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Citation
Bükülmez, Hülya, Fozia Khan, Cynthia F Bartels, Shunichi Murakami, Adriana Ortiz-Lopez, Abdus Sattar, Tariq M Haqqi, and Matthew L Warman. 2014. “Protective Effects of C-Type Natriuretic Peptide on Linear Growth and Articular Cartilage Integrity in a Mouse Model of Inflammatory Arthritis.” Arthritis & Rheumatology (Hoboken, N.j.) 66 (1): 78-89. doi:10.1002/art.38199. http://dx.doi.org/10.1002/art.38199.

Published Version
doi:10.1002/art.38199

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Protective Effects of C-Type Natriuretic Peptide on Linear Growth and Articular Cartilage Integrity in a Mouse Model of Inflammatory Arthritis

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Objective. The C-type natriuretic peptide (CNP) signaling pathway is a major contributor to postnatal skeletal growth in humans. This study was undertaken to investigate whether CNP signaling could prevent growth delay and cartilage damage in an animal model of inflammatory arthritis.

Methods. We generated transgenic mice that overexpress CNP (B6.SJL-Col2a1-NPPC) in chondrocytes. We introduced the CNP transgene into mice with experimental systemic inflammatory arthritis (K/BxN T cell receptor [TCR]) and determined the effect of CNP overexpression in chondrocytes on the severity of arthritis, cartilage damage, and linear growth. We also examined primary chondrocyte cultures for changes in gene and protein expression resulting from CNP overexpression.

Results. K/BxN TCR mice exhibited linear growth delay (P < 0.01) compared to controls, and this growth delay was correlated with the severity of arthritis. Diminished chondrocyte proliferation and matrix production was also seen in K/BxN TCR mice. Compared to non–CNP-transgenic mice, K/BxN TCR mice with overexpressed CNP had milder arthritis, no growth delay, and less cartilage damage. Primary chondrocytes from mice overexpressing CNP were less sensitive to inflammatory cytokines than wild-type mouse chondrocytes.

Conclusion. CNP overexpression in chondrocytes can prevent endochondral growth delay and protect against cartilage damage in a mouse model of inflammatory arthritis. Pharmacologic or biologic modulation of the CNP signaling pathway may prevent growth retardation and protect cartilage in patients with inflammatory joint diseases, such as juvenile idiopathic arthritis.

Linear growth results from the action of multiple signaling pathways. The pathway that includes C-type natriuretic peptide (CNP) contributes to chondrocyte proliferation, differentiation, and matrix synthesis in skeletal growth plates (1–4). Patients with homozygous loss-of-function mutations in natriuretic peptide receptor B (NPR-B), the CNP receptor, have acromesomelic dysplasia (5), and carriers of the heterozygous mutation frequently have short stature (6). Also, patients with chromosome translocations that cause CNP overexpression develop skeletal overgrowth (7). These conditions indicate a dose-dependent effect of CNP signaling on linear growth. Therefore, linear growth may be affected
in patients with acquired diseases that alter the production of CNP or the expression of its receptor, NPR-B. Children with juvenile inflammatory arthritis (JIA) have impaired linear growth, and 40% develop bone degeneration and deformity adjacent to affected joints (8–11). The mechanism by which chronic systemic inflammation suppresses linear growth in children with JIA is not completely understood (12,13). Alterations in growth hormone and insulin-like growth factor (IGF) signaling pathways have been associated with growth delay in children with chronic arthritis. Although serum growth hormone levels were unchanged in patients with JIA, patients did have reduced levels of IGF-1 and IGF binding protein 3 (IGFBP-3) (14), suggesting that inflammation causes growth hormone resistance. The CNP signaling pathway has not been studied in children with JIA.

Animal models of inflammatory arthritis have been used to delineate pathways that contribute to impaired linear growth and joint deformity (15). Local elevations of tumor necrosis factor alpha (TNFα), interleukin-6 (IL-6), and IL-1β levels have been observed in affected growth plates. Furthermore, over-expression of IL-6 (16) or TNFα (17) in transgenic mice causes systemic inflammatory arthritis and growth retardation. By 25 days of age, K/BxN T cell receptor (TCR) mice exhibit pronounced joint inflammation that resembles rheumatoid arthritis (17–19). Herein, we report that these mice also develop linear growth delay and cartilage damage that can be lessened by overexpression of CNP in chondrocytes.

