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Connective Tissue Growth Factor (CTGF) Acts as a Downstream Mediator of TGF-β1 to Induce Mesenchymal Cell Condensation

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Mesenchymal cell (MC) condensation or the aggregation of MCs precedes chondrocyte differentiation and is required for subsequent cartilage formation during endochondral ossification. In this study, we used micromass cultures of C3H10T1/2 cells as an in vitro model system for studying MC condensation and the events important for this process. Transforming growth factor β1 (TGF-β1) served as the initiator of MC condensation in our model system and we were interested in determining whether CTGF functions as a downstream mediator of TGF-β1. CTGF is a matricellular protein that has been found to be expressed in MC condensations and in the perichondrium. Micromass cultures of C3H10T1/2 cells condensed under TGF-β1 stimulation concomitant with dramatic up-regulation of CTGF mRNA and protein levels. CTGF silencing by either CTGF siRNA or CTGF antisense oligonucleotide approaches showed that TGF-β1-induced condensation was CTGF dependent. Furthermore, silencing of CTGF expression resulted in significant reductions in cell proliferation and migration, events that are crucial during MC condensation. In addition, up-regulation of Fibronectin (FN) and suppression of Sox9 expression by TGF-β1 was also found to be mediated by CTGF. Immunofluorescence of developing mouse vertebrae showed that CTGF, TGF-β1 and FN were co-expressed in condensations of MCs, while Sox9 expression was low at this stage. During subsequent chondrogenesis, Sox9 expression was high in chondrocytes while CTGF expression was limited to the perichondrium. Thus, CTGF is an essential downstream mediator of T GF-β1-induced MC condensation through its effects on cell proliferation and migration. CTGF is also involved in up-regulating FN and suppressing Sox9 expression during TGF-β1 induced MC condensation. J. Cell. Physiol. 210: 398–410, 2007.

Endochondral skeletal development begins with the formation of a cartilaginous template, where mesenchymal cells (MCs) aggregate and increase in density prior to their overt differentiation into chondrocytes. Chondrocytes themselves mature through several stages while laying down a matrix. The matrix undergoes mineralization and vessel penetration as the chondrocytes complete their life cycle by undergoing apoptosis. At this point, osteoblasts invade and lay down a bone matrix using the remnants of mineralized cartilage matrix as a template (Kronenberg, 2003). An important stage of endochondral ossification is the initial aggregation of MCs in a process called prechondrogenic or MC condensation. This process is required for subsequent cartilage formation and is regarded as a crucial stage for the commitment of MCs into the chondrocytic lineage (Mariani and Martin, 2003; Shum et al., 2003). The formation of MC condensations are dependent on increased mitotic activity and the migration of cells toward a center (Hall and Miyake, 1992; Hall and Miyake, 1995; Hall and Miyake, 2000).

To model MC condensation in vitro, chick limb bud cells were cultured in high density (micromass) cultures to simulate the process of condensation (Ahrens et al., 1977). However, a draw back of using primary limb bud cultures is that the harvested MCs are already committed toward the chondrocytic lineage and spontaneously condense and differentiate (Ahrens et al., 1977; Zhang et al., 2004). This provides a challenge for studying the contribution of specific growth factors in the process of condensation and/or early differentiation.

The in vitro chondrogenic model has also been successfully applied to C3H10T1/2 cells, a multipotential murine mesenchymal stem cell-line (Denker et al., 1995). C3H10T1/2 cells are capable of differentiating into MC types such as myocytes, adipocytes, osteoblasts, chondrocytes and pericytes in monolayer cultures (Reznikoff et al., 1973; Taylor and Jones, 1979; Denker

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et al., 1995, 1999; Shea et al., 2003; Kale et al., 2005). In addition, when plated in micromass cultures under either TGF-β1 or BMP-2 stimulation, C3H10T1/2 cells condense and differentiate along the chondrocytic lineage (Denker et al., 1995, 1999; Atkinson et al., 1997; Haas and Tuan, 1999). While C3H10T1/2 are multipotential, these cells do not spontaneously differentiate. Thus, the uncommitted nature of these cells and the ability to differentiate into the chondrocytic lineage provides a unique opportunity to study the importance of TGF-β1 and CTGF during the process of condensation and the initial commitment of MCs into the chondrocytic lineage (Denker et al., 1995).

Connective tissue growth factor (CTGF/CCN2) is a 38 kDa cysteine-rich matricellular protein belonging to the CCN family of secreted proteins, whose members include Cyr61, CTGF, Nov, Wisp1, Wisp2, and Wisp3. The highly homologous CCN family members have diverse functions that include cell adhesion, proliferation, differentiation and migration and have been implicated in more complex biological processes including angiogenesis, chondrogenesis/osteogenesis, wound healing, fibrosis and tumorigenesis (Perbal, 2004). CTGF in particular, has been found to be involved in various stages of endochondral ossification (Takigawa et al., 2003). In situ hybridization studies have shown CTGF expression in MC condensations, perichondrium/periosteum, hypertrophic chondrocytes, blood vessels at the growth plate/metaphyseal junction, and osteoblasts lining the metaphyseal trabeculae (Friedrichsen et al., 2003; Ivkovic et al., 2003; Takigawa et al., 2003). In similar fashion, CTGF was also found to be highly expressed in MC condensations and the perichondrium of developing Meckel's cartilage in vivo and recombinant CTGF (rCTGF) treatment promoted adhesion and migration of Meckel's cartilage progenitor cells in vitro (Shimo et al., 2004). CTGF-deficient mice demonstrated various endoskeletal abnormalities resulting from abnormal cartilage/bone development causing a misshapen skeleton (Ivkovic et al., 2003).

