were stored in formalin-fixed paraffin-embedded sections (decalcified before embedding and sectioning) and analysed by conventional staining methods and immunohistochemistry (IHC) assays. Chondrocytes from cartilage were isolated using an inverted microscope and tissue was digested using trypsin and collagenase. Primary chondrocytes were grown to 80-90% confluence. Proliferation assay was performed by counting cells with using automated cell counter. For IHC cells were seed onto coverslips. The following are the antibodies used in this study: anti-osteoponin, anti-fibronectin, anti-collagen type II, anti-Cx43, anti-PCNA and Ki67. Scrape loading assay was used to examine GJIC

Results: Analysis of cartilage sections staining with Safranin O/Fast Green, Hematoxylin-eosin eosin and Toluidine blue did not show significant differences between different phenotypes. However the K258stop/KO cartilage was thinner than wild type and Cx43/KO. Besides cartilage from K258stop/KO showed higher rate of positive cells for PCNA. IHC experiments revealed that K258stop/KO chondrocytes in primary culture contain less levels of collagen type II. K258stop/K258stop was found to have 1.5-fold increase in cell proliferation in comparison with wild type or Cx43/KO. Scrape loading assays suggest that the deletion of CTD slightly reduce GJIC.

Conclusions: We have used a genetically modified murine model to directly characterize, for the first time, the role of the CTD of Cx43 on cartilage structure. Our results strongly support the notion that the CTD of Cx43 plays an important role in chondrocyte phenotype. It is well know that through its CTD, Cx43 serves as scaffolding proteins that associates with structural and signaling molecules leading to regulation of intracellular signaling, independently of channel activity. This study illustrates that a complete isolation of Cx43 from its CTD may have a negative impact on cartilage structure and chondrocyte functions within the tissue.

239
THE EFFECTS OF THREE DIFFERENT CLASSES OF ANTI-INFLAMMATORY AGENTS ON IN VITRO HUMAN OSTEOARTHRITIC CHONDROCYTES EXPOSED TO IL-1β

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Purpose: VA694, a promising cyclooxigenase-2 (COX-2)-inhibiting hybrid drug endowed with nitric oxide (NO) releasing properties (NO-COXIB), showed COX-2-selective inhibitory effects, associated with interesting anti-inflammatory and anti-nociceptive activities. For these reason we performed an in vitro study to explore the effects of VA694 on cartilage metabolism, in comparison with Naproxcinod, a COX inhibitor and NO donor (CINOD), and naproxen, a traditional non steroidal-anti-inflammatory drug (NSAID) on human osteoarthritic chondrocyte cultures.

Methods: Human articular cartilage was obtained from femoral heads of 5 patients (range 63-71 years) with Osteoarthritis (OA) according to ACR criteria undergoing surgery for total hip prostheses. The chondrocytes were isolated from the articular cartilage using sequential enzymatic digestion. The primary cultures so obtained were seeded in 6-well plates until confluence. Cells were then incubated with two concentrations (1μg/ml and 10μg/ml) of VA694, Naproxcinod and naproxen alone or in combination with Interleukin (IL)-1β (5ng/ml) for 48h. In these experimental conditions we evaluated cell viability by MMT assay, release of prostaglandin (PG)E2 through an ELISA kit, COX-2 and metalloproteinases (MMP)-3, -9, -13 gene expression by real time PCR, and activation of nuclear factor (NF)-κb by immunocytochemistry. Data are expressed as the mean ± standard deviation of triplicate values for each experiment. Statistical analysis was performed using an analysis of variance followed by Bonferroni multiple comparison tests.

Results: VA694, Naproxcinod and naproxen tested alone at two different concentrations studied didn't significantly modify cell viability, while IL-1β-stimulated chondrocytes showed a significant decrease in cell viability ($p<0.001$). Both concentrations of all drugs tested restored, in a significant manner ($p<0.01$), cell proliferation.

No significant modification of PGE2 levels was observed in the chondrocyte cultures treated with each of these drugs alone. The presence of IL-1β determined a significant increase ($p<0.001$, $p<0.01$, $p<0.01$ respectively) in PGE2 levels and both concentrations of VA694, Naproxcinod and naproxen, significantly decreased the PGE2 levels in cells damaged with IL-1β ($p<0.001$, $p<0.05$, $p<0.05$ respectively).

We investigated the COX-2, MMP-3, -9, -13 gene expression by real time PCR. VA694, Naproxcinod and naproxen tested at two different concentrations alone not significantly modify the gene expression, while IL-1β-stimulated chondrocytes induced a very significant increase in COX-2, MMP-3, -9, -13 gene expression ($p<0.001$). All the drugs tested in our study significantly inhibited COX-2 and MMP-3, -9, -13 gene expression induced by IL-1β at both concentrations analysed ($p<0.01$, $p<0.05$, $p<0.05$ respectively).

