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C-Type Natriuretic Peptide Regulation of Limb Mesenchymal Chondrogenesis is Accompanied by Altered N-Cadherin and Collagen Type X-Related Functions

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ABSTRACT

AMDM, a form of osteochondrodysplasia, is due to the loss-of-function mutations in NPR-B gene. This study investigated the functional involvement of CNP-3, chick homolog of human CNP, and its receptor NPR-B in chondrogenesis utilizing the micromass culture of the chick limb mesenchymal cells. Results revealed CNP-3 and NPR-B expression in the chick limb bud making stage-specific peak levels first at Hamburger-Hamilton stage 23–24, and second at stage 30–31, corresponding to pre-chondrogenic mesenchymal condensation and initiation of chondrogenic maturation-hypertrophy in vivo, respectively. CNP-3 and NPR-B expression in vitro increased parallel to collagen type X expression, but not to that of collagen type II. Treatment of cultures with CNP significantly increased N-cadherin, and collagen type X expression, glycosaminoglycan synthesis and chondrogenesis. Collagen type II expression was not significantly affected. Thus, results implicated CNP-3/NPR-B signaling in pre-chondrogenic mesenchymal condensation, glycosaminoglycan synthesis and late differentiation of chondrocytes in the process of endochondral ossification. J. Cell. Biochem. 105: 227–235, 2008.

KEY WORDS: CNP; CNP-3; NPR-B; CHONDROGENESIS; ENDOCHONDRAL OSSIFICATION; MICROMASS CULTURE

Chondrogenesis is one of the earliest morphogenetic events of the developing vertebrate limb. The bones of the limb form through endochondral ossification, a process by which mesenchymal cells differentiate into chondrocytes, which proliferate, mature, and undergo hypertrophy and matrix calcification; the calcified cartilage is eventually replaced by bone [Tuan, 2004]. The early process of differentiation of mesenchymal cells into chondrocytes may be divided into three phases: (1) proliferation of the mesenchymal cells originating from the lateral plate mesoderm, (2) condensation of the mesenchymal cells in the core of the limb bud to form cellular aggregates in which cell adhesion molecules N-cadherin and N-CAM are up-regulated, and (3) differentiation of these mesenchymal cells into chondrocytes with the production of a cartilage-specific extracellular matrix rich in collagen type II and sulfated proteoglycans [Tuan, 2004]. Late differentiation of chondrocytes, on the other hand, include the process of maturation and hypertrophy, at which stage the extracellular matrix is rich in collagen type X expression [Tuan, 2004].

Many of the factors regulating endochondral growth have been identified through the study of individuals, who have abnormal growth patterns. A condition known as Acromesomelic dysplasia, type Maroteaux (AMDM; OMIM no. 602875) is an autosomal recessive skeletal dysplasia caused by loss-of-function mutations in the natriuretic peptide receptor (NPR)-B gene [Olney et al., 2006]. It is characterized with severe dwarfism, that is, disproportionate short stature with shortening of the middle and distal segments of the limbs. This finding clearly implicates a role for the natriuretic peptide signaling in the regulation of endochondral ossification.

The family of natriuretic peptides comprises at least three structurally related peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). ANP and BNP act mainly as cardiac hormones, produced respectively by the atrium and the ventricle. CNP, on the other hand, predominates in the central nervous system but is also detected in a wide variety of tissues and cells including cartilage, growth plate cartilage, chondrocytes, osteoblasts, and osteoclasts [Olney, 2006]. C-type natriuretic peptide is the product of a single gene, natriuretic peptide precursor C (Nppc), with a chromosomal location of 2 q24-qter [Potter et al., 2006]. A recent study has identified and characterized the chicken natriuretic peptide precursor gene cluster and found its

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organization to be highly conserved compared with the mammalian Nppb-Nppa cluster [Houweling et al., 2005]. However, phylogenetic analysis indicated that the putative chicken natriuretic peptide precursor genes are the homologues of CNP-3 and Nppb, respectively [Houweling et al., 2005]. In addition, the amino acid sequence of chicken CNP-3 shows high homology to that of human CNP [Houweling et al., 2005].

Natriuretic peptides exert their biological functions by specific binding to cell-surface receptors. Three specific receptors have been identified in mammalian tissues. Two are guanylyl cyclase-coupled receptors (GC-A and GC-B, also called NPR-A and NPR-B, respectively) that act through activation of the cGMP dependent signaling cascade, whereas the third receptor (type C receptor or NPR-C) is not coupled to guanylyl cyclase but is mainly involved in the clearance of the peptides [Olney, 2006; Potter et al., 2006]. Natriuretic peptides bind selectively to these receptors. The rank order of ligand selectivity for NPR-A is ANP > BNP ≫ CNP, for NPR-B is CNP ≫ ANP >BNP, and for NPR-C is ANP > CNP > BNP [Olney, 2006; Potter et al., 2006].

Studies on natriuretic peptides utilizing genetically altered mice revealed that there is no detectable skeletal phenotype in ANP, BNP, and NPR-A knockout mice, whereas CNP and NPR-B knockout mice were severely dwarfed in comparison to control group [Olney, 2006]. Skeletal phenotype of BNP and CNP transgenic mice, on the other hand, revealed significant overgrowth, and thickened trabeculae of the bone, whereas ANP transgenic mice was normal in appearance in comparison to controls [Olney, 2006]. These data together with the information taken from ligand selectivity of NPRs clearly implicate CNP/NPR-B signaling in the regulation of endochondral bone growth [Olney, 2006; Potter et al., 2006].