CTGF is normally not detectable in fibroblastic cells. However, treatment with transforming growth factor β1 (TGF-β1) causes a robust and rapid up-regulation of CTGF expression that is independent of de novo protein synthesis, establishing CTGF as a downstream gene of TGF-β1 (Moussad and Brigstock, 2000). Up-regulation of CTGF by TGF-β1 was found to occur at the transcriptional level by consensus Smad and transcription enhancer elements in the CTGF promoter (Leask and Abraham, 2004). In addition, at low concentrations of TGF-β1, CTGF was found to serve as a chaperone to enhance TGF-β1 binding to its receptor. However, at higher concentrations of TGF-β1 this enhancement did not occur (Abreu et al., 2002). TGF-β1, a multifunctional 25 kDa homodimer, is important in embryonic development, cell proliferation, differentiation, and apoptosis (Shi and Massague, 2003). Detection of TGF-β1 in MC condensations in vivo and the demonstration of condensation in limb bud cultures stimulated with TGF-β1 support the role of TGF-β1 an initiator of MC condensation (Newman, 1988; Hall and Miyake, 1992; Zhang et al., 2004). Furthermore, transfection of a dominant-negative mutant of the TGF-beta type II receptor resulted in the complete inhibition of condensation in undifferentiatied mouse chondrogenic cells (ATDC5) (Kawai et al., 1999).

Since TGF-β1 is a potent stimulator of CTGF expression in cells such as fibroblasts and endothelial cells, and CTGF has biological effects similar to those of TGF-β1, CTGF has been shown to act as an essential downstream mediator of TGF-β1 for some of its effects in those cells (Chen et al., 2000; Gore-Hyer et al., 2002; Leask and Abraham, 2003; Leask and Abraham, 2004). We propose that CTGF may act as a downstream mediator of TGF-β in promoting the condensation of mesenchymal stem cells. In this study, we used micromass cultures of the C3H10T1/2 mesenchymal stem cell line to study the role of CTGF in TGF-β-induced condensation. Silencing of CTGF expression (using siRNA or antisense oligonucleotide approaches) after TGF-β1 stimulation was used to highlight the importance of CTGF in promoting MC condensation and regulating Fibronectin (FN) and Sox9 expression.

MATERIALS AND METHODS

Immunolocalization of CTGF, TGF-β1, FN, and Sox9 during embryonic development

Embryonic day 10.5 and 12.5 murine embryos were fixed with 4% Paraformaldehyde and sectioned using a standard microtome (Leica M) as previously described (Litvin et al., 2004). Sections were stained with Safranin-O to determine the distribution and presence of proteoglycans and counterstained with fast green. For immunofluorescent analyses, tissue sections were deparaffinized and probed with primary antibodies overnight, followed by incubation with Cy2 (green) and Cy3 (red) conjugated secondary antibodies 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA) as previously described (Barr et al., 2003). The sections were mounted using 80% glycerol in PBS and examined using a standard fluorescent microscope (Nikon Eclipse E800). Primary antibodies used: anti-TGF-β1 antibody (R&D Systems, Minneapolis, MN), anti-CTGF antibody (Cambridge Research Biochemicals, Bellingham, UK), anti-Sox-9 antibody (Santa Cruz, Santa Cruz, CA), anti-FN antibody (Santa Cruz).

Cell culture

C3H10T1/2 clone 8 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and expanded in 100 mm standard polystyrene tissue culture dishes (Corning, Corning, NY) in growth media which consisted of Dubecco's Modified Eagles Medium (DMEM) (Cellgro, Herndon, VA) with 10% fetal bovine serum (FBS) (ATCC), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C in a 5% CO_2 humidified incubator.

Transfection efficiency

Twenty-Four hours prior to transfection, 2.5×10^5 cells were plated on 100 mm dishes in growth media without antibiotics. Two hours prior to transfection, the media was changed to Opti-MEM (Gibco, Carlsbad, CA). To determine transfection efficiency, Block-iT transfection kit (Invitrogen, Carlsbad, CA) was used. Block-iT FITC-labeled double stranded oligonucleotides (Block-iT oligos) were diluted in Opti-MEM to a final concentration of 50 nM. The Block-iT oligos were delivered into the cells using Lipofectamine 2000 reagent (Lipofectamine) (Invitrogen) and incubated in a 37°C incubator for 4 h. The cultures were then washed with PBS and transfection efficiency was assessed by counting the fluorescent cells from that of total cells using an inverted fluorescent microscope (TE300 Nikon). Transfection efficiency was calculated as the average of three randomly chosen areas from each culture.

siRNA and antisense oligonucleotide transfection

To down-regulate CTGF expression, a pool of four CTGF siRNA, 5′-GAGGAACTATCCCACCAAA-3′, 5′-GTGAGAACGTTATGTCATG-3′, 5′-CAAAGCAGCTGCAAATACC-3′, 5′-CCATACAAGTAGTCTGTCA-3′ (Dharmacon, Lafayette, CO) was used. C3H10T1/2 cells were transfected with CTGF siRNA (siCTGF), Non-silencing siRNA (siCNTRL) (Qiagen, Valencia, CA) or Lipofectamine alone (LIPO) as indicated above. After 4 h of transfection, FBS was added to bring the culture to 10% FBS and micromass cultured after 12–18 h. For

CTGF antisense oligonucleotide (asCTGF) 5′-GCATT-TCCCGGGCAGCTTGACCCTTCTCGGG-3′, and scrambled oligonucleotide control (scrCNTRL) 5′-TGGGCCTCGACAT-TCGACTATGCCGTGGTCC-3′ were generated with phosphothiolation on every base (Invitrogen). Transfection protocol was identical to siRNA transfection. As indicated above, after 4 h of transfection, FBS was added to bring the culture to 10% FBS and micromass cultured after 12–18 h.

