Arthritis Research & Therapy

arthritis research&therapy

Small molecule inhibitors of WNT/beta-catenin signaling block IL1beta/TNFalpha induced cartilage degradation

Arthritis Research & Therapy 2013, **15**:R93 doi:10.1186/ar4273

Ellie B.M. Landman (ellielandman@gmail.com)
Razvan L. Miclea (r.l.miclea@lumc.nl)
Clemens A. van Blitterswijk (c.a.vanblitterswijk@utwente.nl)
Marcel Karperien (h.b.j.karperien@utwente.nl)

ISSN 1478-6354

Article type Research article

Submission date 11 January 2013

Acceptance date 8 July 2013

Publication date 21 August 2013

Article URL http://arthritis-research.com/content/15/4/R93

Small molecule inhibitors of WNT/β-catenin signaling block IL1β/TNFα induced cartilage degradation

Ellie B.M. Landman[1], Razvan L. Miclea[2], Clemens A. van Blitterswijk[3], Marcel Karperien[1*]

[1] *Dept. of Developmental BioEngineering, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Drienerlolaan 5, 7522 NB Enschede, the Netherlands*

[2] *Dept. of Pediatrics, Leiden University Medical Centre, Einthovenweg 20, 2333 ZC Leiden, the Netherlands*

[3]*Dept. of Tissue Regeneration, MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, Drienerlolaan 5, 7522 NB Enschede, the Netherlands*

Corresponding author:

Prof. dr. Marcel Karperien

Email: h.b.j.karperien@utwente.nl

Abstract

Introduction In this study, we tested the ability of small molecule inhibitors of WNT/β-catenin signaling to block IL1β/TNFα induced cartilage degradation. Pro-inflammatory cytokines like IL1β and TNFα are potent inducers of cartilage degradation by up-regulating MMP expression and activity. Since WNT/β-catenin signaling was found to be involved in IL1β/TNFα induced upregulation of MMP activity, we hypothesized that inhibition of WNT/β-catenin signaling might block IL1β/TNFα induced cartilage degradation. We tested the effect of small molecules that block the interaction between β-catenin and TCF/LEF transcription factors on IL1β/TNFα induced cartilage degradation in mouse fetal metatarsals.

Methods We used mouse fetal metatarsals treated with IL1β and TNFα as an *ex vivo* model for cytokine induced cartilage degradation. Metatarsals were treated with IL1β and TNFα in combination with small molecules PKF115-584, PKF118-310 and CGP049090 at different concentrations and harvested for histology and gene expression analysis.

Results We found that IL1β/TNFα induced cartilage degradation in mouse fetal metatarsals was blocked by inhibiting WNT/β-catenin signaling using small molecules PKF115-584 and partially using CGP049090, dose-dependently. In addition, we found that PKF115-584 blocked IL1β and TNFα induced MMP mRNA expression, but did not reverse the inhibitory effect of IL1β on the expression of cartilage anabolic genes.

Conclusion In this study, we showed that inhibition of WNT/β-catenin signaling by small molecules can effectively prevent IL1β/TNFα induced cartilage degradation, by blocking MMP expression and activity. Furthermore, we elucidate the involvement of WNT/β-catenin signaling in IL1β/TNFα induced cartilage degradation.

Keywords: WNT, cartilage, osteoarthritis.

Introduction

In degenerative cartilage diseases, such as osteoarthritis (OA) or rheumatoid arthritis (RA), the balance between anabolic and catabolic processes is shifted towards breakdown of extracellular cartilage matrix [1-3]. Cartilage destruction is thought to be the result of increased expression and activity of catabolic proteins, such as matrix metalloproteinases (MMPs) [4]. Expression of *MMP1* (collagenase), *MMP3* (stromelysin), *MMP9* (gelatinase) and *MMP13* (collagenase-3) mRNA has been found in chondrocytes in arthritic cartilage [5, 6]. Increased mRNA expression of *MMP1* and *MMP3* was also found in the synovial tissue of OA patients [7]. In agreement, protein expression of MMP1, MMP3 and MMP9 in the synovial fluid of patients with OA in the temporomandibular joint was found to be increased compared to healthy control joints [8]. The essential role of MMPs in cartilage degradation was illustrated by experimental evidence indicating that MMP13 deficient mice were resistant to cartilage damage in medial meniscus destabilization induced cartilage degradation [9]. In addition, cartilage degradation induced by IL1β and oncostatin M in human and bovine articular cartilage explants could be blocked by a specific MMP13 inhibitor [10].

Proinflammatory cytokines like interleukin (IL) 1β and tumor necrosis factor (TNF) α potently induce MMP expression and activity in cartilage and these cytokines are associated with cartilage degradation *in vitro* and *in vivo* [6, 11, 12]. The increased expression of several MMPs in human articular cartilage explants in similar locations where IL1β and TNFα were highly expressed is suggestive for the involvement of IL1β and TNFα in the stimulation of MMP expression [11]. *In vitro* and *in vivo* studies have shown that proinflammatory cytokines, such as IL1β and TNFα are present in both OA and RA joint tissues and synovial fluid [1, 4, 13]. IL1β is associated with cartilage degeneration, whereas TNFα was shown to be involved in driving inflammation [3]. Besides their role in cartilage degradation by

stimulating MMPs, IL1β and TNFα impair the ability of the cartilage to restore the extracellular matrix by blocking the synthesis of new extracellular matrix components [3].

Recently, the canonical WNT/β-catenin signaling pathway has attracted much attention in the pathophysiology of cartilage degenerative disease [14]. The WNT/β-catenin signaling pathway is activated upon binding of WNT to its receptor Frizzled (FZD) and coactivator LRP5/6. Subsequently, the degradation complex for β-catenin is destabilized, resulting in high cytoplasmic levels of β-catenin and translocation of β-catenin to the nucleus where it binds to transcription factors TCF and LEF resulting in activation of target genes [15]. Several lines of evidence, predominantly derived from animal models, support the involvement of WNT/β-catenin signaling in the molecular mechanism underlying cartilage degradation. Conditional activation of β-catenin in articular chondrocytes in adult mice was found to result in articular cartilage destruction with accelerated terminal chondrocyte differentiation [16]. It has also been shown that knock-out of *FRZB*, an antagonist of canonical WNT signaling makes mice more susceptible to chemically induced articular cartilage degradation [17]. Furthermore, increased expression of secreted Frizzled Related Proteins (sFRPs), which prevent binding of WNTs to their receptors, was found in OA synovium, which might be indicative of a compensatory mechanism for increased WNT signaling [18].

Recently, a link between WNT/β-catenin signaling and IL1β-induced cartilage degradation was found. Expression of WNT5a and WNT7a in articular chondrocytes was induced by IL1β [19] and the combination of IL1β and WNT3a induced greater loss of proteoglycans from the extracellular matrix than either alone [12]. In addition, induction of WNT signaling by either recombinant WNT3a or GSK3β inhibitor BIO was shown to induce MMP mRNA expression and proteolytic activity in mouse cartilage explants. The fact that knockdown of TCF4 eliminated this effect, indicates the involvement of TCF4 in WNT-induced MMP expression

[20]. In addition, involvement of LEF1 was found in increased MMP13 expression upon IL1β stimulation [21].

Since pro-inflammatory cytokine induced cartilage degradation appears to involve WNT/β-catenin signaling and increased WNT/β-catenin signaling has been implicated in the initiation and progressive deterioration of cartilage degeneration, we hypothesized that small molecule inhibitors of the interaction between β-catenin and TCF4 and LEF1 could be used to prevent cytokine induced cartilage degradation. The aim of this study is to assess the potential effects of small molecules that inhibit the WNT/β-catenin signaling pathway on the degeneration of cartilage. We have selected small molecules PKF115-584, PKF118-310 and CGP049090 that block the binding of β-catenin to its transcription factor TCF4. PKF115-584 and CGP049090 also block the binding between β-catenin and transcription factor LEF1 [22-24]. In addition, PKF115-584 not only blocks, but also disrupts the binding between β-catenin and TCF [22-24]. To study the potential effect of these WNT inhibitors, we used explanted mouse fetal metatarsals in which we induced cartilage degradation by adding IL1β and TNFα.

