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Molecules and Cells

TGF-β3 Inhibits Chondrogenesis by Suppressing Precartilage Condensation through Stimulation of N-cadherin Shedding and Reduction of *cRREB-1* Expression

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Transforming growth factor-β (TGF-β) plays crucial roles in controlling cell differentiation and maintaining tissue integrity. Previously we reported that TGF-β3 treatment decreased the mRNA expression of the gap junction protein, connexin 43 as well as cell number, which lead to the inhibition of chondrogenic condensation in cultured chick leg bud mesenchymal cells. The present study demonstrates that TGF-β3 can induce cleavage in the ectodomain of neuronal cadherin (N-cadherin) at the initiation stage of chondrogenesis and reduce cell numbers, cellular adhesion and the expression level of connexin 43. Differential displayed PCR (DD-PCR) comparison of adherent- and non-adherent chick leg chondrogenic progenitor cells showed increased expression of the chick ras-responsive element binding transcription factor, *cRREB-1*, in adherent cells. In chick leg bud mesenchymal cells, *cRREB-1* transcription was inhibited by TGF-β3 at the early stage of chondrogenesis. Small interfering RNA (siRNA)-mediated knockdown of *cRREB-1* reduced cell numbers, cellular adhesion, and the expression level of connexin 43 resulting in the inhibition of precartilage condensation. Taken together, these findings indicate that TGF-β3 mediates the inhibitory signal necessary for precartilage condensation by stimulating N-cadherin shedding and reducing *cRREB-1* expression levels.

INTRODUCTION

Chondrogenesis of mesenchymal cells is a prerequisite for cartilage formation in the developing limb, wherein cell proliferation is followed by precartilage aggregation or condensation, leading to the formation of the cartilaginous template for the future skeleton (Goldring et al., 2006; Olsen et al., 2000). Precartilage condensation is associated with an increase in cell-to-cell contacts and interactions through cell-cell adhesion molecules such as N-cadherin (DeLise and Tuan, 2002; Oberlander and Tuan, 1994), and N-CAM (Leckband and Parksam, 2006; Widelitz et al., 1993) and cartilage specific extracellular matrix (ECM) components including fibronectin, collagen, laminin, tenascin, and hyaluronan (Bang et al., 2000; DeLise et al., 2000; Downie and Newman, 1995; Knudson and Knudson, 2001).

N-cadherin is one of several characterized classical cadherins which contains multiple extracellular calcium binding domains that participate in calcium-mediated cell-to-cell adhesion (Gumbiner, 1996; Takeichi et al., 1990). The presence of a single dimerization tripeptide domain (HAV; histidine, alanine, valine) distinguishes the classical cadherins from the non-classical members of this family of calcium-mediated adhesion molecules (Takeichi, 1988). N-cadherin-mediated cell adhesion is the principal factor governing the tissue formation process by controlling cell migration, proliferation, cellular differentiation, and the regulation of specific genes (Goichberg et al., 2001; Linask et al., 1998; Redfield et al., 1997; Seghatoleslami et al., 2000). N-cadherin is transiently upregulated by chondrifying mesenchymal cells and induces cell-to-cell adhesion, an essential event for precartilage condensation (Oberlander and Tuan, 2000). In addition, linkage to the actin cytoskeleton and calcium-mediated lateral condensation of classical cadherins results in a change of the cellular shape, a morphological event that plays an important role in the regulation of chondrogenesis (Zanetti and Solursh, 1984; 1986). Although precartilage condensation through regulation of cell-to-cell adhesions is essential for chondrogenic differentiation, the biochemical signaling pathways are not yet fully understood.

The transforming growth factor (TGF)-β superfamily, comprised of TGF-βs, bone morphogenetic proteins, activins and related proteins, plays a key role in development, cellular differentiation, apoptosis, and carcinogenesis (Derynck and Zhang, 2003; Massague, 1990; 2008). TGF-β isoforms have been

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shown to have stimulatory or inhibitory effects on cartilage cells depending on the developmental origin and stage of the cells. Exposure of chick mesenchymal cells of the embryonic forelimbs of stages 23/24 and 22/23 to TGF-β1, TGF-β2 and TGF-β3 promotes the process of cellular condensation by stimulating expression of cell adhesion molecules as well as ECM molecules (Carrington and Reddi, 1990; Downie and Newman, 1994; Jiang et al., 1993; Kulyk et al., 1989; Leonard et al., 1991; Roark and Greek, 1994; Tsonis et al., 1994).

Previously our laboratory showed that TGF-β3 functions differentially in establishing fore- and hind-limb identity: in chick wing bud mesenchymal cells, it stimulates chondrogenesis via upregulation of Wnt-5a (Jin et al., 2006), but in leg bud mesenchymal cells, it inhibits chondrogenesis through an inhibitory action on precartilage condensation by downregulating connexin 43 (Jin et al., 2008). In this report, we present data showing that TGF-β3 treatment of chick leg bud mesenchymal cells decreases cell-to-cell adhesions by promoting ectodomain shedding of N-cadherin. Moreover, for the first time, we found that the chick ras-responsive element binding transcription factor, *cRREB-1*, is one of the key functional genes in N-cadherin-mediated cell-to-cell adhesions among leg bud mesenchymal cells. The stimulation of N-cadherin ectodomain shedding downregulates *cRREB-1* mRNA level, and it also results in decreased cell adhesion during chondrogenic differentiation of chick leg bud mesenchymal cells.