TGF-β1 treatment

Unless otherwise stated, 1 ng/ml of TGF-β1 (Calbiochem, San Diego, CA) was supplemented to the differentiation media which consisted of Ham's F12 media (Gibco) plus 10% FBS to initiate the MC condensation process in the micromass cultures of C3H10T1/2. TGF-β1 was also supplemented to the growth media of monolayer cultures to perform both the migration and proliferation assays.

Micromass culture

Micromass culturing technique was performed as previously described (Denker et al., 1995). Briefly, 2×10^5 C3H10T1/2 cells were plated as a 20 μl drop at the center of a 24-well tissue culture plate and allowed to adhere for 1 h at 37°C prior to flooding with 1 ml of differentiation media (with or without TGF-β1 stimulation). For transfection experiments, C3H10T1/2 cells were transfected in monolayer and 12–18 h post-transfection, the cells were plated in micromass cultures, followed by the addition of differentiation media with or without TGF-β1 stimulation. Micromass cultures were viewed using a TE300 Nikon inverted microscope. In addition, total RNA or cell lysates were collected from the micromass cultures after 48 h.

Western blot analysis

Total protein was prepared using RIPA buffer and Western blot analyses were performed as previously described (Safadi et al., 2003). For micromass cultures, six micromass cultures were pooled in 200 μl of RIPA buffer. Forty micrograms total protein were loaded and resolved on a 10% SDS-PAGE gel. The protein was transferred to a PVDF membrane and probed with the following primary antibodies: CTGF 1:100 (Santa Cruz), β-Actin 1:5,000 (Sigma, St. Louis, MO), Sox9 1:5,000 (Abcam, Cambridge, MA), FN 1:100 (BD, Franklin Lakes, NJ). Membranes were washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz). Signal was detected by enhanced chemiluminescence (Pierce, Rockford, IL). Three independent experiments were performed for all Western blot analyses, showing similar results. Representative Western blot analyses are shown in Figures 1–3 and 6.

RNA isolation and reverse transcription

Total RNA was isolated using RNeasy kit (Qiagen) and DNase treated using DNA-free kit (Ambion, Austin, TX). RNA quality and quantity was determined using spectrophotometry. RNA quality was further confirmed by agarose gel electrophoresis. For cDNA synthesis, 1 μg total RNA was reverse transcribed using First Strand Stratascript kit (Stratagene, La Jolla, CA).

Semi-quantitative PCR (sqPCR) and quantitative PCR (qPCR)

Gene expression for CTGF, Sox9, and FN was determined by qPCR analysis using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA) using 1 μl cDNA. Reactions were run on a 7,400 Real-Time PCR system (Applied Biosystems). Ten-fold serially diluted CTGF plasmid was used to generate the standard curve and all the samples were normalized to GAPDH. Three independent experiments were performed for each gene and each experiment was performed in triplicate. All experiments showed similar results and a representative experiment is shown in Figures 2, 4 and 6. For CTGF gene expression after antisense oligonucleotide treatment, sqPCR analysis was performed using the GeneAmp PCR system 9700 (Applied Biosystems). CTGF primers for sqPCR are as follows: 5′-CCCTGACCCAACTATGATGC-3′, 5′-GGGA-CAGTTGTAATGGCAGG-3′. The cycling program was as follows: 95°C for 3 min, 25 cycles of 94°C, 60°C, 72°C for 30 sec each, followed by 72°C for 2 min. Samples were resolved using 1.5% agarose gel and densitometric analysis was performed using Alpha Imager (Alpha Innotech, San Leandro, CA). Samples were normalized to GAPDH. Three independent experiments were performed and each PCR reaction was performed in triplicate. All experiments showed similar results and a representative experiment is shown in Figure 3.

Cell proliferation assay

Subconfluent C3H10T1/2 cultures were transfected with Lipofectamine alone, 20 nM siCTGF or siCNTRL prior to being plated at 5,000 cells/well in six replicates on a 96-well microtiter plate (Fisher). The cells were cultured in growth media with or without (negative control) TGF-β1 (1 ng/ml) for 24 h before proliferation was determined using the CyQuant cell proliferation kit (Molecular Probes, Carlesbad, CA). Fluorescence was measured using a fluorometer (Wallac 1420, Perkin Elmer, Wellesley, MA) with filters set for fluorescein. A total of six measurements were taken per treatment group. Three independent experiments were performed with similar results and a representative experiment is shown in Figure 5.

Cell migration assay

Cell migration assay was adapted from the wounding assay as previously described (Luo et al., 2004). C3H10T1/2 cells were transfected with Lipofectamine alone, 20 nM siCTGF or siCNTRL prior to being plated at 10,000 cells/well in triplicate on an 8-well chamber slide (Labtek, Campbell, CA). The cells were cultured in growth media for 24 h prior to performing a migration assay. To perform the migration assay, an area on the plate was cleared of cells using a pipette tip. Next, the cultures were incubated for 8 h in growth media supplemented with 1 ng/ml TGF-β1 at 37°C to allow for migration. Cultures were viewed using a TE300 Nikon inverted microscope and photographed at zero and 8 h after clearing. Cell migration was determined by measuring the difference between the cleared area prior to migration from the cleared area remaining after migration (area restored) using the BioQuant software (Bioquant Image Analysis Corporation, Nashville, TN). Two measurements on each of the triplicate samples were taken for a total of six measurements per treatment group. Three independent experiments were performed with similar results. A representative experiment is shown in Figure 5.

Confocal microscopy

C3H10T1/2 cells were micromass cultured on coverglass chamber slides as described above. Next, the cell membrane was labeled using 1 μM SP-DiOC$_{18}$(3) membrane dye (Molecular Probes) for 5 min at 37°C, then placed in 4°C for an additional 15 min. The cells were then washed with PBS and resuspended in differentiation media supplemented with or without 1 ng/ml TGF-β1. Cultures were protected from light and incubated for 24 h. Next, the cells were fixed using 4.0% formaldehyde in PBS at 37°C for 10 min and permeabilized in acetone for 10 min at −20°C. The cells were observed using a Confocal Microscope (Olympus Fluoview) at a wavelength of 488 nm.