**MATERIALS AND METHODS**

These experiments were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University.

**Generation of CNP-transgenic mice.** We cloned full-length human CNP (NPPC) complementary DNA (cDNA) into the pKN185 vector, which drives CNP expression under the control of Col2a1 promoters and enhancers (20). Col2a1-NPPC–transgenic mice were produced following pronuclear injection into zygotes on a mixed C57BL/6J-SJL background. One founder animal that had ~4 copies of the CNP transgene integrated into a single locus, as determined by Southern blotting and Mendelian segregation of the transgene, was then backcrossed for >20 generations onto the C57BL/6J background. C57BL/6J mice were also used as wild-type (WT) controls.

**Development of the K/BxN TCR mouse model of arthritis.** KRN TCR–transgenic mice, as well as K/BxN TCR mice that develop spontaneous arthritis, have been previously described (21–23). KRN TCR–transgenic mice were a gift from CBDM Lab (Joslin Diabetes Center/Harvard Medical School). K/BxN TCR mice were obtained by crossing KRN TCR–transgenic mice with nonobese diabetic mice (NOD/ShiLtJ; The Jackson Laboratory). Only offspring that inherited the TCR transgene developed inflammatory arthritis on this background; mice that did not inherit the TCR transgene were used as nontransgenic controls.

**Breeding the CNP transgene into the mice with experimental arthritis.** We bred Col2a1-NPPC–transgenic mice with KRN TCR mice. Offspring that carried both the Col2a1-NPPC and the TCR transgenes were then bred with NOD mice. The phenotypes of offspring that were double heterozygous for the TCR and Col2a1-NPPC transgenes (K/BxN TCR,Col2a1-NPPC) were compared to the phenotypes of offspring that only inherited the TCR transgene (K/BxN TCR). Other offspring resulting from this cross included mice heterozygous for the Col2a1-NPPC transgene only and mice that inherited neither transgene (BxN). Offspring were examined at birth and weekly, from 3 weeks of age until 20 weeks of age.

**Small-animal radiography.** A Faxitron radiographic inspection unit (model 8050-010; Field Emission) was used to obtain radiographic images of the mice postmortem. Legs were exposed for 1.5 minutes at 35 kVp; the entire body was exposed for 1.5 minutes at 30 kVp.

**Clinical scoring of arthritis severity.** The severity of clinical arthritis was determined using a previously described scoring system (24,25), with modifications (available online at http://www.case.edu/artsci/biol/skeletal/24114569s.html). Higher scores indicate increased severity of clinical arthritis.

**Sample collection.** Mice were bled under sedation and then euthanized. Samples were collected from mice that were 1, 2, 3, 4, 6, 8, 12, 16, and 24 months old. To evaluate the growth plates, one hind leg (femur and tibia) from each mouse was dissected, fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 24 hours at 4°C, decalcified with 0.5M EDTA for 1 week, and then embedded in paraffin. Coronal sections measuring 4 μm across the femorotibial joint were stained with Safranin O–fast green or hematoxylin and eosin and analyzed by immunohistochemical staining or in situ hybridization. For accurate measurement of articular cartilage and growth plate cartilage, sections were obtained at the point where the anterior cruciate ligament inserts into the tibia.

**Histologic scoring system.** Histologic evaluation of inflammation in the knee joint and cartilage integrity was performed using two scoring systems. The histologic scoring system described by Pettit et al (26) was used to assess the severity of inflammatory arthritis. The International Cartilage Repair Society scoring system (27) was used to assess the status of the extracellular matrix repair in joint cartilage (available online at http://www.case.edu/artsci/biol/skeletal/24114569s.html). An investigator who was not otherwise involved with the study evaluated the mouse knee sections under blinded conditions and assigned a score based on these systems.

**Cell proliferation analysis by bromodeoxyuridine (BrdU) labeling.** BrdU (300 mg/kg dose; Zymed) was injected intraperitoneally. Mice were euthanized 2 hours after injection. Tissue was processed and embedded in paraffin as
described above. BrdU incorporation was detected using a BrdU-staining kit according to the instructions of the manufacturer (Zymed). The percentage of BrdU-positive cells was determined by dividing the number of BrdU-positive chondrocytes by the total number of chondrocytes that were counted in multiple sections of the growth plate.