VA694, Naproxcinod and naproxen pre-treatment were able to inhibit IL-1β-induced NFκB activation, as measured by its nuclear translocation (p50 and p65 subunits). Naproxcinod and naproxen pre-treatment didn't affect cytoplasmic NFκB levels, while VA694 was able to decrease the cytoplasmic levels of both subunits (Figure 1, Figure 2).

Conclusions: The results of the present study suggest that VA694, Naproxcinod and naproxen exert anti-inflammatory and chondroprotective effects. These drugs in fact inhibit the production of PGE2, COX-2 and MMP-3, -9, -13 gene expression, stimulated by IL-1β.

However, VA694 seems to have a more efficient effect on NF-κB inhibition.

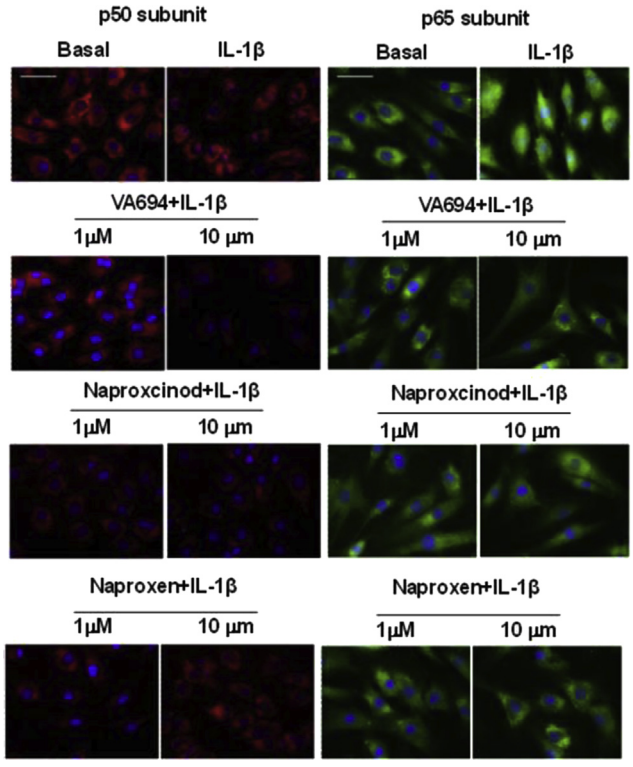


Fig 1. Localization of NFκB, red (p50), green (p65) subunits by Immunocytochemistry. Scalebar 50μm.

