Interaction of fibroblast growth factor and C-natriuretic peptide signaling in regulation of chondrocyte proliferation and extracellular matrix homeostasis

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Accepted 8 August 2005
Journal of Cell Science 118, 5089-5100 Published by The Company of Biologists 2005
doi:10.1242/jcs.02618

Summary

Overexpression of C-natriuretic peptide (CNP) in cartilage partially rescues achondroplasia in the mouse. Here, we studied the interaction of fibroblast growth factor (FGF) and CNP signaling in chondrocytes. CNP antagonized FGF2-induced growth arrest of rat chondrosarcoma (RCS) chondrocytes by inhibition of the Erk mitogen activated protein kinase pathway. This effect of CNP was protein kinase G-dependent and was mimicked by the cGMP analog pCPT-cGMP. FGF2-mediated activation of both MEK and Raf-1 but not Ras or FRS2 was abolished by CNP demonstrating that CNP blocks the Erk pathway at the level of Raf-1. CNP also counteracted the FGF2-mediated degradation of RCS extracellular matrix. CNP partially antagonized FGF2-induced expression, release and activation of several matrix-remodeling molecules including matrix metalloproteinase 2 (MMP2), MMP3, MMP9, MMP10 and MMP13. In addition, CNP compensated for FGF2-mediated matrix loss by upregulation of matrix production independent of its interference with FGF signaling. We conclude that CNP utilizes both direct and indirect ways to counteract the effects of FGF signaling in a chondrocyte environment.

Key words: Fibroblast growth factor, C-natriuretic peptide, Mitogen-activated protein kinase, Growth arrest, Extracellular Matrix, Chondrocyte

Introduction

Activating mutations in fibroblast growth factor receptor-3 (FGFR3) result in human dwarfisms ranging from the mildest condition, hypochondroplasia, to lethal thanatophoric dysplasia (Passos-Bueno et al., 1999). Although the mechanism of FGFR3 action remains obscure, both chondrocyte proliferation and differentiation appear to be disturbed leading to remarkably short growth plate cartilage (Wilcox et al., 1998). Recently, several reports have shed light on FGFR3 action in cartilage by demonstrating that sustained activation of the Erk MAP kinase pathway accounts for the inhibitory effect of FGF signaling on chondrocyte proliferation and cartilage matrix production (Raucci et al., 2004; Krejci et al., 2004; Murakami et al., 2004; Yasoda et al., 2004).

Natriuretic peptides comprise a family of three structurally related peptides: atrial natriuretic peptide, brain natriuretic peptide and C-natriuretic peptide (CNP) (Rosenzweig and Seidman, 1991). Unlike atrial and brain natriuretic peptides that act via the bloodstream (Mukoyama et al., 1991), CNP acts locally in a variety of tissues (Koller et al., 1992). CNP has recently emerged as an important regulator of longitudinal bone growth. Genetic ablation of CNP or its signaling components results in severe skeletal dysplasias caused by reduced chondrocyte proliferation and differentiation (Chusho et al., 2001; Miyazawa et al., 2002; Tamura et al., 2004). Loss-of-function mutations in the CNP cognate receptor, natriuretic peptide receptor (NPR)-B, causes an achondroplasia-like dwarfism in mice and acromesomelic dysplasia, type Maroteaux in humans (Tsuji and Kunieda, 2005; Bartels et al., 2004). In limb organ culture, CNP upregulates chondrocyte proliferation and cartilage matrix production (Yasoda et al., 1998; Mericq et al., 2000). However, the molecular basis of CNP action in cartilage remains unclear. Since the effects of CNP and FGF on endochondral bone growth appear to oppose each other, it is reasonable to propose a direct interaction of their signaling pathways. In fact, Yasoda et al. (Yasoda et al., 2004) demonstrated that overexpression of CNP in the cartilage of a mouse model for achondroplasia partially rescues the disease phenotype by correcting the decreased extracellular matrix synthesis through inhibition of the Erk pathway.

Although the CNP-mediated reversal of FGF signaling in cartilage was demonstrated (Yasoda et al., 2004), the underlying mechanism remains unclear. In this study, we determined the molecular mechanism of FGF and CNP signaling interactions in a chondrocyte environment as well as

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Zydus EX1019
U.S. Patent No. 12,233,106

the effects of such interactions on chondrocyte proliferation and extracellular matrix homeostasis.

Materials and Methods

Cell growth, cell cycle and cGMP analyses

Cells were propagated in DMEM media (Gibco, Gaithersburg, MD) containing 10% FBS (Atlanta Biological, Nordcross, GA) and antibiotics. For proliferation studies, cells were seeded in 24-well plates and treated as desired for 30 minutes prior to FGF2 (R&D Systems, Minneapolis, MN) addition, cultivated for 72 hours and counted. Chlorpromazine, EHNA, 4-{[3′,4′-(methylenedioxy)-benzyl]amino}-6-methoxyqinazoline, zaprinast, IBMX, CNP and Rp-8-pCPT-cGMPS were from Calbiochem (San Diego, CA); pCPT-cGMP and Rp-8-Br-PET-cGMPS were from Sigma (St Louis, MO); and heparin was from Gibco. For cell cycle analysis, cells treated with FGF2, CNP and pCPT-cGMP for 24 hours were analyzed for DNA content as described elsewhere (Krejci et al., 2004). Intracellular cGMP concentration was determined by ELISA (R&D Systems).

Western immunoblotting (WB) and immunoprecipitation

Cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 25 mM NaF, 0.1 mM DTT, 1 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 1 mM PMSF, 8 mM β-glycerolphosphate, 10 mM Na_3VO_4, 1 μg/ml aprotinin). Lysates (20 μg) were resolved by SDS-PAGE and transferred onto a PVDF membrane. Blots were visualized by luminescence (Amersham, Piscataway, NJ). The following antibodies were used: actin, Erk2, MEK1, B-Raf, FRS2, NPR-B, NPR-C, MMP9, MMP13 (Santa Cruz Biotechnology, Santa Cruz, CA); Raf-1 (Transduction Laboratories, Lexington, KY); P-Raf-1^{S338}, Erk1/2, P-Erk1/2$^{T183/Y185}$, P-MEK$^{S217/221}$, P-FRS2^{Y196} (Cell Signaling, Beverly, MA); Ras, 4G10 (Upstate Biotechnology, Lake Placid, NY); MMP10 (Lab Vision, Fremont, CA); MMP2 and MMP3 (Chermicon, Temecula, CA). For immunoprecipitation, 500 μg of total protein was incubated with 2 μg of Raf-1 (Transduction Laboratories), B-Raf or FRS2 antibody (Santa Cruz) for 2 hours at 4°C. Immunocomplexes were purified using A/G-agarose (Santa Cruz). Western blotting signal was quantitated by determining the integrated optical density (I.O.D.) of given band using the Scion Image software (Scion Corporation, Frederick, MA).

Signal transduction studies

Cells were serum starved for 12 hours before treatment with FGF2 for 30 minutes. When CNP, pCPT-cGMP, KT5823 (Calbiochem) or U0126 (Cell Signaling) were used, cells were pretreated for 30 minutes prior to FGF2 treatment. The phosphorylation status of Raf-1, Erk and MEK was detected by WB using the antibodies described above. Erk activity was determined using an Erk kinase assay kit (Cell Signaling). Briefly, Erk was immunoprecipitated from 200 μg of total protein and incubated with recombinant Elk-1 in the presence of ATP. Phosphorylation of Elk-1 at Ser383 was determined by WB. For the Raf kinase assay, Raf-1 or B-Raf immunocomplexes were washed with kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$). The kinase reaction was performed for 30 minutes at 30°C in the presence of 20 μM ATP and 500 ng of recombinant MEK1 (Santa Cruz) in 40 μl of kinase buffer. MEK1 phosphorylation at Ser217/221 was determined by WB. Ras activation was determined using Ras activation assay (Upstate Biotechnology). Briefly, active Ras was purified from cell lysates using the GST fusion protein containing the Ras-binding domain of Raf-1, and detected by WB.

RT-PCR and real-time RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) including DNase I treatment. cDNA was synthesized from 2 μg of total RNA. Real-time RT-PCR conditions are described in detail elsewhere (Krejci et al., 2004). The authenticity of real-time RT-PCR products was verified by agarose electrophoresis. Table 1 lists the PCR primers used.