The objective of this study was to further dissect out the implication of the CNP-3/NPR-B signaling in the process of chick limb mesenchymal chondrogenesis, and to identify the specific phase(s) of this process that CNP-3/NPR-B signaling is putatively involved in. Thus, the correlation of the expression patterns of CNP-3 and NPR-B with those of known markers of mesenchymal condensation (N-cadherin), chondrogenic differentiation (collagen type II), and maturation and hypertrophy (collagen type X) has been analyzed on the basis of RT-PCR at mRNA level in chick limb mesenchymal micromass cultures. In addition, the chondrogenic response to CNP treatment in these cultures was analyzed on the basis of alterations at the mRNA level of above-mentioned molecules involved in specific phases of this process.

MATERIALS AND METHODS

CHICKEN EMBRYOS

Fertilized chicken eggs (the ''Cock of Denizli'' strain) were obtained from The Directorate of the Ministry of Agriculture and Village Affairs, The Cock of Denizli Production Unit and Hatchery (Denizli, Turkey) and incubated at 37.5°C in a humidified egg incubator for the desired period of time. The Ethical Review Committee of the Pamukkale University, School of Medicine, Denizli, Turkey, approved the procedures used in this study.

MICROMASS CULTURE OF EMBRYONIC CHICK LIMB BUD MESENCHYMAL CELLS

Limb buds of Hamburger and Hamilton (HH) stage 23/24 embryos [Hamburger and Hamilton, 1992] were isolated and digested using the technique described by Ahrens et al. [1977] and modified by San Antonio and Tuan [1986]. In brief, limb buds were dissected from embryos at stage 23/24 in Ca^{2+}/Mg^{2+} free saline with glucose (CMFSG) and enzymatically dissociated for 1 h at 37°C in 0.1% trypsin (Type II-S, Sigma, St. Louis, MO), 0.1% collagenase (Worthington, Lakewood, NJ), and 10% chick serum (Sigma) in CMFSG. The enzymatic digestion was stopped with 10% calf serum (Hyclone, Logan, UT) in CMFSG. Cells were passed through a cell strainer (Falcon, Franklin Lakes, NJ), subsequently pelleted and resuspended in 10% calf serum in CMFSG, and counted with a hemocytometer in the presence of trypan blue (>95% dye exclusion), and cell concentration was adjusted to $10–30 \times 10^6$ cells/ml, that is, 30×10^6 cells/ml for the study of expression patterns presented in figure 2, and 10×10^6 cells/ml for the study of CNP treatment of micromass cultures presented in Figures 3 and 4. The cell suspension was gently mixed by trituration and then plated as 15-μl drops in the center of each well of a 12-well tissue culture plate (Corning Glassworks, Corning, NY). Following a 1.5- to 2-h attachment period, each well was filled with 1 ml of Ham's F-12 culture medium (Gibco-BRL, Gaithersburg, MD); supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), penicillin (50 U/ml), and streptomycin (50 μg/ml); and incubated at 37°C, 5% CO_2. During the CNP treatment experiments the same culture medium was additionally supplemented with 10^{-7} M CNP (32–53; Human, Porcine, AnaSpec, Inc., San Jose, CA). Culture medium was replaced daily.

QUANTITATION OF CHONDROGENESIS BY ALCIAN BLUE STAINING

At every 24 h of culture, the cultures were rinsed with phosphate-buffered saline (PBS), fixed for 20 min in 4% paraformaldehyde, and rinsed again with PBS. These cultures were stained overnight with Alcian blue (Sigma), pH 1.0 [Lev and Spicer, 1964]; viewed; and photographed. The quantity of cartilage was estimated from the amount of dye, measured as A_{650}, extracted in 6 M guanidine–HCl (1 ml/culture) for overnight at room temperature [San Antonio and Tuan, 1986]. Eight cultures per group were utilized for each experiment and experiment was repeated three times. Results presented are the mean ± SEM of these three experiments.

ANTIBODIES

6B3, monoclonal antibody against chick N-cadherin [George-Weinstein et al., 1997], II-II6B3, monoclonal antibody against chick collagen type II [Linsenmayer and Hendrix, 1980], and X-AC9 monoclonal antibody against chick collagen type X [Schmid and Linsenmayer, 1985] were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA).

IMMUNOHISTOCHEMISTRY

Micromass cultures were fixed at the appropriate times with 4% paraformaldehyde in PBS and rinsed in PBS. They were processed for routine histology, paraffin embedded, blocked, and 10 μm sections were taken for immunohistochemistry. Endogenous

peroxidase was first quenched with 30% H_2O_2/methanol (1:9). After rinsing and blocking with serum blocking solution (Zymed, San Francisco, CA), cultures were incubated with the appropriate primary antibodies (6B3 diluted 1:125 in PBS; II-II6B3 diluted 1:250 in PBS; X-AC9 diluted 1:250 in PBS). Immunodetection was done by using the Zymed Histostain-Plus kit, containing biotinylated broad-spectrum secondary antibody (Zymed) and streptavidin conjugated with horseradish peroxidase (HRP), and developed using the aminoethyl carbazole (AEC) chromogen substrate mixture.

