miRNA sequences. Inhibition of NF-κB and p38-MAPK activation with specific inhibitors down-regulated the expression of COX-2 but up-regulated the expression of mir-101_3, mir-199a* in human OA chondrocytes suggesting negative regulation of mir-101_3, mir-199a* by the activation of these pathways.

Conclusions: Our data implicate mir-199a* in the post transcriptional regulation of COX-2 expression in human OA chondrocytes. These results also identify mir-199a* as a novel therapeutic target for the treatment of OA.

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ROLE OF C-TYPE NATRIURETIC PePTIDE (CNP) IN OSTEOGENESIS AND TREATMENT OF OSTEOPOROSIS

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Purpose: The recently described C-type natriuretic peptide (CNP) is critical for normal longitudinal growth in humans and plays a central role in osteoblast regulation in bone formation and regeneration. The purpose of this study was to determine whether CNP has therapeutic potential in osteoporosis or osteopenia secondary to inflammatory arthritis. Our goal was to test whether CNP overproduction can reverse the osteoporosis that develops in mouse disease models due to a lack of proper numbers and function of osteoblasts to balance the resorptive effect of osteoclasts.

Methods: CNP(COL2A1TG) mice have been generated by cloning human CNP cDNA into a construct that contained the mouse collagen type II (COL2A1) promoter and enhancer (GenBank #m65161). We initially assessed the bone phenotype of mice that over-express CNP in chondrocytes in vivo. We then made primary chondrocyte and osteoblast cultures from mouse rib cartilage and calvarial osteoblasts. RNA and protein collected were used to measure osteoblastic activity. We induced osteoporosis/osteopenia by inducing chronic inflammatory arthritis in mice that over expressed CNP compared to wild type in vivo.

Results: Phenotypic characteristics of the CNP over-expressing mice became evident at 3-4 weeks of age. There was excessive elongation and noticeable widening of the distal ends of long bones. Increased bone trabeculation in both primary and secondary ossification centers was obvious in histology, at 8-9 weeks of age in CNP(COL2A1TG) mice. Micro-CT analysis of age- and sex-matched littermates (4 female, 7 male), both wild type and CNP(COL2A1TG), at various ages were analyzed. CNP(COL2A1TG) mice showed higher bone mineral density (BMD) and content (BMC) in the subchondral bone in proximal tibia (Table 1). Trabeculation in the subchondral bone and under the growth plate in CNP(COL2A1TG) mice was also increased. CNP(COL2A1TG) mouse serum showed significantly higher osteocalcin levels compared to wild type from 8-13 weeks (p<0.005).

Table 1. Spine m-CT

<table>
<thead>
<tr>
<th>Condition</th>
<th>Avg TbTh (μm)</th>
<th>Trabecular BMC (mg)</th>
<th>Cortical BMC (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 wk F1 WT (n=2)</td>
<td>50.57 (±1.02)</td>
<td>4.80 (±0.18)</td>
<td>7.65 (±0.2)</td>
</tr>
<tr>
<td>8 wk F1 CNP(COL2A1TG) (n=2)</td>
<td>52.44 (±0.12)</td>
<td>6.95 (±0.03)</td>
<td>9.08 (±0.8)</td>
</tr>
<tr>
<td>14 wk F1 WT (n=4)</td>
<td>77.70 (±1.2)</td>
<td>3.51 (±0.80)</td>
<td>9.16 (±0.9)</td>
</tr>
<tr>
<td>14 wk F1 CNP(COL2A1TG) (n=2)</td>
<td>91.40 (±2.4)</td>
<td>7.43 (±1.20)</td>
<td>13.57 (±1.3)</td>
</tr>
</tbody>
</table>

Quantitative RT-PCR analysis showed that, in both primary chondrocytes and osteoblast cultures, collagen II, X, I, OPG, osteocalcin were expressed at significantly higher levels in the CNP(COL2A1TG) mouse chondrocytes or when calvarial osteoblasts were treated with exogenous CNP (10ng/ml > 3wk). However, RANKL mRNA was found to be decreased 2.5 fold in CNP(COL2A1TG) mouse chondrocytes and osteoblasts. When we induced systemic arthritis using the K/BxN TCR serum model in mice (n=32) that were either CNP(COL2A1TG) or wild type, the CNP(COL2A1TG) mice did not develop endochondral delay or periarticular bone loss/osteoporosis even after 4 weeks of systemic arthritis compared to wild type. Serum osteocalcin levels, measured after the K/BxN TCR arthritis developed, showed decreased levels at 2.5, 3.5 and 5 months-of-age (Fig. 1). Interestingly, CNP(COL2A1TG) mice continued to maintain high osteocalcin levels even after arthritis developed. Micro-CT analysis suggested that CNP(COL2A1TG) mice that developed arthritis were resistant to both trabecular and cortical bone mineral density loss due to inflammatory changes in the joint. Trabecular thickness, inter-trabecular space and the connectivity of the trabecular structure were not different in wild type (WT) and K/BxN TCR mice.

Conclusions: Our observations from in vivo and in vitro experiments suggest a clear effect of CNP signaling in osteogenesis and suggest a role in osteoporosis treatment during systemic arthritis.