**In situ hybridization.** Slides were deparaffinized and fixed in 4% formaldehyde. Sections were digested with proteinase K (1 µg/ml) for 20 minutes at 37°C and acetylated in 0.25% acetic anhydride in 0.1M triethanolamine HCl. After fixation in 4% formaldehyde, sections were hybridized with 35S-labeled riboprobes in hybridization buffer (50% deionized formamide, 300 mM NaCl, 20 mM Tris HCl, pH 8.0, 5 mM EDTA, 0.5 mg/ml yeast transfer RNA, 10% dextran sulfate, and 1× Denhardt’s solution) in a humidified chamber at 55°C overnight. After hybridization, slides were washed with 5× saline–sodium citrate (SSC) at 50°C, 50% formamide, 2× SSC at 65°C, and 1× NTE (0.5 M NaCl, 10 mM Tris HCl, pH 8.0, 5 mM EDTA) at 37°C, and then treated with RNase A (20 µg/ml) and RNase T1 (1 unit/µl) in 1× NTE at 37°C for 20 minutes. Slides were further washed in 1× NTE at 37°C, 50% formamide, 2× SSC at 65°C, 2× SSC, 0.1× SSC, and then dehydrated with graded concentrations of ammonium acetate and ethanol. Slides were dipped in NTB emulsion (Kodak) and exposed to the emulsion for 6 weeks, after which they were developed and counterstained with Hoechst 33258 (Sigma). The NPRC probe was made from the pKN185-hCNP vector construct that was originally used to create the transgenic mice. A 5’ 280-bp fragment of the human CNP cDNA sequence plus 118 bp of upstream vector sequence was used for sense and antisense probes (398 bp).

**Primary chondrocyte isolation and cytokine treatment.** Primary chondrocytes from rib cages of ~5-day-old mice were isolated by enzymatic digestion using standard methods (28). Briefly, rib cages were collected in sterile PBS. The ribs were rinsed several times before digestion with 10 ml of 3 mg/ml collagenase B ( Worthington) in Dulbecco’s modified Eagle’s medium (DMEM) for ~1 hour at 37°C. The ribs were then washed several times with PBS and digested overnight at 37°C with collagenase solution (2 ml of 3 mg/ml collagenase B/DMEM plus 10 ml DMEM with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). Viable cell counts were determined by trypsin blue staining, and cells were seeded at a density of 1 × 106 cells per 35-mm culture plate in 2.5 ml DMEM (~1.2 × 105 cells/cm²). Cells were grown in culture medium until ~90% confluent, with a change of medium every 3 days. Once cells became ~90% confluent, chondrocyte cultures were serum-starved and treated with cytokines (10 ng/ml TNFα or 10 ng/ml IL-1β; both from R&D Systems) overnight (18 hours) and messenger RNA (mRNA) and proteins were extracted as previously described (29).

**Western blotting.** Cells were lysed in 500 µl of buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, and Complete protease inhibitor cocktail), and total lysate protein (60 µg/lane) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad), blocked with nonfat dry milk, resuspended in Tris buffered saline, and probed with primary and secondary antibodies as previously described (29,30). Primary antibodies included anti-phosphorylated and anti-nonphosphorylated p38 MAPK and ERK-1/2 (p38 MAPK phosphorylated #4631, p38 MAPK nonphosphorylated #9212, phospho–ERK-1/2–phospho-MAPK [Thr202/Ty204] [Cell Signaling Technologies]) and anti–NPR-B (sc-25486; Santa Cruz Biotechnology). Immuno-reactive proteins were visualized by using 1:1,000 diluted horseradish peroxidase (HRP)–linked secondary antibodies and enhanced chemiluminescence (GE Healthcare) as described (31). HRP-conjugated goat anti-rabbit IgG at a dilution of 1:2,000 (sc-2004; Santa Cruz Biotechnology) was used as the secondary antibody for NPR-B.

**RNA extraction and cDNA synthesis.** Total cytoplasmic RNA was prepared from primary chondrocytes using an RNeasy kit (Qiagen). RNA (2 µg) was reverse transcribed using a SuperScript II reverse-transcriptase kit (Invitrogen), and the cDNA mixture was diluted 5-fold in nuclease-free water; 5 µl was used for real-time quantitative polymerase chain reaction (qPCR).