Extracellular matrix studies

For Alcian blue staining, cells were treated with CNP and pCPT-cGMP for 30 minutes prior to FGF2 treatment for 72 hours, fixed with 4% paraformaldehyde and stained with Alcian blue for 30 minutes. For MMP activity, cells were stimulated with FGF2 and CNP for 48 hours in media containing 10% FBS followed by 24 hours of cultivation in serum-free media. Conditioned culture media was normalized to cell number, concentrated ten times and subjected to non-reducing SDS-PAGE using gels containing either 1 mg/ml gelatin (Sigma) or casein (Bio-Rad, Hercules, CA). Resolved proteins were renatured by 2.5% Triton X-100 and incubated in activation buffer (50 mM Tris-HCl pH 7.5, 10 mM $CaCl_2$) for 19 hours (gelatin) or 96 hours (casein) at 37°C. Gels were stained with Coomassie Brilliant Blue R250 (Bio-Rad). MMP activity was confirmed by incubation of gels in activation buffer containing either the MMP inhibitor EDTA (30 mM) or the serine protease inhibitor PMSF (2 mM). For evaluation of matrix synthesis, cells were treated with FGF2 and CNP for 48 or 72 hours in presence of 10 μCi/ml of [^{35}S]sulfate or 5 μCi/ml of [^{3}H]proline (Perkin Elmer, Boston, MA). For pulse-chase experiments, cells were labeled with [^{35}S]sulfate or [^{3}H]proline for 12 hours, washed and treated with FGF2 and CNP for 48 or 72 hours. Following the cultivation period, cells were harvested and incorporated radioactivity was determined by liquid scintillation. For evaluation of extracellular matrix mass, cells were treated with CNP for 48 hours, scraped or detached by Trypsin-EDTA (Gibco; 10 minutes, 37°C) and both the wet and dry mass of the cell pellets were determined.

Mass spectrometry

The protein band was excised from the acrylamide gel and subjected to tandem mass spectrometric analysis after in-gel trypsin digestion. Recovered peptides were analyzed by nanobore LC (Vydac C8 MS 0.3 mm × 15 cm; Vydac, Hesperia, CA) with electrospray sample introduction into an ion-trap mass spectrometer (Thermo Finnigan LCQ Deca XP; Thermo, San Jose, CA). To identify peptides, the tandem spectra were analyzed by searching against a National Center for Biotechnology Information-derived database.

Results

CNP counteracts FGF2-mediated growth arrest of RCS cells via inhibition of the Erk MAP kinase pathway

Rat chondrosarcoma (RCS) cells respond to FGF by arresting their growth in the G1 phase of cell cycle (Aikawa et al., 2001). Treatment of cells with FGF2 together with CNP led to partial reversal of FGF2-mediated growth arrest as evidenced by both cell counting and measurement of the cell cycle distribution (Fig. 1). C-natriuretic peptide binds and activates NPR-B guanylyl cyclase resulting in rapid elevation of intracellular cGMP (Suga et al., 1992; Hagiwara et al., 1994). Consequently, the biological actions of CNP appear to be mediated by cGMP (Drewett et al., 1994). We therefore asked whether the CNP effect on FGF2-mediated growth arrest can be reproduced by a membrane permeable cGMP analog pCPT-cGMP (Miller et al., 1973). Fig. 1 shows that pCPT-cGMP reversed, similarly to CNP, the FGF2-mediated growth arrest. For the subsequent experiments, 10 ng/ml of FGF2, 0.2 μM CNP and 200 μM pCPT-cGMP were used unless otherwise noted.

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Table 1. PCR primers used for RT-PCR and real-time RT-PCR analyses

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Product (bp)	Annealing temperature (°C)
Adamts1	GGTCACATCATTCCTGGACAACG	TGTTCACACACTTGCCGCTG	293	65
Adamts4	AGTCCCATTTCCCGCAGAAC	TGGTTTGTCTAAGAGGCAGTGTCG	216	65
Adamts5	AACGAGTTTACGGGGATGGGTC	ATGGAAGAGTCAGCCACCAAGAGG	301	65
Aggrecan	TGGGTGGATGCAGAGAGACG	TTCGAGGCTCTTCCCAGTCC	481	63
Biglycan	AACCGTATCCGCAAAGTGCC	AGTTCATTCAGGGTCTCAGGGAG	197	63
Brevican	TCAAAGGGGTCGTCTTCCTCTAC	AACAGGCTTCTCGTGGGTTCTG	213	63
Clusterin	CTGGCATCATACATACGCTTTTCC	ACTGTCGGGTCATCTTCACCTTC	301	63
Cspg3	GGAACCTACAGTGGCTTTGGAAG	TATCAGAGTGCTGGGGATGTCC	334	63
Cspg4	ACCCTCATCAGGAAACCCTCAC	TCACCGCCAATGTGGATAGG	310	63
Cspg5	TGACTTCACCCCCTTTGATGAG	CCAGAGTATGCTGTGGAGGAACTG	221	63
Cspg6	TTCGTCCAGAGCAGCGATTG	CCAGCACTTTCAAGGAGATTCATC	220	63
Decorin	TGATTGTCATAGAACTGGGCGG	ATTCCTTTCAGGCTGGCTGC	205	63
Fibronectin	GCTCCTTCACAGATGTCCGAAC	CCGTTTGAGTTGCCACCGTAAG	220	60
Frs2α	GAGCCCGTTGTAGAAAGGAATAGTC	ATCGCTGTCATAGCCAGTGTCC	537	65
Frs2β	CCCTCCACAACAACAACAACG	GGTTTCATCTTCCTCACCATCAGG	305	65
Gapdh	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	452	65
Glypican	TCTGCTGCCCGATGACTATCTG	TGACAGCCCGAGAACATTCTG	206	63
Lumican	CAACAATTTGACCGAGTCCGTG	TTAAATGAGTTTCCAGGCACGC	381	63
Mmp2	GACCTTGACCAGAACACCATCG	GCTGTATTCCCGACCGTTGAAC	474	63
Mmp3	ACCAAGAGAGAGTGTGGATTCTGC	TTGAGAGAGATGGAAACGGGC	385	65
Mmp7	AAGAGGGTTAGTTGGGGGACTGC	GGAGTAAGTGTGGCTCAGGAGG	419	63
Mmp8	GGACATTCCTTGGGACTCTCTCAC	TCAAATCTCAGGTGGGGGTCAC	200	63
Mmp9	AGAGATGTGCGTCTTCCCCTTC	TCAGGTTTAGAGCCACGACCATAC	318	55
Mmp10	CCCTGGATTTTATGGAGATGCTC	TTGGCTCGTGGAGAACCTGTAGAC	175	60
Mmp11	TTCAAGGCGGGCTTTGTGTG	TCCACGAAGGAAGTAGGCATAGC	426	63
Mmp12	AGATTCATAAAGGCGAGGCTGAC	TTTCACTGGGGCTCCATAGAGG	350	60
Mmp13	GGTCTTCTGGCACACGCTTTTC	ATGGCATCAAGGGATAGGGCTG	332	63
Mmp14	AGGAATAACCAAGTGATGGACGG	ATGAATGAGCCTCTGGGAGACTC	371	60
Perlecan	CAATACGGAGCCTACTTTCAGGAC	CTAATGAAGTCTTTGCTGCGGC	179	63
Syndecan1	TGACTTCACTGCTCGGGACAAG	GTTTCATCCTCCACAACCTCTTTG	261	63
Syndecan2	TCTGACAACATCCCAACTGATTCC	AACTTCCGTCCGCTTGAACAG	235	63
Syndecan4	GGACCTTCCCTGAGGTGATTTC	TGCTGGACATGGACACTTTGTTG	183	63
Versican	TAACTGTGAAGCCAGCCGTAGC	TCCCCCATCTGTGAAACTTGG	293	63

Since FGF-mediated growth arrest of RCS cells requires Erk MAP kinase (Raucci et al., 2004; Krejci et al., 2004), we determined the effect of CNP treatment on FGF2-mediated activation of Erk. Cells were pretreated with either CNP or pCPT-cGMP for 30 minutes prior to FGF2 addition and Erk phosphorylation and kinase activity was assayed 30 minutes later. Both CNP and pCPT-cGMP abolished FGF2-mediated Erk activation (Fig. 2A,B).

FGF2 treatment of RCS cells elicits sustained Erk activation lasting at least 24 hours (Krejci et al., 2004). We thus determined the effect of CNP and pCPT-cGMP on long-term FGF2-mediated Erk activation. Cells were treated with FGF2 alone or together with CNP or pCPT-cGMP for different times up to 12 hours and analyzed for Erk phosphorylation by WB. In short-term FGF2 treatment (up to 1 hour), Erk phosphorylation was nearly completely inhibited by CNP or pCPT-cGMP in contrast to longer FGF2 treatment (2-12 hours), where both CNP and pCPT-cGMP lowered Erk activation by approximately 50% (Fig. 2C).