RNA ISOLATION AND REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total RNA was isolated for each experiment either from whole limb buds of embryos in between HH stages 18–35 or from micromass cultures by using Tri-Reagent (1 ml/4–20 limb buds depending on the stage of the embryo, or 1 ml/8 cultures) (Sigma). All experiments were repeated three times. Results presented are the mean ± SEM of these three experiments. Total RNA was quantified on the basis of A_{260} and examined for integrity by agarose gel electrophoresis and ethidium bromide staining. One microgram of template RNA was processed for RT and amplified by PCR using the Qiagen OneStep RT-PCR with Q-solution kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Briefly, 10 μl of 5× Qiagen OneStep RT-PCR Buffer containing 12.5 mM $MgCl_2$, 2 μl of dNTP mix containing 10 mM of each dNTP, 10 μl of 5× Q-solution, 3 μl of each gene-specific primer mix containing 10 μM of each gene-specific forward and reverse primer (Metabion International AG, Martinsried, Germany; see Table I for selected genes and primers used), 2 μl of Qiagen OneStep RT-PCR enzyme mix and 22 μl of RNase-free distilled H_2O were added to template RNA in a total volume of 50 μl. RT-PCR reactions were performed under the following conditions: 1 cycle of 50°C for 30 min (reverse transcription), 95°C for 15 min (initial PCR activation step); 25–30 cycles of 94°C for 1 min (denaturation), 55–58°C for 1 min (annealing), 72°C for 1 min (extension); and reactions were prolonged at 72°C for 10 min, terminated at 4°C, and stored at −20°C. According to our PCR optimization results (data not presented) 25 cycles in these PCR protocols was corresponding to the linear range of amplification. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining. Densitometric analyses of the gels were performed using the GelQuant Ver.2.7 (DNR Bio-Imaging Systems, Jerusalem, Israel) software.

STATISTICAL ANALYSIS

Statistical analyses for quantitation of chondrogenesis, and densitometric analyses of the gels were performed using the non-parametric Mann–Whitney's *U*-test and $P < 0.05$ was taken as statistically significant.

RESULTS

TEMPORAL EXPRESSION OF CNP-3 AND NPR-B IN THE CHICK LIMB BUD

Analysis of the temporal expression patterns of CNP-3 and NPR-B during the development and patterning of the chick limb bud on the basis of RT-PCR revealed that both CNP-3 and NPR-B are expressed at all stages examined, that is, in between Hamburger-Hamilton (HH) stages 18–35 (Fig. 1A). However, when the level of stage specific expression of CNP-3 and NPR-B were normalized to that of GAPDH, the densitometric analysis revealed a peak at HH stage 23–24 and a higher peak, that is, almost 2 times increase from the base line level, at HH stage 30–31 for CNP-3 mRNA (Fig. 1B). NPR-B mRNA, on the other hand, showed a sharp increase, that is, 6.5 times, at HH stage 23–24, decreased 2.7 times until HH stage 28–29, followed by a second peak, that is, 2.1 times increase, at HH stage 30–31, and reached its plateau level after a slight decrease, at HH stages 32–35 (Fig. 1B).

ANALYSIS OF THE SEQUENCE AND TIMING OF EVENTS DURING CHONDROGENESIS IN MICROMASS CULTURES

RT-PCR analysis of the RNA isolated from micromass cultures at specific time points during the course of chondrogenesis with gene specific primers against N-cadherin, collagen type II, and collagen type X (Table I) supported the protein expression data obtained from immunohistochemical analyses of these molecules on sections of micromass cultures. In brief, on day-1 mesenchymal cells showed islands of condensation (Fig. 2A, Day-1) and N-cadherin expression in these condensation areas was down regulated on day-2 with only few cells remaining N-cadherin positive (Fig. 2B,C,E). The density of Alcian blue staining in these micromass cultures increased in parallel to chondrogenic differ-

TABLE I. Primers for Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression

Gene	Primer sequence (5′–3′)	Product (bp)	GenBank accession numbers (Ref.)
N-cadherin	F: AGATTCTGGAAATCCACATGC, R: CTTCCTTCATAGTCAAAGACT	540	NC006089.2 [Zhang et al., 2003]
Collagen type II	F: CATCAAACTCTGCCATCCCG, R: TGAAGTGGAAACCGCCGTTG	224	M17866 [Young et al., 1984]
Collagen type X	F: ATTGCCAGGGATGAAGGGACATAG, R: AGGTATTCCTGAAGGTCCTCTTGG	439	M13496 [Ninomiya et al., 1986]
CNP-3	F: AAGAGAGGGATCGTGAGCAA, R: TCCTGGACAGACCCTTTTTG	201	XM417643 [Houweling et al., 2005]
NPR-B	F: GCCTGCTCCGAGTACGTG, R: GTGCAGGTGGGAGACGAA	252	XM427055 (this study)
GAPDH	F: AGTCATCCCTGAGCTGAATG, R: AGGATCAAGTCCACAACACG	330	J00849 [Dugaiczyk et al., 1983]

F, forward primer; R, reverse primer.