Materials and methods

Luciferase assay

HEK293t cells were seeded at 7500 cells/cm^2 in 96-wells plates (Nunc International) and cultured for 24 hours in DMEM supplemented with 10% FBS and 100 U Penicillin/Streptomycin (Gibco), prior to transfection with the TopFlash TCF/LEF luciferase reporter construct (Millipore) and pRL-CMV control (Promega). Cells were stimulated with the GSK3β inhibitor BIO (Sigma Aldrich) to stimulate the WNT/β-catenin pathway in combination with the inhibitors, 24 hours after transfection. After 24 hours of stimulation, luminescence was measured using the Dual-Glo luciferase assay (Promega).

Metabolic activity

To study the effect of small molecules on metabolic activity, the pre-osteoblast cell line KS483-4C3 was used [25]. Twenty-four hours after seeding, KS483-4C3 cells were stimulated with different concentrations of the compounds and 24 hours later, the metabolic activity was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were incubated with MTT for 4 hours and after stopping the reaction by adding DMSO, optical density was measured at 540 nm.

Immunofluorescence staining for nuclear accumulation of β-catenin

Nuclear accumulation of β-catenin was detected by immunofluorescence staining. KS483-4C3 cells were seeded on glass slides (Nalgene Nunc International) and treated with LiCl both with and without small molecule inhibitors. After 3 hours, cells were washed in PBS and fixed in 3,7% buffered formalin. Subsequently, cells were quenched in 50 mM NH_4Cl for 10 minutes and incubated overnight at 4^oC in NETGEL (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% NP-40, 0.25% gelatin, and 0.02% azide). The next day, cells were incubated with anti-β-catenin antibody (1:500 in NETGEL, BD Transduction Laboratories) for 1 hour at room temperature. Then cells were incubated with anti-mouse FITC- labeled secondary antibody (1:250 in NETGEL, Sigma Aldrich) for 1 hour at room temperature and mounted using Vectashield mounting medium (VECTOR Labs).

Mouse fetal metatarsals

Mouse fetal metatarsals were isolated from FVB mouse embryos (time-paired, Harlan) at day 17,5 of gestation [26, 27]. After isolation, metatarsals were individually cultured in 24-wells plates in 200 µl per well in Minimal Essential Medium (MEM) alpha, supplemented with 10% Fetal Bovine Serum (FBS), 100 U Penicillin/Streptomycin (Gibco) and 1% Glutamax (Invitrogen) for 48 hours. After this equilibration period, metatarsals were treated with several concentrations of the small molecules either alone or in combination with 10 ng/ml TNFα or IL1β (R&D Systems) or a combination of both for 1, 4 or 7 days. Animal experiments were approved by the ethical committee of the University Medical Centre Utrecht.

Morphometric and histological analysis

Optical microscopy was performed at different time points and the lengths of the metatarsals were determined along the sagittal axis of the bone, using image analysis software (ImageJ). For histological examination, metatarsals were fixed in 10% formalin and dehydrated in ethanol series before embedding in paraffin. Five micrometer sections were cut using a rotary microtome (HM355S Microm International). Sections were stained for glycosaminoglycans using 0,5% Alcian Blue (Sigma Aldrich) in H_2O (pH set to 1 using HCl) for 30 minutes and counterstained in 1% Nuclear Fast Red (Sigma Aldrich) for 5 minutes. For immunohistochemical staining of collagen type II, sections were pre-incubated in 5 µg/ml Proteinase K (Sigma Aldrich) for 10 minutes followed by 1 mg/ml Hyaluronidase (Sigma Aldrich) for 30 minutes, both at 37^{o}C. Rabbit polyclonal collagen type II primary antibody (Abnova) was diluted 1:1000 and incubated overnight at 4^{o}C. For visualization, the EnVision®+ System-HRP kit (Dako) was used.

Gene expression analysis

Five metatarsals were pooled and lysed in Trizol for RNA isolation, using the Nucleospin RNA II kit (Bioke) according to manufacturer's protocol. Subsequently, cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Quantitative polymerase chain reaction (qPCR) was performed using iQ SYBR Green Supermix (Bio-Rad) on MiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). Gene expression was normalized using GAPDH and expressed as fold induction compared to controls. Primer sequences are listed in table 1.

Statistical analysis

Results were expressed as mean values +/- 95% confidence intervals (CI) and statistical significance was tested using ANOVA (PASW Statistics 18).

Results

Effect of small molecules on TCF/LEF reporter activity, nuclear translocation of β-catenin and metabolic activity

We first tested the efficacy and specificity of the small molecule compounds to inhibit canonical WNT/β-catenin signaling in HEK293t cells transiently transfected with the TopFlash TCF/LEF reporter. The compounds were tested in the presence or absence of BIO, a potent activator of WNT/β-catenin signaling by blocking GSK3β [28]. We found a dose-dependent decrease in reporter activity when the cells were treated with BIO and the small molecule inhibitors. PKF115-584 treatment resulted in a six-fold decrease in reporter activity at a concentration of 1,0 µM. At a concentration of 3,0 µM, luciferase reporter activity increased again most likely due to notable cell death upon visual inspection of the cultures.

PKF118-310 and CGP049090 were slightly less effective. Maximal inhibition was found at 3,0 µM, which decreased reporter activity by four-fold and six-fold respectively (Figure 1A).

The effect of small molecules on the metabolic activity of cells was tested in KS483-4C3 cells using an MTT assay [25]. No significant effects on metabolic activity were found when cells were treated with lower concentrations of the compound, however, 1,0 µM or 3,0 µM of PKF115-584 and PKF118-310 and 3,0 µM of CGP049090 did cause a significant decrease in metabolic activity (Figure 1B).

Since β-catenin can only effectively activate the WNT/β-catenin pathway after nuclear translocation, the cellular localization of β-catenin was determined by immunofluorescence staining. Figure 1C shows that stimulation of the WNT/β-catenin pathway by LiCl, which also inhibits GSK3β, resulted in translocation and accumulation of β-catenin in the nucleus. At 1,0 µM, CGP049090 reduced the intensity of β-catenin staining, but did not inhibit nuclear translocation induced by LiCl, whereas PKF118-310 reduced intensity of β-catenin staining and also inhibited LiCl induced nuclear translocation. In contrast, PKF115-584 did not affect the intensity of the β-catenin staining under basal conditions. In the presence of LiCl, β-catenin membrane staining was increased but nuclear translocation was markedly inhibited by PKF115-584.

TNFα and IL1β induce cartilage degradation in mouse fetal metatarsals

Metatarsals were cultured in medium containing IL1β or TNFα or a combination of both at concentrations of 10 ng/ml. TNFα tended to blunt longitudinal bone growth although this did not reach significance. In contrast, IL1β alone induced bone and cartilage resorption resulting in a significant reduction in bone length after 4 and 7 days of treatment. Co-treatment of IL1β with TNFα caused even more abundant bone and cartilage resorption resulting in a significant

reduction in bone length (Figure 2A/B). Since IL1β and TNFα were more effective than either IL1β or TNFα alone, we used the combination of IL1β and TNFα to induce cartilage degradation in further experiments.

Inhibitory effect of small molecules on cartilage degradation in mouse fetal metatarsals

Since visual inspection of cell cultures treated with concentrations higher than 1,0 µM showed increased cell death, most likely due to toxic side-effects, we chose to test the effect of the three WNT inhibitors in a dose range from 0,1 µM to 1,0 µM. When metatarsals were treated with the small molecules only, no effect was observed on the *in vitro* growth of the metatarsals at any of the concentrations that were tested (Supplementary Figure 1). The decrease in growth and resorption of the metatarsals when treated with TNFα and IL1β was counteracted when metatarsals were treated with small molecules. At a concentration of 1,0 µM, PKF115-584 blocked bone and cartilage resorption most effectively. Also CGP049090 counteracted the detrimental effects of TNFα and IL-1β on explant resorption, albeit less effective than PKF115-584, whereas PKF118-310 had no significant effect (Figure 3A).