Duration of the cGMP response in CNP-treated RCS cells

Having found that CNP inhibits FGF2-mediated long-term Erk activation only partially, we determined the time duration of the CNP signal. CNP treatment led to rapid accumulation of intracellular cGMP followed by a slow decline and stabilization from two to 12 hours (Fig. 3A). This decline was not due to the lack of CNP stability since treatment of naïve cells with CNP-containing media used previously for five hours accumulated cGMP similar to fresh CNP (not shown). Addition of fresh CNP to cells chronically treated with CNP failed to elevate cGMP levels (Fig. 3B). The protein quantities of the CNP cognate receptor, NPR-B, and the clearance receptor, NPR-C (Suga et al., 1992), were unaffected by prolonged CNP treatment (not shown) suggesting that NPR-B desensitization accounts for the decline in cGMP levels in cells chronically treated with CNP.

The duration of cGMP signal is limited by its rapid degradation by phosphodiesterases (PDE) including PDE1-3, 5, 6, and 9-11 (Lucas et al., 2000). We therefore evaluated a panel of PDE inhibitors for their effect on both CNP-mediated cGMP accumulation and reversal of FGF2 growth arrest. Cells were pretreated with inhibitors of PDE1 (chlorpromazine; 1-100 μM) (Marshak et al., 1985), PDE2 (EHNA; 1-100 μM), PDE5 (4-{[3′,4’-(methylenedioxy)benzyl]amino}-6-methoxyqinazoline; 1-100 μM) (Takase et al., 1994), PDE5, PDE6 and PDE9 (zaprinast; 1-100 μM), or the nonselective PDE inhibitor IBMX (0.01-1.0 mM) (Dousa et al., 1999) for 30 minutes before addition of CNP. The intracellular cGMP was determined five hours later. With the exception of chlorpromazine, all the PDE inhibitors increased cGMP levels when compared with CNP alone (Fig. 3C). However, no additional effect of PDE inhibitors on CNP-mediated reversal of FGF2 growth arrest was observed (not shown).

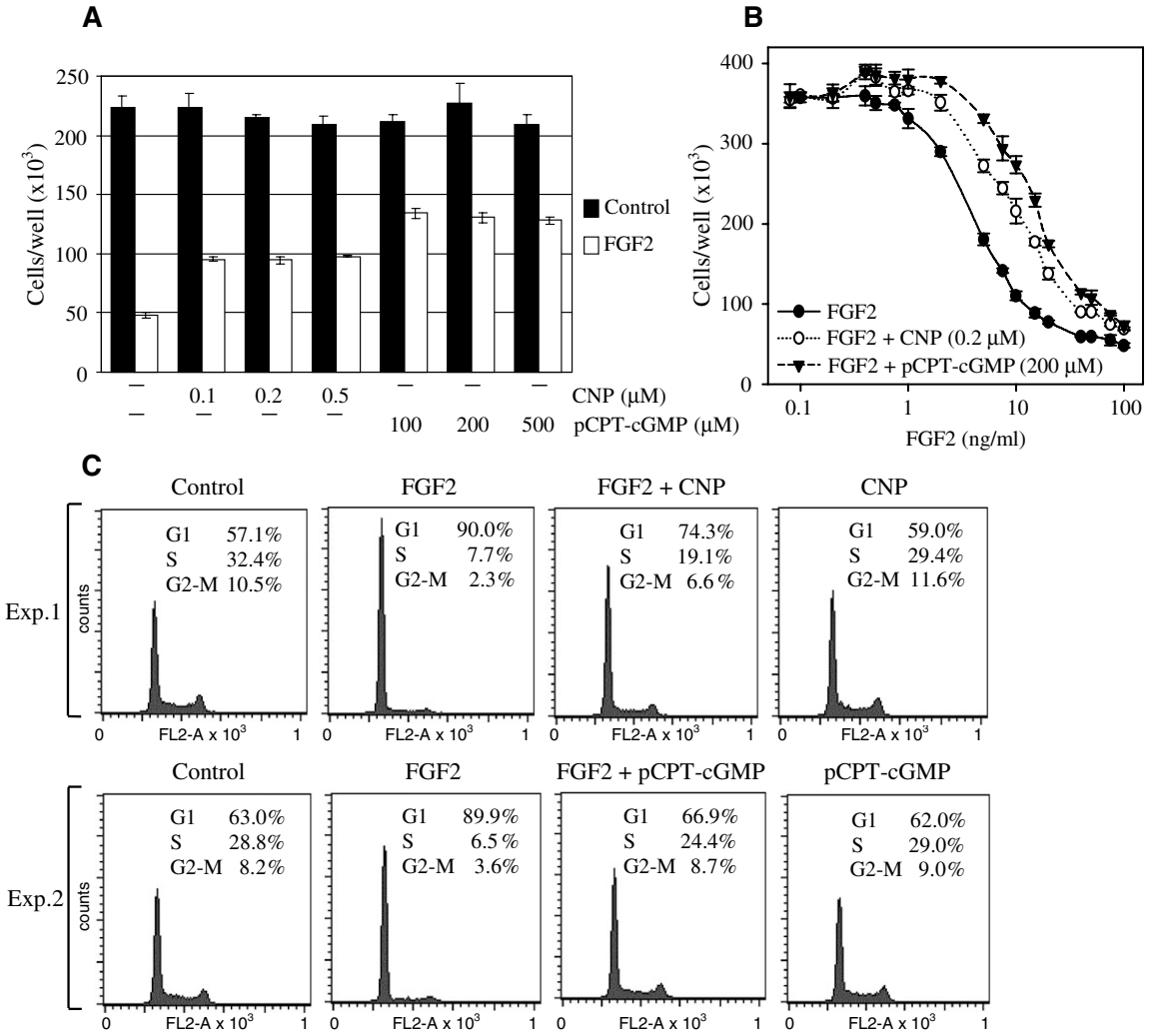

Fig. 1. CNP antagonizes FGF2-mediated inhibition of RCS proliferation. (A,B) Cells were treated with FGF2 and either CNP or pCPT-cGMP for 72 hours and counted. Data represent the average from four wells with the indicated standard deviation. (B) Cells not treated with FGF2 were given an artificial value of 0.08 on logarithmic *x*-axis. (C) Cells were treated with FGF2 and either CNP (Experiment 1) or pCPT-cGMP (Experiment 2) for 24 hours and analyzed for DNA content by flow-cytometry. Note the FGF2-mediated accumulation of cells in the G1 phase of cell cycle that was partially reversed by CNP or pCPT-cGMP.

CNP inhibition of FGF2-mediated Erk activation is dependent on protein kinase G (PKG)

Cyclic GMP signals through activation of PKG, cGMP-gated ion channels (CGI) or cGMP-regulated PDE (Lucas et al., 2000). Chemical inhibition of PKG by specific inhibitor KT5823 (Kase et al., 1987) fully restored FGF2-mediated Erk activation in the presence of CNP (Fig. 4A). Of the synthetic cGMP analogs, only pCPT-cGMP (activator of PKG and CGI but not of PDE) antagonized FGF2 growth arrest in contrast to both Rp-8-pCPT-cGMPS (inhibitor of PKG and activator of CGI with no effect on PDE) and Rp-8-Br-PET-cGMPS (inhibitor of PKG and CGI and stimulator of PDE) (Sirotkin et al., 2000) that had no effect even at 500 μM (Fig. 4B).