**Real-time qPCR.** Using a StepOnePlus thermocycler (Applied Biosystems), reactions were performed in a 20-µl volume with 0.5 µM of each primer and SYBR Green Master Mix according to the instructions of the manufacturer (Qiagen). The expression level of the housekeeping gene Rpl7 was used to normalize mRNA expression. Information on the primers used to amplify specific cDNA sequences is available online at http://www.case.edu/artsci/biol/skeletal/24114569s.html. All reactions were performed in duplicate.

**Statistical analysis.** Disease severity and histopathologic scores were compared between groups, using chi-square tests for categorical variables (clinical and histologic scores) and t-tests for continuous variables. Results are expressed as the mean ± SD. To test differences between the groups of mice, data on the nose-to–tail tip length of mice were examined by analysis of variance (ANOVA). ANOVA F test results were reported. P values less than 0.05 were considered significant. We also used Pearson correlation matrices to test for correlations between the clinical and histopathologic data. The data were analyzed using SAS 9.0 and Stata 11.0.

**RESULTS**

**Col2a1-NPPC–transgenic mice exhibit linear bone overgrowth.** Mice carrying the CNP transgene driven by the Col2a1 promoter/enhancer (Col2a1-NPPC) demonstrated skeletal overgrowth at 4 weeks of age (available online at http://www.case.edu/artsci/biol/skeletal/24114569s.html). Overgrowth affected the long bones and the vertebrae (Figures 1A and B) and was associated with increases in growth plate width, the size of hypertrophic chondrocytes (P < 0.01 by F test) (Figures 1C and D), nose-to–tail tip length (Figure 1E),
and the number of proliferating chondrocytes \((P < 0.01\) by F test) (Figure 1F). The mean \pm SD width of the growth plate was 148 \pm 14 \mu m in Col2a1-NPPC–transgenic mice \((n = 12)\) and 99 \pm 5.3 \mu m in nontransgenic control mice \((n = 5)\) at 4 weeks of age \((P < 0.05\) by Student’s \(t\)-test). CNP transgene expression in growth plate and articular chondrocytes was detected by in situ hybridization (Figures 1G and H). Col2a1-NPPC–transgenic mice developed thoracic kyphosis and joint dislocations over time, but no abnormalities outside of the skeletal system were detected.

**Arthritic K/BxN TCR mice exhibit linear growth delay and articular cartilage damage.** K/BxN TCR mice developed arthritis by age 3 weeks and exhibited visible growth delay by age 12 weeks when compared to littermates that did not inherit the TCR transgene \((BxN)\) (Figure 2A). Significant differences in linear growth (as determined by nose-to–tail tip length) between K/BxN TCR mice and BxN littermates were detected by 4 weeks of age \((P < 0.05\) by F test) (Figure 2B). To prevent length discrepancies, we did not obtain tissue for DNA isolation by clipping tails in order to genotype the mice. In the
K/BxN TCR mice, the severity of arthritis was inversely correlated with longitudinal growth; this correlation was strongest at age 14 weeks (available online at http://www.case.edu/artsci/biol/skeletal/24114569s.html). The growth plates in the K/BxN TCR mice were narrower and had fewer cells than growth plates in the BxN littermates (Figure 2C). The articular cartilage in K/BxN TCR mice also became less cellular, had less cartilage matrix, and had a more irregular surface than the articular cartilage in BxN mice (Figures 2D and E).

CNP overexpression in chondrocytes improves linear growth and reduces articular cartilage damage in K/BxN TCR mice. Since CNP overexpression enhanced linear growth in Col2a1-NPPC–transgenic mice, we sought to determine whether overexpression could protect against growth impairment in mice with inflammatory arthritis. We first bred Col2a1-NPPC–transgenic mice onto the KRN TCR background and then onto the K/BxN background (Figure 3A).

In K/BxN TCR mice that overexpressed CNP...
(K/BxN TCR,Col2a1-NPPC–transgenic mice), no growth retardation was seen (P < 0.05 by F test) (Figure 3B), growth plates were increased in width (Figure 3C and online at http://www.case.edu/artsci/biol/skeletal/24114569s.html), and long bones were increased in length (Figure 3D) compared to K/BxN TCR arthritic mice. The increase in growth plate width in mice with the Col2a1-NPPC transgene was associated with an increase in BrdU incorporation (http://www.case.edu/artsci/biol/skeletal/24114569s.html). CNP overexpression reduced the clinical arthritis score in the K/BxN TCR mice, with a mean ± SD arthritis score of 4.37 ± 1.38 in CNP-overexpressing mice (n = 8) compared to 8.66 ± 3.26 in the K/BxN TCR mice (n = 14) (P < 0.05 by t-test).