Alcian Blue staining for glycosaminoglycans demonstrated that the glycosaminoglycan content of the cartilaginous matrix of IL1β/TNFα-treated metatarsals was decreased. In line with the effect of IL1β and TNFα on glycosaminoglycans in the extracellular matrix, Alcian Blue staining was partially preserved by co-treatment with PKF115-584 and PKF118-310, but not CGP049090 (Figure 4A/B). Also, Collagen II staining of the extracellular matrix was completely lacking after treatment with IL1β and TNFα, whereas co-treatment with PKF115-584 but not PKF118-310 or CGP049090, could prevent loss of Collagen II from the extracellular matrix (Figure 4C).

Small molecule WNT/β-catenin inhibitors decrease expression of matrix catabolic genes

Since the catabolic effect of cytokines on cartilage consists of both induction of MMP expression as well as downregulated expression of cartilage matrix genes, we tested the effect of small molecule inhibitors on mRNA expression of these genes. In line with previous studies [6, 12], IL1β and TNFα significantly induced expression of *Mmp3*, *Mmp9* and *Mmp13*. Small molecule PKF115-584 significantly downregulated IL1β/TNFα induced expression of *Mmp9* and *Mmp13*, after 4 days of treatment, whereas CGP049090 only blocked the IL1β/TNFα induced upregulation of *Mmp9* after 4 days (Figure 5A/B). The expression of cartilage matrix genes *Acan* and *Col2a1* was significantly downregulated and the expression of *Sox9* tended to decrease upon treatment with IL1β and TNFα after 1 and 4 days of treatment. PKF115-584 and CGP049090 and to a lesser extent PKF118-310 also decreased the mRNA expression of *Acan* and *Col2a1* from day 1 on. Neither compound was able to counteract IL1β/TNFα induced reduction in gene expression neither at day 1, nor at day 4. The three inhibitors did not affect *Sox9* nor counteracted the effect of IL1β/TNFα on *Sox9* expression after 1 day of treatment. Prolonged treatment with small molecules, with the exception of PKF118-310, decreased *Sox9* expression.

Discussion

We hypothesized that inhibition of WNT/β-catenin signaling in cartilage might be an effective therapeutic strategy for the treatment of cytokine induced cartilage degradation. Therefore, in this study we have tested this hypothesis by assessing the effect of recently identified small molecule inhibitors of WNT/β-catenin signaling on cartilage degradation in the absence or presence of the pro-inflammatory cytokines IL1β and TNFα, which are known to potently

stimulate cartilage degradation by upregulating the expression of MMPs and aggrecanases [1, 11].

Using the TopFlash reporter experiments, we have shown that the small molecule inhibitors effectively block WNT/β-catenin signaling, whilst having only a minor unfavorable effect on metabolic activity of KS483-4C3 cells at higher concentrations, as measured by MTT assay. Immunofluorescence staining for β-catenin revealed that PKF115-584 blocked nuclear translocation of β-catenin upon LiCl stimulation, without altering total β-catenin expression in basal conditions or after stimulation. PKF118-310 and CGP049090 slightly decreased the expression of β-catenin under basal conditions as well as upon LiCl stimulation. PKF118-310 blocked β-catenin translocation after LiCl stimulation, whereas CGP049090 did not affect nuclear translocation of β-catenin. Taking together these findings, we conclude that the small molecule inhibitors we selected can be used for further experiments to assess the effect on *in vitro* cartilage degradation. The discrepancy in the effect of the different small molecule inhibitors on nuclear translocation of β-catenin, might indicate different mechanisms of action between these compounds.

To study the effects of the compounds on IL1β and TNFα induced cartilage degradation, we used an *ex vivo* model consisting of mouse fetal metatarsals [29, 30]. In degenerative cartilage disease, not only chondrocytes, but also osteoblasts in the underlying bone are involved. The organ culture system that we used, includes the primary center of ossification and the developing bone collar as well as the cartilage template, providing chondrocytes as well as osteoblasts. Previously, it has also been shown that in this model system immune cells including macrophages and osteoclasts reside in the perichondrium [31]. This allows for communication between different cell types implemented in degenerative joint diseases, that cannot be mimicked in other *in vitro* models, such as cartilage explants. Furthermore, in this system chondrocytes and osteoblasts are in their natural environment allowing the different

cell types to interact with each other and with the extracellular matrix like they would do *in vivo*. In line with previous studies, we found that IL1β and TNFα are potent inducers of cartilage and bone degradation in mouse fetal metatarsals [32-34]. Therefore, we considered this model to be suitable for studying the effect of small molecule inhibitors of the WNT/β-catenin signaling pathway on cartilage degradation induced by proinflammatory cytokines. In line with literature, IL1β alone demonstrated a mild effect on explant degradation. TNFα did not have an effect but acted synergistically with IL1β [29]. We therefore have chosen the combination of these cytokines to induce explant degradation and to evaluate the potential effect of the WNT/β-catenin inhibitors. Cartilage degradation is mainly due to increased expression and activity of MMPs, which can be induced by, amongst others, IL1β and TNFα [1, 11]. Indications of the involvement of WNT/β-catenin in IL1β/TNFα induced upregulation of MMPs were found [12]. Based on morphometric and histological examination, we have shown that inhibition of WNT/β-catenin signaling by PKF115-584 can prevent the catabolic effects of IL1β and TNFα on cartilage. CGP049090 prevented degradation of the extracellular matrix as well, albeit less effective, whereas PKF118-310 did not have an anti-catabolic effect.

Gene expression analysis revealed that the compounds, particularly PKF115-584 and CGP049090, inhibit IL1β/TNFα induced expression of catabolic genes *Mmp3, Mmp9* and *Mmp13*. This indicates that inhibition of WNT/β-catenin signaling has an anti-catabolic effect by blocking the induction of MMPs by IL1β and TNFα. In line with previous studies [12], this further indicates that WNT/β-catenin signaling is involved in IL1β/TNFα induced MMP expression. As mentioned before, the catabolic effect of inflammatory cytokines consists on the one hand of the increased expression and activity of matrix degrading proteins, and on the other hand of decreased expression of cartilage anabolic genes. For the WNT/β-catenin inhibitors to effectively block cartilage degradation, they should interfere with both

components of cartilage destruction. We found that small molecule inhibitors do block the catabolic process induced by IL1β and TNFα. However, we did not find an effect of WNT/β-catenin inhibition on recovery of basal gene expression levels of extracellular matrix components *Acan* and *Col2a1* after the use of IL1β/TNFα, indicating that the synthesis of new extracellular matrix is not stimulated by small molecule inhibition. In addition, small molecules seem to have a repressive effect on bone growth, indicating a combined inhibitory effect on differentiation. These findings implicate that WNT/β-catenin signaling is involved in the IL1β/TNFα induced effect on catabolic genes, but not in the effect on cartilage anabolic genes. In skeletal development, low levels of β-catenin are thought to promote chondroprogenitor differentiation, whereas in later stages, high levels of β-catenin promote chondrocyte hypertrophic differentiation and subsequent endochondral ossification [35-37]. Based on these findings, inhibition of WNT/β-catenin signaling could be expected to induce cartilage matrix formation. However, low levels of WNT/β-catenin signaling seem not to have a stimulating effect on extracellular matrix formation after IL1β and TNFα induced cartilage degradation. Other pathways, such as the MAPK/ERK pathway [38] and the NFκB pathway [39], were suggested to regulate the IL1β induced inhibition of gene expression of *ACAN* and *COL2A1*. Furthermore, immunofluorescence staining of β-catenin indicated that PKF115-584 might stabilize β-catenin in the cytosol, allowing for β-catenin to exert alternative effects, such as direct binding to SOX9 and sequestering of SOX9 in the cytoplasm, thereby inhibiting expression of matrix genes.

Both PKF115-584 and PKF118-310 inhibit WNT/β-catenin signaling by blocking the binding of β-catenin to the transcription factor TCF4 [22, Wei, 2010 #118]. We found differential effects of PKF115-584 and PKF118-310 on IL1β/TNFα induced cartilage degradation, which might be due to the fact that PKF115-584 inhibits translocation of β-catenin to the nucleus upon LiCl stimulation without affecting the basal amount of β-catenin, whereas PKF118-310

reduced both. In addition, CGP049090, which blocks the binding of β-catenin to TCF4 and LEF1 [24], was not as effective as PKF115-584. This might be due to the fact that PKF115-584 not only blocks binding of β-catenin to TCF4, but also disrupts binding of TCF4 to DNA [24].