CNP inhibits FGF2-mediated activation of the Erk pathway at the level of Raf-1

We next determined the level at which CNP targets the Erk pathway. Cells were treated with FGF2 and CNP for 30 minutes and probed for MEK, Raf and Ras activation. Upon FGF2 addition, MEK phosphorylation at Ser217/221 was completely abolished by CNP (not shown). Treatment with FGF2 led to Raf-1 activatory phosphorylation at Ser338 (Dhillon and Kolch, 2002) that was abolished by CNP (Fig. 5A). We were unable to detect Raf-1 kinase activity in cells treated with FGF2 alone (not shown). Since addition of heparin potentiates FGF2-mediated Erk activation in RCS cells (Krejci et al., 2004) the Raf-1 kinase assay was performed in cells treated with FGF2 together with heparin. FGF2-mediated Raf-1 kinase activity was only partially inhibited by CNP under these conditions (Fig. 5B). We also tested whether B-Raf is activated by FGF2. We found high B-Raf kinase activity in naïve cells even after 24 hours of serum starvation. However, no change in B-Raf activity was found when cells were treated with FGF2 and/or CNP, nor when heparin was used to increase FGF2 action (not shown). FGF2 treatment led to potent

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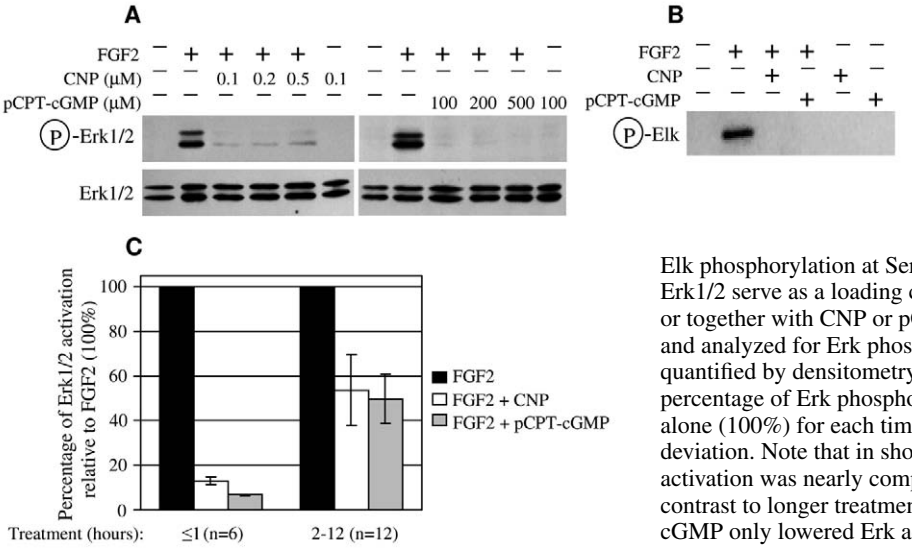

Fig. 2. CNP inhibits FGF2-mediated activation of Erk MAP kinase. (A,B) Serum-starved cells were treated with FGF2 and either CNP or pCPT-cGMP for 30 minutes and analyzed for Erk1/2 phosphorylation by WB (A) or Erk activity by kinase assay using Elk as a substrate (B). Elk phosphorylation at Ser383 was determined by WB. Levels of total Erk1/2 serve as a loading control. (C) Cells were treated with FGF2 alone or together with CNP or pCPT-cGMP for different times up to 12 hours and analyzed for Erk phosphorylation by WB. The WB signal was quantified by densitometry and normalized to total Erk expression. The percentage of Erk phosphorylation relative to cells treated with FGF2 alone (100%) for each time point was graphed with the indicated standard deviation. Note that in short-term FGF2 treatment (≤1 hour), Erk activation was nearly completely inhibited by CNP or pCPT-cGMP in contrast to longer treatments (2-12 hours) where both CNP and pCPT-cGMP only lowered Erk activation by approximately 50%.

activation of Ras that was not abolished by CNP treatment (Fig. 5C).

Kinase active FGFRs recruit the majority of their downstream signaling pathways via tyrosine phosphorylation of the docking protein FRS2 (Kouhara et al., 1997). Immunoprecipitation of FRS2 followed by WB with the 4G10 phosphotyrosine antibody showed increased signal upon FGF2 treatment that was not decreased by CNP (Fig. 6A). However, the prominent FRS2 band showed ~20 kDa upstream mobility shift in FGF2-treated cells that was reduced in FGF2/CNP-treated cells (Fig. 6A). Inhibition of FGF2-mediated Erk activation by the MEK inhibitor U0126 also eliminated the FRS2 mobility shift suggesting that Erk phosphorylates FRS2 (Fig. 6B). Interestingly, cells treated with CNP alone also showed a small but distinct FRS2 mobility shift that could be abolished by the PKG-specific inhibitor KT5823 (Fig. 6B,C).

Fig. 6A shows that a portion of FRS2 was visualized by the 4G10 antibody in untreated cells. Probing of cell lysates with an antibody that recognizes FRS2 only when phosphorylated at Tyr196 showed a uniform signal regardless of treatment with FGF2. By contrast, no signal was detected in untreated NIH3T3 cells whereas strong Tyr196 phosphorylation was induced by FGF2 (not shown).

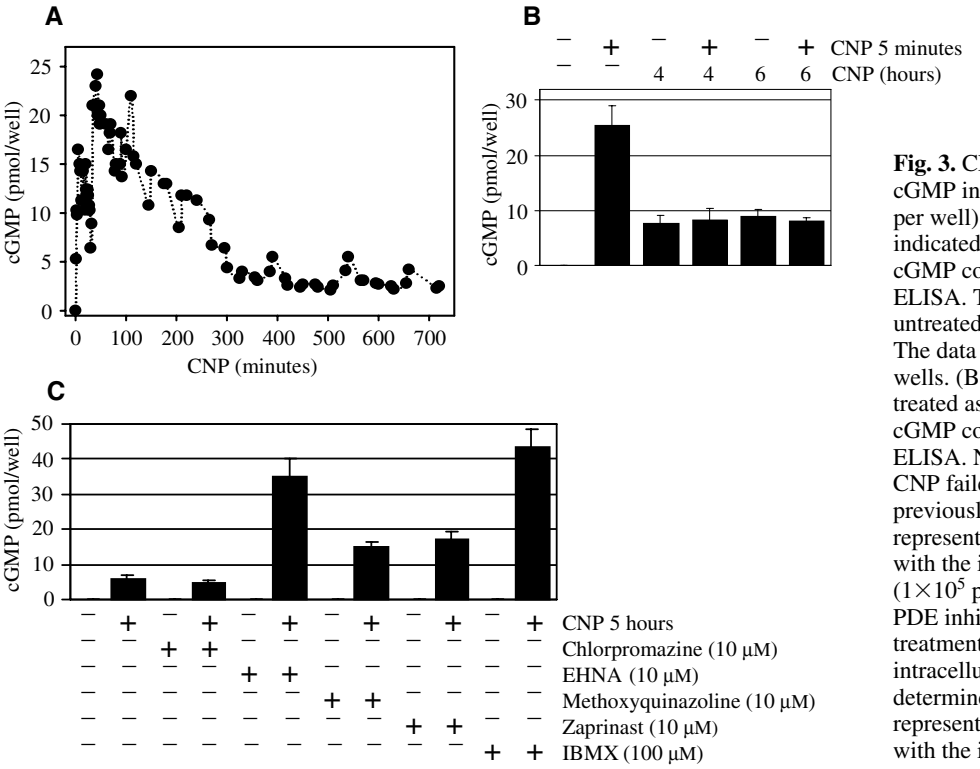

Fig. 3. CNP-mediated generation of cGMP in RCS cells. (A) Cells (1×10^5 per well) were treated with CNP for the indicated times and the intracellular cGMP concentration was determined by ELISA. The cGMP concentration in untreated cells was 0.023 pmol/well. The data represent the average from two wells. (B) Cells (1×10^5 per well) were treated as indicated and the intracellular cGMP concentration was determined by ELISA. Note that the addition of fresh CNP failed to induce cGMP in cells previously treated with CNP. The data represent the average from two wells with the indicated range. (C) Cells (1×10^5 per well) were pretreated with PDE inhibitors 30 minutes before treatment with CNP (5 hours) and the intracellular cGMP concentration was determined by ELISA. The data represent the average from two wells with the indicated range.

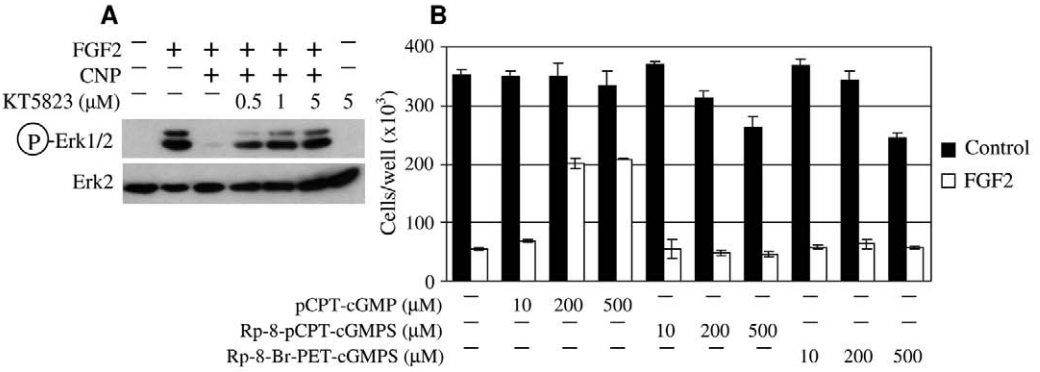

Fig. 4. CNP inhibits FGF2-mediated Erk MAP kinase activation via PKG. (A) Serum-starved cells were pre-treated with the PKG inhibitor KT5823 for 30 minutes before treatment with FGF2 and CNP (30 minutes) and analyzed for Erk phosphorylation by WB. Levels of total Erk2 serve as a loading control. (B) Cells were treated with FGF2 and cGMP analogs that either activate PKG (pCPT-cGMP) or inhibit PKG (Rp-8-pCPT-cGMPS; Rp-8-Br-PET-cGMPS) for 72 hours and counted. The data represent the average from four wells with the indicated standard deviation.