Statistical analysis

Univariate ANOVA was used to analyze differences in CTGF mRNA expression between the negative control (no TGF-β1 treatment) and the transfected groups which consisted of TGF-β1 stimulated C3H10T1/2 cultures transfected with either Lipofectamine transfection control, non-silencing siRNA control, or CTGF siRNA (either 10, 20, or 50 nM doses). Univariate ANOVAs were also used to analyze FN or Sox9 mRNA expression differences between the negative control and the same transfected groups as above, except that only the 20 nM dose was used for CTGF siRNA. Univariate ANOVA was followed by the Bonferroni post-hoc test which compared mRNA expressions in the transfected groups with that of the

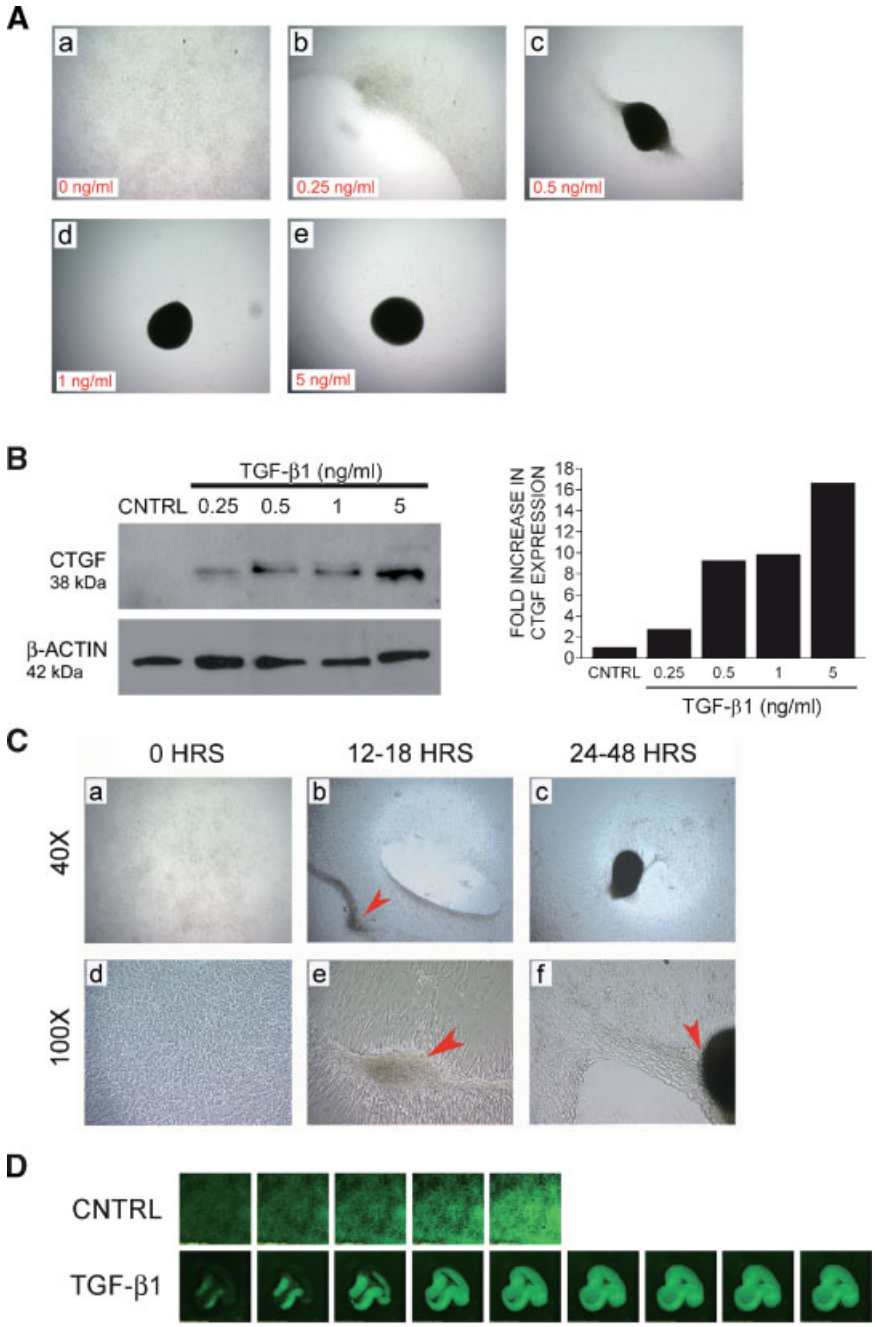

Fig. 1. CTGF up-regulation by TGF-β1 occurs concurrently with induction of MC condensation. **A**: Micromass cultures were stimulated with 0.25, 0.5, 1, and 5 ng/ml of TGF-β1 (Ab–Ae) for 48 h. MC condensation resulted in the formation of prechondrogenic nodules at TGF-β1 concentrations of 0.5 ng/ml and above (Ac–Ae), compared to the untreated control cultures (Aa) in which there was no evidence of MC condensation. All panels are 40× magnification. **B**: Representative Western blot analysis using 40 μg total proteins from micromass cultures treated with TGF-β1 shows CTGF expression increased in a dose-dependent manner. CTGF expression was normalized to β-Actin and data are expressed as fold increase compared to the untreated control (CNTRL). **C**: Micromass cultures stimulated with 1 ng/ml TGF-β1 were followed with time after treatment. There was clear evidence of MC aggregation towards a center (arrowheads) by 12–18 h (Cb and Ce) resulting in the formation of nodules by 24–48 h (Cc and Cf); 40× magnification (Ca–Cc); 100× magnification (Cd–Cf). **D**: Micromass cultures stimulated with 1 ng/ml TGF-β1 for 24 h were optically sectioned via confocal microscopy. The results show that cells were uniformly distributed in non-TGF-β1 treated control (CNTRL) cultures (10 μm sections) compared to the nodules consisting of large aggregations of cells (50 μm sections) for TGF-β1 stimulated cultures. All experiments were repeated three times with similar results.

