has prompted the search for a strategy based on specific

selective inhibition of BMP receptors, combined with restricted local exposure. In this context, substantial consideration has been dedicated to inhibiting BMP by selective modulation of type I BMP receptors (ALK1, -2, -3 and -6; also known as ACVRL1, ACVR1, BMPR1A and BMPR1B, respectively) (Occhetta et al., 2018).

Based on literature evidence, we hypothesized that inhibition of BMP signalling by targeting ALK2 and ALK3 with small molecules (i.e. Compound A from Novartis) or LDN-193189, a commercially available BMP type I receptor kinase (Cuny et al., 2008), would prevent hypertrophy in an *in vitro* model using human chondrocytes from OA patients. To test this hypothesis, we first developed and characterized a 3D *in vitro* OA micro-cartilage model (namely, OA micro-cartilage) using minimally expanded (named P0) human OA articular chondrocytes (obtained following a 10 day short expansion protocol using freshly isolated cells) and small cell aggregates. The developed system was able to recapitulate specific OA traits *in vitro* (cellular hypertrophy, chondrocytes clustering and expression of hypertrophic markers). The established model was then used to assess the effect of BMP signalling inhibition in counteracting hypertrophic phenotype features typical of OA cartilage.

RESULTS

Establishment of a 3D *in vitro* micro-cartilage model displaying OA cartilage features

PolyHema coated plates were used to induce condensation and 3D culture of minimally expanded human OA articular chondrocytes. After 3 days of culture in chondrogenic differentiation medium (ChM), compact spheroidal aggregates (namely OA micro-cartilage) were reproducibly obtained. Histological and immunohistochemical

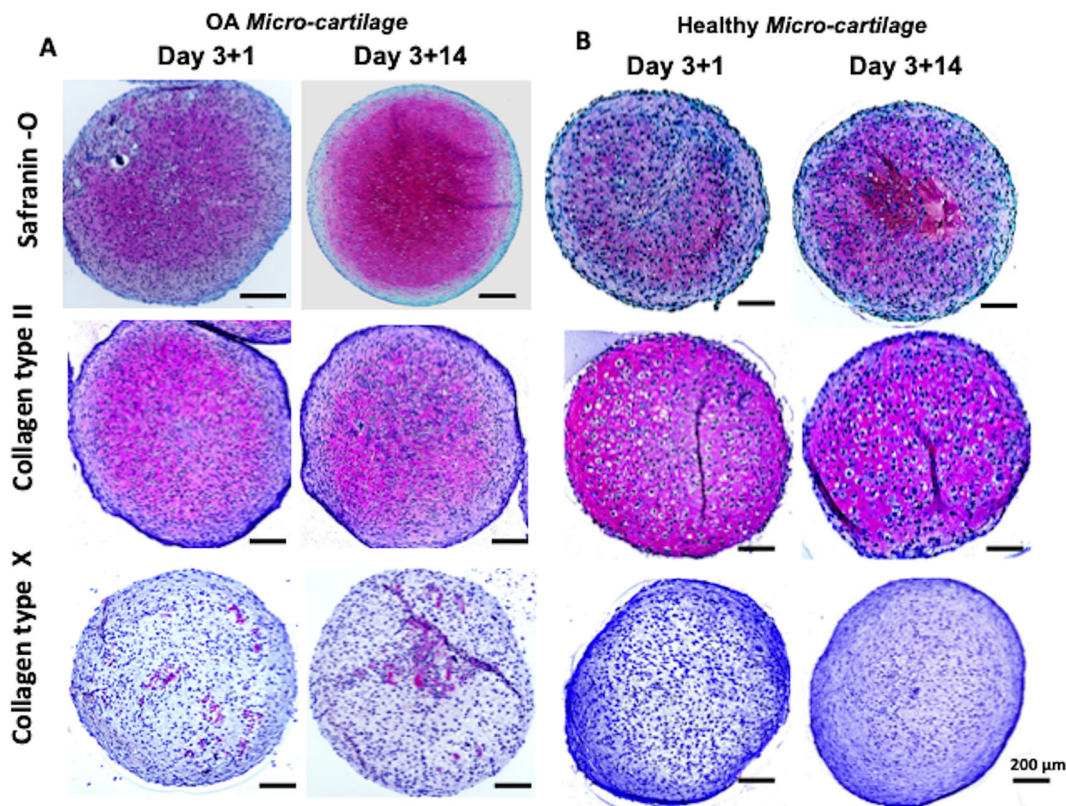


Fig. 1. Maturation of micro-cartilage models. (A,B) Maturation of OA (A) and healthy (B) micro-cartilage models at early (day 3+1) and late (day 3+14) time points, generated with minimally expanded OA articular chondrocytes. Although both models exhibit chondrogenic traits (deposition of GAG and COL-II), deposition of COL-X was detected only in the OA micro-cartilage (A). Samples were histologically analysed for GAG (as represented by Safranin-O staining), collagen type II deposition and collagen type X deposition. All images were acquired at the same magnification.

analysis (Fig. 1A) revealed that even at an early time point (1 day after start of culture, i.e. day 3+1) OA chondrocytes accumulated a cartilaginous matrix rich in glycosaminoglycans (GAG) and collagen type II (COL-II). Interestingly, collagen type X (COL-X) immunoreactivity was clearly detected in specific clusters within the OA micro-cartilage (Fig. 1A). Stable accumulation of GAG and COL-II was observed even at the later time point (day 3+14), together with the presence of abundant and larger COL-X-positive clusters (Fig. 1A).

Healthy micro-cartilage generated with passage 0 (P0) healthy chondrocytes also accumulated abundant cartilaginous matrix rich in GAG (similar to OA micro-cartilage) and COL-II (more intense than OA micro-cartilage) at both time points (days 3+1 and 3+14) (Fig. 1B). Nevertheless, healthy micro-cartilage did not express COL-X at either time point. Notably, no significant difference was observed in the proliferation rate of OA and healthy chondrocytes (data not shown). Thus, the developed OA micro-cartilage retained key hypertrophic features of OA cartilage *in vitro*, which were absent in the healthy micro-cartilage.

Finally, further supplementation of BMP-2 to healthy micro-cartilage did not induce expression of hypertrophic markers (*COL10A1* and *MMP13*), but promoted abundant accumulation of cartilaginous matrix rich in GAG (Fig. S1). Thus, interestingly, the healthy status of the micro-cartilage was maintained even upon BMP-2 treatment, suggesting the specificity of our model in maintaining the traits of the tissue from where chondrocytes were taken.

Specificity of the established OA micro-cartilage in retaining OA features

To confirm the importance of using minimally expanded OA chondrocytes to establish an OA micro-cartilage that retained key hypertrophic OA features, another micro-cartilage was developed using OA chondrocytes expanded for two passages (P2), following a standard protocol. Longer *in vitro* chondrocyte expansion resulted in the development of micro-aggregates, characterized by poor GAG and COL-II accumulation and absence of COL-X deposition at both early (day 3+1) and late (day 3+14) time points (Fig. S2).