MATERIALS AND METHODS

Cell culture and treatment

Mesenchymal cells derived from the distal tips of Hamburger-Hamilton (HH) stage 22/23 embryo leg buds of fertilized White Leghorn chicken eggs were micromass cultured as previously described (Jin et al., 2006). Briefly, the cells were suspended at a density of 2×10^7 cells/ml in Ham's F-12 medium containing 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco Invitrogen, USA). The cells were plated in three drops (15 μl each) onto 35-mm Corning culture dishes, and incubated for 1 h at 37°C under 5% CO_2 to allow attachment. The cells were maintained in 1 ml of culture medium for the indicated time periods in the absence or presence of 5 ng/ml of TGF-β3 (R&D Systems, USA) or other reagents.

Analysis of cell differentiation and precartilage condensation

Chondrogenic differentiation was measured by Alcian blue staining of sulfated cartilage glycosaminoglycans (Lev and Spicer, 1964). Alcian blue-bound sulfated glycosaminoglycans were extracted with 6 M guanidine-HCl, and quantified by measuring the absorbance of the extracts at 600 nm.

Binding of peanut agglutinin (PNA) was used as a specific marker for precartilage condensation (Maleski and Knudson, 1996). Briefly, cultures were rinsed twice with 0.02 M PBS, pH 7.2, fixed in methanol:acetone (1:1) for 1 min, air-dried, and then incubated with 100 μg/ml biotinylated PNA (Sigma, USA) for 1 h. PNA binding was visualized with VECTASTAIN ABC and DAB substrate solution kit (Vector Laboratories Inc., USA). Quantification of the density of PNA staining in the various areas was carried out with Image J (version 1.36 b, freeware downloaded from http://rsb.info.nih.gov/ij/, an image processing and analysis program developed at the National Institutes of Health, USA).

Cell proliferation and cell adhesion assay

Proliferation of mesenchymal cells was determined by direct counting of cells from micromass cultures. Control and treated cultures were maintained for the indicated days, detached with trypsin/EDTA solution, and counted in triplicate using a hemacytometer. Alternatively, cell proliferation was determined by incubating cells with 2 mCi/ml/dish of [^{3}H]thymidine (Amersham, UK) and the amounts of incorporated [^{3}H]thymidine was measured.

For cell adhesion assay, primary cultured cells were incubated for 2 h with agitation in the absence or presence of reagents and seeded with 5×10^5 cells on each 35 mm Corning culture dish and incubated in the absence or presence of reagents. The adherent cells were counted at 6, 12 and 24 h after seeding in triplicate by hemacytometer (Santiago and Erickson, 2002).

Functional blocking of N-cadherin

A 16-mer synthetic peptide (HLRAHAVDIN GNQVEN; Peptron, Korea) containing the conserved histidine-alanine-valine (HAV) cadherin recognition sequence present within the portions of the distalmost N-cadherin ectodomain (EC1) presumptive binding domain of all type I classic cadherins, was generated with flanking amino acids corresponding to the mouse N-cadherin sequence (Miyatani et al., 1989). Previous studies have shown that the flanking sequence confers cadherin-type specificity, suggesting that the HAV peptide should be specific for N-cadherin (Noe et al., 1999). A scrambled peptide (ARLQHDV-NANVHEING) was applied to cultures as a control. Peptides were used at a final concentration of 100 μg/ml.

Differential display PCR

Total cellular RNA was extracted with TriZol (Gibco Invitrogen, USA) according to the manufacturer's recommended procedure. To identify differentially expressed genes, we used a Gene Fishing DEG kit (SeeGene, Korea) with the 40 arbitrary Annealing Control Primers (ACPs). cDNA (4 μl) was amplified by PCR in 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.0, containing 0.2 mM dNTPs, 125 ng of each primer and 2.5 units of Taq DNA polymerase (Promega, USA) (total volume 50 μl). Samples were subjected to PCR amplification for 40 cycles as follows: 94°C denaturing for 40 s, 65°C annealing for 40 s and 72°C extension for 40 s. PCR products were electrophoresed on a 2.0% agarose gel. DNA fragments from DD-PCR were isolated using a gel extraction kit (Strata gene, USA) according to the manufacturer's protocol, and ligated into the cloning vector pCR II (Gibco Invitrogen). The cDNA inserts were sequenced by the dideoxynucleotide chain termination method (Sequenase 2.0, USB, USA).

RT-PCR

Complementary DNA (cDNA) was synthesized using 1 μg of RNA and 20 μl of master mix for reverse transcription reaction containing 200 unit/μl Superscript III (Gibco Invitrogen), 5 mM $MgCl_2$, PCR buffer II, 1 mM dNTP, 1 U/μl RNase inhibitor, and 2.5 mM oligo dT in DEPC-treated distilled water. The master mix was incubated in a Perkin-Elmer GeneAmp PCR system 9600 (USA) at 42°C for 55 min and then at 99°C for 5 min. After reverse transcription, 2 μl of cDNA was mixed with 40 μl of PCR master mix containing 2 mM $MgCl_2$, PCR buffer II, 2.5 U/ml Taq DNA polymerase (Takara, Japan), and 0.15 μM primers. The oligonucleotides used as primers were as follows: cRREB-1, 5′-CATTTCATCTGTGTCGTCTG-3′, 5′-AAGTGTT-CCTCTTCCCTCTC-3′; GAPDH, 5′-GATGGGTGTTCAACCA-TGAG AAA-3′, 5′-ATCAAAGGTGGAAGAATGGCT G-3′. The cDNAs in samples were subjected to PCR amplification for 40 cycles (for cRREB) or 25 cycles (for GAPDH) as follows: 94°C denaturing for 40 s, 65°C annealing for 40 s and 72°C exten-