CNP overexpression also reduced the severity of articular cartilage inflammation and damage in the K/BxN TCR mice (Figure 3E). Thirteen-week-old male mice with CNP overexpression had better articular cartilage chondrocyte distribution and organization and cartilage matrix content than the controls. Although CNP overexpression appeared to protect articular cartilage against damage caused by inflammation (Figures 4A–C), overexpression did not reduce inflammation-
related changes that occurred in the synovium (Figure 4D).

CNP overexpression enhances signal transduction via NPR-B and reduces the sensitivity of this signaling pathway to the proinflammatory cytokines TNFα and IL-1β. We performed cGMP enzyme-linked immunosorbent assays, real time qPCR, and Western blotting using cultures of rib cartilage primary chondrocytes harvested from Col2a1-NPPC–transgenic and WT control mice. The mean ± SD intracellular cGMP level in the Col2a1-NPPC–transgenic mouse chondrocytes was ~8 ± 0.7-fold higher than in the WT mouse chondrocytes, confirming enhanced CNP signaling. In Col2a1-NPPC–transgenic mouse chondrocytes, levels of protein and mRNA for NPR-B (Npr2) were increased (Figures 5, 6A, and online at http://www.case.
C-TYPE NATRIURETIC PEPTIDE PROTECTS CARTILAGE DURING ARTHRITIS

In Col2a1-NPPC–transgenic mouse chondrocytes, TNFα exposure had no effect on p38 MAPK phosphorylation, whereas IL-1β reduced p38 MAPK phosphorylation (Figure 6B). In contrast, both treatment with TNFα and treatment with IL-1β increased p38 MAPK phosphorylation in WT mouse chondrocytes. The expression of mRNA for the MMPs was either the same or less elevated in Col2a1-NPPC–transgenic mouse chondrocytes compared to WT mouse chondrocytes following exposure to these cytokines ($P > 0.05$) (Figure 6C).

It has been reported that IGF-1, IGF receptor I, and IGFBP-3 levels are diminished in serum of children with JIA, accounting for the delay in longitudinal growth. To understand whether CNP overexpression affects Igf1, Igf1r, and Igfbp3 mRNA expression, we performed real-time qPCR using primary chondrocyte cultures of rib cartilage harvested from Col2a1-NPPC–transgenic and WT mice. Col2a1-NPPC–transgenic mouse primary chondrocytes did not have increased levels of mRNA for Igf1 or Igf1r, but did have ~6–7-fold increased levels of mRNA for Igfbp3 compared to WT mouse chondrocytes. When exposed to TNFα (10 ng/ml) and IL-1β (10 ng/ml) overnight, Igf1 and Igf1r expression levels in chondrocytes were suppressed significantly in both Col2a1-NPPC–transgenic and WT mouse chondrocytes. Interestingly, Igfbp3 expression levels did not differ between Col2a1-NPPC–transgenic and WT mouse chondrocytes exposed to proinflammatory cytokines; in addition, Igfbp3 expression remained up-regulated in the Col2a1-NPPC–transgenic mouse chondrocytes (available online at http://www.case.edu/artsci/biol/skeletal/24114569s.html).

Earlier reports indicate that TNFα and IL-1β inhibit chondrocyte differentiation and DNA synthesis in growth plate and costal chondrocytes by suppressing Sox9 expression (33). It is known that CNP up-regulates Sox9 expression in chondrocytes (34,35). Thus, we sought to determine whether CNP overexpression in primary chondrocytes from Col2a1-NPPC–transgenic mice also up-regulates Sox9 mRNA expression. Results showed that in primary chondrocytes from Col2a1-NPPC–transgenic mice, Sox9 expression is up-regulated...

Figure 5. C-type natriuretic peptide overexpression in chondrocytes increases the expression of genes associated with cartilage anabolism.