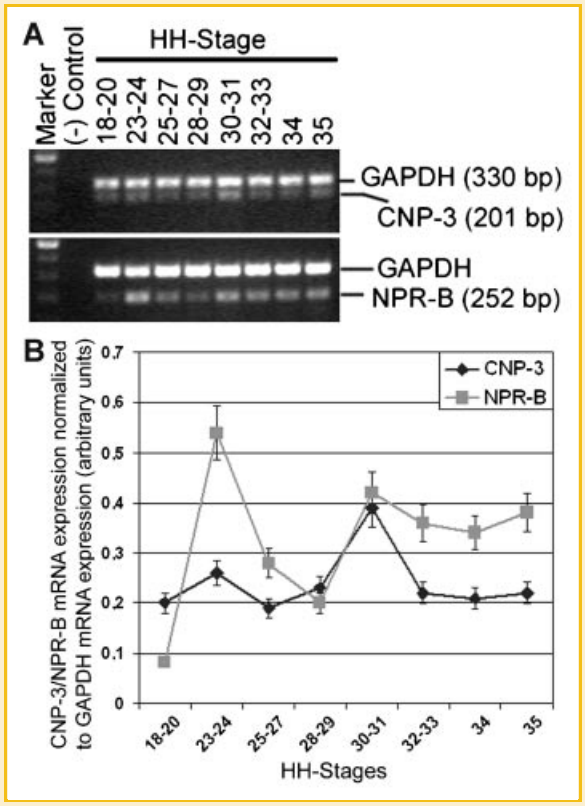

Fig. 1. Temporal expression of CNP-3 and NPR-B in the chick limb bud. A: Analysis of the temporal expression patterns of CNP-3 and NPR-B during the development and patterning of the chick limb bud on the basis of RT-PCR (using 25 cycles) in between HH stages 18–35. B: Densitometric analysis of the level of stage specific expression of CNP-3 and NPR-B normalized to that of GAPDH. See text for details. HH, Hamburger-Hamilton; GAPDH, Glycer-aldehyde-3-phosphate dehydrogenase; bp, base pair.

entiation and secretion of extra cellular matrix (ECM) rich in sulfated proteoglycans (Fig. 2A, Day-2–14). During this process collagen type II expression in ECM showed its peak level on day 1 and decreased gradually thereafter (Fig. 2B,C,F). Collgen type X expression in the ECM, on the other hand, was detectable on day 2 by RT-PCR (Fig. 2B,C) and on day 3 by immunohistochemistry (Fig. 2G), and gradually increased in parallel to maturation and hypertrophy of chondrocytes (Fig. 2B,C,G). Both collagen type II and X reached their plateau level at the end of first week, on day 7, in these cultures (Fig. 2B,C).

ANALYSIS OF THE CNP-3 AND NPR-B EXPRESSION DURING CHONDROGENESIS IN VITRO

RT-PCR analysis with gene specific primers against CNP-3 and NPR-B (Table I) within this well-established model system, on the other hand, revealed almost undetectable mRNA levels for CNP-3, and NPR-B under the given RT-PCR conditions for this experiment at the stage of chondrogenic differentiation, when collagen type II expression showed its peak level (Fig. 2B, Day-1). In this experiment NPR-B expression became more detectable on day 2 of culture in parallel to that of collagen type X, and gradually increased during the maturation and hypertrophy of chondrocytes (Fig. 2B,C). The magnitude of NPR-B mRNA expression level was significantly lower than those of collagen type II and X during this process (Fig. 2B,C). CNP-3 expression in this experiment was detectable only with higher PCR cycle number (25 cycles for Fig. 2B vs. 30 cycles for Fig. 2D) in parallel to NPR-B expression (Fig. 2D).

ANALYSIS OF THE EFFECT OF CNP TREATMENT ON CHONDROGENESIS AND ITS STAGE SPECIFIC MARKERS IN VITRO

Treatment of the micromass cultures with 10^{-7} M human CNP on day 1–7 significantly increased the glycosaminoglycan synthesis and chondrogenesis in comparison to control cultures analyzed on the basis of Alcian blue staining (Fig. 3A,B; $P < 0.001$). Collagen type X immunohistochemistry on day 7 showed significant increase of this protein in CNP treated group in comparison to control group (Fig. 3C). Further analysis of the mechanism(s) of action of CNP induction in these cultures revealed that normalized mRNA level of N-cadherin in control group decreased 34% from day 1 to day 2 ($P < 0.001$), where as that in CNP treated group showed an increase of 17% ($P < 0.03$) during the same time period (Fig. 4A,B). The normalized mRNA level of N-cadherin in CNP treated group was significantly higher than that of control group on day 2 (Fig. 4A,B; $P < 0,001$). However, this increase in N-cadherin expression due to CNP treatment was transient, and the level of normalized N-cadherin mRNA decreased to control levels on days 3 and 4 (Fig. 4A,B). In general, N-cadherin expression showed a significant decrease in both groups on days 3 and 4 ($P < 0.001$). Normalized collagen type X mRNA expression in CNP treated group was comparable to that in control group up to day 4 in these cultures. However, on days 6 and 7 normalized collagen type X mRNA expression in CNP treated group was significantly higher than that in control group (Fig. 4D,E; $P < 0.001$). Normalized collagen type II expression levels on days 1–4, on the other hand, showed comparable decreasing patterns in both groups. Although there was a slight increase in normalized collagen type II expression levels in CNP treated cultures versus controls during this time period, the difference between two groups was statistically not significant (Fig. 4A,C).