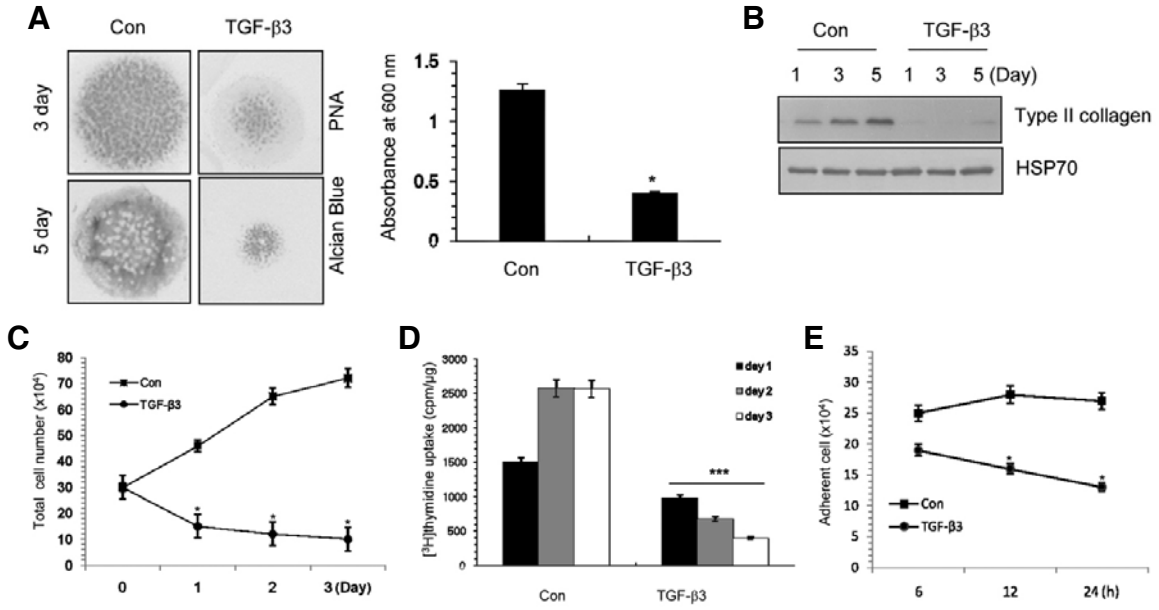

Fig. 1. TGF-β3 inhibits cellular condensation during the chondrogenesis of leg bud mesenchymal cells. Cells were cultured at a density of 2×10^7 cells/ml with or without 5 ng/ml of TGF-β3 for 24 h. (A) Cells were stained with PNA at culture day 3 and Alcian blue at culture day 5 (left panel). Chondrogenesis was quantified by measuring the absorbance of bound Alcian blue at 600 nm (right panel). (B) Changes in the level of type II collagen in control and TGFβ3-treated cultures were determined by Western blot analysis at the indicated days. HSP70 was used as a loading control. The cell numbers cells were counted (C) and thymidine incorporation was measured (D) at the indicated days. (E) Cells were cultured for 2 h with agitation in the absence or presence of 5 ng/ml of TGF-β3, seeded at a density of 5×10^5 cells/ml and cultured in the absence or presence of TGF-β3. Finally, adherent cells were counted at 6, 12, and 24 h after culture. The data shown are representative of at least four independent experiments. *, statistically different from control cells ($P < 0.005$).

sion for 40 s. The RT-PCR products were separated on a 2% agarose gel.

Western blot analysis

Proteins (30 μg) or conditioned media were separated by 10% polyacrylamide gel electrophoresis containing 0.1% SDS and transferred to nitrocellulose membrane (Schleicher and Schuell, Germany). The membranes were incubated for 1 h at room temperature in blocking buffer (20 mM Tris-HCl, 137 mM NaCl, pH 8.0, containing 0.1% Tween and 3% non-fat dry milk), and individually probed with antibodies against N-cadherin (sc-7939), Type II collagen (sc-7764), connexin43 (sc-9059) or phosphorylated form of Connexin43 (sc-12900) purchased from Santa Cruz Biotechnology Inc. (USA), or HSP70 (spa822) from Stressgen Bioreagents Corp. (Canada). The blots were developed with a peroxidase-conjugated secondary antibody and reactive proteins were visualized by the ECL system (Pierce Biotechnology Inc., USA).

Transfection of cRREB-1-specific small interfering RNA (siRNA)

Three different *RREB-1* specific antisense ; siRNA-1; 5′-GAGATCCCACTGGAAAGCCTTTCAT-3′ (starting nucleotide no. 1113), siRNA-2; 5′-GAAGAGAGGTAGAAAGAAA-3′ (starting nucleotide no. 3649), siRNA-3; 5′-GGGAAGGCACAGAGAGAAA-3′ (starting nucleotide no. 4557) were obtained from Gibco Invitrogen. Isolated leg bud mesenchymal cells were transfected with the RNA oligonucleotides (final RNA concentration, 50 nM) using lipofectamine 2000 (Gibco Invitrogen) according to the manufacturer's recommended procedure and cells were incubated for 2 h with agitation in the absence or presence of RNA oligonucleotides, plated on 35 mm Corning culture dishes as 3 drops of 15 μl cell suspensions each in the absence or presence of RNA oligonucleotides. To analyze the efficiency of siRNA oligonucleotides against to cRREB, total RNA was extracted from cells cultured for 1 day using TriZol (Gibco Invitrogen) for RT-PCR analysis.