Conclusion

In conclusion, this study provides evidence for the involvement of WNT/β-catenin signaling in MMP mediated cartilage degradation induced by IL1β and TNFα. Furthermore, we show that WNT/β-catenin signaling is not involved in the repressive effects of IL1β and TNFα on cartilage matrix proteins like ACAN and COL2A1. Instead, we provide evidence that WNT/β-catenin signaling may be directly involved in the regulation of the expression of these extracellular matrix proteins via an as yet unknown mechanism.

Abbreviations

ACAN, Aggrecan; cDNA, Coding DesoxyriboNucleic Acid; Col2a1, Collagen type 2a1; FZD, Frizzled; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GSK3β, Glycogen synthase kinase 3β; IL1β, Interleukin 1β; LEF1, Lymphoid enhancer-binding factor 1; LiCl, Lithium Chloride; LRP, LDL receptor-related protein; MMP, Matrix metalloproteinase; mRNA, Messenger RiboNucleic Acid; NFκB, Nuclear Factor κB; OA, Osteoarthritis; RA, Rheumatoid arthritis; sFRP, Secreted Frizzled related protein; TCF4, Transcription factor 4; TNFα, Tumor necrosis factor α

Competing interests

The authors declare that they have no competing interests.

Author contributions

EL performed luciferase and MTT assays, analyzed data and drafted the manuscript. RM performed immunofluorescent staining for β-catenin. Both EL and RM performed *ex vivo* experiments. MK and CB contributed extensively to the discussion of experimental design and data interpretation. All authors read and approved the final manuscript.

References

1. Fernandes JC, Martel-Pelletier J, Pelletier JP: **The role of cytokines in osteoarthritis pathophysiology**. *Biorheology* 2002, **39**:237-246.
2. Hedbom E, Hauselmann HJ: **Molecular aspects of pathogenesis in osteoarthritis: the role of inflammation**. *Cell Mol Life Sci* 2002, **59**:45-53.
3. Kapoor M, Martel-Pelletier J, Lajeunesse D, Pelletier JP, Fahmi H: **Role of proinflammatory cytokines in the pathophysiology of osteoarthritis**. *Nat Rev Rheumatol* 2011, **7**:33-42.
4. Pelletier JP, Martel-Pelletier J, Abramson SB: **Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets**. *Arthritis Rheum* 2001, **44**:1237-1247.
5. Freemont AJ, Hampson V, Tilman R, Goupille P, Taiwo Y, Hoyland JA: **Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific**. *Ann Rheum Dis* 1997, **56**:542-549.
6. Reboul P, Pelletier JP, Tardif G, Cloutier JM, Martel-Pelletier J: **The new collagenase, collagenase-3, is expressed and synthesized by human chondrocytes but not by synoviocytes. A role in osteoarthritis**. *J Clin Invest* 1996, **97**:2011-2019.
7. Keyszer GM, Heer AH, Gay S: **Cytokines and oncogenes in cellular interactions of rheumatoid arthritis**. *Stem Cells* 1994, **12**:75-86.
8. Kanyama M, Kuboki T, Kojima S, Fujisawa T, Hattori T, Takigawa M, Yamashita A: **Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids of patients with temporomandibular joint osteoarthritis**. *J Orofac Pain* 2000, **14**:20-30.
9. Little CB, Barai A, Burkhardt D, Smith SM, Fosang AJ, Werb Z, Shah M, Thompson EW: **Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development**. *Arthritis Rheum* 2009, **60**:3723-3733.
10. Piecha D, Weik J, Kheil H, Becher G, Timmermann A, Jaworski A, Burger M, Hofmann MW: **Novel selective MMP-13 inhibitors reduce collagen degradation in bovine articular and human osteoarthritis cartilage explants**. *Inflamm Res* 2010, **59**:379-389.
11. Tetlow LC, Adlam DJ, Woolley DE: **Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes**. *Arthritis Rheum* 2001, **44**:585-594.
12. Yuasa T, Otani T, Koike T, Iwamoto M, Enomoto-Iwamoto M: **Wnt/beta-catenin signaling stimulates matrix catabolic genes and activity in articular chondrocytes: its possible role in joint degeneration**. *Lab Invest* 2008, **88**:264-274.
13. Caron JP, Fernandes JC, Martel-Pelletier J, Tardif G, Mineau F, Geng C, Pelletier JP: **Chondroprotective effect of intraarticular injections of interleukin-1 receptor antagonist in experimental osteoarthritis. Suppression of collagenase-1 expression**. *Arthritis Rheum* 1996, **39**:1535-1544.
14. Wu Q, Zhu M, Rosier RN, Zuscik MJ, O'Keefe RJ, Chen D: **Beta-catenin, cartilage, and osteoarthritis**. *Ann N Y Acad Sci* 2010, **1192**:344-350.
15. Clevers H: **Wnt/beta-catenin signaling in development and disease**. *Cell* 2006, **127**:469-480.
16. Zhu M, Tang D, Wu Q, Hao S, Chen M, Xie C, Rosier RN, O'Keefe RJ, Zuscik M, Chen D: **Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice**. *J Bone Miner Res* 2009, **24**:12-21.
17. Lories RJ, Peeters J, Bakker A, Tylzanowski P, Derese I, Schrooten J, Thomas JT, Luyten FP: **Articular cartilage and biomechanical properties of the long bones in Frzb-knockout mice**. *Arthritis Rheum* 2007, **56**:4095-4103.
18. Imai K, Morikawa M, D'Armiento J, Matsumoto H, Komiya K, Okada Y: **Differential expression of WNTs and FRPs in the synovium of rheumatoid arthritis and osteoarthritis**. *Biochem Biophys Res Commun* 2006, **345**:1615-1620.