Finally, the OA micro-cartilage was compared with an OA macro-cartilage, also obtained with minimally expanded OA chondrocytes. The OA micro-cartilage showed higher expression of chondrogenic (*COL2A1*) and hypertrophic (*COL10A1*) genes than OA macro-cartilage at both time points (Fig. S3A). At the

protein level, the OA micro-cartilage showed a denser matrix deposition rich in COL-II and GAG than the OA macro-cartilage (Fig. S3B,C), even at an early time point (day 3+1). The presence of COL-X-expressing areas was also more evident in the OA micro-cartilage (Fig. S3B) than in the OA macro-cartilage (Fig. S3C).

Collectively, our results demonstrate that OA micro-cartilage generated with minimally expanded OA human chondrocytes and based on small-size cell aggregates enables the maintenance of hypertrophic features characteristic of OA cartilage. Conversely, these features are not retained following long-term chondrocyte expansion and are reduced and delayed in a conventional macroscale model.

OA chondrocytes express ALK2 and ALK3

The expression of *ALK1*, *ALK2* and *ALK3* was measured both in a pool of freshly isolated human OA chondrocytes and after the short P0 expansion. RT-qPCR analysis revealed that although *ALK2* and *ALK3* were expressed at relatively high levels in both fresh and P0 OA chondrocytes, *ALK1* was undetectable (Fig. 2A), indicating the absence of ALK1-based signalling in OA chondrocytes. Collectively, these results confirm the possibility of modulating BMP signalling by targeting ALK2 and ALK3 receptors in OA micro-cartilage.

Selectivity profiles of Compound A and LDN-193189

The activities of Compound A and LDN-193189 in biochemical kinase assays and cellular reporter assays specific for TGF- β and BMP signalling are listed in Table S1. Both compounds strongly inhibit ALK2 without being particularly selective for the other tested BMP type I receptors. As observed from peptide phosphorylation assay results (Table S1), LDN-193189 at 50 nM is likely to inhibit ALK1, ALK2 and ALK6. Although Compound A at 500 nM is likely to inhibit ALK1 and ALK2, a higher concentration (i.e. 1000 nM) would be required to inhibit ALK6. Specifically, the selectivity for ALK2 over ALK1 is twofold and fivefold for LDN-193189 and Compound A, respectively, and the selectivity of ALK2 over ALK3 is 2.6-fold and 4.3-fold for LDN-193189 and Compound A, respectively. Moreover, TGF- β type I receptor ALK5 (also known as TGFBR1) is poorly inhibited by either compound in biochemical kinase assays. This activity in biochemical assays and the preferential inhibition of BMP signalling was confirmed by cellular reporter gene assays for TGF- β and BMP signalling.

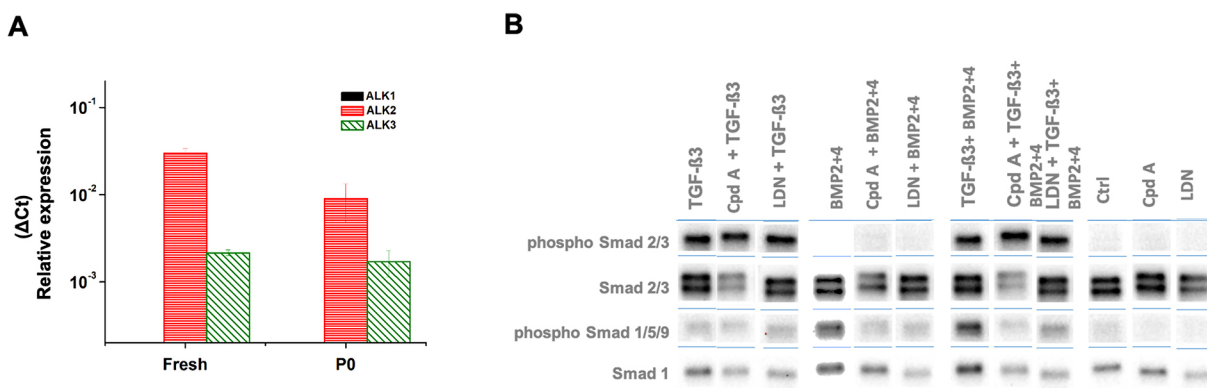


Fig. 2. In OA chondrocytes, *ALK2* and *ALK3* are expressed, and Compound A and LDN-193189 are selective for BMP signalling. (A) Gene expression levels of *ALK1*, *ALK2* and *ALK3*, measured both in a pool of freshly isolated OA chondrocytes and after the short P0 expansion. (B) Activation of BMP and TGF- β pathways in P0 OA articular chondrocytes assessed in response to treatment with BMP inhibitors (Compound A and LDN-193189) by western blot analysis of SMAD1/5/9 and SMAD2/3 phosphorylation, respectively.

The optimal concentration of Compound A had already been defined in our previous study (Occhetta et al., 2018). Specifically, Compound A showed maximum specificity and selectivity to inhibit BMP receptor type I at 500 nM in hMSCs. Thus, the same concentration was used for the OA micro-cartilage. In the current study, two different concentrations of LDN-193189 were tested (5 and 50 nM), based on the LDN-193189 IC_{50} values shown in Table S1. Gene expression analysis of OA micro-cartilage treated with 50 nM LDN-193189 revealed a statistically significant ($P<0.05$) higher expression of *COL2A1* compared with the 5 nM condition, at both time points. With respect to the expression of hypertrophic genes, statistically significant lower expression of *COL10A1* was observed at the late time point ($P<0.05$), and at the early time point for *MMP13* ($P<0.05$), when the OA micro-cartilage was treated with 50 nM LDN-193189. Therefore, 50 nM LDN-193189 was used in subsequent experiments (Fig. S4A).

The specificity and selectivity of both LDN-193189 (50 nM) and Compound A (500 nM) in inhibiting BMP over TGF- β signalling was tested in human OA articular chondrocytes. Both Compound A and LDN-193189 decreased SMAD 1/5/9 phosphorylation in comparison with controls, whereas neither compound caused any difference in the phosphorylation of SMAD2/3 compared with controls (Fig. 2B; Fig. S4B), confirming the specificity and selectivity of both compounds in targeting the BMP pathway.

Compound A and LDN-193189 treatments induce a stable cartilage gene expression profile in OA micro-cartilage

Treatment of OA micro-cartilage with Compound A or LDN-193189 did not significantly affect the expression of *COL2A1*, as compared with controls (Fig. 3), at either late or early time points. Instead, a reduction in the expression levels of the hypertrophic marker *COL10A1* was observed upon treatment with Compound A or LDN-193189 at both early (not significant) and late ($P<0.0001$ for both treatments) time points. Similarly, the expression of other hypertrophic cartilage markers such as *MMP13* and *IHH* was reduced in OA micro-cartilage generated in the presence of both BMP inhibitors ($P<0.0001$ for *MMP13* at both time points; $P<0.01$ for day 3+14 for *IHH*). The BMP pathway antagonist *GREM1* was significantly ($P<0.0001$) upregulated upon both Compound A and LDN-193189 treatment as compared with control OA micro-cartilage at both time points. Expression of genes known to reduce chondrocyte hypertrophy *in vivo* (Zhong et al., 2016), namely the Wnt signalling antagonists *FRZB* and *DKK1*, was assessed. Upregulated expression of both *FRZB* ($P<0.0001$ at the early time point, $P<0.01$ at the late time point) and *DKK1* ($P<0.0001$ at the early time point) was detected upon both treatments as compared with control. Taken together, these data show that selective inhibition of both ALK2 and ALK3 receptors leads to reduced expression of hypertrophy genes in human OA chondrocytes.