RESULTS

TGF-β3 inhibits precartilage condensation by downregulating cell-to-cell adhesion in chick leg bud mesenchymal cell

We have previously reported that TGF-β3 enhanced chondrogenesis in wing bud mesenchymal cells, whereas TGF-β3 inhibited chondrogenesis in leg bud mesenchymal cells from stage 22/23 chick embryos, which suggests that TGF-β3 functions differently in establishing fore- and hind-limb identity (Jin et al., 2006; 2007a). Consistent with this observation, the degree of precartilage condensation and chondrogenesis as assessed by peanut agglutinin (PNA) binding at day 3 and by Alcian blue staining for sulfated proteoglycans at day 5 were significantly decreased in leg bud mesenchymal cell culture with treatment of 5 ng/ml TGF-β3 (Fig. 1A). The level of a chondrogenic protein, type II collagen was also dramatically decreased by TGF-β3 treatment (Fig. 1B). We have also shown previously that TGF-β3 increased apoptosis of leg bud mesenchymal cells through the downregulation of connexin43 (Cx43) and integrin β4 (Jin et al., 2008) suggesting an inhibitory role of TGF-β3 on the modulation of chondrogenic competent cell density. Indeed, the total number of cells (Fig. 1C), thymidine incorporation (Fig. 1D) and adherent cells (Fig. 1E) were significantly reduced in the cultures treated with TGF-β3 compared to the control cultures.