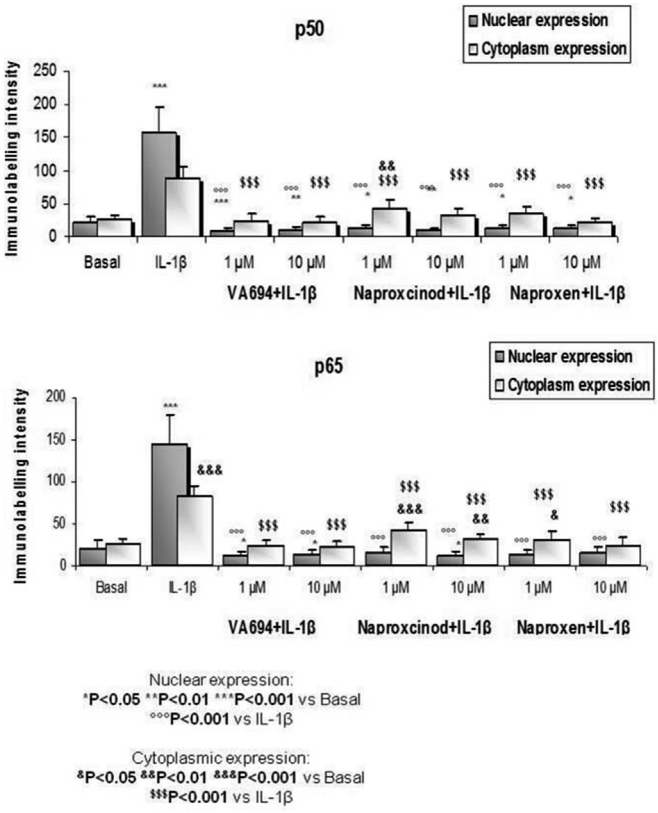
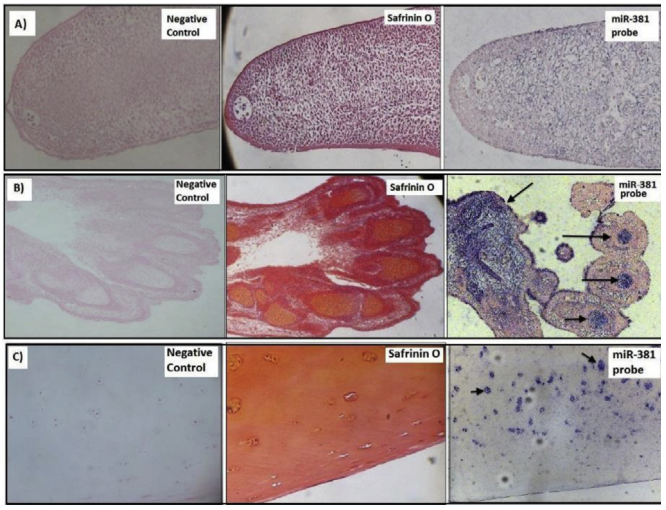
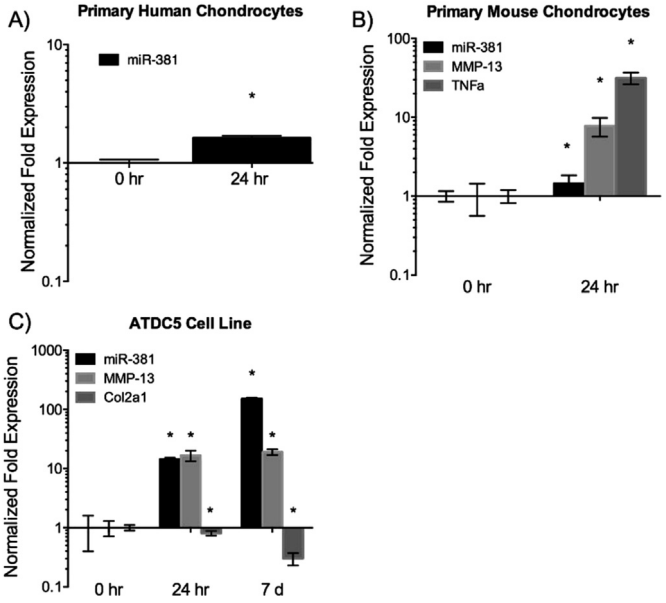
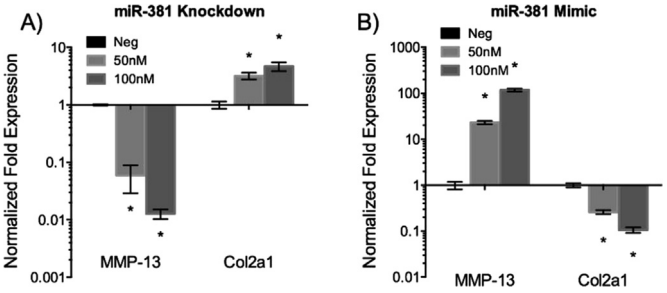


Fig 2. Immunolabelling intensity forp50andp65 subunits, calculated using the Image J software.







240
MICRORNA-381 IN CHONDROGENESIS AND INTERLEUKIN-1-β INDUCED CHONDROCYTE RESPONSES

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Purpose: Osteoarthritis is a debilitating joint condition characterized by cartilage degradation. The pathways that regulate cartilage degradation are still unclear. We previously identified miR-381 as a putative regulator of chondrogenesis related genes. Therefore, the aim of this study was to determine the role of miR-381 in chondrogenesis and cartilage degeneration.

Methods: miR-381 expression was assessed in vitro by qRT-PCR in response to IL-1β stimulation in primary human (PHC) and mouse (PMC) chondrocytes, and the chondrocyte-like ATDC5 cell induced to differentiate to chondrocytes with insulin, transferrin, and selenous acid (ITS) + premix. miR-381 expression was assessed in vivo in mouse embryos and osteoarthritic cartilage by in situ hybridization. The effect of miR-381 on chondrogenesis was assessed using a synthetic RNA mimic or inhibitor. Luciferase assays were used to assess the role of miR-381 in regulating NF-kB signaling. Upstream regulators were probed using siRNA or overexpression plasmids for Sox9 and Runx2.

Results: miR-381 expression was elevated in chondrogenic and hypertrophic ATDC5 cells. IL-1β-induced miR-381 expression in ATDC5 cells, PMCs, and PHCs. miR-381 was also expressed areas of cartilage degradation absorption in mouse embryos and human osteoarthritic cartilage. miR-381 expression was increased in ATDC5 cells over-expressing Runx2 or Sox9. miR-381 suppressed the expression of Col2a1 (collagen, type II, alpha 1) and enhanced the expression of metalloproteinase-13 (MMP-13), but did not regulate NFKBIA and NKRF activity.

Conclusions: miR-381 is highly expressed during chondrogenesis and in arthritic cartilage. It is likely regulated by Sox9 and Runx2. miR-381 may contribute to absorption of the cartilage matrix by repressing collagen II and inducing MMP-13.