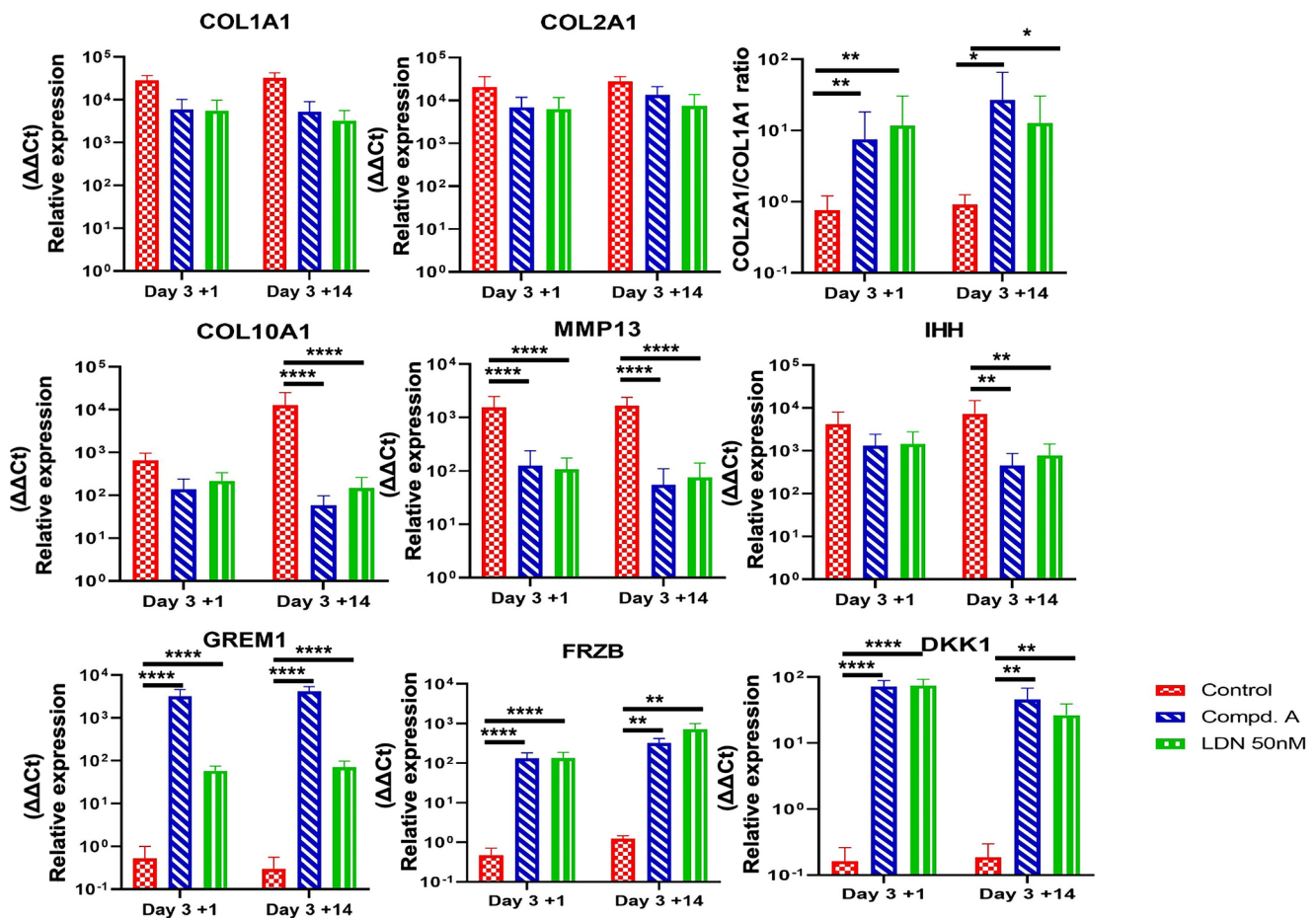


Fig. 3. Compound A and LDN-193189 induce a stable cartilage gene expression profile in OA micro-cartilage. Quantitative RT-PCR analyses showing the gene expression levels of chondrogenic de-differentiation gene *COL1A1*; articular cartilage specific gene *COL2A1*; hypertrophy specific genes *COL10A1*, *MMP13* and *IHH*; BMP pathway antagonist gene *GREM1*; and Wnt pathway-specific antagonist genes *FRZB* and *DKK1*. *GAPDH* was considered as the reference gene for all ΔΔCt calculations and normalized to day 1 basal expression in P0 articular chondrocytes. The fold changes are represented at logarithmic scale. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ and **** $P<0.0001$.

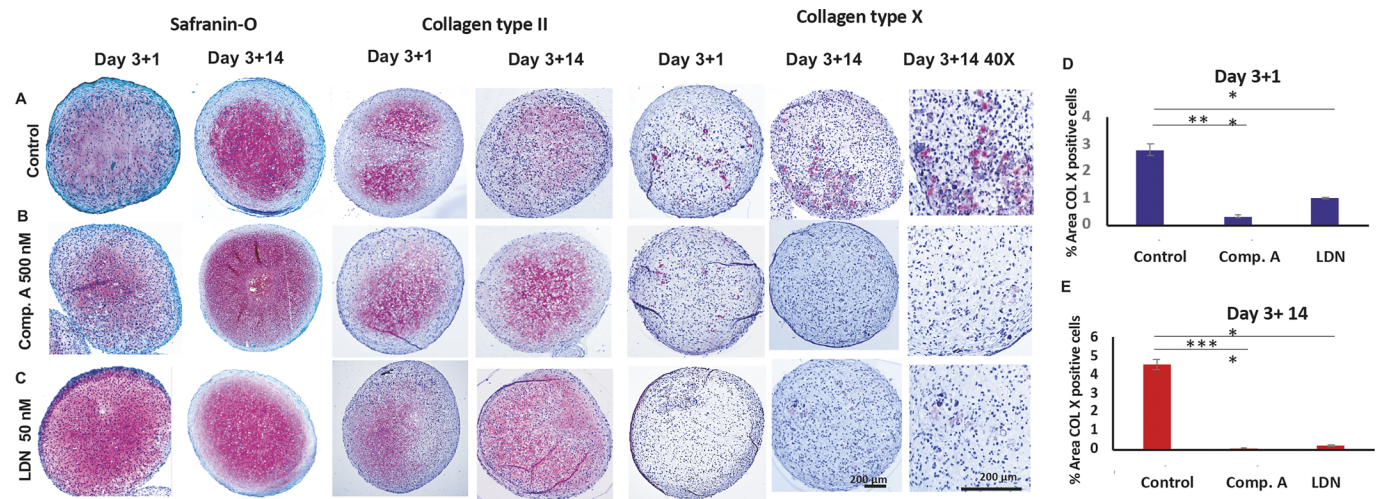


Fig. 4. Compound A and LDN-193189 induce a stable cartilage matrix deposition in OA micro-cartilage. (A–C) OA micro-cartilage was cultured for (3+1) or (3+14) days in ChM alone (A) or supplemented with Compound A (B) or LDN-193189 (C). Samples were histologically assessed for the deposition of GAG (Safranin-O), COL-II and COL-X. All images were acquired at the same magnification unless specified. (D) Percentage area of COL-X-positive cells at day 3+1. (E) Percentage area of COL-X-positive cells at day 3+14, calculated using ImageJ. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Compound A and LDN-193189 treatments favour a stable cartilage matrix and reduce chondrocyte clustering