DISCUSSION

The objective of this study was to further investigate the proposed implication of the CNP-3/NPR-B signaling in the process of chick limb mesenchymal chondrogenesis. More specifically, it was aimed to identify the specific phase(s) of this process that CNP-3/NPR-B signaling is most likely involved in.

A commonly used in vitro model system to analyze the effects of many signaling and regulatory molecules on chondrogenesis has been the chick limb bud mesenchymal micromass culture [Tufan and Tuan, 2001]. It has been shown in the literature [DeLise et al., 2000; Daumer et al., 2004] and in this study that the in vivo chondrogenic sequence can experimentally be reproduced in this culture system. This system has the advantage of analyzing the phases of chondrogenesis on the basis of phase specific markers. Most commonly used markers in these cultures include (but are not limited to) N-cadherin expression for the condensation of mesenchymal cells, collagen type II expression and Alcian blue

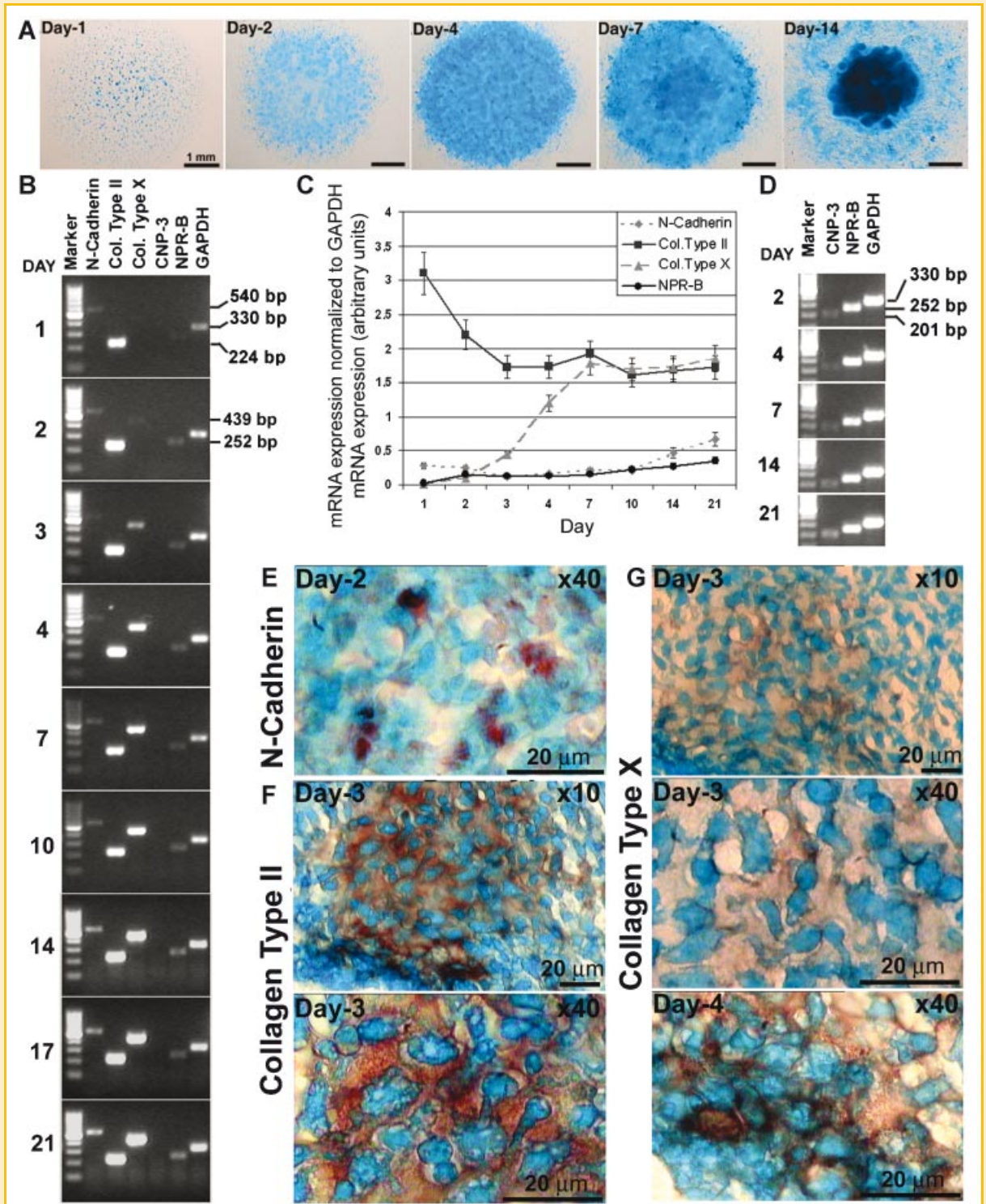

Fig. 2. Analysis of the sequence and timing of events during chondrogenesis in micromass cultures. A: Alcian blue staining of whole micromass cultures at specific time points. B: RT-PCR analysis of N-cadherin, collagen type II, collagen type X, CNP-3, NPR-B, and GAPDH on the basis of 25 cycles. C: Densitometric analysis of the level of stage specific expression of N-cadherin, collagen type II, collagen type X, and NPR-B normalized to that of GAPDH. D: RT-PCR analysis of CNP-3, NPR-B, and GAPDH on the basis of 30 cycles. E–G: Immunohistochemical analysis of N-cadherin (E), collagen type II (F), and collagen type X (G) on sections of micromass cultures. See text for details. Scale bar = 1 mm for all panels in (A), and 20 μm for all panels in (E–G). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