19. Hwang SG, Ryu JH, Kim IC, Jho EH, Jung HC, Kim K, Kim SJ, Chun JS: **Wnt-7a causes loss of differentiated phenotype and inhibits apoptosis of articular chondrocytes via different mechanisms**. *J Biol Chem* 2004, **279**:26597-26604.
20. Ma B, van Blitterswijk CA, Karperien M: **A Wnt/beta-catenin negative feedback loop inhibits IL-1-induced MMP expression in human articular chondrocytes**. *Arthritis Rheum* 2012.
21. Yun K, Im SH: **Transcriptional regulation of MMP13 by Lef1 in chondrocytes**. *Biochem Biophys Res Commun* 2007, **364**:1009-1014.
22. Lepourcelet M, Chen YN, France DS, Wang H, Crews P, Petersen F, Bruseo C, Wood AW, Shivdasani RA: **Small-molecule antagonists of the oncogenic Tcf/beta-catenin protein complex**. *Cancer Cell* 2004, **5**:91-102.
23. Wei W, Chua MS, Grepper S, So S: **Small molecule antagonists of Tcf4/beta-catenin complex inhibit the growth of HCC cells in vitro and in vivo**. *Int J Cancer* 2010, **126**:2426-2436.
24. Gandhirajan RK, Staib PA, Minke K, Gehrke I, Plickert G, Schlosser A, Schmitt EK, Hallek M, Kreuzer KA: **Small molecule inhibitors of Wnt/beta-catenin/lef-1 signaling induces apoptosis in chronic lymphocytic leukemia cells in vitro and in vivo**. *Neoplasia* 2010, **12**:326-335.
25. van der Horst G, van der Werf SM, Farih-Sips H, van Bezooijen RL, Lowik CW, Karperien M: **Downregulation of Wnt signaling by increased expression of Dickkopf-1 and -2 is a prerequisite for late-stage osteoblast differentiation of KS483 cells**. *J Bone Miner Res* 2005, **20**:1867-1877.
26. van Beek E, Oostendorp-van de Ruit M, van der Wee-Pals L, Bloys H, van de Bent C, Papapoulos S, Lowik C: **Effects of experimental conditions on the release of 45calcium from prelabeled fetal mouse long bones**. *Bone* 1995, **17**:63-69.
27. Haaijman A, Karperien M, Lanske B, Hendriks J, Lowik CW, Bronckers AL, Burger EH: **Inhibition of terminal chondrocyte differentiation by bone morphogenetic protein 7 (OP-1) in vitro depends on the periarticular region but is independent of parathyroid hormone-related peptide**. *Bone* 1999, **25**:397-404.
28. Yasuhara R, Yuasa T, Williams JA, Byers SW, Shah S, Pacifici M, Iwamoto M, Enomoto-Iwamoto M: **Wnt/beta-catenin and retinoic acid receptor signaling pathways interact to regulate chondrocyte function and matrix turnover**. *J Biol Chem* 2010, **285**:317-327.
29. Van Bezooijen RL, Van Der Wee-Pals L, Papapoulos SE, Lowik CW: **Interleukin 17 synergises with tumour necrosis factor alpha to induce cartilage destruction in vitro**. *Ann Rheum Dis* 2002, **61**:870-876.
30. Miclea RL, Siebelt M, Finos L, Goeman JJ, Lowik CW, Oostdijk W, Weinans H, Wit JM, Robanus-Maandag EC, Karperien M: **Inhibition of Gsk3beta in cartilage induces osteoarthritic features through activation of the canonical Wnt signaling pathway**. *Osteoarthritis Cartilage* 2011, **19**:1363-1372.
31. van der Pluijm G, Deckers M, Sijmons B, de Groot H, Bird J, Wills R, Papapoulos S, Baxter A, Lowik C: **In vitro and in vivo endochondral bone formation models allow identification of anti-angiogenic compounds**. *Am J Pathol* 2003, **163**:157-163.
32. Lefebvre V, Peeters-Joris C, Vaes G: **Modulation by interleukin 1 and tumor necrosis factor alpha of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes**. *Biochim Biophys Acta* 1990, **1052**:366-378.
33. Martensson K, Chrysis D, Savendahl L: **Interleukin-1beta and TNF-alpha act in synergy to inhibit longitudinal growth in fetal rat metatarsal bones**. *J Bone Miner Res* 2004, **19**:1805-1812.
34. MacRae VE, Farquharson C, Ahmed SF: **The restricted potential for recovery of growth plate chondrogenesis and longitudinal bone growth following exposure to pro-inflammatory cytokines**. *J Endocrinol* 2006, **189**:319-328.
35. Day TF, Guo X, Garrett-Beal L, Yang Y: **Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis**. *Dev Cell* 2005, **8**:739-750.

36. Tamamura Y, Otani T, Kanatani N, Koyama E, Kitagaki J, Komori T, Yamada Y, Costantini F, Wakisaka S, Pacifici M, Iwamoto M, Enomoto-Iwamoto M: **Developmental regulation of Wnt/beta-catenin signals is required for growth plate assembly, cartilage integrity, and endochondral ossification**. *J Biol Chem* 2005, **280**:19185-19195.
37. Hill TP, Spater D, Taketo MM, Birchmeier W, Hartmann C: **Canonical Wnt/beta-catenin signaling prevents osteoblasts from differentiating into chondrocytes**. *Dev Cell* 2005, **8**:727-738.
38. Wang X, Li F, Fan C, Wang C, Ruan H: **Effects and relationship of ERK1 and ERK2 in interleukin-1beta-induced alterations in MMP3, MMP13, type II collagen and aggrecan expression in human chondrocytes**. *Int J Mol Med* 2011, **27**:583-589.
39. Yuasa T, Kondo N, Yasuhara R, Shimono K, Mackem S, Pacifici M, Iwamoto M, Enomoto-Iwamoto M: **Transient activation of Wnt/{beta}-catenin signaling induces abnormal growth plate closure and articular cartilage thickening in postnatal mice**. *Am J Pathol* 2009, **175**:1993-2003.

Figure legends

Figure 1: Small molecule inhibitors of WNT/β-catenin signaling effectively block TCF/LEF mediated activity of β-catenin. **A.** Small molecules dose-dependently inhibit TCF/LEF reporter activity in HEK293t cells, induced by the GSK3β inhibitor BIO (1,0 µM). Data represent the mean of three independent experiments +/- CI. **B.** Metabolic activity, as measured using an MTT assay in KS483-4C3 cells, was not affected by small molecules at lower concentrations, however, at 1,0 µM (except for CGP049090) and 3,0 µM, metabolic activity was significantly decreased. Data represent the mean of three independent experiments +/- CI. **C.** Treatment with 50 mM LiCl induced nuclear translocation of β-catenin. Small molecules by themselves had no effect on cellular localization of β-catenin, whereas PKF118-310 and PKF115-584 blocked LiCl-induced translocation of β-catenin to the nucleus. CGP049090 did not affect nuclear accumulation of β-catenin after LiCl treatment. A representative example of three independent experiments is shown. Scale bar represents 10 µm. (* $p < 0,05$)

Figure 2: Combined treatment with IL1β and TNFα caused cartilage degradation in mouse fetal metatarsals. **A.** Mouse fetal metatarsals treated with a combination of IL1β and TNFα exhibit abundant cartilage resorption, whereas treatment with IL1β alone had minor effects and TNFα tended to blunt longitudinal growth only. A representative picture of six independent experiments is shown. Scale bar represents 500 µm. **B.** Treatment with IL1β or a combination of IL1β and TNFα significantly decreased bone length after 4 days and after 7 days of treatment. Data represents the mean of six independent experiments +/- CI. (* $p < 0,05$)

Figure 3: Cartilage degradation induced by IL1β and TNFα in mouse fetal metatarsals can be blocked by small molecule WNT inhibitors. **A.** Morphological changes of metatarsals caused by IL1β and TNFα (10 ng/ml each) can be blocked by co-treatment with PKF115-584 at a

concentration of 1,0 µM. CGP049090 partially blocks resorption of the metatarsals, whereas PKF118-310 did not have an effect. A representative picture of three independent experiments is shown. Scale bar represents 500 µm. **B.** PKF115-584 dose-dependently blocked a decrease in bone length caused by IL1β/TNFα (10 ng/ml each) treatment over time (indicated by red arrow). Other compounds and other concentrations did not counteract detrimental effects of IL1β/TNFα treatment. Data represents the mean of three independent experiments +/- CI.

Figure 4: IL1β/TNFα induced loss of glycosaminoglycans and Collagen II was blocked by co-treatment with PKF115-584 **A.** Metatarsals were treated with IL1β and TNFα (10 ng/ml each) in combination with small molecule WNT/β-catenin inhibitors (1,0 µM). PKF115-584 preserved morphology and glycosaminoglycan staining. A representative picture of two independent experiments is shown. Scale bar represents 500 µm. **B.** Magnification of the boxed region in **A**. Scale bar represents 100 µm. **C.** After 7 days of treatment, PKF115-584 (1,0 µM) prevented IL1β and TNFα (10 ng/ml each) induced loss of Collagen II staining. A representative picture of three independent experiments is shown. Scale bar represents 100 µm.