negative control and also the non-silencing siRNA control to the other experimental groups. A P-value ≤ 0.05 was considered significant for all analyses. An unpaired, two-tailed t-test was used to analyze effects of CTGF siRNA on either cell migration or cell proliferation and was compared to the non-silencing siRNA control. A P-value ≤ 0.05 was considered significant for the t-tests.

RESULTS

Induction of mesenchymal stem cell condensation and up-regulation of CTGF by TGF-β1

To study the role of CTGF as a potential downstream mediator of mesenchymal stem cell (MC) condensation, an in vitro model was established using micromass

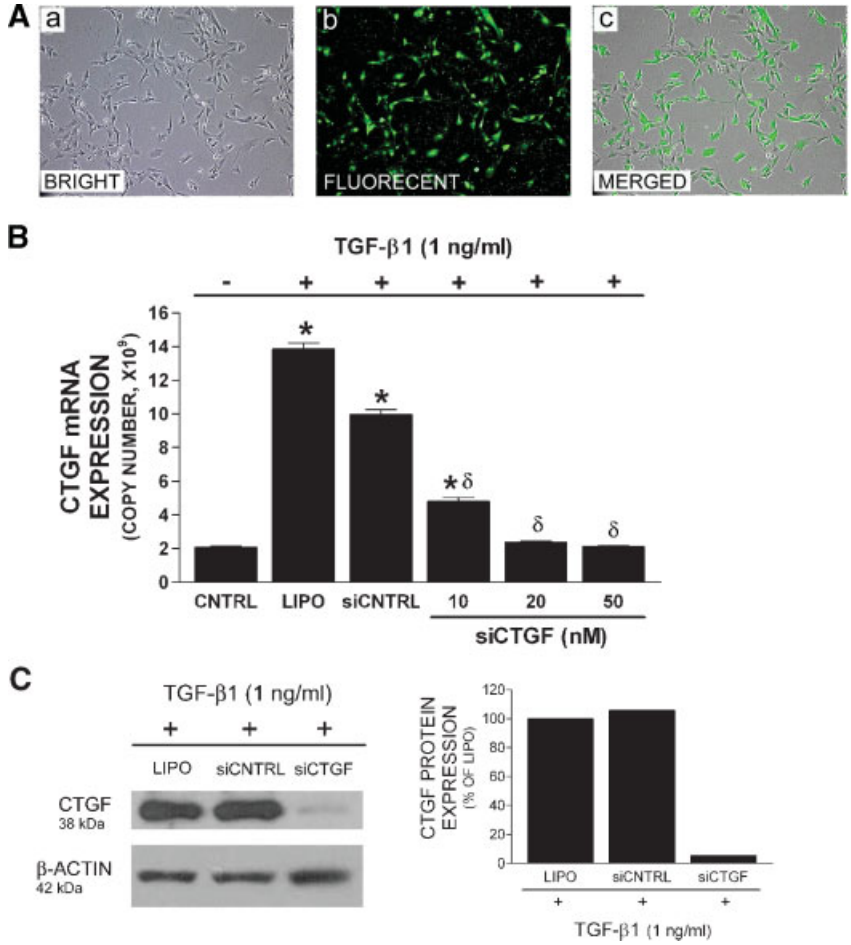

Fig. 2. Down-regulation of CTGF expression by CTGF siRNA. **A**: High levels of transfection efficiency were achieved using 50 nM of FITC-labeled double-stranded oligonucleotides; 150× magnification. **B**: CTGF mRNA expression was down-regulated by CTGF siRNA (siCTGF) in a dose-dependent manner. cDNA was obtained from micromass cultures transfected with 10, 20, and 50 nM siCTGF, 50 nM non-silencing siRNA control (siCNTRL) or Lipofectamine transfection control (LIPO). These cultures were treated with TGF-β1 (1 ng/ml) for 48 h and compared to non-TGF-β1-treated cultures (CNTRL). Each condition was run in triplicate and CTGF mRNA expression was normalized to GAPDH. CTGF expression was up-regulated by TGF-β1 in the LIPO and siCNTRL cultures (*$P < 0.0001$ compared to CNTRL). This up-regulation was effectively blocked in cultures transfected with siCTGF ($^{\delta}P < 0.001$ compared to siCNTRL). **C**: Representative Western blot analysis using 40 μg total protein obtained from micromass cultures transfected with 20 nM siCTGF or siCNTRL or Lipofectamine only (LIPO) followed by 1 ng/ml TGF-β1 stimulation for 48 h. CTGF protein expression was normalized to β-Actin before percent expression was determined. The up-regulation of CTGF protein expression by TGF-β1 was inhibited (>95%) by siCTGF compared to siCNTRL or LIPO. All experiments were performed three times with similar results.

cultures of C3H10T1/2 cells. These cells have been shown to form precartilaginous nodules following TGF-β1 treatment (Denker et al., 1995), making this an attractive culture system to study MC condensation. Micromass cultures of C3H10T1/2 cells treated with increasing doses of TGF-β1 resulted in nodule formation (MC condensation) at doses equal to or greater than 0.5 ng/ml TGF-β1 (Fig. 1A). This aggregation of MCs occurred concomitantly with a dose-dependent increase in CTGF expression (Fig. 1B). The induction of CTGF protein expression levels by TGF-β1 ranged from a 3-fold increase at 0.25 ng/ml to a greater than 16-fold increase at the maximum dose of 5 ng/ml. Collectively, these results show that the prechondrogenic condensation of MCs is coincident with the up-regulation of CTGF expression, both being initiated by TGF-β1 stimulation.