OA micro-cartilage treated with either of the BMP inhibitors exhibited a cartilaginous ECM rich in GAG and COL-II, similar to the control OA micro-cartilage during the whole culture period, as demonstrated by analysis of immunohistochemical staining of three biological replicates (Fig. 4A–C; Fig. S5A–F). However, a noticeable difference in the accumulation of COL-X was observed. The control OA micro-cartilage showed an increase in the specific clustered expression of COL-X over time. In contrast, treatment with either Compound A or LDN-193189 resulted in a drastic reduction in the amount of chondrocyte clusters positive for COL-X. Quantitative analysis revealed 2.79% COL-X-positive cells at day 3+1 in the control (Fig. 4D), which increased to 4.55% at day 3+14 (Fig. 4E). Treatment with BMP inhibitors reduced the percentage of COL-X-positive cells to 0.31% at day 3+1 (Fig. 4D) and 0.07% at day 3+14 (Fig. 4E) for Compound A, and to 1% at day 3+1 (Fig. 4D) and 0.24% at day 3+14 for LDN-193189 (Fig. 4E). Immunofluorescence analysis of samples treated with the BMP inhibitors also showed the reduction of proteins related to cartilage degradation, such as MMP13 and DIPEN (an aggrecan MMP-generated C-terminal neopeptide, co-localized within MMP13-positive cells) (Fig. 5). Conversely, a drastic increase in the co-localized cluster of cells expressing MMP13 and DIPEN was observed in control samples. Quantitative analysis revealed maximum fluorescence intensity of MMP13 and DIPEN in the controls, whereas a reduction in fluorescence intensity was observed after treatment with Compound A or LDN-193189 (Fig. 5).

Recently, co-localized expression of ALK6 and ITGA4 has been reported for hypertrophic chondrocytes in OA cartilage samples (Ferguson et al., 2018). In accordance with these findings, we observed a high abundance of ALK- and ITGA4-positive cells in the control samples. Conversely, upon treatment with either Compound A or LDN-193189, a reduction in the number of cells expressing ALK6 and ITGA4 was observed (Fig. 6). Quantitative analysis revealed maximum fluorescence for ALK6 and ITGA in the control samples and reduced levels upon Compound A or LDN-193189 treatment (Fig. 6). Therefore, continuous inhibition of BMP signalling with either 500 nM Compound A or 50 nM LDN-193189 was sufficient to prevent the development of OA-like features

(hypertrophy, degradation of cartilage matrix and chondrocyte cluster formation) in the OA micro-cartilage.

DISCUSSION

In the current study we investigated the potential of BMP inhibition to prevent the development of hypertrophic features typical of OA cartilage, as a potential strategy to counteract hypertrophic traits typical of OA cartilage. To this aim, first a 3D *in vitro* OA micro-cartilage model was developed, using minimally expanded human OA articular chondrocytes, which specifically captures typical OA-like hypertrophic features. We then used this model to demonstrate that selective inhibition of BMP signalling, not affecting TGF- β signalling, results in a reduction of hypertrophic features while maintaining the cartilaginous nature of the tissue.

The key elements needed to maintain OA features in the developed *in vitro* OA micro-cartilage model are (1) short *in vitro* expansion phase (important to preserve the native features of the chondrocytes) (Mao et al., 2019) and (2) small size of the formed aggregates. In particular, this latter feature resulted in faster kinetics of chondrogenic differentiation in the new micro-cartilage model as compared with the traditional macro-cartilage model, in accordance with a recent study where a similar trend was observed between MSC-based micro- and macro-pellets (Pamela et al., 2019 preprint). The decision to use micro-aggregates to assess chondrogenic differentiation *in vitro* was driven by the idea of allowing cells to homogeneously sense and respond to applied soluble factors. The size of macro-cartilage models resulted in oxygen and nutrient diffusion gradients, ultimately leading to spatiotemporal heterogeneity, as exemplified by non-uniform GAG distribution. Conversely, the uniform matrix deposition in the micro-cartilage model suggested a homogenous distribution of exogenous factors.

The newly developed OA micro-cartilage model was then exploited to test the hypothesis that selective inhibition of BMP signalling pathways, using two BMP receptor type I biased inhibitors (Compound A and LDN-193189), would prevent the development of hypertrophic OA features. LDN-193189 has been reported to inhibit BMP signalling in a Fibrodysplasia ossificans progressiva mouse model (Mohedas et al., 2013). A previous study from our group showed how simultaneous inhibition of ALK2 and ALK3 through