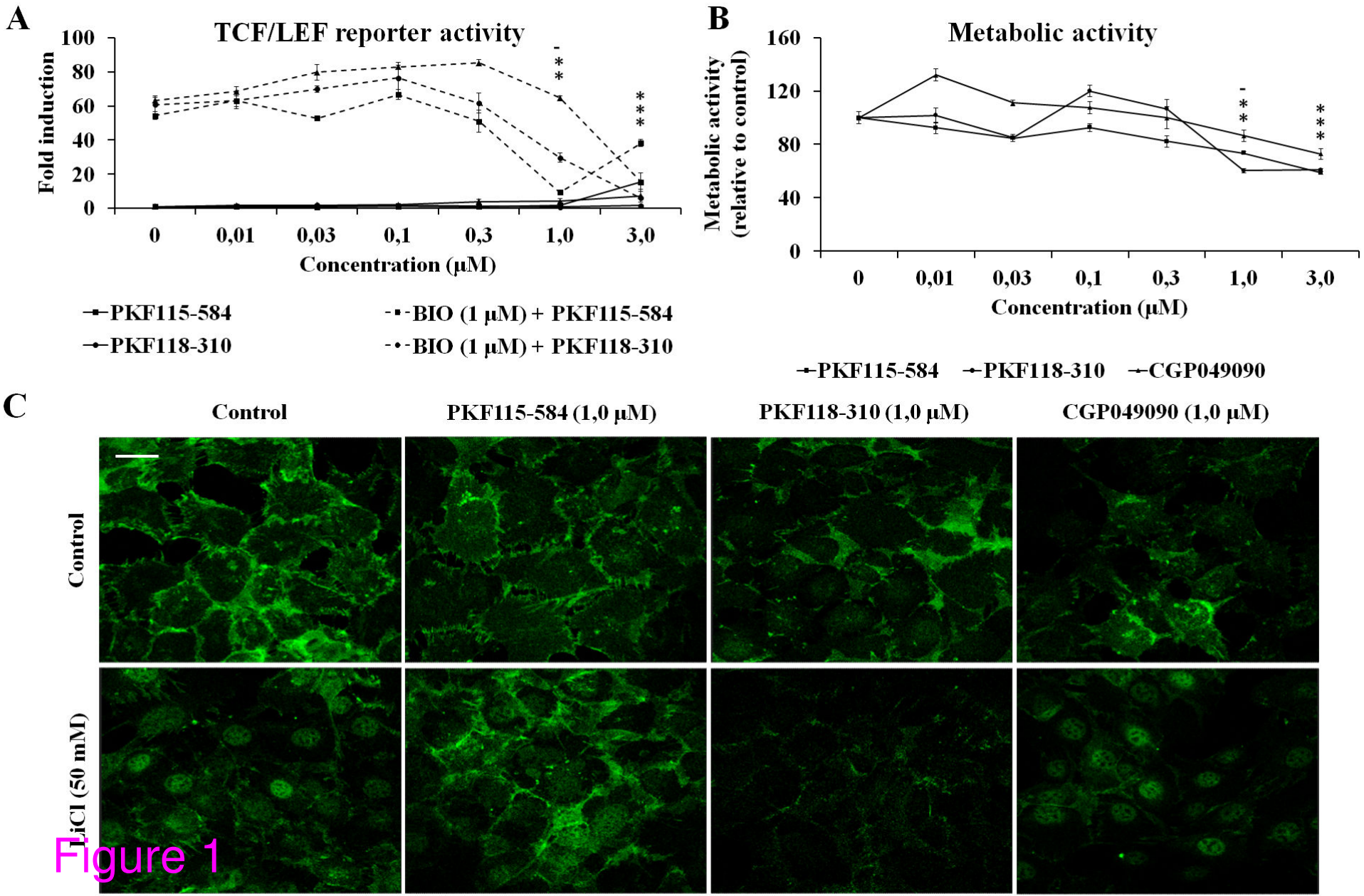
Figure 5: Small molecule inhibitors block IL1β/TNFα induced expression of MMPs, without affecting the IL1β/TNFα induced decrease in mRNA expression of cartilage markers. **A.** Significant upregulation of *Mmp3* expression was found when metatarsals were treated with IL1β and TNFα. Both PKF115-584 and CGP049090 decreased this upregulation after 4 days of co-treatment, whereas PKF118-310 did not have an effect. *Mmp9* expression was significantly downregulated by PKF115-584 after 1 day and IL1β/TNFα induced upregulation was prevented by both PKF115-584 and CGP049090, but not by PKF118-310, after 4 days of culture. Expression of *Mmp13* was significantly upregulated by IL1β and TNFα, whereas this effect was blocked by co-treatment with PKF115-584 or CGP049090, but not PKF118-310. **B.** *Acan* expression is significantly decreased by IL1β and TNFα at both day 1 and day 4.

Also, small molecules PKF115-584 and CGP049090 downregulated expression of *Acan* by themselves. Expression of *Col2a1* is downregulated by IL1β and TNFα and this effect could not be counteracted by small molecules. No significant effects on *Sox9* expression were found. Data represents the mean of two independent experiments +/- CI (*p < 0,05).

Supplemental figure 1: Small molecule inhibitors do not affect mouse fetal metatarsals. **A.** No morphological changes were found in metatarsals treated with small molecules PKF115-584, PKF118-310 or CGP049090 at a concentration of 1,0 µM. A representative picture of two independent experiments is shown. Scale bar represents 500 µm. **B.** No significant differences were found in the bone length when metatarsals were treated with small molecules. Data represent the mean +/- C.I. of three independent experiments.

Table 1 Primer sequences for qPCR

Gene name	Primer sequence	Product size	Annealing temperature
ACAN	For 5' AGGCAGCGTGATCCTTACC 3‘ Rev 5' GGCCTCTCCAGTCTCATTCTC 3'	136 bp	60^{o}C
COL2A1	For 5' CGTCCAGATGACCTTCCTACG 3‘ Rev 5' TGAGCAGGGCCTTCTTGAG 3'	122 bp	60^{o}C
SOX9	For 5' TGGGCAAGCTCTGGAGACTTC 3‘ Rev 5' ATCCGGGTGGTCCTTCTTGTG 3'	98 bp	60^{o}C
MMP3	For 5' TGGCATTCAGTCCCTCTATGG 3‘ Rev 5' AGGACAAAGCAGGATCACAGTT 3'	116 bp	60^{o}C
MMP9	For 5' GGTGATTGACGACGCCTTTGC 3‘ Rev 5' CGCGACACCAAACTGGATGAC 3'	115 bp	60^{o}C
MMP13	For 5' AAGGAGCATGGCGACTTCT 3‘ Rev 5' TGGCCCAGGAGGAAAAGC 3'	72 bp	60^{o}C
GAPDH	For 5' CGCTCTCTGCTCCTCCTGTT 3‘ Rev 5' CCATGGTGTCTGAGCGATGT 3'	82 bp	60^{o}C
B2M	For 5' GACTTGTCTTTCAGCAAGGA 3‘ Rev 5' ACAAAGTCACATGGTTCACA 3'	106 bp	60^{o}C


A
TCF/LEF reporter activity
Fold induction
100
80
60
40
20
0
0
0,01
0,03
0,1
0,3
1,0
3,0
Concentration (µM)
***
***
PKF115-584
PKF118-310
BIO (1 µM) + PKF115-584
BIO (1 µM) + PKF118-310
B
Metabolic activity
Metabolic activity (relative to control)
160
120
80
40
0
0
0,01
0,03
0,1
0,3
1,0
3,0
Concentration (µM)
***
***
PKF115-584
PKF118-310
CGP049090
C
Control
PKF115-584 (1,0 µM)
PKF118-310 (1,0 µM)
CGP049090 (1,0 µM)
Control
LiCl (50 mM)
Figure 1