Aggregation towards a center, a characteristic of MC condensation, was demonstrated in micromass cultures stimulated with 1 ng/ml TGF-β1 stimulation during a 48 h period (Fig. 1C). Nodule formation resulted from the proliferation of cells and their migration toward a focal point. The movement of cells was clearly evident between 12 and 18 h (Fig. 1Cb,Ce) and culminated in the formation of large, dense cell masses (nodules) by 24–48 h following TGF-β1 treatment (Fig. 1Cc,Cf). It is important to note that in the absence of TGF-β1, the cells did not exhibit any evidence of condensation or nodule formation despite the fact that they were overcrowded (cultured in micromass). To better visualize these nodules, optical sections were taken by confocal microscopy (Fig. 1D). The optical sections confirmed the condensed, cellular nature of the nodules which spanned several hundred microns in diameter (from top to bottom) as compared to the uncondensed control cultures (Fig. 1D).

Silencing of CTGF expression by siRNA or antisense oligonucleotide transfection

For subsequent experiments examining the importance of CTGF in TGF-β1-mediated MC condensation, we tested the use of siRNA to selectively silence CTGF expression. Prior to determining gene expression levels,

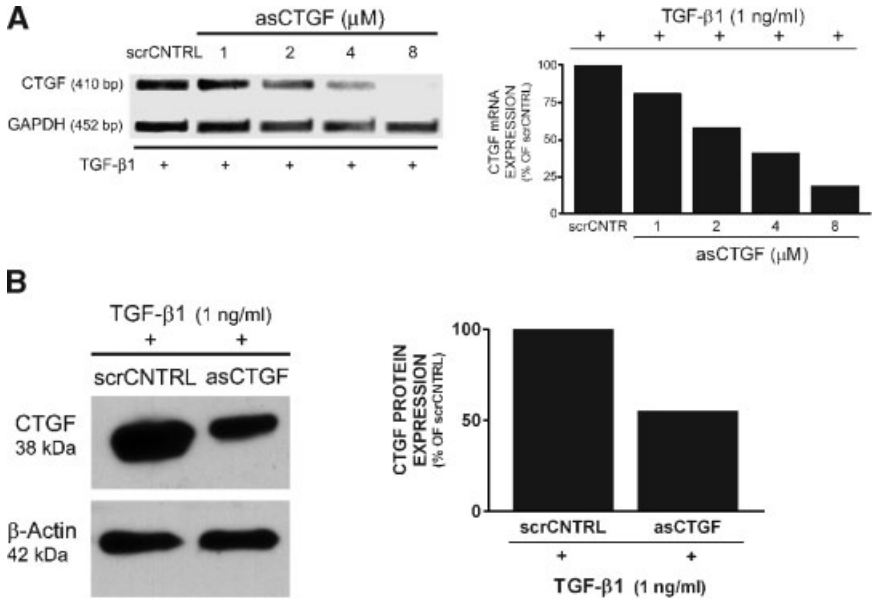

Fig. 3. Down-regulation of CTGF expression by antisense oligonucleotide. **A**: Semi-quantitative PCR analysis shows transfection with CTGF antisense oligonucleotide (asCTGF) decreases CTGF expression in a dose-dependent manner compared to scrambled oligonucleotide control (scrCNTRL) in cultures stimulated with TGF-β1 (1 ng/ml) for 48 h. CTGF mRNA expression was normalized to GAPDH and data are expressed as percent of scrCNTRL. **B**: Representative Western blot analysis using 40 μg total protein from micromass cultures transfected with 8 μM asCTGF or scrCNTRL followed by TGF-β1 (1 ng/ml) stimulation for 48 h. CTGF expression was normalized to β-Actin and data are presented as percent of scrCNTRL. The up-regulation of CTGF protein expression by TGF-β1 was inhibited (approximately 50%) by asCTGF. All experiments were repeated three times with similar results.

siRNA transfection conditions were optimized. Transfection efficiency was determined to range from 90 to 95% using 50 nM fluorescent siRNA (Fig. 2A). At this dose, cell toxicity was not evident as determined by MTT assay for cell number (data not shown) (Goodwin et al., 2004). Furthermore, siRNA did not induce apoptosis as determined by TUNEL assay (data not shown). Using these conditions, increasing doses of CTGF siRNA (siCTGF) were transfected into C3H10T1/2 cells; the positive controls were transfected with either Lipofectamine alone (LIPO) or with 50 nM non-silencing siRNA (siCNTRL). All transfected cultures were then stimulated with 1 ng/ml TGF-β1 for 48 h and CTGF gene expression was determined by quantitative real-time PCR (qPCR) analysis. Negative control cultures (CNTRL) did not receive TGF-β1 stimulation. CTGF mRNA levels were significantly up-regulated in the TGF-β1 stimulated positive controls (LIPO and siCNTRL). This effect was blocked in cultures transfected by siCTGF in a dose-dependent manner and the CTGF mRNA expression levels for cultures transfected with 20 and 50 nM doses of siCTGF were not significantly different from CNTRL (Fig. 2B). Western blot analysis of micromass cultures transfected with 20 nM siCTGF showed that up-regulation of CTGF protein levels by TGF-β1 was also blocked (Fig. 2C). Thus, siCTGF (at doses of 20 nM and greater) effectively blocked TGF-β1-mediated up-regulation of CTGF protein and mRNA in micromass cultures, with expression levels similar to the negative control (non-TGF-β1-treated) cultures.