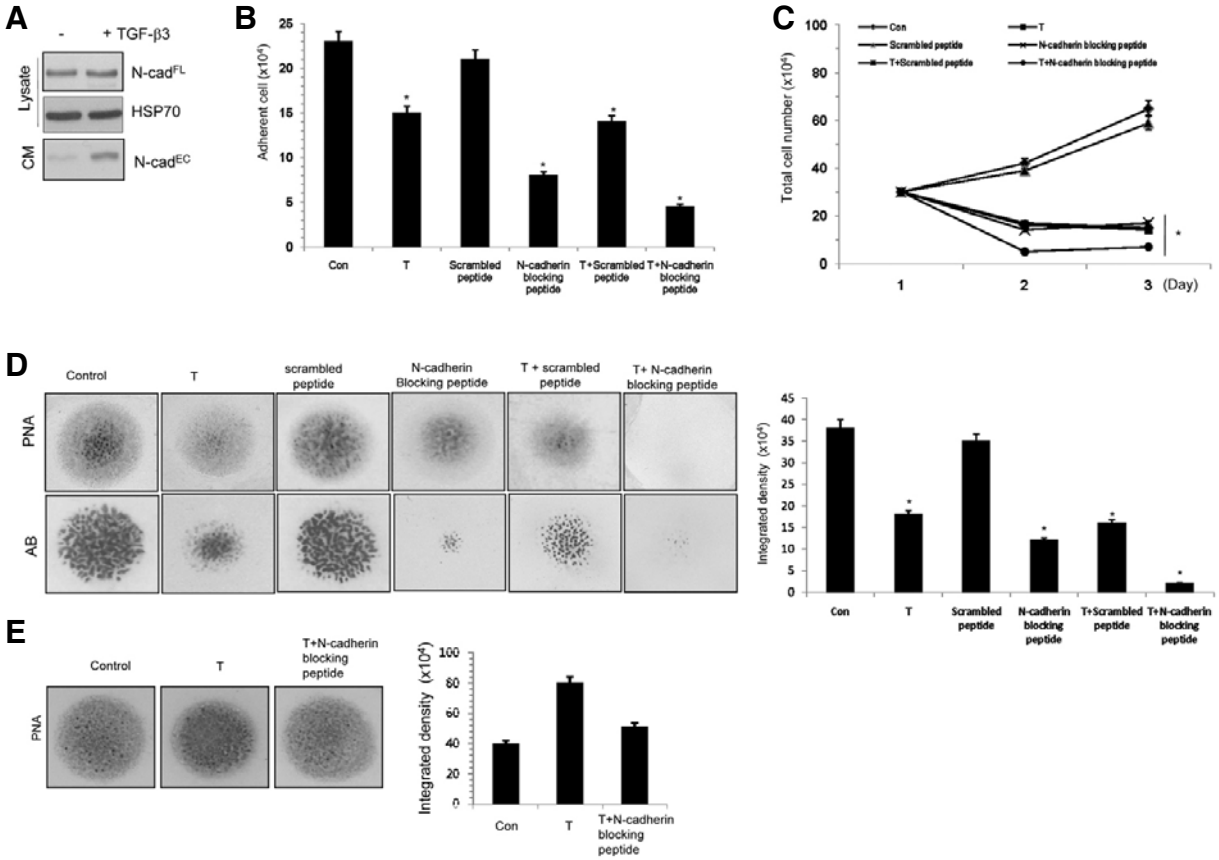

Fig. 2. TGF-β3 inhibits cellular condensation via the upregulation of N-cadherin ectodomain shedding. (A) Leg bud mesenchymal cells were cultured at a density of 2×10^7 cells/ml with or without 5 ng/ml of TGF-β3. Cell lysates and conditioned media (CM) obtained from 24 h-culture were subjected to Western blot analysis using an antibody against the full-length N-cadherin (N-cad^{FL}) and an antibody against the ectodomain of N-cadherin (N-cad^{EC}), respectively. (B-D) Cells were cultured with or without 5 ng/ml of TGF-β3 (T) and/or 100 μg of the N-cadherin blocking peptide (HLRAHAVDINGN-QVEN). A scrambled peptide (ARLQHDVNANVHEING) was used as a control. Cells were cultured for 2 h with agitation in the absence or presence of reagents, seeded with 5×10^5 cells/ml, cultured in the absence or presence of reagents, and adherent cells were counted after 6 h of culture (B). The cell numbers were counted at the indicated days (C). Cells were stained with PNA at culture day 3 and Alcian blue at culture day 5 and the staining density of PNA was quantified with Image J (D). Wing bud mesenchymal cells were cultured with or without 5 ng/ml of TGF-β3 (T) and/or 100 μg of the N-cadherin blocking peptide (HLRAHAVDINGNQ-VEN). A scrambled peptide (ARLQHDVNANVHEING) was used as a control. Cells were stained with PNA at culture day 3 and staining density of PNA was quantified with Image J (E). The data represent the average values of five independent experiments, with standard deviations shown. *, statistically different from control cells ($P < 0.005$).

TGF-β3 induces ectodomain shedding of N-cadherin

The cadherin superfamily includes cadherins, protocadherins, desmogleins, desmocollins, and additional proteins. Structurally, they share "cadherin repeats", which are the extracellular Ca^{2+}-binding domains. N-cadherin plays an important role in mesenchymal cell condensation, a prerequisite for chondrogenesis (Tavella et al., 1994). To assess the implication of N-cadherin involvement in the chondro-inhibitory action of TGF-β3, chick chondrogenic progenitor cells were cultured in the absence or presence of 5 ng/ml of TGF-β3 and subjected to Western blot analysis with antibody against the full-length or the C-terminal part of N-cadherin. The level of full-length N-cadherin protein was not affected by TGF-β3 treatment. However, Western blot analysis of conditioned medium (CM) from chondrogenic progenitor cells showed that TGF-β3-treatment increased the levels of the soluble 35 kDa fragment of N-cadherin at day 1 of culture (Fig. 2A) suggesting that the extracellular domain of the 135 kDa full-length N-cadherin protein was cleaved at the initiation stage of chondrogenesis.

To determine the function of N-cadherin shedding during chondro-inhibition by TGF-β3, the effect of the synthetic peptide (HLRAHAVDINGNQVEN) which contains the presumptive N-cadherin interaction region (HAV), was examined and compared to a non-active scrambled control peptide (ARLQHDV-NANVHEING). Cultured chick leg chondrogenic progenitor cells were incubated for 2 h with agitation in the absence or presence of the peptides with or without TGF-β3 and then seeded for the adherent assay. As shown in Fig. 2B, the number of adherent cells was reduced in cultures containing TGF-β3 or the N-cadherin blocking peptides in comparison to the control. The most drastic effect was seen in cultures co-treated with TGF-β3 and N-cadherin blocking peptides (Fig. 2B). The total number of cells was also reduced in those cultures (Fig. 2C). Cells treated with TGF-β3 or co-treated with TGF-β3 and N-cadherin blocking peptides showed a decrease in the area and intensity of precartilage condensation (Fig. 2D). The chondro-inhibitory action of N-cadherin blocking peptides was also observed in a culture of wing chondroblasts stimulated by TGF-β3

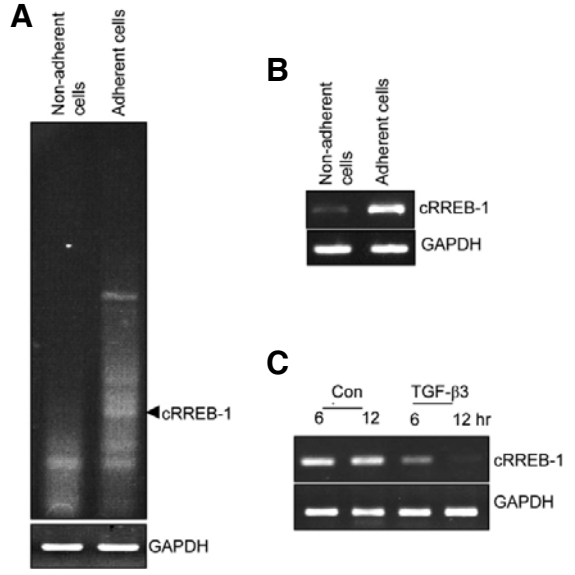

Fig. 3. TGF-β3 suppresses *cRREB-1* mRNA expression early in chondrogenesis. Leg bud mesenchymal cells were seeded at a density of 2×10^7 cells/ml and cultured for 1 h, and adherent and non-adherent cells were harvested. (A) cDNAs generated from adherent and non-adherent cells were amplified with 40 different ACPs, and differentially expressed genes were cloned and identified by sequence analysis. In adherent cells, several genes amplified with ACP15 were significantly increased, one of which was c*RREB-1* as confirmed by sequencing analysis. (B) RT-PCR using cRREB-1-specific primer was performed on RNA from adherent and non-adherent cells. GAPDH was used as a loading control. (C) RT-PCR using cRREB-1-specific primers was performed on RNA from cells treated with or without TGF-β3. The data shown are representative of at least four independent experiments.