CNP counteracts the FGF2-mediated loss of RCS extracellular matrix

In thanatophoric dysplasia, an increased cell to matrix ratio can be observed in the growth plate cartilage, suggesting decreased matrix production (W.R.W., unpublished). Similarly, the mouse model of achondroplasia has decreased matrix synthesis that is increased by CNP or Erk inhibition (Yasoda et al., 2004). Treatment of RCS cells with FGF2 also results in a loss of their extracellular matrix (Krejci et al., 2004) suggesting that, apart from inhibition of proliferation, FGF signaling affects the homeostasis of chondrocyte extracellular matrix. We used the RCS model to discover how CNP and FGF signaling affect the extracellular matrix.

Cells were treated with FGF2 and CNP for 72 hours and the amount of extracellular matrix was assessed by Alcian blue staining. The loss of Alcian blue staining in FGF2-treated cells was restored by CNP (Fig. 7A) or pCPT-cGMP (not shown). During FGF2 treatment, a protein with molecular mass of approximately 300 kDa was abundantly released into the media by RCS cells. Using protein mass spectrometry, we identified

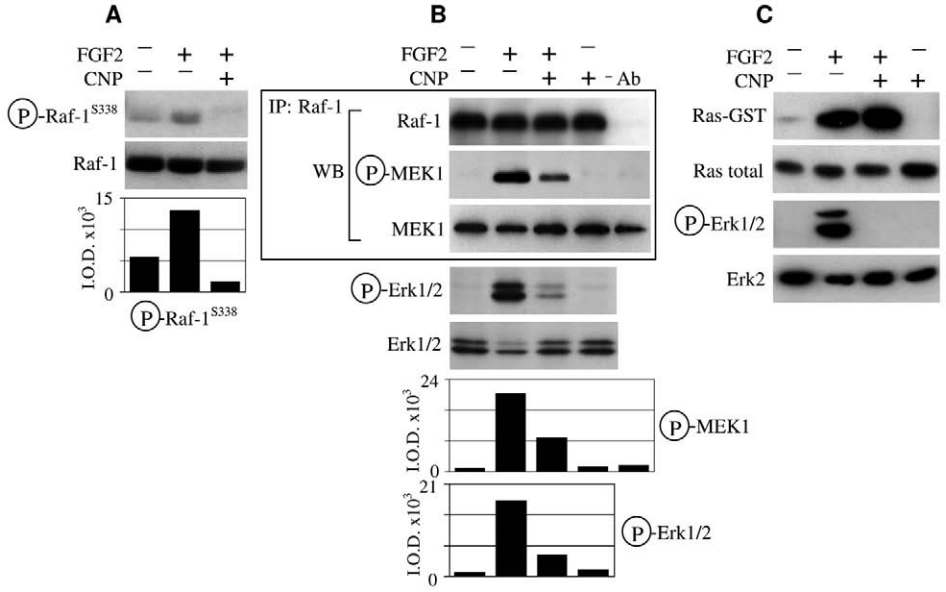

Fig. 5. CNP inhibits FGF2-mediated activation of the Erk MAP kinase pathway at the level of Raf-1. (A) Serum-starved cells were treated with FGF2 and CNP for 30 minutes and Raf-1 activation (phosphorylation at Ser338) was determined by WB. Levels of total Raf-1 serve as a loading control. The WB signal was quantified by densitometry and graphed. (B) Serum-starved cells were treated as indicated for 30 minutes in media containing 1 μg/ml of heparin. Raf-1 was immunoprecipitated (IP) and its kinase activity was determined by kinase assay using MEK1 as a substrate. MEK1 phosphorylation at Ser217/221 was determined by WB. Phosphorylated Erk1/2 was determined in total cell lysates used for Raf-1 IP. Levels of total Raf-1, MEK1 and Erk1/2 serve as a loading controls. '–Ab', no antibody used for IP. The WB signal was quantified by densitometry and graphed. (C) Serum-starved cells were treated as indicated for 30 minutes and activated Ras (Ras-GST) was determined as described in the Materials and methods. Erk1/2 phosphorylation was detected in total cell lysates used for the Ras activity assay. Levels of total Ras and Erk2 serve as loading controls.

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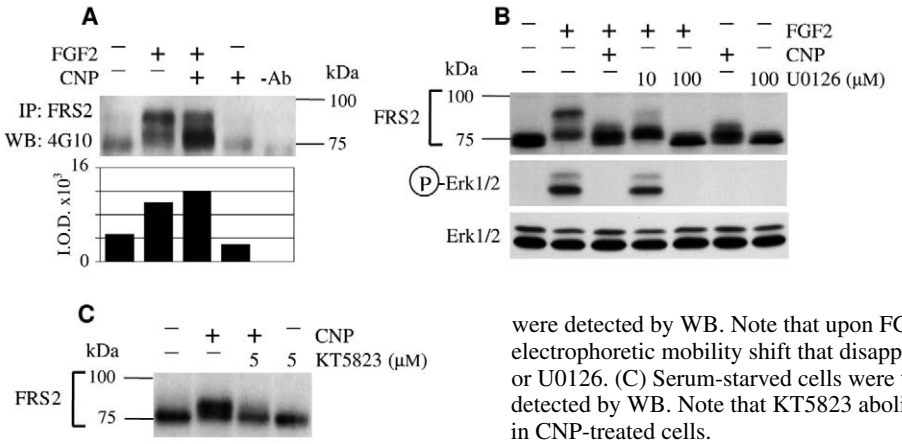

Fig. 6. Modulation of FRS2 phosphorylation by FGF2 and CNP. (A) Serum-starved cells were treated with FGF2 and CNP for 30 minutes and immunoprecipitated FRS2 was analyzed for tyrosine phosphorylation by WB with the 4G10 antibody. The WB signal was quantified by densitometry and graphed. (B) Serum-starved cells were treated as indicated for 30 minutes and FRS2, phosphorylated Erk1/2, and total Erk1/2 were detected by WB. Note that upon FGF2 treatment, FRS2 underwent an electrophoretic mobility shift that disappeared when Erk activation was prevented by CNP or U0126. (C) Serum-starved cells were treated as indicated for 30 minutes and FRS2 was detected by WB. Note that KT5823 abolishes the FRS2 electrophoretic mobility shift seen in CNP-treated cells.

this protein as fibronectin (Fig. 7B). Quantification of fibronectin mRNA by real-time RT-PCR revealed its potent upregulation by FGF2, which was significantly reversed by CNP (Fig. 7C).

We next investigated the mechanism of the FGF2-mediated matrix loss and its inhibition by CNP. We hypothesized that FGF2 triggers the degradation of the RCS extracellular matrix. Therefore, we asked whether FGF2 elevates expression and activity of molecules known to degrade the matrix. As determined by RT-PCR, 4 hours of treatment with FGF2 increased the quantity of matrix metalloproteinase 3 (*Mmp3*), *Mmp10*, *Mmp13* and a disintegrin-like and metalloproteinase with thrombospondin motif 1 (*Adamts1*) and *Adamts5* transcripts (Fig. 8A). The quantities of *Mmp8*, *Mmp11* and *Adamts4* transcripts were unchanged, and transcripts of *Mmp7*, *Mmp12* and *Mmp14* were not detected (not shown). The *Mmp2* and *Mmp9* mRNAs were also upregulated by FGF2 but this induction required at least 24 hours of treatment. We next used real-time RT-PCR to determine whether CNP interferes with the FGF2-mediated induction of these matrix-remodeling molecules. The MEK inhibitor U0126 was used to inhibit FGF2-mediated Erk activation completely. FGF2-mediated accumulation of *Mmp3*, *Mmp10* and *Mmp13* mRNAs was only weakly antagonized by CNP but was abolished by U0126 indicating the involvement of the Erk pathway. By contrast, induction of *Adamts1* was only partially sensitive to CNP and U0126; *Adamts5* induction was insensitive to both (Fig. 8B).