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THE ROLE OF FIBROBLAST GROWTH FACTOR 2 (FGF2) IN THE CELLULAR RESPONSE TO INJURY FOLLOWING MURINE CARTILAGE EXPLANTATION

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Purpose: Studies from our lab have previously demonstrated that there is rapid activation of a number of intracellular signalling pathways upon explantation of porcine articular cartilage including the three mitogen activated protein kinases (MAPKs); c-Jun N terminal kinase (JNK), p38, and the extracellularly regulated kinase (ERK) (Gruber, 2004), as well as activation of NFkB, p38 and jak/Stat (unpublished). Re-cutting cartilage that has been rested in serum free medium for 48h results in activation of the ERK pathway only, and is due to release of FGF2 from the pericellular matrix. In order to determine the relative contribution of FGF2 to the explantation response, we set up signalling assays and studied gene induction in injured murine hip cartilage from wild type (WT) and FGF2 null mice.

Methods: Murine cartilage from 4-6 week old WT or FGF2 null mice was explanted from the intact hip by blunt dissection, and was either lysed immediately, or was cultured in serum free medium for up to 24h. Some explanted cartilage was rested in serum free medium (48h), then was re-cut. Protein was extracted from the hips (3-5 for signalling experiments) for phospho-western blotting for pERK, pJNK, p38 and pikappaB. Equal protein loading was confirmed by re-blotting for total ERK, IkB or tubulin. Gene analysis was performed on 5-8 hip explants (either explanted, re-cut or stimulated with FGF2). Messenger RNA was extracted using Trizol and purified. Quantitative RT-PCR for 48 pre-selected candidate genes using Taqman microfluidic cards was performed.

Results: Cartilage explantation led to early activation of MAPK pathways as well as causing phosphorylation of IkB, indicative of activation of NFkB. A very weak activation of inflammatory pathways was apparent when rested cartilage was re-cut. When FGF2 null cartilage was explanted there was an approximate 50% reduction in MAPK activation. A number of genes (selected from an unpublished murine OA microarray study) were regulated in the chondrocytes following explantation. These are listed in Table 1. Some of these genes were also regulated when the tissue was re-cut (rather than explanted), and those same genes were shown to be strongly induced by stimulation with recombinant FGF2. CCL2, arginase 1, IL-1α, CCL7 and

Figure 1. Osteocalcin (ng/ml)

Legend: Arg1 - arginase 1; CCL - CC chemokine ligand; HAS1 - hyaluronan synthase 1; CD14 - cluster differentiation 14; TNFRSF12a - TNF Receptor superfamily member 12a; TSG6 - TNF stimulated gene 6; TIMP1 - tissue inhibitor of metalloproteinase 1; PtgE - prostaglandin E synthase.
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IL-1BETA TRANSLOCATES THE PROTEIN DIMETHYLRARGININASE 2 (DDAH2) TO THE MITOCHONDRION OF HUMAN NORMAL CHONDROCYTES

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Purpose: Mitochondria is acquiring an important role in the osteoarthritis pathology (OA). IL-1beta is one of the main cytokines related to the inflammation and the destruction of the cartilage which is known to regulate mitochondrial functionality and to produce nitric oxide (NO). Previously, we have described the total proteome of chondrocytes regulated by IL-1beta identifying some mitochondrial proteins. Nevertheless, the exact role that IL-1beta has in the regulation of mitochondrial protein expression and their implication in the OA process, is not well understood. For this reason, we analyzed the mitochondrial protein expression profile by the action of IL-1beta, in order to understand the development of different rheumatic pathologies.

Methods: Human normal chondrocytes were isolated from the cartilage of knees of autopsies from donors without previous history of joint disease. The cells were incubated for 48 hours in basal conditions or with IL-1beta (5 ng/ml) and mitochondrial proteins were purified. A pool of these mitochondrial proteins of 4 different donors in each condition, was resolved by bidimensional electrophoresis (2-DE). Proteins were visualized with Sypro stain. The qualitative and quantitative analysis were carried out with PD-QUEST software. After that, proteins were identified by mass spectrometry (MS) using the MALDI-TOF/TOF technology. The validation of the results and the study of interesting proteins were made by real-time PCR, western blot and microscopy. Total NO quantification was evaluated with Griess reagent assays.

Results: The comparative analysis of mitochondrial proteome of chondrocytes stimulated with IL-1beta for 48 hours with respect to the basal condition revealed a differential expression of signal transduction proteins, regulators of cytoskeleton, transcription, metabolic and stress related pathways. IL-1beta increased with respect to the basal condition different proteins like annexin A2 (ANXA2) or mitochondrial superoxide dismutase (SOD2). Another protein that increased its expression 2.59 times was dimethylarginine dimethylaminohydrolase 2 (DDAH2). This protein has an important role as regulator of NO synthesis when it hydrolyzes the inhibitor of NO synthase, ADMA. DDAH2 did not show any regulation at mRNA nor total protein expression after the stimulation with IL-1beta, nevertheless the study of mitochondrial extracts showed a clear increase of DDAH2 in the condition of IL-1beta with respect to the basal condition (4.18±1.41 vs. basal-1, n=4; *P<0.05). By means of techniques of conventional immunofluorescence and confocal microscopy, we observed that DDAH2 was located again in the mitochondria of IL-1beta-stimulated chondrocytes. These results were also reproducible in cartilage slices treated with IL-1beta. In addition to this we demonstrated that DDAH2, which didn’t have been before described in chondrocytes nor cartilage, regulated the NO production induced by IL-1beta.

Conclusions: Our studies indicate that IL-1beta increased the expression of certain proteins with a mitochondrial localization such as DDAH2 that was identified for the first time in human normal chondrocytes and cartilage. It seemed to have an important role in the IL-1beta-NO production. Its specific localization in the mitochondria could help to understand the role of this organell and NO in rheumatic pathologies.