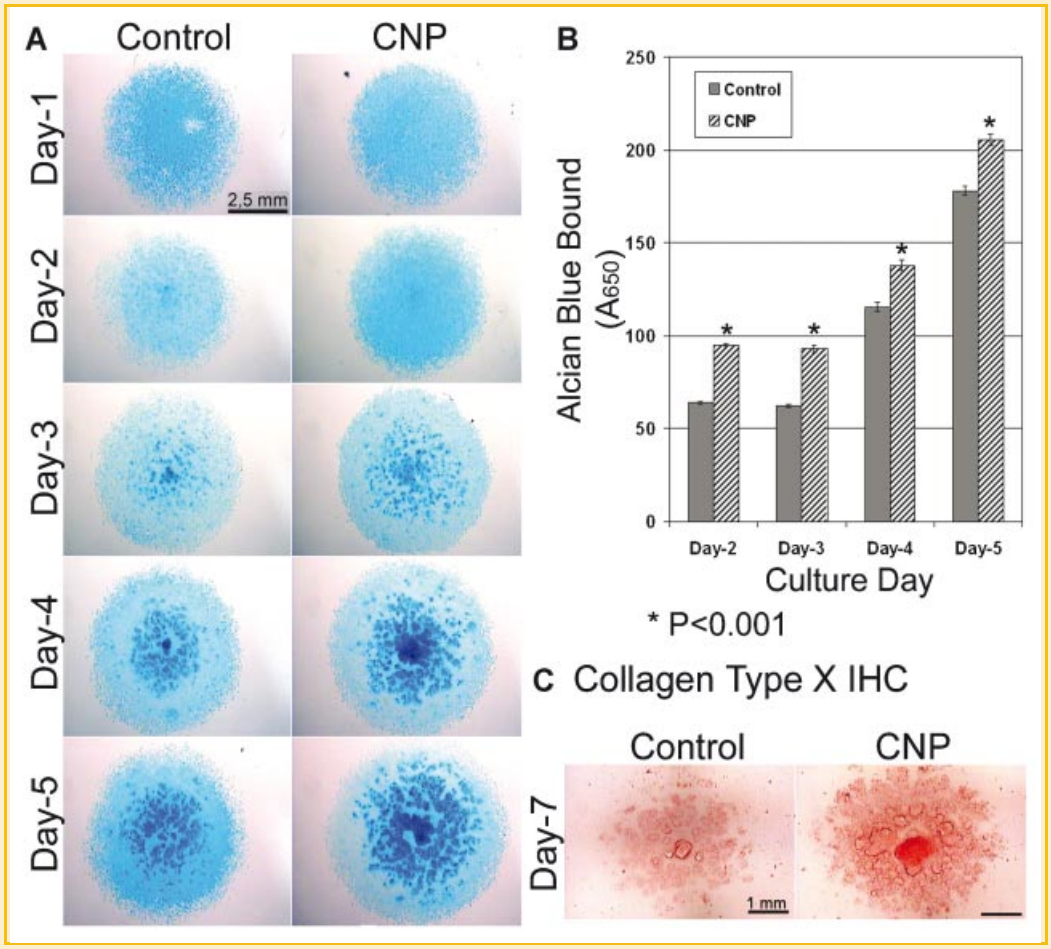

Fig. 3. Analysis of the effect of CNP treatment on chondrogenesis in vitro. A,B: Quantitation of chondrogenesis by Alcian blue staining. Scale bar = 2.5 mm for all panels in A; $^{*}P < 0.001$ in panel B. C: Collagen type X immunohistochemistry (IHC). See text for details. Scale bar = 1 mm for both panels in (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

staining for the chondrogenic differentiation of limb mesenchymal cells, and glycosaminoglycan synthesis, and collagen type X expression for the maturation and hypertrophy of differentiated chondrocytes. Thus, in this study the correlation of the expression patterns of CNP-3 and NPR-B with those of mentioned known markers has been analyzed on the basis of RT-PCR at mRNA level in chick limb mesenchymal micromass cultures. Furthermore, the alterations of these markers in response to CNP treatment during this process were evaluated.

The known events during CNP-3/NPR-B signaling may be summarized as follows [Olney, 2006; Potter et al., 2006]: upon binding of CNP-3 to its receptor NPR-B the intracellular second messenger cGMP production is increased. Cyclic GMP activates a number of proteins, including cGMP-dependent protein kinases I and II (cGK-I and cGK-II), cyclic nucleotide-regulated ion channels, and cGMP-regulated phosphodiesterases. A number of studies have shown the experimental and clinical results of the impairment of this signaling cascade in terms of dwarfism and osteochondrodysplasias, namely AMDM [Olney, 2006; Potter et al., 2006]. However, investigation of the mechanism(s) of action of this signaling pathway within the process of endochondral ossification, that is, more specifically within the process of limb mesenchymal chondrogenesis, has been limited in literature.