(Fig. 2E). However, the inhibitory effect of N-cadherin blocking peptides on the chondrogenesis of wing chondroblasts was less dramatic than that of leg chondroblasts. These data collectively suggest that the downregulation of cell adhesion due to N-cadherin shedding by TGF-β3 is responsible for suppression of precartilage condensation in leg bud mesenchymal cells.

TGF-β3 inhibits cellular condensation by downregulating the chick ras-responsive element binding transcription factor, cRREB-1

To identify genes involved in the adhesion of chondrogenic-competent cells, differential display PCR (DD-PCR) was performed. The isolated mesenchymal cells were plated and incubated for 1 h, and adherent- and non-adherent cells were harvested. Total RNA was subjected to ACP-based RT-PCR, using a combination of 40 arbitrary primers and two anchored oligo (dT) primers from the ACP-based GeneFishing PCR kit. The resulting amplimers were isolated and sequenced, and the nucleotide sequences were used to search the GenBank database. The DD-PCR yielded 40 differentially expressed amplicons (Fig. 3A), one of which displayed considerable homology to the chick ras-responsive element binding transcription factor, *cRREB-1*. RT-PCR analysis further confirmed that the expression level of the mRNA encoding *cRREB-1* was much higher in adherent versus non-adherent leg bud mesenchymal cells (Fig. 3B). Furthermore, *cRREB-1* expression was substantially increased in control cultures in comparison to the TGF-β3-treated cultures at the beginning of the culture period (Fig. 3C). These data suggest that RREB-1 might be involved in condensational changes of chondrogenic-competent cells during TGF-β3-induced chondro-inhibition.

To investigate the role of RREB-1 during chondrogenesis, vectors containing three different RREB-1-specific small interfering RNAs (siRNAs) were constructed and used to transfect mesenchymal cells. RT-PCR analysis 48 h after transfection revealed that siRNA effectively knocked down the endogenous cRREB-1 mRNA level by cRREB-1 siRNA-1 but not by siRNA-2

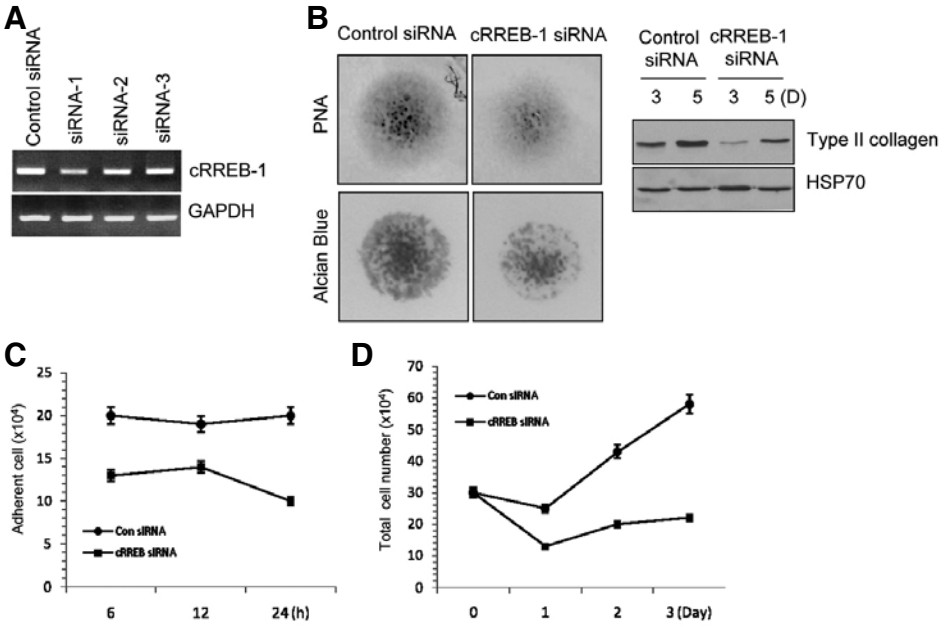

Fig. 4. siRNA-directed knockdown of *cRREB-1* inhibits cellular condensation during chondrogenesis. Leg bud mesenchymal cells were transfected with either control (control siRNA-1, 5′-ATGAAAGGCTTTCCAGTGGGAGCTC-3′) or cRREB-1-specific siRNAs (siRNA-1, -2, -3). (A) The knock-down efficiency was confirmed by RT-PCR using *cRREB-1* specific primers at 48 h. (B) The cells were stained for PNA binding on culture day 3 or with Alcian blue on culture day 5 (left panel) and changes in the level of type II collagen were determined by Western blot ana-lysis at the specified time points (right panel). (C) Adherent cells at 6, 12 and 24 h after transfection were counted. (D) The cell numbers were counted at the indicated days. *, statistically different from control cells ($P < 0.005$).