Results of quantitative real-time polymerase chain reaction analysis of primary chondrocyte cultures recovered from Col2a1-NPPC–transgenic mice and control mice with the proinflammatory cytokines TNFα and IL-1β. TNFα and IL-1β each significantly reduced Npr2 mRNA expression in both Col2a1-NPPC–transgenic and WT mouse chondrocytes. However, Npr2 mRNA expression in Col2a1-NPPC–transgenic mouse chondrocytes after TNFα or IL-1β treatment was comparable to Npr2 expression in untreated WT mouse chondrocytes (Figure 6A).

In Col2a1-NPPC–transgenic mouse chondrocytes, TNFα exposure had no effect on p38 MAPK phosphorylation, whereas IL-1β reduced p38 MAPK phosphorylation (Figure 6B). In contrast, both treatment with TNFα and treatment with IL-1β increased p38 MAPK phosphorylation in WT mouse chondrocytes. The expression of mRNA for the MMPs was either the same or less elevated in Col2a1-NPPC–transgenic mouse chondrocytes compared to WT mouse chondrocytes following exposure to these cytokines ($P > 0.05$) (Figure 6C).

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Figure 5. C-type natriuretic peptide overexpression in chondrocytes increases the expression of genes associated with cartilage anabolism.

Results of quantitative real-time polymerase chain reaction analysis of primary chondrocyte cultures recovered from Col2a1-NPPC–transgenic mice and control mice for several genes associated with cartilage anabolism are shown. Values are the mean ± SD. * = $P < 0.05$.

Since CNP overexpression in Col2a1-NPPC–transgenic mice appeared to protect articular cartilage in vivo against the damaging effect of inflammation, we treated primary chondrocytes from Col2a1-NPPC–transgenic mice and control mice with the proinflammatory cytokines TNFα and IL-1β. TNFα and IL-1β each significantly reduced Npr2 mRNA expression in both Col2a1-NPPC–transgenic and WT mouse chondrocytes. However, Npr2 mRNA expression in Col2a1-NPPC–transgenic mouse chondrocytes after TNFα or IL-1β treatment was comparable to Npr2 expression in untreated WT mouse chondrocytes (Figure 6A).

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5-fold as compared to WT mouse chondrocytes. As expected, when chondrocytes were exposed to TNFα overnight, Sox9 expression was reduced in both WT and Col2a1-NPPC–transgenic mouse chondrocytes, but in Col2a1-NPPC–transgenic mouse chondrocytes that reduction brought Sox9 expression to the level seen in untreated WT mice (Figure 6D). We suggest that CNP overexpression in Col2a1-NPPC–transgenic mice protected against down-regulation of Sox9 expression levels during TNFα exposure.

**DISCUSSION**

It has been posited that in children with JIA the inhibitory effects of proinflammatory cytokines (IL-6, TNFα, and IL-1β) on growth plate chondrocytes contribute to growth suppression (36,37). Elevation of proinflammatory cytokines (e.g., TNFα and IL-1β) in synovial fluid has also been associated with destruction of growth plates adjacent to an arthritic joint. Proinflammatory cytokines decrease the width of the proliferating zone in growth plate cartilage, which leads to a decrease in endochondral bone growth. IL-1β has been shown to reduce expression of cartilage collagens and proteoglycans (13,38).

In this study, we tested whether the growth-promoting effects of the CNP/NPR-B signaling pathway would lessen the severity of growth failure and cartilage degeneration in a mouse model of inflammatory arthri-
CNP overexpression protects growth and prevents joint damage by diminishing chronic inflammation. Consequently, studies investigating the antiinflammatory effects of CNP in models of arthritis are warranted. While our data demonstrate that linear growth retardation in K/BxN TCR mice can be prevented by CNP overexpression, it remains to be determined whether this protective mechanism is relevant in human arthritis. The role of CNP in cartilage integrity and growth is an area of active research, and further studies are needed to elucidate its exact role in the pathophysiology of arthritis.
effect will be observed in other animal models of inflammation and whether pharmacologic manipulation of the CNP signaling pathway will have similar effects.