CTGF antisense oligonucleotide (asCTGF) was used as an alternative method to silence CTGF expression. Antisense conditions were optimized with transfection efficiency and cell toxicity that were comparable to those for siCTGF (data not shown). With the conditions optimized, cultures were transfected with increasing doses of asCTGF followed by treatment with 1 ng/ml TGF-β1 for 48 h; the positive controls included TGF-β1 stimulated micromass cultures transfected with Lipofectamine alone or a scrambled antisense oligonucleotide (scrCNTRL). The positive controls demonstrated similar inductions of CTGF mRNA levels (8- to 12-fold increase) over the negative (non-TGF-β1 treated) control, and the Lipofectamine transfection control was similar to the scrCNTRL (data not shown). The TGF-β1 induced up-regulation of CTGF mRNA levels was inhibited in a dose-dependent manner in cultures transfected with the asCTGF to approximately 20% of scrCNTRL values at the 8 μM dose (Fig. 3A). Cell lysates from cultures transfected with 8 μM scrCNTRL or asCTGF and treated with 1 ng/ml TGF-β1 were used for Western blot analysis. The induction of CTGF protein expression was inhibited by 50% in micromass cultures transfected with the asCTGF compared to the scrCNTRL (Fig. 3B). These results show that transfection with the asCTGF inhibits TGF-β1 induced up-regulation of CTGF expression albeit not as efficiently as the CTGF siRNA approach.

Silencing of CTGF expression inhibits mesenchymal cell condensation induced by TGF-β1

To explore the possibility that CTGF functions as a downstream mediator of TGF-β1 in MC condensation, micromass cultures were transfected with siCTGF, asCTGF or appropriate controls, and then stimulated to condense with TGF-β1 (1 ng/ml) for 48 h. Down-regulation of CTGF expression by either siCTGF or asCTGF inhibited the aggregation of MCs thereby preventing nodule formation (Fig. 4Ad and Ah, respectively), while their corresponding non-silencing controls (siCNTRL or scrCNTRL) (Fig. 4Ac,Ag, respectively) demonstrated condensation and nodule formation in a manner similar to the Lipofectamine transfection control (Fig. 4Ab,Af). This inhibition of condensation was dependent on the duration of the CTGF knockdown; as

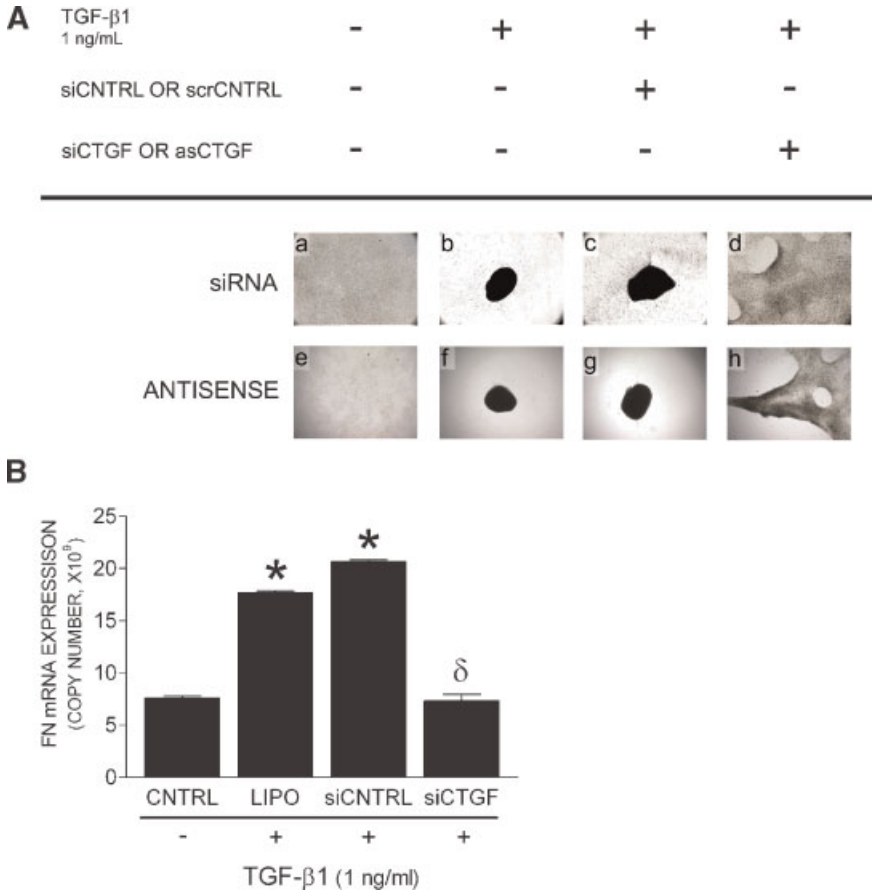

Fig. 4. Down-regulation of CTGF expression inhibits MC condensation and FN expression. **A**: Micromass cultures transfected with 20 nM siCTGF or 8 μM asCTGF inhibited MC condensation (Ad and Ah, respectively). Micromass cultures transfected with non-silencing siRNA (siCNTRL) (Ac), scrambled antisense (scrCNTRL) (Ag), or Lipofectamine only (Ab and Af) did not inhibit nodule formation (MC condensation). All cultures except the negative control (Aa and Ae) received 1 ng/ml TGF-β1 stimulation for 48 h; negative controls did not condense. All panels are 40× magnification. **B**: Quantitative real-time PCR analysis was performed on cDNA obtained from cultures transfected with 20 nM CTGF siRNA (siCTGF), 20 nM non-silencing siRNA control (siCNTRL), or Lipofectamine only (LIPO) followed by TGF-β1 (1 ng/ml) for 48 h, and compared to the untreated control (CNTRL) which did not receive TGF-β1. Each condition was run in triplicate and FN mRNA expression was normalized to GAPDH. FN expression was up-regulated by TGF-β1 in LIPO and siCNTRL cultures ($^*P < 0.0001$ compared to CNTRL). The up-regulation of FN expression was inhibited in siCTGF transfected cultures ($^\delta P < 0.001$ compared to siCNTRL), demonstrating that FN mRNA up-regulation by TGF-β1 is CTGF dependent. All experiments were performed three times with similar results.