To identify which MMPs are likely to be involved in the

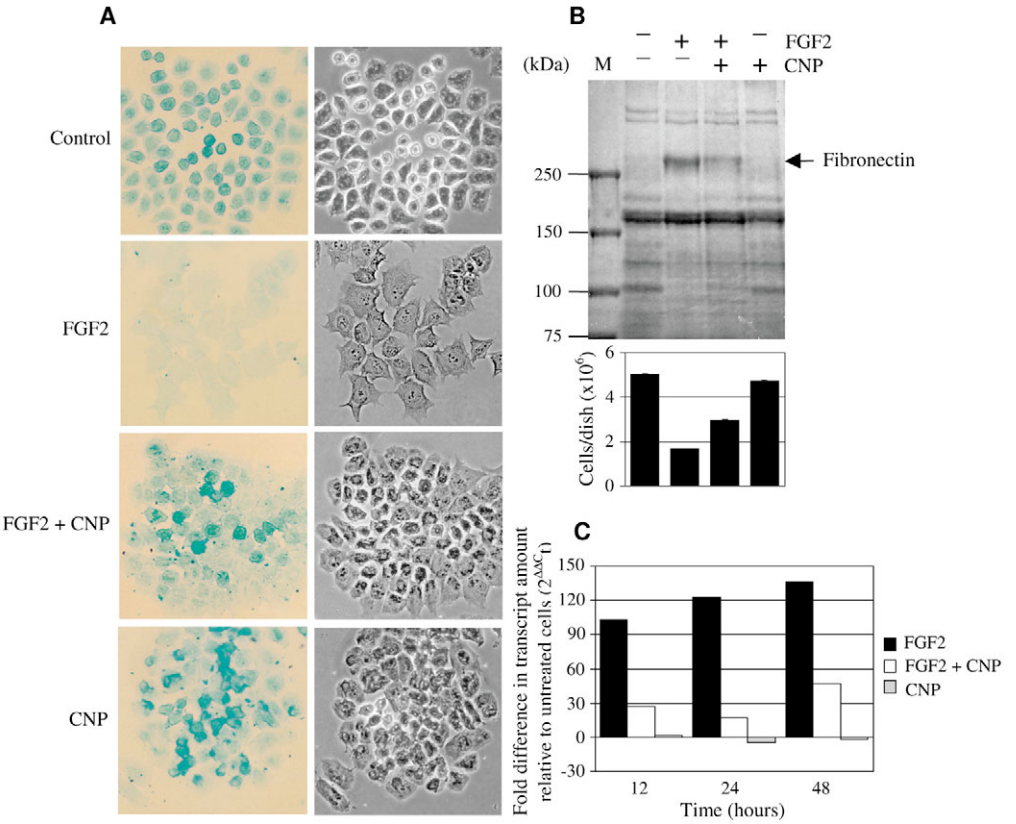

Fig. 7. CNP counteracts FGF2-mediated loss of the extracellular matrix and fibronectin induction in RCS cells. (A) Cells were treated with FGF2 and CNP for 72 hours and the amount of extracellular matrix was determined by Alcian blue staining (left panel, 200×). The corresponding darkfield photograph is also shown (right panel, 200×). (B) Cells were treated with FGF2 and CNP for 72 hours and the culture media, conditioned by cells for last 24 hours of treatment, was resolved by reducing SDS-PAGE followed by Coomassie Brilliant Blue staining. Note that the volume of the sample loaded corresponds to the 1/100th of the total media conditioned by the amount of cells indicated. (C) Cells were treated with FGF2 and CNP for up to 48 hours and the amount of fibronectin mRNA was determined by real-time RT-PCR. Quantities of the transcript are relative to untreated cells at each time point.

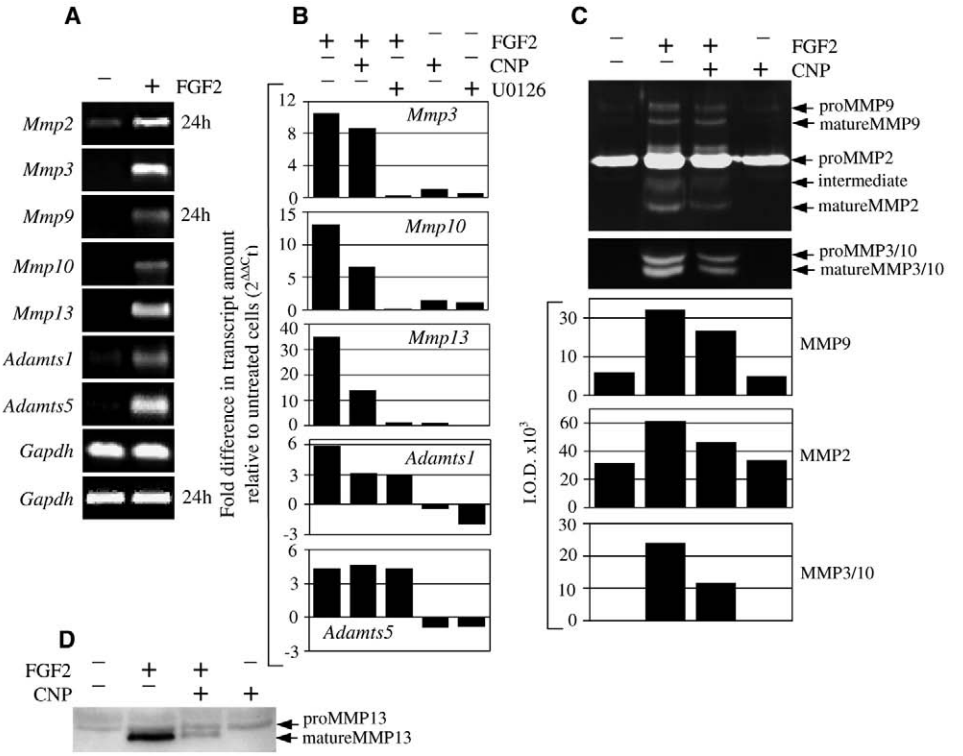

Fig. 8. CNP effects on FGF2-mediated induction and activation of matrix-remodeling molecules. (A) Cells were treated with FGF2 for 4 or 24 hours and the expression of the listed molecules was assayed by RT-PCR. Levels of *Gapdh* serve as a control for template quantity. (B) Cells were treated with FGF2 and either CNP or the MEK inhibitor U0126 for 4 hours and the amounts of *Mmp3*, *Mmp10*, *Mmp13*, *Adamts1* and *Adamts5* mRNAs were determined by real-time RT-PCR. Transcript quantities are relative to untreated cells. *Mmp2* and *Mmp9* were not studied due to the possible nonspecific actions of U0126 during the prolonged cultivation time necessary for their induction. (C,D) Cells were treated with FGF2 and CNP for 72 hours and the conditioned culture media was analyzed for MMP activity (C) by zymography using gelatin (upper figure) or casein (lower figure) as a substrate or (D) for presence of MMP13 by WB. The zymography signal was quantified by densitometry and graphed.

matrix degradation induced by FGF2, we performed gelatin and casein zymography on media conditioned by cells treated with FGF2 and CNP. Gelatin zymography showed a 72 kDa gelatinolytic band corresponding to pro-MMP2 in untreated cells but no pro-MMP9 was detected (Fig. 8C). FGF2 increased the quantity of pro-MMP2 and also induced both intermediate (Okada et al., 1997) and mature forms of MMP2. Similarly, pro-MMP9 and its mature form were induced by FGF2. CNP partially counteracted the FGF2 effect on both MMP2 and MMP9 induction (Fig. 8C). The zymography data were confirmed by MMP2 and MMP9 WB (not shown). Using casein zymography, both pro- and mature forms of MMP3 and/or MMP10 (Nakamura et al., 1998) were detected in media conditioned by FGF2-treated cells; this induction was partially reversed by CNP (Fig. 8C). In the same conditioned media, MMP10 but not MMP3 was detected by WB (not shown) suggesting that MMP activity detected by casein zymography corresponds to MMP10. Finally, the quantity of MMP13 increased in culture media conditioned by FGF2-treated cells and this increase was reversed by CNP (Fig. 8D).