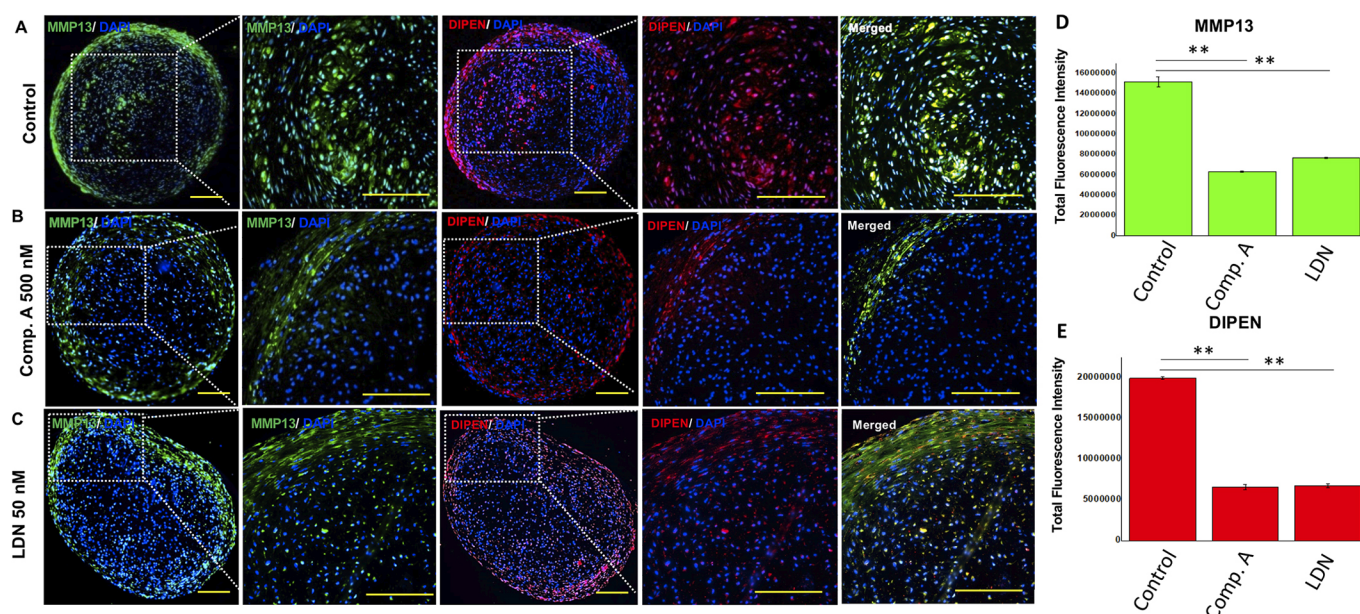


Fig. 5. Compound A and LDN-193189 reduce the expression of cartilage hypertrophic markers MMP13 and DIPEN in OA micro-cartilage. (A-C) OA micro-cartilage was cultured for (3+14) days in ChM alone (A) or supplemented with Compound A (B) or LDN-193189 (C). Expression of MMP13 (green) and DIPEN (red) proteins was assessed by immunofluorescence. All images were acquired at the same magnification. (D,E) Total fluorescence intensity of MMP13 (D) and DIPEN (E) as calculated by ImageJ. ** $P<0.01$. Scale bars: 200 μ m.

Compound A treatment led to development of stable articular cartilage without ossification *in vivo* in an ectopic mouse model (Occhetta et al., 2018). In a similar fashion, inhibiting ALK2 and ALK3 mitigated hypertrophic features in the OA micro-cartilage model. Specific downregulation of hypertrophic genes such as *COL10A1*, *MMP13* and *IHH* was observed, together with a reduction of clustered expression of COL-X at the protein level, in accordance with previous data for hMSCs (Occhetta et al., 2018). This suggests a similarity between hMSCs and human OA articular chondrocytes with respect to the role of BMP signalling in regulating hypertrophic

differentiation. The observed reduction in the expression of hypertrophic markers also correlates with previous *in vivo* studies in which conditional deletion of ALK3 in developing joints resulted in the downregulation of COL-X, MMP13 and IHH (Lin et al., 2016). MMP13 was also previously shown to be overexpressed in *in vitro* cultured articular chondrocytes treated with BMP-2 (Aref-Eshghi et al., 2015) and to be upregulated in precise spatial locations (i.e. both as fibrillated matrix in the superficial zones and in the chondrocyte lacunae) in an ACLT-induced OA mouse model (Appleton et al., 2007). Accordingly, in our OA model, higher

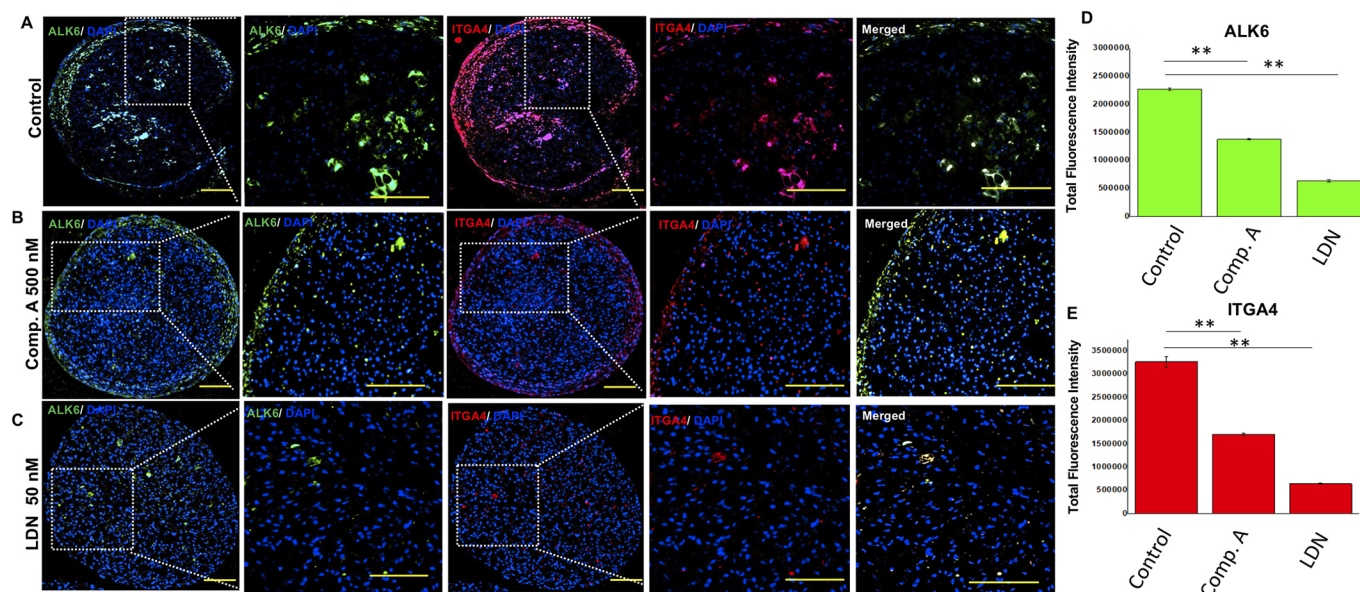


Fig. 6. Compound A and LDN-193189 reduce the clustered expression of ALK6 and ITGA4 in OA micro-cartilage. (A-C) OA micro-cartilage was cultured for (3+14) days in ChM alone (A) or supplemented with Compound A (B) or LDN-193189 (C). Expression of ALK6 (green) and ITGA4 (red) proteins was assessed by immunofluorescence. All images were acquired at the same magnification. (D,E) Total fluorescence intensity of ALK6 (D) and (E) ITGA4 as calculated by ImageJ. ** $P<0.01$. Scale bars: 200 μ m.

expression of MMP13 was observed in the outer periphery (i.e. fibrillated matrix) of the OA micro-cartilage as well as in correspondence to lacunae of clustered chondrocytes. Conversely, treatment with BMP inhibitors drastically reduced the expression of MMP13 and COL-X in chondrocytes lacunae, while maintaining only sparse expression at the periphery of the micro-cartilage. Because these clusters represent a hallmark of OA and have been reported to be a key contributor in OA progression (Lotz et al., 2010), we can postulate that BMP inhibition counteracted the onset of morphological and molecular features typical of OA cartilage (Jayasuriya et al., 2018). Interestingly, treatment with the two BMP inhibitors (Compound A and LDN-193189) led to downregulation of hypertrophic markers, whereas chondrogenic matrix production was preserved at both the gene and protein level. By contrast, strategies based on BMP inhibition by treatment with a BMP antagonist (e.g. Noggin) or soluble BMP receptors have been reported to block cartilage matrix production, thus limiting their translational potential (Oshin et al., 2007). This observation highlights the potential translational superiority of using the proposed strategy, based on specific and selective inhibition of BMP receptor-based signalling, to mitigate OA-like features while preserving cartilage matrix.