The most commonly utilized in vitro system to analyze the effects of natriuretic peptides on chondrogenesis has been the clonal mouse embryonic cell line ATDC5 known to be chondroprogenitor-like when induced with insulin [Fujishige et al., 1999; Suda et al., 2002]. The main outcomes of a few in vitro studies on CNP/NPR-B signaling with ATDC5 cells may be summarized as follows: (1) insulin induces chondrogenic differentiation of these cells analyzed on the basis of collagen type-II expression [Fujishige et al., 1999], (2) CNP and NPR-B expressions also increase significantly in parallel to collagen type-II expression [Fujishige et al., 1999], and (3) CNP treatment of these chondrifying cells reduces the proliferation rate and increases the expression of collagen type-X, a well known maturation and hypertrophy marker of chondrocytes [Suda et al., 2002]. A very recent study published during the period of analysis of results of our present study, on the other hand, suggested that CNP increases the number of chondrogenic condensations, induces the expression of N-cadherin, stimulates glycosaminoglycan synthesis, but does not alter the expression of the chondrogenic transcription factors Sox9, -5, and -6, or of the main ECM genes encoding

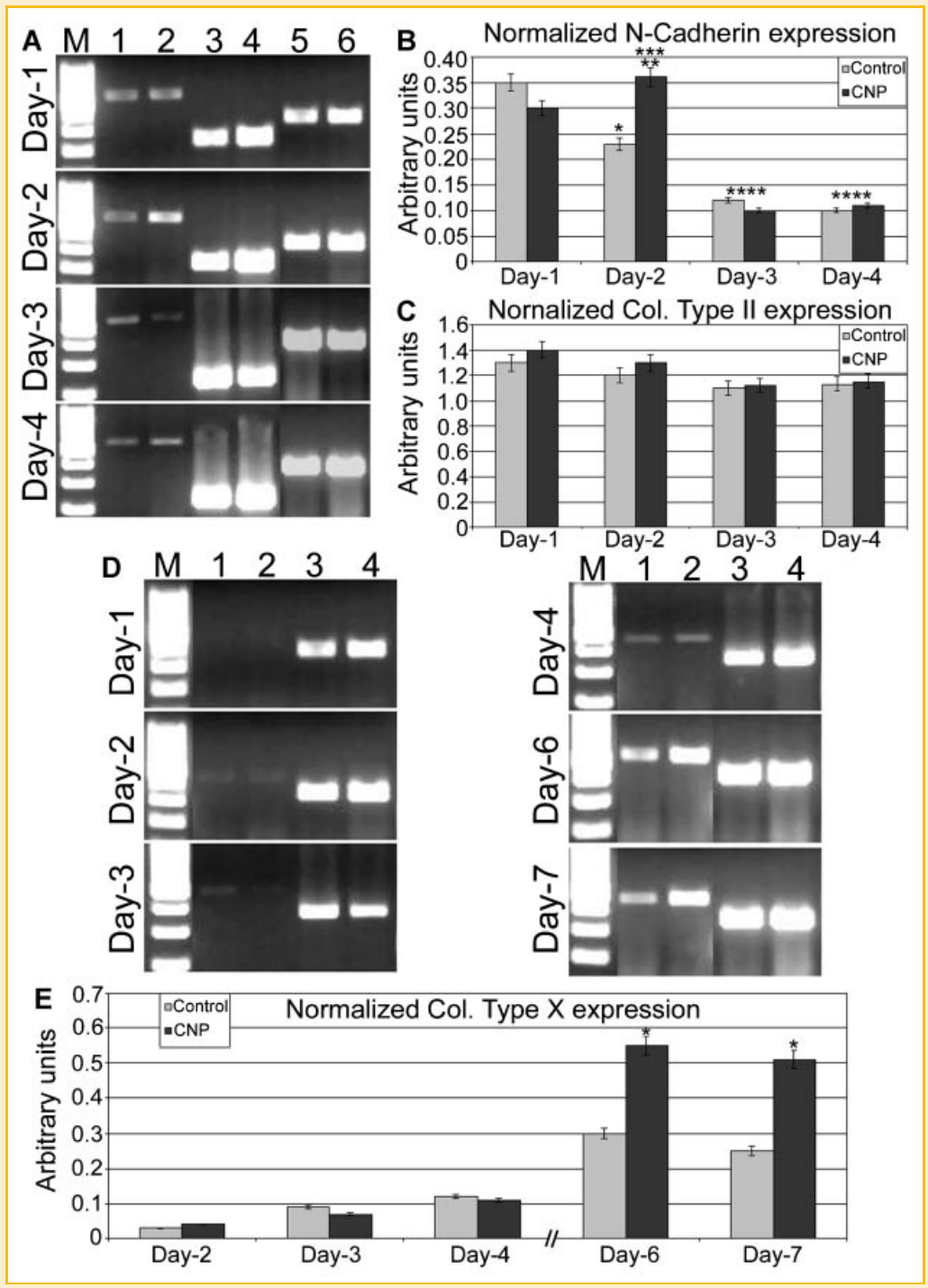

Fig. 4. Analysis of the functional involvement of CNP in chondrogenesis in vitro. A: RT-PCR analysis of N-cadherin (Lanes 1 and 2), collagen type II (Lanes 3 and 4), and GAPDH (Lanes 5 and 6) expressions in control (Lanes 1, 3, and 5) and CNP treated (Lanes 2, 4, and 6) cultures on the basis of 25 cycles on days 1–4. B: Densitometric analysis of the level of days in culture specific expression of N-cadherin normalized to that of GAPDH in control and CNP treated cultures on days 1–4. $^{*}P < 0.001$ for N-Cadherin-Control-Day-1 versus N-Cadherin-Control-Day-2; $^{**}P < 0.03$ for N-Cadherin-CNP-Day-1 versus N-Cadherin-CNP-Day-2; $^{***}P < 0.001$ for N-Cadherin-Control-Day-2 versus N-Cadherin-CNP-Day-2; $^{****}P < 0.001$ for both group results on days 3 and/or 4 versus day 2. C: Densitometric analysis of the level of days in culture specific expression of collagen type II normalized to that of GAPDH in control and CNP treated cultures on days 1–4. D: RT-PCR analysis of collagen type X (Lanes 1 and 2), and GAPDH (Lanes 3 and 4) expressions in control (Lanes 1 and 3) and CNP treated (Lanes 2 and 4) cultures on the basis of 25 cycles on days 1–7. E: Densitometric analysis of the level of days in culture specific expression of collagen type X normalized to that of GAPDH in control and CNP treated cultures on days 1–7. $^{*}P < 0.001$ for Collagen type X-Control-Day-6 or 7 versus Collagen type X-CNP-Day-6 or 7, respectively. See text for details.

collagen type II and aggrecan of mouse embryonic limb bud cells in micromass culture [Woods et al., 2007]. The second conclusion by Fujishige et al. [1999] of the summary given above has been considered as an indication of involvement of CNP/NPR-B signaling in the regulation of early differentiation of mesenchymal cells in to chondrocytes. However, later studies from the literature [Suda et al., 2002; Woods et al., 2007] and this present study do not support this hypothesis.