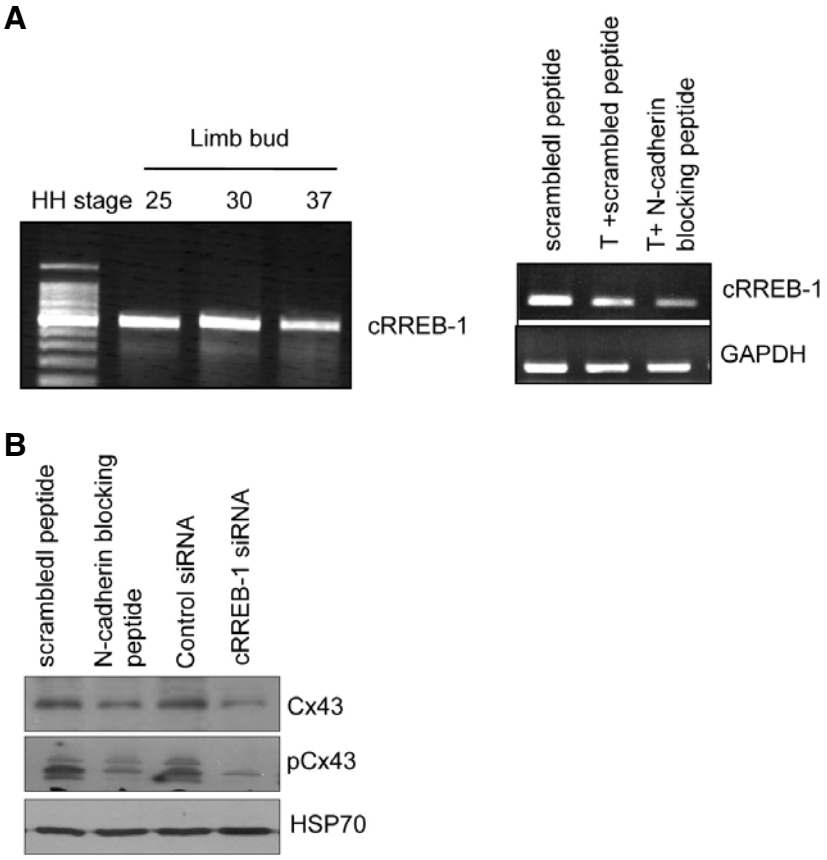

Fig. 5. TGF-β3 inhibits cellular condensation through downregulation of cRREB-1 and Cx43 by enhancing N-cadherin ectodomain shedding. (A) RT-PCR using cRREB-1-specific primer was performed on RNA from either developing limb buds from HH stage 25, 30, and 37 chick embryos (left panel) or cells treated with 5 ng/ml TGF-β3 (T) and/or 100 μg of N-cadherin blocking peptide and then harvested at day 1 (right panel). RT-PCR was performed using RREB-1-specific primers. GAPDH was used as a loading control. (B) Cells were treated with 100 μg of N-cadherin blocking peptide or transfected with cRREB-1 specific siRNA (cRREB-1 siRNA) and the changes in the levels of conexin43 (Cx43) and phospho-connexin43 (pCx43) were determined by Western blot analysis at culture day 2. The data shown are representative of at least four independent experiments.

or siRNA-3 (Fig. 4A). Knockdown of the endogenous *cRREB-1* in these cells inhibited precartilage condensation, Alcian blue uptake and the expression of type II collagen (Fig. 4B). The numbers of adherent cells (Fig. 4C) and total cells (Fig. 4D) were also reduced in cultures transfected with cRREB-1 siRNA in comparison to the control. We also used Runx2 siRNA as control to rule out any unspecific effects of the siRNA. Runx2 siRNA did not show any of effect (data not shown).

The level of cRREB transcription was also observed in chick limb bud of HH stage 25, 30, and 37 indicating that it has a putative role in limb development (Fig. 5A, left panel). Treatment of cells with TGF-β3 and/or the N-cadherin blocking peptides suppressed the level of *cRREB-1* transcription at culture day 1 (Fig. 5A, right panel). These data indicate that cRREB-1 acts at the early condensation stage of chondrogenic competent cells and TGF-β3-induced N-cadherin shedding might be responsible for the downregulation of *cRREB-1*.

Since cell-cell contact and cell-cell communication is a prerequisite for cell adhesion and precartilage condensation, the negative modulation on cell adhesion and precartilage condensation by the knockdown of endogenous *cRREB-1* or treatment with N-cadherin blocking peptides could affect the expression or activation levels of cell-cell communication proteins such as connexins. Previously, our laboratory showed that blockage of gap junctional communication, particularly of connexin 43 (Cx43), inhibited cell proliferation and precartilage condensation of leg bud mesenchymal cells (Jin et al., 2008). The unphosphorylated and phosphorylated forms of the Cx43 protein were reduced by the transfection of cRREB-1 specific siRNA or treatment with N-cadherin blocking peptides (Fig. 5B) suggesting the possible involvement of Cx43 on the chondro-inhibitory action of TGF-β3.

DISCUSSION

Chondrogenesis is the earliest phase of skeletal development involving the condensation of mesenchymal cells, the differentiation of mesenchymal cells into chondrocytes, and patterning of chondrifying tissues into skeletal structures (Hoffman et al., 2003). Mesenchymal condensation results from an active cell movement that causes an increase in mesenchymal cell packing density in the core of the limb bud (Cottrill et al., 1987; DeLise et al., 2000; Knudson and Knudson, 2001). This event is associated with an increase in cell-to-cell contacts and interactions through cell-cell adhesion molecules and gap junctions that initiates chondrogenic differentiation (Kang, 2008). In this study, we found that TGF-β3 triggers N-cadherin ectodomain shedding and results in a reduction of cell-to-cell adhesion at the initiation stage of chondrogenesis in chick leg bud mesenchymal cells. N-cadherin belongs to a family of intercellular adhesion molecules that mediate calcium-dependent cell-to-cell adhesions through homophilic interactions. Therefore, the release of the homophilic binding site-containing extracellular domain is functionally important for the regulation of cell adhesion and cell migration (Paradies and Grunwald, 1993). Since changes in and modification of the extracellular composition by cell-cell and cell-matrix interaction are crucial for differentiation of mesenchymal cells into chondrocytes and the maturation of chondrocytes (DeLise et al., 2000; Tuan, 2004), inhibition of cell-to-cell adhesion by TGF-β3-mediated N-cadherin impairment could be at least partially responsible for the chondro-inhibitory effect.