The BMP antagonist *GREM1* and Wnt signalling antagonists, such as *FRZB* and *DKK1*, have previously been defined as natural brakes in hypertrophic differentiation of articular cartilage. *In vivo*, the expression of these markers has been inversely correlated to the onset of OA (Leijten et al., 2012), confirmed by the low expression of these markers in the reported OA micro-cartilage. Also, in a previous study from our group, downregulation of such hypertrophy brakes was obtained by mechanically challenging a cartilage-on-chip model with hyperphysiological compression, which finally resulted in induction of OA features (Occhetta et al., 2019). Treatment of the OA micro-cartilage with the optimized concentration of BMP inhibitors led to significant upregulation of these markers, suggesting multiple molecular routes activated by BMP inhibitor treatment towards counteracting clinically relevant OA traits in the model. In contrast to our results, previous studies demonstrated a reduction in the expression levels of *DKK1* as a result of *ALK2* or *ALK3* deficiency (Kamiya et al., 2011). Nevertheless, these studies refer to osteoblast-targeted conditional knockout of *ALK2* and *ALK3*, and the different targeted cell type considered in this work may explain the observed differences in results, as cell context might play a crucial role. In addition, the small molecule BMP-receptor inhibitors inhibit several BMP-type receptors at the employed concentration simultaneously, which could further explain the seemingly conflicting results.

Notably, this OA micro-cartilage model allowed us to preserve *in vitro* the OA-like traits (e.g. hypertrophic foci or islands) of patient-derived cells, without the need to ‘artificially’ induce them in the system before applying the selected therapeutic treatment. We thus believe that, in this model, the timing of inhibitor administration mimics a possible therapeutic treatment at middle-late OA stage (i.e. when phenotypical manifestations of the pathology, such as COL-X foci, are already present in the cartilage). To mimic a treatment at an early stage (namely, before phenotypical manifestations of the pathology, such as COL-X foci), a different model should be considered, for instance starting with healthy cells, inducing an OA phenotype by usage of inflammatory cytokines (Francioli et al., 2011) or mechanical overload (Occhetta et al., 2019) and simultaneously starting the treatment.

The observed intense expression of *ALK6* and *ITGA4* in the clustered chondrocytes in the OA micro-cartilage correlates with the results of a recent study showing co-localized expression of *ALK6* and

ITGA4 in cells within pathological cartilage tissue (Ferguson et al., 2018). Upregulated *ITGA4* expression has also been observed explicitly in OA cartilage tissue, whereas it is absent in healthy cartilage (Ostergaard et al., 1998). Thus, the expression of these markers in the OA micro-cartilage is suggestive of the recapitulation of *in vivo*-like OA traits. The reduction of these markers upon treatment of the OA micro-cartilage with the two BMP inhibitors further validates the potential of the proposed strategy in preventing the appearance of OA-like features. Nevertheless, it is important to mention here that although dominant *ALK1* signalling has been associated with OA in earlier reports, showing an increase in the *ALK1:ALK5* ratio in human OA samples (Blaney Davidson et al., 2009), *ALK1* was not expressed in the expanded chondrocytes of our study; thus, this particular reported feature of OA cannot be recapitulated in our proposed model.

Despite the promising results, translation of this strategy into preclinical and clinical applications requires addressing key issues. First, there is strict need for a well-defined delivery system that can be utilized for localized delivery of such small molecule inhibitors *in vivo*, in order to avoid potential on-target toxicity in other tissues. Moreover, signalling pathways (e.g. BMP signalling) are tightly spatiotemporally regulated during articular cartilage development and potentially play multiple roles (Ray et al., 2015). A strategy enabling recapitulating such spatiotemporal regulation would thus be required to specifically counteract hypertrophic events, while avoiding altering other BMP-regulated processes. To achieve such drug release, the use of biomaterials to which small molecules could be crosslinked to allow controlled release (Lo et al., 2014) could open new treatment options. It should also be noted that the OA micro-cartilage model developed in this study is simplified and lacks several features of a diseased joint as a multi-tissue organ.

As a future perspective, the level of complexity of the current 3D *in vitro* OA micro-cartilage model may be increased by integrating mechanical stimulation, inflammatory cytokines and vascularization-promoting growth factors to address the current limitations. Furthermore, because articular cartilage, subchondral bone and synovial membrane act as one functional joint organ, targeting only articular cartilage might not be sufficient to counteract the progression of OA. The level of complexity of the proposed system can thus be extended by modelling the cross-talk between cartilage, bone and synovial compartments. Moreover, future studies exploring the mechanisms and/or physiological and functional significance of the changes observed in the current study will potentially open the path towards their clinical translation. Finally, the long-term effects of the proposed BMP inhibitors (LDN-193189 and Compound A) need to be monitored in order to extend the therapeutic potential of the observed effects. In summary, this study shows that specific inhibition of BMP signalling by targeting *ALK2* and *ALK3* receptors can counteract hypertrophy in a human articular chondrocyte-based *in vitro* model. The established human 3D micro-cartilage, based on minimally expanded OA chondrocytes and small-size cell aggregates, successfully retains several key features of hypertrophic OA cartilage *in vitro* with high levels of reproducibility. Although we exploited the developed micro-cartilage model to screen the effect of small molecules in this study, such a human OA model holds the broader potential to generate further understanding of cartilage development and OA pathogenesis.

MATERIALS AND METHODS

OA articular cartilage sample collection

Macroscopically fibrillated human articular cartilage was obtained from the knee joints of 13 donors with clinical history of OA who were undergoing

total knee replacement, after informed consent from patients and in accordance with the Institutional Ethics Committee (University Hospital Basel, Switzerland). OA cartilage samples from 10 donors (mean donor age 74 years, range 55–82 years, 6 females and 4 males) were used to generate a pool of OA native chondrocytes for RT-qPCR analysis (Occhetta et al., 2019). The remaining cartilage samples ($n=3$, age range 48–79, all males) were used for the development and validation of the OA micro-cartilage.

Healthy articular cartilage sample collection

Macroscopically healthy human articular cartilage was obtained from the knee joint of two cadavers with unknown clinical history of joint disorders (41 and 54 years, males), after informed consent by relatives and in accordance with the Institutional Ethics Committee (University Hospital Basel, Switzerland) and used to generate the healthy micro-cartilage.

OA and healthy human articular chondrocyte isolation and expansion

Both OA and healthy cartilage biopsies were minced into small pieces and enzymatically digested to release chondrocytes. Specifically, cartilage specimens were incubated with 0.15% type II collagenase (10 ml solution/g tissue, 300 U/mg; Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37°C for 22 h under agitation (Barbero et al., 2004). Released cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 4.5 mg/ml D-glucose, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.29 mg/ml L-glutamine (complete medium) and expanded following an optimized short cell expansion protocol in tissue culture dishes. Specifically, freshly isolated chondrocytes (both OA and healthy) were plated in tissue culture flasks at a cell density of 2×10^4 cells/cm² in complete medium supplemented with 5 ng/ml FGF-2 (R&D Systems) and 1 ng/ml TGF-β1 (R&D Systems) for the first 7 days. Medium was then switched to complete medium only (in the absence of FGF-2 and TGF-β1) for the following 3 days. Medium was changed twice a week and minimally expanded chondrocytes were used at passage 0 (P0) for further experiments unless specified. As additional control, a standard cell expansion protocol was used, where the OA chondrocytes were cultured for two passages (P2) in complete medium continuously supplemented with 5 ng/ml FGF-2 and 1 ng/ml TGF-β1 (Centola et al., 2015).