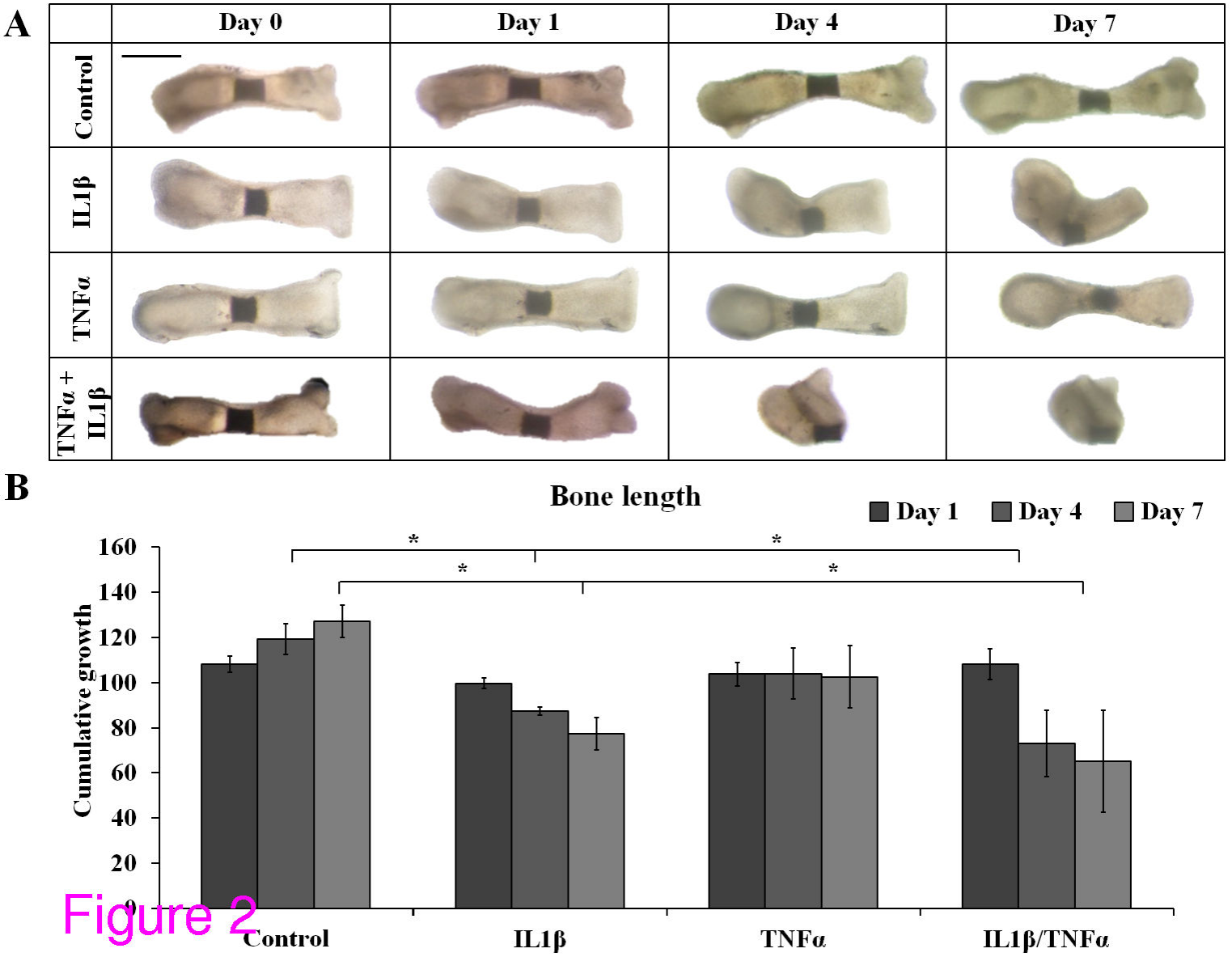
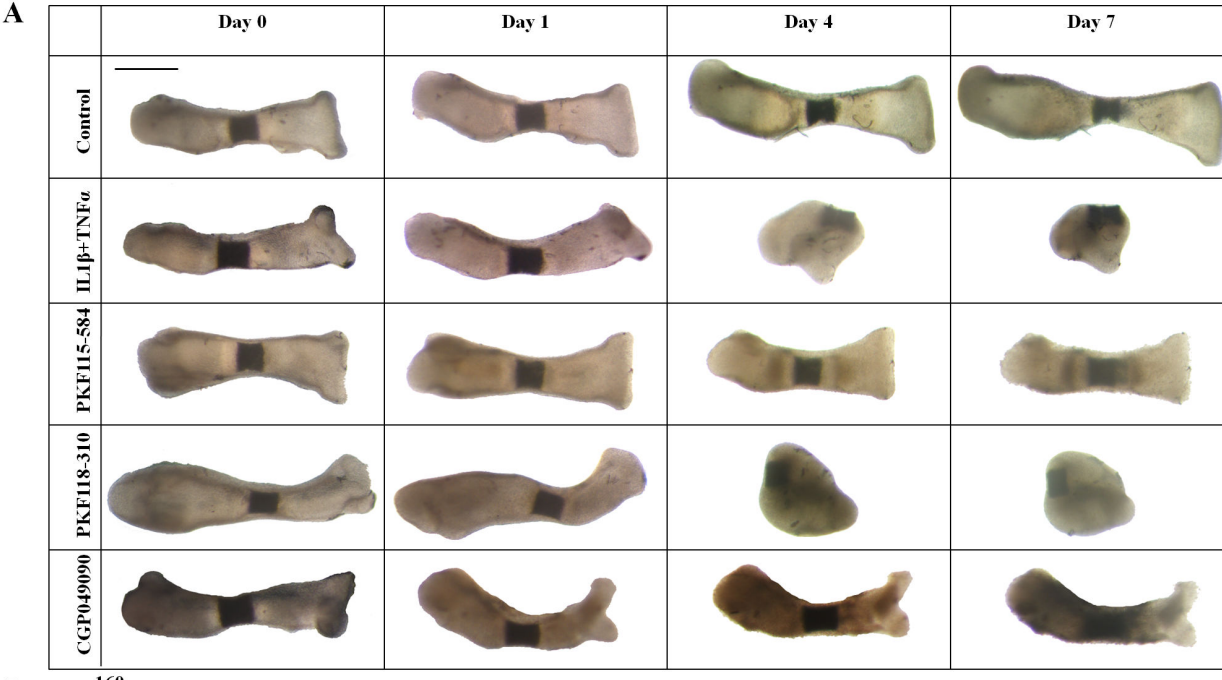

A
Day 0
Day 1
Day 4
Day 7
Control
IL1β
TNFα
TNFα + IL1β
B
Bone length
Day 1
Day 4
Day 7
Cumulative growth
160
140
120
100
80
60
40
20
0
*
*
*
*
Control
IL1β
TNFα
IL1β/TNFα

Figure 2


A
Day 0
Day 1
Day 4
Day 7
Control
IL1β+TNFα
PKF115-584
PKF118-310
CGP049090

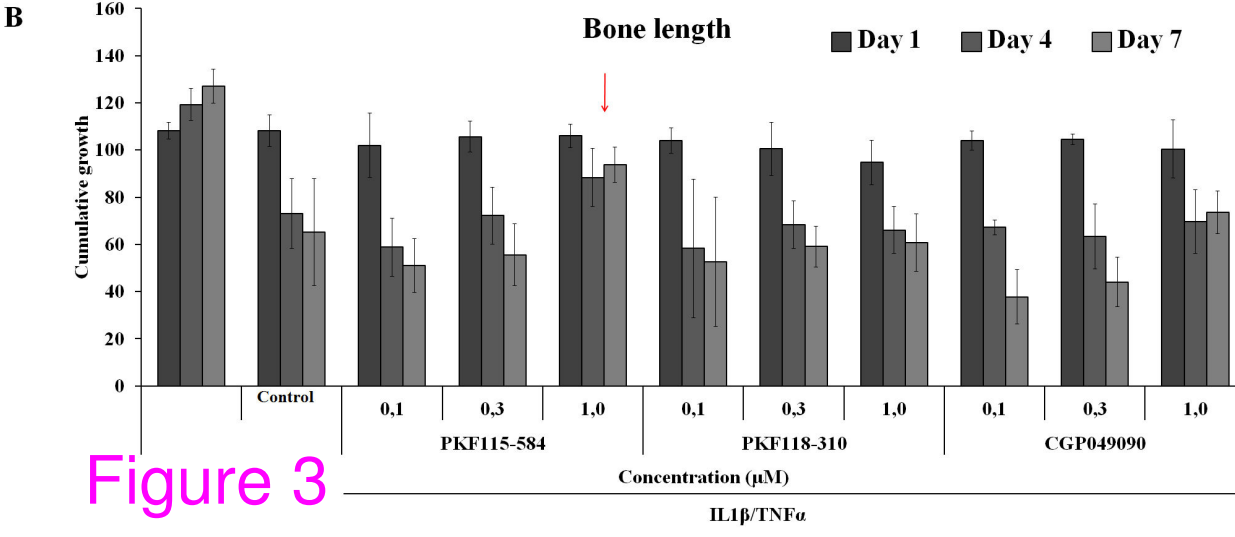

B
Bone length
Day 1
Day 4
Day 7
Cumulative growth
160
140
120
100
80
60
40
20
0
Control
0,1
0,3
1,0
0,1
0,3
1,0
0,1
0,3
1,0
PKF115-584
PKF118-310
CGP049090
Concentration (μM)
IL1β/TNFα