Ectodomain shedding is a process by which the extracellular domain of a transmembrane molecule is proteolytically removed from the cell surface. In the case of N-cadherin, ectodomain shedding results in a soluble N-cadherin fragment, which inhibits normal N-cadherin function in cell adhesion, as shown in this study. Thus far, induction of cellular proteases such as several matrix-metalloproteinases (MMPs), γ-secretase, and ADAMs (A Disintegrin And Metalloproteases) might implicate ectodomain shedding of N-cadherin. Previously our laboratory also showed that TGF-β3 regulates the mRNA levels of *ADAM-10* which has been shown to mediate both basal and inducible cadherin ectodomain shedding (Jin et al., 2007b). Reiss et al. (2006) showed that ADAM-10-mediated cleavage of cadherin represents a potent mechanism for regulating the cell-cell adhesion, motility, and proliferation of epithelial cells. In contrast to epithelial cells, TGF-β3 suppresses induction of ADAM-10 and inhibits cell proliferation in chick leg bud mesenchymal cells (Jin et al., 2007b) suggesting that TGF-β3-mediated N-cadherin shedding and impaired cell adhesion are induced by cellular proteases other than ADAM-10.

The exact mechanism involved in the disruption of cell adhesion via the ectodomain shedding of N-cadherin has not been studied. One possibility is the internalization of N-cadherin via endocytosis. The residual membrane-tethered cleavage product of N-cadherin might potentially be targeted by tyrosine kinases to induce endocytosis and/or further cleaved intracellularly and released as a soluble fragment into the cytosol in a complex with β-catenin. This question will be an interesting challenge to study in future research.

In the present study, we further showed the novel finding that *cRREB-1* is likely to function in N-cadherin-mediated cell-to-cell adhesion. RREB-1, a zinc finger protein, was first cloned from a human medullary thyroid carcinoma cell line and is implicated in Ras responsiveness (Thiagalingam et al., 1996). Ras-responsive transcriptional element (RRE) in the promoter of the human *calcitonin* gene binds RREB-1, and RREB-1 was shown to be activated by Raf, the immediate downstream effector of Ras in receptor protein tyrosine kinase cascade (Thiagalingam et al., 1996). However, its exact function has not been elucidated. Here, we found that the adherent chondrogenic progenitor cells showed higher levels of *cRREB-1* mRNA than non-adherent chondrogenic progenitor cells, and that RREB-1 knockdown in chondrogenic progenitor cells decreased cell-to-cell adhesions and subsequently inhibited precartilage condensation of chick leg bud mesenchymal cells. Moreover, treatment with TGF-β3 or the addition of functional N-cadherin blocking peptides inhibited transcriptional expression of *cRREB-1* levels. This is the first study showing the involvement of cRREB-1 in cellular condensation and chondrogenesis and that cRREB-1 signaling might be responsible for TGF-β3-induced reduction of cellular adhesion.

Herein, we showed that TGF-β3 inhibits cell-to-cell adhesion during chondrogenesis of leg bud mesenchymal cells though the stimulation of N-cadherin ectodomain shedding and the reduction of *cRREB-1* mRNA levels. Apoptosis induced by a loss in cell adhesion has been described in a wide variety of adherent cell types (Grossmann, 2002). It can be postulated that TGF-β3-induced ectodomain shedding resulting in decreased chondroblast condensation may also lead to an increase in apoptosis. Previously, our laboratory showed that in cultured chick leg bud mesenchymal cells, TGF-β3 treatment downregulates connexin 43 (Cx 43) and induces apoptotic cell death via the downregulation of integrin β4 (Jin et al., 2008). Since cadherin expression is necessary for the formation of tight, adherent gap junctions (Gumbiner et al., 1988), the inhibitory effect of TGF-β3 observed in this study might be the result of Cx43 downregulation. The adherent junction could be acting as a node for the transmission of signals that emanate from the cell-to-cell contacts.

Our study helps to verify the regulatory molecules and signaling mechanism involved in establishing fore-limb and hind-limb identity induced differentially by TGF-β3. Treatment of chick wing bud mesenchymal cells with TGF-β3 increases cell-to-cell adhesion by suppressing of N-cadherin ectodomain shedding, which is in contrast to leg bud mesenchymal cells (unpublished data), in which TGF-β3 regulation of N-cadherin ectodomain shedding has the opposite effect on cellular adhesion. This study suggests a possible interaction between N-cadherin and cRREB, since the functional blocking of N-cadherin suppressed cellular condensation by inhibiting cRREB levels. Therefore, we hypothesize that the opposite action of N-cadherin shedding by TGF-β3 may lead opposite expression level of cRREB in wing- vs. leg bud mesenchymal cells, which could contribute to establishing fore-limb and hind-limb identity. However, it will need to be investigated more thoroughly. In summary, we have provided evidence that of N-cadherin ectodomain shedding and the downregulation of *cRREB-1* are key mediators of the chondro-inhibitory action of TGF-β3 during chondrogenesis of chick leg bud mesenchymal cells.

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