Establishment of OA and healthy micro-cartilage models and an OA macro-cartilage model

For the establishment of the OA and healthy micro-cartilage models, OA and healthy chondrocytes were seeded at a cell density of 0.5×10^5 cells per well in complete medium in 96-well U bottom plates, pre-coated with 2% w/v poly(2-hydroxyethyl methacrylate) (Sigma-Aldrich) to avoid cell adhesion to the surface of the plate (Chameettachal et al., 2016; Chawla et al., 2017). Three days after seeding, upon formation of micro-cartilage aggregates, the medium was changed to chondrogenic differentiation medium (ChM; DMEM containing 4.5 g/l D-glucose with 100 mM HEPES buffer, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml glutamate, 1.25 µg/ml human serum albumin, insulin-transferrin-selenium, 4.7 µg/ml linoleic acid, supplemented with 0.1 mM ascorbic acid 2-phosphate, 10^{-7} M dexamethasone and 10 ng/ml TGF-β3) or to ChM supplemented with either LDN-193189 (5 or 50 nM as specified; Sigma-Aldrich) or Compound A (500 nM; Novartis). Micro-cartilage was cultured for 14 days and the medium was changed every 3 days. Samples were collected at defined time points for further analyses. Specifically, two experimental time points were considered: an early time point at day 4 (i.e. day 1 after addition of ChM; day 3+1) and a late time point at day 18 (i.e. day 14 after addition of ChM; day 3+14).

Further, to assess whether OA-like phenotype associated with hypertrophy can be induced in the healthy micro-cartilage model upon addition of BMPs, the healthy micro-cartilage was treated with BMP-2 (100 ng/ml) after day 1 and day 14 of adding ChM.

For the establishment of a control OA macro-cartilage model, aliquots of 0.25×10^6 OA chondrocytes were centrifuged for 5 min at 250 g to form macro-pellets in complete medium in 2 ml tubes. After 3 days, the medium was changed to ChM and the culture continued for 14 days.

Western blot analysis

Minimally expanded OA chondrocytes were cultured in monolayer till confluency; starvation was induced 12 h before experimentation. OA chondrocytes were treated with Compound A (500 nM) or LDN-193189 (50 nM) for 60 min prior to stimulation with TGF-β3 (10 ng/ml; Novartis) and/or BMP-2 (10 ng/ml; R&D systems) and/or BMP-4 (10 ng/ml; R&D systems) for an additional 30 min. For protein extraction, cells were washed with PBS and the proteins extracted using lysis buffer containing 25 mM Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 5% glycerol and supplemented with phosphatase and protease inhibitor mixture (Roche). Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific). Proteins were subjected to 4–15% SDS-PAGE and then transferred onto PVDF membranes. After blocking (5% fat-free powdered milk in TBS with 0.1% Tween for 1 h), membranes were probed with primary antibodies against phospho-Smad1/5/9, phospho-Smad2/3, Smad1 and Smad2/3 (Cell Signaling Technology). Anti-GAPDH antibody (Origen) was used as loading control. Blots were then exposed to appropriate peroxidase-coupled secondary antibodies (Southern Biotech). Protein detection was done using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and signal was acquired with the Gel Doc XR+ Imager system (Bio-Rad).

Biochemical kinase selectivity assays

Kinase activity was determined either using an auto-phosphorylation assay (ALK2 and ALK3) or a peptide phosphorylation assay (ALK1, ALK2, ALK5 and ALK6). The assays were performed in 384-well, low volume microtiter assay plates in a final reaction volume of 6 µl. For all biochemical assays, human recombinant proteins were expressed in and purified from baculovirus-transfected insect cells. The constructs comprised the GS domain and kinase domain of ALK1 (amino acids 166–493), ALK2 (amino acids 172–499), ALK3 (amino acids 198–525), ALK5 (amino acids 162–503) or ALK6 (amino acids 168–495). Dose-response curves were generated by incubating 10 nM of each kinase in 50 mM HEPES pH 7.5, 0.02% Tween 20, 0.02% BSA, 1 mM DTT, 10 µM Na₃VO₄, 10 mM β-glycerolphosphate, 1 mM MgCl₂, 12 mM MnCl₂ and 15 µM ATP for 60 min at 32°C in the presence or absence of Compound A or LDN-193189 diluted in DMSO. The amount of generated ADP is a measure of kinase activity and was quantified using the ADP-Glo Kinase Assay (Promega) according to the manufacturer's instructions. ADP is converted to ATP, which is subsequently converted into a bioluminescent signal by the luciferase assay reagents. The luminescent signal was measured using a PHERAstar Multilabel Reader (BMG Labtech) and positively correlates with kinase activity.

An additional kinase selectivity panel, which measures phosphorylation of a fluorescein-labelled peptide substrate, was set-up for ALK1, ALK2, ALK5 and ALK6. A fluorescein-labelled peptide substrate is phosphorylated by the respective kinase, which results in the introduction of two additional negative charges. Due to this difference in charge, the phosphorylated and unphosphorylated peptides migrate with different velocities in an electrical field. In the applied method, the separation takes place inside a chip that contains a complex capillary system for simultaneous analysis of 12 samples (12-Sipper Chip, Caliper Technologies, Mountain View, CA, USA). The separated labelled peptides can be quantified by fluorescence intensity through the laser and detection system of the instrument (LC3000, Caliper Life Sciences). The assays were performed in 384-well, low volume microtiter assay plates in a final reaction volume of 9 µl. Dose-response curves were generated by incubating 10 nM of each kinase together with 2 µM of the fluorescently labelled substrate peptide 5-Fluo-Ahx-KKYQAEEN-T-YDEYENKK-amid (10 mM stock solution in DMSO) in 50 mM HEPES pH 7.5, 0.02% Tween 20, 0.02% BSA, 1 mM DTT, 10 µM Na₃VO₄, 10 mM β-glycerolphosphate, 1 mM MgCl₂, 12 mM MnCl₂ and 15 µM ATP for 60 min at 30°C in the presence or absence of Compound A or LDN-193189 diluted in DMSO. Kinase reactions were terminated by adding 15 µl STOP buffer (100 mM HEPES pH 7.5, 5% DMSO, 0.1% Caliper coating reagent, 10 mM EDTA and 0.015% Brij35). Plates with terminated kinase reactions were transferred to the Caliper LC3000 workstation (Caliper Technologies, Mountain View, CA, USA) for reading. The relative amount of phosphorylated peptide, r , was calculated using the heights of the substrate peak, s , and the product peak, p , such that $r = p/(p+s)$.

BMP signalling reporter gene assay

A human liver hepatocellular carcinoma cell line (HuH7) stably transfected with a reporter plasmid consisting of the BMP response element (BRE) from the Id1 promoter fused to a luciferase reporter gene was generated through lentiviral transduction. This cell line was used to determine the inhibitory effect of the compounds on BMP signalling. Cells were maintained in DMEM (high glucose plus L-glutamine; Gibco), 10% FCS (Amimed), 1% penicillin/streptomycin (Amimed) and 5 µg/ml blasticidin (InvivoGen) at 37°C and 5% CO₂. Assays were performed in sterile 384-well flat bottom polystyrene microtiter plates. The cells were starved through medium exchange in blasticidin- and FCS-free medium 16 h before the assay. Some 2×10⁴ cells in a total volume of 40 µl were added to each well of a plate already containing serial dilutions of each compound in DMSO (final DMSO concentration 0.5%). Cells and compound were incubated for 1 h at 37°C and 5% CO₂ before stimulation with 5 µl/well recombinant BMP-6 (R&D Systems) at a final concentration of 100 ng/ml. Assay plates were incubated for another 5 h at 37°C and 5% CO₂ before luciferase levels were quantified using the Steady-Glo Luciferase Assay System (Promega). Steady-Glo Reagent (5 µl) was added to each well and the samples mixed through vigorous shaking of the plate before measuring the luminescence in a PHERAstar Multilabel Reader.