Reduction in linear growth is one complication of joint inflammation. Another common complication is widening of the metaphysis, which has been attributed to neovascularization and VEGF overexpression in the growth plate. CNP may play a role in this neovascularization, since chondrocytes that overexpress CNP were shown to produce increased Vegf mRNA levels (Figure 5), and VEGF immunostaining revealed an area that encompasses hypertrophic and proliferating chondrocytes, as well as the trabecular bone where the blood vessels reside (available online at http://www.case.edu/artsci/biol/skeletal/24114569s.html). Consequently, Col2a1-NPPC–transgenic mice developed widening at the ends of their long bones. We did not observe worsening periarticular overgrowth in the K/BxN TCR, Col2a1-NPPC–transgenic mice, perhaps because of the protective effect of CNP overexpression on the chondrocyte response to inflammatory cytokines. However, further studies are needed. An analog of CNP has been shown to improve skeletal growth in mice harboring a knockin Fgfr3 allele that causes thanatophoric dysplasia in humans (48). It will be interesting to determine whether this analog will be able to improve the cartilage phenotype in K/BxN TCR mice.

CNP overexpression by mouse chondrocytes prevented endochondral growth delay and reduced articular cartilage damage in a mouse model of systemic inflammatory arthritis. The likely mechanism for this effect is a cell-autonomous increase in chondrocyte differentiation, proliferation, hypertrophy, and matrix production, and a cell-autonomous resistance to the growth-suppressive effects of proinflammatory cytokines. These data suggest that the CNP/NPR-B pathway may represent a novel therapeutic target to preserve growth plate and joint cartilage integrity during systemic inflammatory diseases. Although our findings are of particular relevance to JIA, their impact may extend to other forms of inflammatory arthritis and other inflammatory diseases of childhood that stunt longitudinal growth, such as inflammatory bowel disease.

ACKNOWLEDGMENTS

We thank Diana Mathis, PhD, and Christophe Benoist, PhD (Joslin Diabetes Center/Harvard Medical School) for the generous gift of K/BxN T cell receptor mice. We also thank Yoshihiko Yamada, PhD (National Institute of Dental and Craniofacial Research) for the Col2a1 promoter and enhancer construct pKNI85. We gratefully acknowledge Arnold Kaplan, PhD for use of the laboratories at the Skeletal Research Center and for discussing study results, providing help with data interpretation, and for critical review of the manuscript. We are also thankful for the generous assistance of Jean F. Welter, MD, PhD for scoring mouse joint cartilage, reviewing data, and helping with the manuscript.

REFERENCES

1. Chusho H, Tamura N, Ogawa Y, Yasoda A, Suda M, Miyazawa T, et al. Dwarfism and early death in mice lacking C-type natriuretic peptide. Proc Natl Acad Sci U S A 2001;98:4016–21.
2. Yasoda A, Komatsu T, Chusho H, Miyazawa T, Ozasa A, Miura M, et al. Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. Nat Med 2004;10:80–6.
3. Miyazawa T, Ogawa Y, Chusho H, Yasoda A, Tamura N, Komatsu Y, et al. Cyclic GMP-dependent protein kinase II plays a critical role in C-type natriuretic peptide-mediated endochondral ossification. Endocrinology 2002;143:3604–10.
4. Tsuji T, Kunieda T. A loss-of-function mutation in natriuretic peptide receptor 2 (Npr2) gene is responsible for disproportionate dwarfism in cn/cn mouse. J Biol Chem 2005;280:14288–92.
5. Bartels CF, Bukulmez H, Padayatti P, Rheke DK, van Ravenswaaij-Arts C, Pauli RM, et al. Mutations in the transmembrane natriuretic peptide receptor NPR-B impair skeletal growth and cause acromesomelic dysplasia, type Maroteaux. Am J Hum Genet 2004;75:27–34.
6. Olney RC, Bukulmez H, Bartels CF, Prickett TC, Espiner EA, Potter LR, et al. Heterozygous mutations in natriuretic peptide receptor-B (NPR2) are associated with short stature. J Clin Endocrinol Metab 2006;91:1229–32.
7. Bocciardi R, Giorda R, Buttgereit J, Gimelli S, Divizia MT, Beri S, et al. Overexpression of the C-type natriuretic peptide (CNP) is associated with overgrowth and bone anomalies in an individual with balanced t(2;7) translocation. Hum Mutat 2007;28:724–31.
8. Dartoft G, Ostlund H, Jorgensen PH, Andrressen TT. Glucocorticoid treatment or food deprivation counteract the stimulating effect of growth hormone on rat cortical bone strength. Acta Paediatr 1992;81:912–7.
9. Kaufmann S, Jones KL, Wehenrebng WB, Culler FL. Inhibition by prednisone of growth hormone (GH) response to GH-releasing hormone in normal men. J Clin Endocrinol Metab 1988;67:1258–61.
10. White PH. Growth abnormalities in children with juvenile rheumatoid arthritis. Clin Orthop Relat Res 1990;259:46–50.
11. Bacon MC, White PH, Raiten DJ, Craft N, Margolis S, Levander OA, et al. Nutritional status and growth in juvenile rheumatoid arthritis. Semin Arthritis Rheum 1990;20:97–106.
12. Liem JJ, Rosenberg AM. Growth patterns in juvenile rheumatoid arthritis. Clin Exp Rheumatol 2003;21:663–8.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Bukulmez had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Bukulmez, Khan, Haqqi, Warman.