The moderate inhibition of both FGF2-mediated induction and activation of matrix-remodeling molecules by CNP (Fig. 8) is unlikely to fully account for the rather significant effect of CNP on FGF2-mediated matrix loss (Fig. 7). Therefore, to gain a better insight into the regulation of RCS extracellular matrix turnover, we treated cells with FGF2 and CNP for 48 or 72 hours and assessed the rate of matrix production and degradation by radioactive labeling both the sulfated proteoglycan and collagen compartments of the matrix. The [^{35}S]sulfate uptake was significantly inhibited by FGF2 (Fig. 9A) similar to the results obtained in pulse-chase experiment (Fig. 9B) suggesting that FGF2 inhibits synthesis and triggers degradation of the proteoglycan matrix. Addition of CNP partially reversed the effects of FGF2 on the proteoglycan matrix (Fig. 9A,B). Moreover, CNP alone significantly increased [^{35}S]sulfate incorporation (Fig. 9A). Although CNP increased both wet and dry weight of the cell pellet obtained by scraping, no effect was observed when cells were harvested by trypsinization (Fig. 9E), confirming that CNP increases the mass of the RCS extracellular matrix. FGF2 did not affect [^{3}H]proline uptake (Fig. 9C). After 48 hours of treatment, FGF2 weakly decreased the amount of incorporated [^{3}H]proline in one of five replicate pulse-chase experiments (not shown) in contrast to a 72 hour treatment leading to an invariant 30% loss of [^{3}H]proline incorporation in all three replicate experiments (Fig. 9D).

To test whether the CNP-mediated increase of RCS extracellular matrix mass correlates with increased proteoglycan expression, cells were treated with CNP for 0.5, 1, 2, 4, 6, 8 and 24 hours and the expression of a panel of proteoglycans (aggrecan, biglycan, brevican, chondroitin-sulphate proteoglycan 3 (*Cspg3*), *Cspg4*, *Cspg5*, *Cspg6*, clusterin, decorin, glypican, lumican, perlecan, syndecan-1, syndecan-2, syndecan-4 and versican) was determined by real-time RT-PCR. Although all of the molecules studied were expressed by RCS cells, CNP did not upregulate the transcript amounts of any of them (not shown).

Discussion

In this study, we investigated the FGF and CNP signaling interactions in a chondrocyte environment. We found that: (A) FGF signaling affects chondrocyte behavior by two principal phenotypes, inhibition of proliferation and degradation of the extracellular matrix; (B) both phenotypes require, at least partially, activation of Erk MAP kinase and (C) can be reversed

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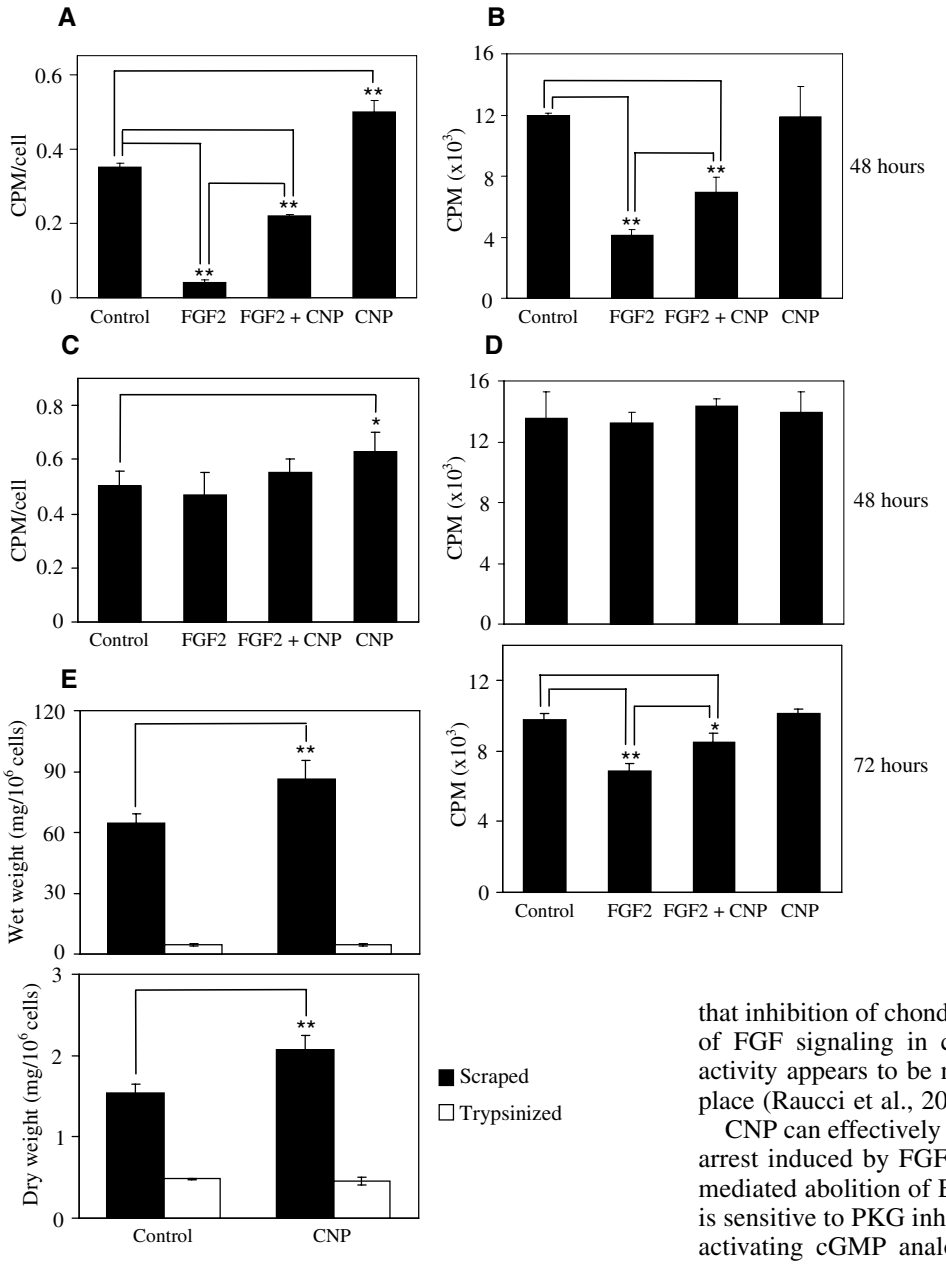

Fig. 9. FGF2 and CNP effects on extracellular matrix homeostasis. (A,C) Cells were treated with FGF2 and CNP for 48 hours in the presence of [^{35}S]sulfate (A) or [^{3}H]proline (C) and the amount of incorporated radioactivity in the cell layer was determined by liquid scintillation. Note the potent regulation of [^{35}S]sulfate incorporation by both FGF2 and CNP in contrast to [^{3}H]proline incorporation that was only mildly enhanced by CNP. (B,D) Cells were pulsed with [^{35}S]sulfate (B) or [^{3}H]proline (D) for 12 hours prior to 48 or 72 hours of treatment with FGF2 and CNP. The remaining incorporated radioactivity was determined by liquid scintillation. Note that the FGF2-mediated decrease of incorporated [^{3}H]proline required 72 hours of treatment. The data represent the average from six wells with the indicated standard deviations. (E) Cells were treated with FGF2 and CNP for 48 hours, harvested by either scraping or trypsinization and both the wet and dry mass of the pellets was determined. The data represent the average from eight wells with the indicated standard deviations. Statistically significant differences are highlighted (ANOVA; *P<0.05, **P<0.01).

by CNP-mediated inhibition of the Erk pathway; (D) CNP inhibits Erk activation via PKG and targets the Erk pathway at the level of Raf-1; and (E) CNP increases extracellular matrix mass independent of its interference with FGF signaling. Thus, CNP utilizes both direct and indirect ways to antagonize the outcomes of FGF signaling in chondrocytes (Fig. 10).

Interaction of FGF and CNP signaling in the regulation of chondrocyte proliferation

In the FGFR3-related dwarfisms, the proliferating cartilage is significantly shortened and cell cycle inhibitors, such as ink4 proteins and p21^{Waf1}, are upregulated (Li et al., 1999; Legeai-Mallet et al., 2004). These observations have been experimentally confirmed in CFK2 and RCS chondrocyte models (Henderson et al., 2000; Aikawa et al., 2001), showing that inhibition of chondrocyte proliferation is a major outcome of FGF signaling in cartilage. Prolonged Erk MAP kinase activity appears to be necessary for this growth arrest to take place (Raucci et al., 2004; Krejci et al., 2004).