TGF-β signalling reporter gene assay

Human HEK293 cells were transiently transfected with a reporter plasmid consisting of a TGF-β response element (CAGA12) from the PAI-1 promoter fused to a luciferase reporter gene. These cells were used to determine the inhibitory effect of the inhibitor compounds on TGF-β signalling. Cells were maintained in DMEM (high glucose plus L-glutamine; Gibco), 10% FCS (Amimed) and 1% penicillin/streptomycin (Amimed) at 37°C and 5% CO₂. Assays were performed in sterile 384-well flat bottom polystyrene microtiter plates. The cells were starved through medium exchange in FCS-free medium 16 h before the assay. Some 2×10⁴ cells in a total volume of 40 µl were added to each well of a plate already containing serial dilutions of each compound in DMSO (final DMSO concentration 0.5%). Cells and compound were incubated for 1 h at 37°C and 5% CO₂ before stimulation with 5 µl/well recombinant TGF-β2 (amino acids 303–414, dimer) at a final concentration of 9 ng/ml. Assay plates were incubated for another 5 h at 37°C and 5% CO₂ before luciferase levels were quantified using the Steady-Glo Luciferase Assay System (Promega # E2520). Steady-Glo Reagent (5 µl) was added to each well and the samples mixed through vigorous shaking of the plate before measuring the luminescence in a PHERAstar Multilabel Reader.

Quantitative real-time RT-PCR

Total RNA extraction using TRIzol (Sigma-Aldrich), complementary DNA synthesis and quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR; 7300 AB Applied Biosystems) were performed according to standard protocols. Expression levels of the following genes of interest were specifically quantified (Applied Biosystems): *ALK1* (Hs00163543_m1), *ALK2* (Hs00153836_m1), *ALK3* (Hs01034913_g1), *COL1A1* (Hs00164004_m1), *COL2A1* (Hs00264051_m1), *COL10A1* (Hs00166657_m1), *MMP13* (Hs00233992_m1), *IHH* (Hs01081800_m1), *GREM1* (Hs01879841_s1), *FRZB* (Hs00173503_m1) and *DKK1* (Hs00183740_m1). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as reference housekeeping gene (Hs02758991_g1). Minimally expanded OA chondrocytes were considered as reference to calculate the basal levels for all the $\Delta\Delta C_t$ calculations. Six biologically independent samples from three different donors were considered for each condition and time point.

Histological and immunohistochemical analysis

Samples of healthy and OA micro-cartilage and OA macro-cartilage were harvested, washed with PBS and fixed in 4% paraformaldehyde overnight at 4°C. Post fixation aggregates were dehydrated, embedded in paraffin and 5 µm sections obtained. Sections were stained with Safranin-O or Fast Green and counterstained with Haematoxylin (J.T. Baker) nuclear stain to evaluate cartilage tissue formation. Representative samples were analysed

immunohistochemically for deposition of collagen type II (COL-II; MP Biomedicals) and collagen type X (COL-X; Abcam). In both immunohistological procedures, after rehydration in ethanol series, sections were treated as previously described (Occhetta et al., 2018) or according to the manufacturer's instructions. Sections were incubated with a goat anti-mouse IgG biotinylated secondary antibody (Dako). Following colour development using Vectastain ABC/alkaline phosphatase kit (Linaris), sections were counterstained with Haematoxylin. Negative staining controls were performed during each analysis by omitting primary antibodies. Images for histological and immunohistochemical sections were acquired using the Eclipse Ti2 inverted microscope (Nikon). The percentage of COL-X-positive cells was calculated using ImageJ (NIH).

Immunofluorescence analysis

Samples of OA micro-cartilage were harvested, washed with PBS and fixed in 4% paraformaldehyde overnight at 4°C. Post fixation, aggregates were dehydrated, embedded in paraffin and 5 µm sections obtained. Sections were permeabilized with 0.3% Tween 20 (Sigma-Aldrich) in PBS solution for 10 min. A blocking solution (3% bovine serum albumin and 0.5% Triton in PBS) was applied for 1 h at room temperature to block nonspecific bindings. Samples were incubated overnight at 4°C with the following primary antibodies: mouse anti-human COL-II (1:200; Abcam), rabbit anti-human MMP13 (1:200; Abcam), mouse anti-human DIPEN (1:200; MD Biosciences), goat anti-human BMPR-IB/ALK6 (1:200; R&D Biosystems) and mouse anti-human ITGA4 (1:200; R&D Biosystems). As appropriate, secondary antibodies labelled with Alexa Fluor 488, Alexa Fluor 546 or Alexa Fluor 647 (Invitrogen) were used and DAPI was used to stain the cell nuclei. Negative controls were performed during each analysis by omitting the primary antibodies. Fluorescence images were acquired using the Eclipse Ti2 inverted microscope (Nikon). The total fluorescence was calculated as a measure of fluorescence intensity using ImageJ (NIH) (Chawla and Ghosh, 2017).

Statistical analysis

Results of RT-qPCR quantification are presented as mean±s.d. The number of donors tested and analysed is indicated by *n*. Statistical analysis was performed using GraphPad Prism 8.00 for Windows. Normal distribution of data populations was assessed using the D'Agostino–Pearson Test. Two-tailed Student's *t*-test (normal distributions) and Mann–Whitney test (non-normal distributions) were used when comparing two populations. Multiple comparisons were realized using ordinary one-way ANOVA. When comparing normally distributed variables, Bonferroni's or Dunnett's multiple comparison tests with single pooled variance were used for small or large numbers of populations; statistical significance is indicated by **P*<0.05 or ***P*<0.01, respectively. When comparing non-normally distributed variables, the Kruskal–Wallis test with Dunn's multiple comparison test was used; statistical significance is indicated by ****P*<0.00 or *****P*<0.0001.

Competing interests

C.H., S.G.-G. and I.K. are full-time employees of Novartis Pharma AG and are Novartis shareholders.

Author contributions

Conceptualization: S.C., C.H., S.G.-G., S.G., I.M., A.B., P.O.; Methodology: S.C., M.H.M.B., B.D., I.K., P.O.; Validation: S.C.; Formal analysis: S.C., P.O.; Investigation: S.C., M.H.M.B., B.D., C.H., S.G.-G., I.K.; Data curation: A.B.; Writing - original draft: S.C., P.O.; Writing - review & editing: C.H., S.G.-G., I.K., S.G., I.M., A.B., P.O.; Supervision: I.M., A.B.; Project administration: I.M., P.O.; Funding acquisition: S.G., I.M., A.B., P.O.

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Supplementary information

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