Results of our present study showed the increased expression of CNP-3 and NPR-B during the pre-chondrogenic condensation stage, that is, HH stage 23–24, and later during the maturation and hypertrophy stage of chondrocytes, that is, HH stage 30–31 onward, in vivo. In vitro results, on the other hand, showed increased expression of these molecules in parallel to that of collagen type-X at the maturation and hypertrophy stage of chondrogenesis. Since we were unable to detect the very earliest pre-chondrogenic condensation phase in vitro, where N-cadherin expression is high and collagen type II expression is low, we were also unable to speculate on the expression profile of CNP-3 and NPR-B at this stage in vitro. Yet, CNP treatment significantly induced chondrogenesis, and transiently reversed the decreasing profile of N-cadherin expression from day 1 to 2 in to an increasing one. In consequence of this effect the level of N-cadherin expression in CNP treated cultures was significantly higher than that of control cultures on day 2. In addition, CNP treatment significantly induced expression of collagen type X only after 4–5 days in culture, that is, on days 6 and 7 (collagen type X reached its plateau level of expression in control cultures at this stage in vitro as shown in Fig. 2C). Thus, this study added to our knowledge by resolving the conflict in the literature regarding the implication of CNP/NPR-B signaling either in the condensation of mesenchymal cells and glycosaminoglycan synthesis [Woods et al., 2007], or in the early differentiation of mesenchymal cells in to chondrocytes [Fujishige et al., 1999], or in the late differentiation process, that is, maturation and hypertrophy of chondrocytes [Suda et al., 2002]. Our results implicated CNP in both pre-chondrogenic condensation and late differentiation stages as an inducing factor. Glycosaminoglycan synthesis is also increased significantly due to CNP induction. The study by Komatsu et al. [2002] showing reduction of the rate of cell differentiation into hypertrophic chondrocytes in CNP knockout mice also supports our in vitro findings in terms of in vivo studies.

The mechanism(s) of action of CNP induction of chondrogenesis does unlikely to include the induction of collagen type II expression during the process of early chondrogenic differentiation. Although there is an increase in collagen type II expression in CNP treated cultures in comparison to controls, the difference is statistically insignificant and also the decreasing profile of collagen type II expression in control cultures is not changed in CNP treated cultures.

An important point that also deserves attention is the very low levels of CNP-3 mRNA that was detectable only with high PCR cycle numbers when compared to that of NPR-B within the chondrogenic mesenchyme of the limb. It is clear that as the receptor in this signaling pathway NPR-B has to be expressed within the mesenchymal cells and later in chondrocytes, where it is implicated as a regulator of mesenchymal condensation and maturation and hypertrophy. The ligand of this receptor, CNP, on the other hand, has been suggested to act through an autocrine/paracrine mechanism, since the blood levels of this protein are very low [Olney et al., 2006]. In situ hybridization analysis of spatial CNP-3 expression within the developing chick limb bud at HH stage 36 revealed its main expression within the mesenchyme deep to the dermis outside the chondrogenic mesenchymal chore region [Houweling et al., 2005]. RT-PCR results at mRNA level shown in this study support this information from the literature.

In conclusion, this study established the basis of the future studies that may investigate the mechanism(s) of action of CNP-3/NPR-B signaling in the process of endochondral ossification, specifically limb mesenchymal chondrogenesis. Results implicate CNP-3 as an autocrine/paracrine factor acting trough NPR-B and involving in the process of early pre-chondrogenic mesenchymal condensation, glycosaminoglycan synthesis, and late differentiation, that is, maturation and hypertrophy, of chondrocytes.

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