CNP can effectively prevent both Erk activation and growth arrest induced by FGF2 in RCS cells (Figs 1, 2). The CNP-mediated abolition of Erk activation depends on PKG since it is sensitive to PKG inhibition and can be mimicked by a PKG activating cGMP analog but not by PKG-inhibiting cGMP analogs (Figs 1, 2, 4). Since CNP only partially prevented FGF2-mediated growth arrest (Fig. 1), we investigated the mechanisms that limit CNP signaling in the RCS system. The duration of the CNP signal can be restricted by limited stability or clearance of CNP, desensitization of the NPR-B receptor, and PDE-mediated degradation of cGMP (Dousa et al., 1999; Silberbach and Roberts, 2001). In RCS cells, cGMP accumulation was rapidly downregulated by decreased NPR-B activity and PDE-mediated hydrolysis (Fig. 3). Although they increased steady state cGMP levels (Fig. 3C), PDE inhibitors failed to augment CNP's influence on FGF2-induced growth arrest (not shown). In addition, FGF2-induced growth arrest and long-term Erk activation was only partially prevented by PDE-resistant pCPT-cGMP (Fig. 1, Fig. 4B). We thus conclude that CNP's potential to antagonize FGF signaling is limited, and the limiting factor(s) lie downstream of cGMP. Additional experiments are ongoing to identify such factors in RCS cells.

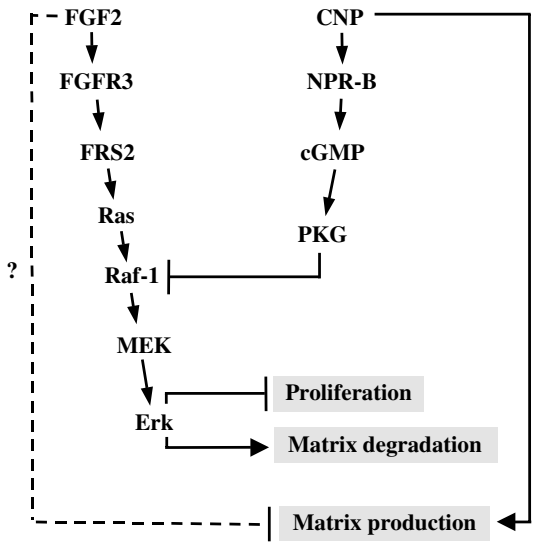

Fig. 10. Interaction of FGF2 and CNP signaling in RCS cells. FGF2 inhibits proliferation and triggers matrix degradation in RCS cells through activation of the Erk MAP kinase pathway. CNP signaling counteracts FGF2 effects by inhibiting the Erk pathway at the level of Raf-1. In addition, CNP directly stimulates RCS matrix production thereby further compensating for the FGF2-mediated matrix loss. The inhibitory effect of FGF2 on RCS matrix production is not presently clear (dashed line).

Molecular mechanisms of FGF and CNP signaling interaction in chondrocytes

In RCS cells, FGF2-mediated activation of MEK and Raf-1 was abolished by CNP whereas activation of Ras and tyrosine phosphorylation of FRS2 were unaffected indicating that CNP signaling inhibits the Erk pathway downstream of Ras, i.e. at the level of Raf-1 (Fig. 5). Since the CNP effect on Erk activation required PKG activity (Fig. 4A), it is very likely that Raf-1 was inhibited by PKG. Interestingly, PKG is able to phosphorylate Raf-1 at Ser43, resulting in the uncoupling of the Ras/Raf-1 interaction and inactivation of the Erk pathway (Suhasini et al., 1998).

FGF-receptors recruit the Erk MAP kinase module via the membrane-anchored adaptor protein FRS2 (Kouhara et al., 1997). Kinase-active FGFRs phosphorylate FRS2 on multiple tyrosines, creating binding sites for Grb2 and Shp-2 proteins that relay the signal to the Erk pathway. Erk, in turn, limits the duration of its own activity by phosphorylating FRS2 on several threonine residues resulting in reduction of its tyrosine phosphorylation, diminished recruitment of Grb2, and ultimately downregulation of Ras/Erk activity (Lax et al., 2002). In FGF2-treated RCS cells, FRS2 appears to be phosphorylated by Erk without diminishing the duration of Erk signal, which lasts at least 24 hours (Fig. 6B) (Krejci et al., 2004). One likely explanation for this phenomenon is the presence of both FRS2α and FRS2β isoforms in RCS cells, as determined by RT-PCR (not shown). Although FRS2α contains eight Erk phosphorylation sites, FRS2β does not, being insensitive to Erk-mediated inhibition (Lax et al., 2002). Sustained Erk activity underlies FGF-mediated growth arrest in chondrocytes in contrast to most cells where Erk is transiently activated by FGFs leading to a mitogenic outcome (Raucci et al., 2004; Krejci et al., 2004; Pages et al., 1993). Thus, the ability of chondrocytes to evade negative feedback by Erk may be critical for maintenance of sustained Erk activity resulting in the unique cellular interpretation of the FGF stimulus in cartilage.

We also identified a mobility shift in FRS2 induced by CNP in a PKG-dependent manner suggesting that PKG phosphorylates FRS2 (Fig. 6B,C). There is one canonical PKG phosphorylation site (RRXS motif) in both rat FRS2α (amino acids 425-428) and FRS2β (amino acids 178-181). It is, however, unlikely that PKG-mediated phosphorylation of FRS2 contributed to the CNP-mediated inhibition of the Erk pathway since both FGF2-mediated tyrosine phosphorylation of FRS2 and activation of Ras were unaffected by CNP (Figs 5, 6).

Interaction of FGF and CNP signaling in extracellular matrix homeostasis

The cartilaginous matrix consists predominantly of sulfated proteoglycans, as well as type II and other collagens. Evidence is emerging that the production of the chondrocyte extracellular matrix is modulated by FGF signaling. First, the growth plate cartilage of individuals suffering from thanatophoric dysplasia or murine model to achondroplasia shows an increased cell to matrix ratio, suggesting decreased matrix production (W.R.W., unpublished) (Yasoda et al., 2004). Second, the treatment of RCS chondrocytes with FGF2 results in a loss of their extracellular matrix (Krejci et al., 2004).

We show that FGF2-mediated loss of the RCS extracellular matrix (Fig. 7) is primarily due to a reduction in its sulfated proteoglycan content (Fig. 9). Although FGF2 triggers degradation of the proteoglycan matrix (Fig. 9B), we can not clearly conclude that it inhibits proteoglycan matrix synthesis since the concomitant degradation may partially account for the decreased [^{35}S]sulfate uptake (Fig. 9A). In an attempt to identify the molecules involved in FGF2-mediated degradation of RCS matrix, we found that FGF2 upregulated expression, release, and activation of several matrix metalloproteinases including MMP2, 3, 9, 10 and 13 (Fig. 8). Although the contribution of individual MMPs to matrix degradation was not determined here, all of the induced MMPs are capable of degrading cartilage proteoglycans (Fosang et al., 1991; Fosang et al., 1992; Little et al., 2002; Stickens et al., 2004).

The significant CNP reversal of RCS extracellular matrix loss, triggered by FGF2 (Fig. 7A), appears to originate from both CNP-mediated inhibition of MMP induction (Fig. 8) and CNP stimulation of extracellular matrix synthesis (Fig. 9). It is unclear how CNP achieves the latter effect. RCS cells do not express FGF1-13 (P.K. and W.R.W., unpublished) and show rapid basal growth in contrast to potent growth arrest when exposed to minute amount of FGFs (Krejci et al., 2004). Moreover, CNP increased RCS matrix production without an increase in their proliferation as expected when antagonizing FGF signaling (Figs 1, 9). We thus conclude that CNP does not increase the amount of the RCS extracellular matrix through the interference with endogenous FGF signaling. In RCS cells, CNP elevated [^{35}S]sulfate incorporation in an amount roughly proportional to the increase in extracellular matrix mass (Fig. 9A,E) suggesting that it upregulates proteoglycan production. However, the transcription of a panel of proteoglycans tested here was not altered by CNP thus leaving the mechanism of

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CNP-mediated increase in the RCS extracellular matrix open to future investigation.

Upon FGF2 treatment, we found elevated expression and release of fibronectin into the culture media (Fig. 7B,C). We presently do not know the effect of increased fibronectin on RCS cells. Addition of fibronectin to hepatic stellate or lung carcinoma cells results in Erk activation (Poulos et al., 1997; Han et al., 2005) and thus FGF2-induced fibronectin may contribute to the maintenance of long-term Erk activity in RCS cells and thereby to FGF2-mediated growth arrest. Additional experiments are now ongoing to test this hypothesis.

We are grateful to P. Lin and R. Radha for assistance with the FACS data analysis. This work was supported by Yang Seng Tang USA Company and NIH 5P01-HD22657.

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