Figure 3

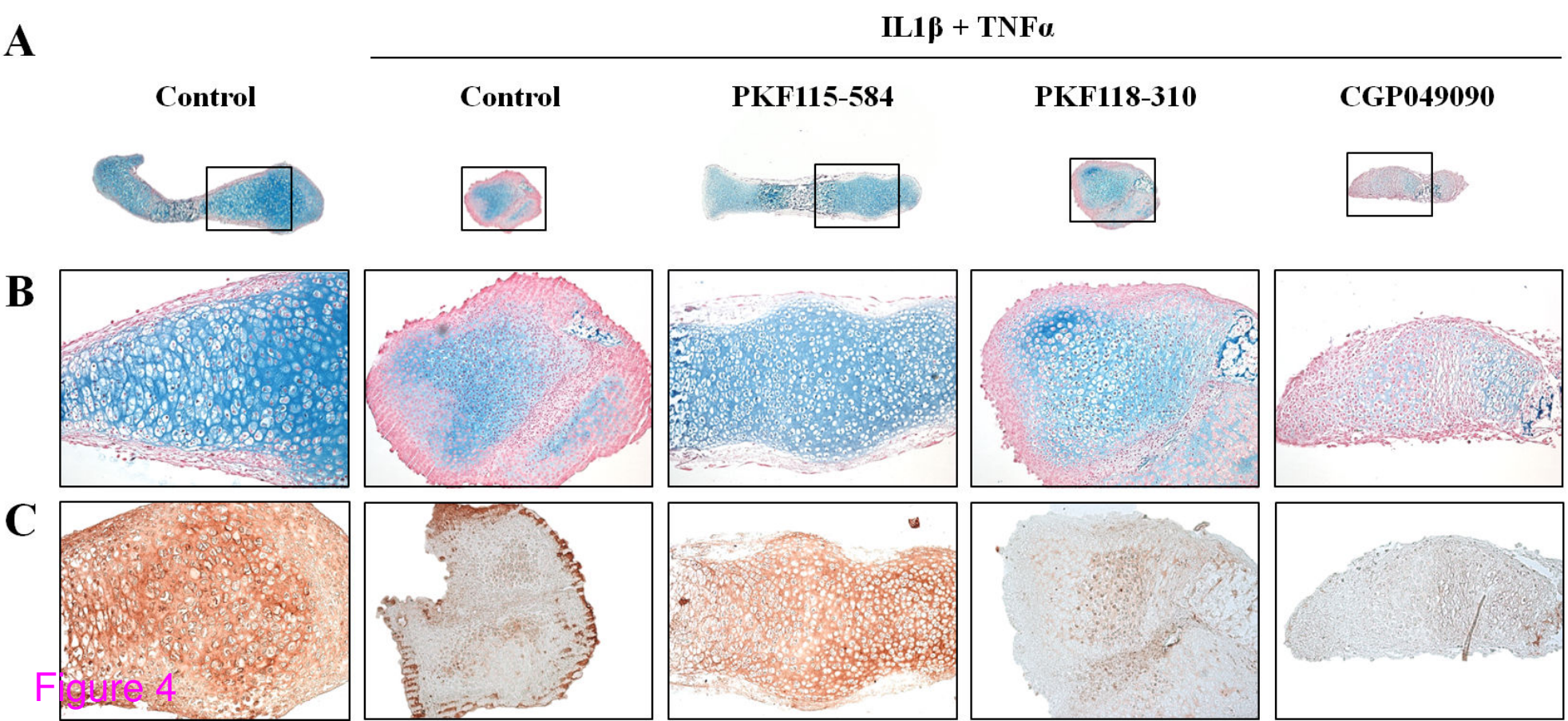
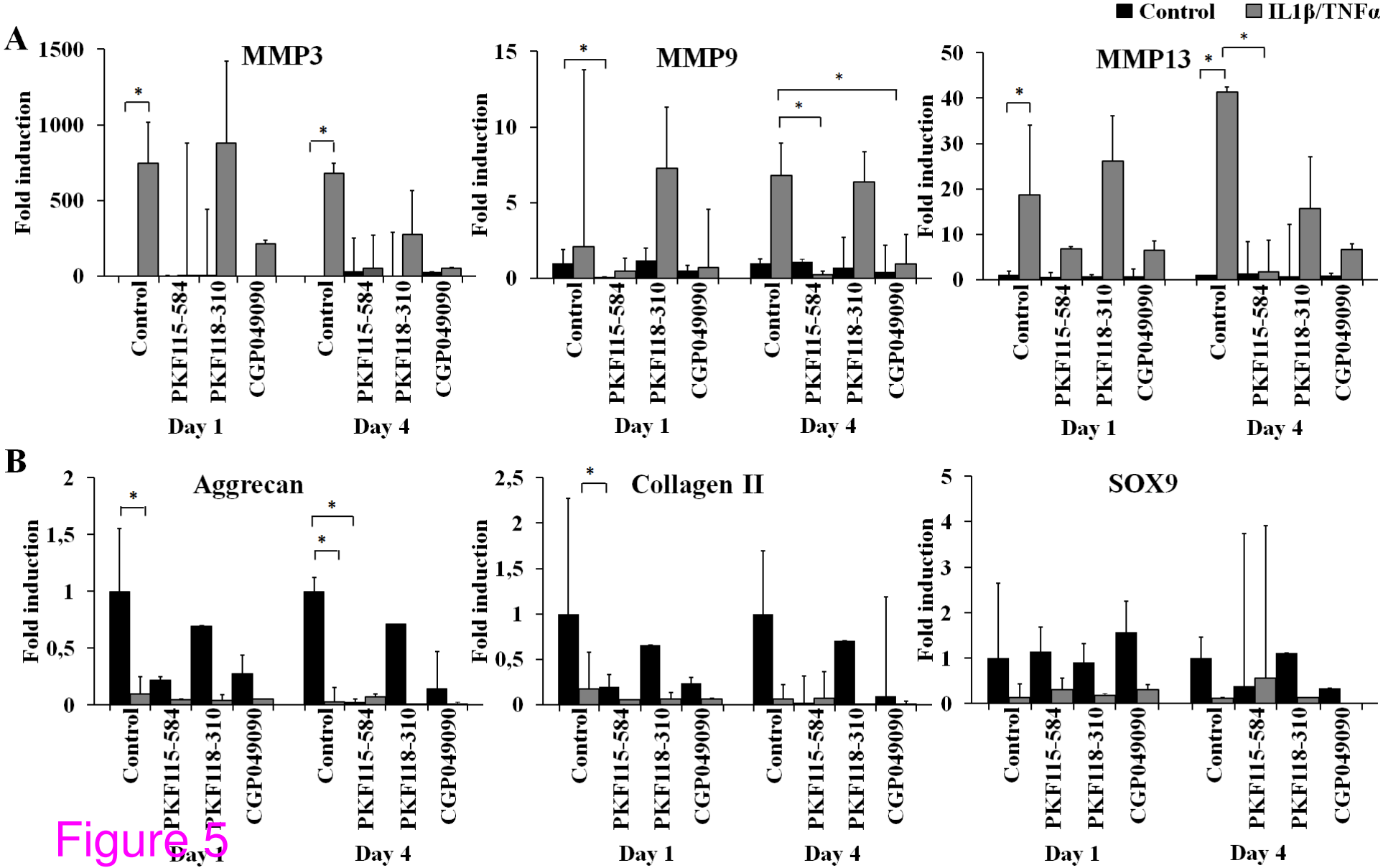

A
IL1β + TNFα
Control
Control
PKF115-584
PKF118-310
CGP049090
B
C

Figure 4


Control
IL1β/TNFα
A
MMP3
MMP9
MMP13
Fold induction
1500
1000
500
0
15
10
5
50
40
30
20
10
Control
PKF115-584
PKF118-310
CGP049090
Day 1
Day 4
B
Aggrecan
Collagen II
SOX9
2
1,5
1
0,5
2,5
5
4
3
Figure 5

Additional files provided with this submission:

Additional file 1: sup1.png, 491K
http://arthritis-research.com/imedia/1399349726106226/supp1.png