Acquisition of data. Bukulmez, Khan, Bartels, Ortiz-Lopez.

Analysis and interpretation of data. Bukulmez, Khan, Murakami, Sattar, Haqqi, Warman.
13. MacRae VE, Farquharson C, Ahmed SF. The pathophysiology of the growth plate in juvenile idiopathic arthritis. Rheumatology (Oxford) 2006;45:11–9.

14. Simon D, Lucidarme N, Pricer AM, Ruiz JC, Czernichow P. Treatment of growth failure in juvenile chronic arthritis. Horm Res 2002;58 Suppl 1:28–32.

15. Takah K, Hashimoto J, Hayashida K, Shi K, Takano H, Tsuboi H, et al. Early closure of growth plate causes poor growth of long bones in collagen-induced arthritis rats. J Musculoskelet Neuronal Interact 2002;2:344–51.

16. De Benedetti F, Mezza C, Martini A. Role of interleukin-6 in growth failure: an animal model. Horm Res 2002;58 Suppl 1:24–7.

17. Mangialiao S, Ji H, Korganow AS, Kouskoff V, Benoist C, Mathis D. The arthritogenic T cell receptor and its ligand in a model of spontaneous arthritis. Arthritis Rheum 1999;42:2517–23.

18. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-specific disease provoked by systemic autoimmunity. Cell 1996;87:811–22.

19. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. A new mouse model of rheumatoid arthritis: organ-specific disease provoked by systemic autoimmunity. Rymuuchi 1997;37:147.

20. Yamada Y, Miyashita T, Savagner P, Horton W, Brown KS, Mathis D, et al. Regulation of the collagen II gene in vitro and in transgenic mice. Ann N Y Acad Sci 1990;580:81–7.

21. Ji H, Guaguier D, Ohmura K, Gonzalez A, Duchatelle V, Danoy P, et al. Genetic influences on the end-stage effector phase of arthritis. J Exp Med 2001;194:321–30.

22. Ji H, Ohmura K, Mahmood U, Lee DM, Hofhuis FM, Boackle SA, et al. Arthritis critically dependent on innate immune system players. Immunity 2002;16:57–68.

23. Ji H, Pettit A, Ohmura K, Ortiz-Lopez A, Duchatelle V, Degott C, et al. Critical roles for interleukin 1 and tumor necrosis factor α in antibody-induced arthritis. J Exp Med 2002;196:76–87.

24. Monach P, Hattori K, Huang H, Hyatt E, Morse J, Nguyen L, et al. The K/BxN mouse model of inflammatory arthritis: theory and practice. Methods Mol Med 2007;136:269–82.

25. Monach PA, Mathis D, Benoist C. The K/BxN arthritis model. Curr Protoc Immunol 2008;15:15.22.

26. Pettit AR, Ji H, von Stechow D, Muller R, Goldring SR, Choi Y, et al. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. Am J Pathol 2001;159:1689–99.

27. Mainil-Varlet P, Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. Potent inhibition of early closure of growth plate causes poor growth of long bones in collagen-induced arthritis rats. J Musculoskelet Neuronal Interact 2002;2:344–51.

28. Gartland A, Mechler J, Mason-Savas A, MacKay CA, Mailhot G, Oppeneer T, et al. Evaluation of the therapeutic potential of a CNP analog in a Fgfr3 mouse model recapitulating achondroplasia. Am J Hum Genet 2012;91:1108–14.