the effect of the siCTGF abated and CTGF levels began to increase at 72 and 96 h post-transfection, the cultures began to condense and eventually formed nodules in the continuous presence of TGF-β1. The inhibitory effect of the siCTGF was more dramatic than that of the asCTGF (compare Fig. 4Ad vs. Ah), consistent with the finding that the siCTGF was more robust than the asCTGF in blocking CTGF up-regulation induced by TGF-β1. These results demonstrate that the condensation of mesenchymal stem cells induced by TGF-β1 is CTGF dependent and supports a role for CTGF as an essential downstream mediator of TGF-β1 in this process.

Up-regulation of FN expression by TGF-β1 is CTGF dependent

FN is a large extracellular matrix glycoprotein that actively promotes cellular aggregation during MC condensation and it is also present in early developing chondrocytes (Melnick et al., 1981; Kosher et al., 1982; Tomasek et al., 1982; Glant et al., 1985). FN expression serves as a marker for prechondrogenic condensation and its expression is up-regulated in several cell types including MCs by TGF-β1 (Ignotz and Massague, 1986; Ignotz et al., 1987; Kulyk et al., 1989b). In our cultures, FN mRNA up-regulation by TGF-β1 was found to be CTGF dependent. Micromass cultures transfected with Lipofectamine alone or a siCNTRL followed by 1 ng/ml TGF-β1 for 48 h demonstrated a significant increase in FN mRNA expression (2- to 3-fold) compared to the negative (non-TGF-β1 treated) control (Fig. 4B). CTGF silencing using 20 nM siCTGF abrogated the TGF-β1 induction of FN mRNA expression, demonstrating that FN mRNA up-regulation by TGF-β1 was CTGF dependent (Fig. 4B).

Silencing of CTGF expression inhibits TGF-β1 induced migration and proliferation of C3H10T1/2 cells

Cell migration and proliferation are central to the process of MC condensation. Since CTGF has been shown to stimulate migration and proliferation of various cell types, we sought to determine whether CTGF mediated the effects of TGF-β1 on migration and/or proliferation of C3H10T1/2 cells. To assess migration, cultures of C3H10T1/2 cells transfected with Lipofectamine alone, 20 nM siCTGF or 20 nM siCNTRL were cultured in the presence of TGF-β1 (1 ng/ml) for 24 h. After 24 h, each culture was cleared using a pipette tip resulting in a well-demarcated area of plastic devoid of cells stretching across each well (0 h; Fig. 5A). Cells were

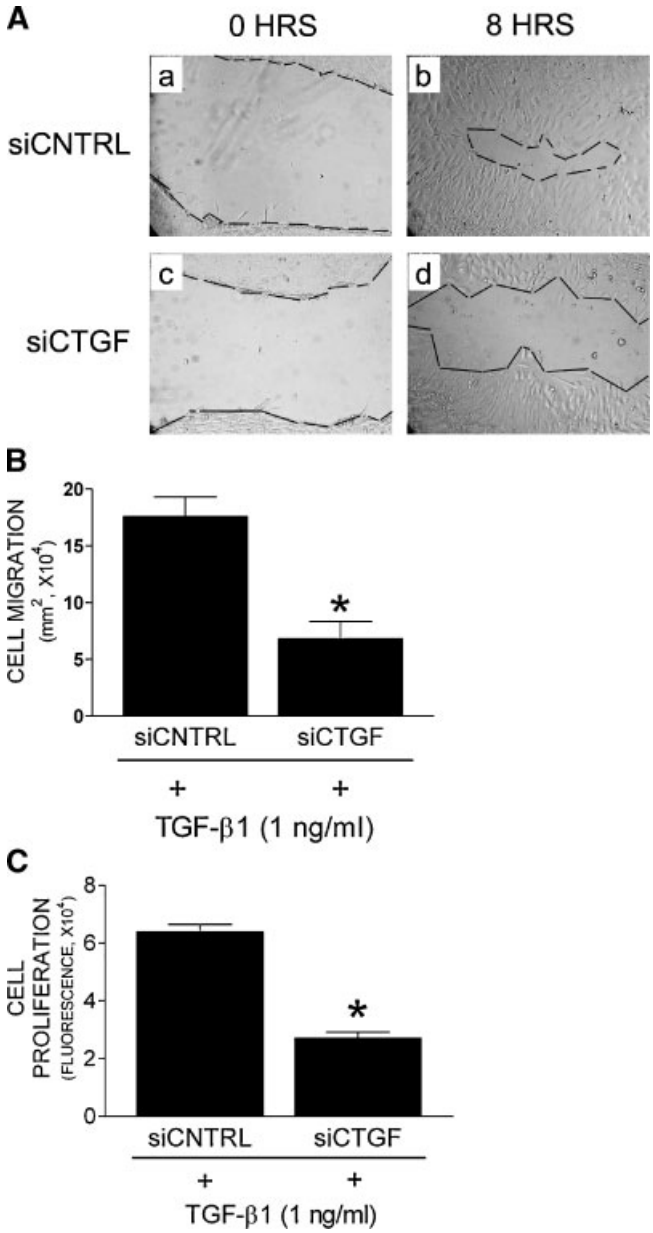

Fig. 5. Down-regulation of CTGF expression inhibits cell migration and proliferation. **A**: Cultures of C3H10T1/2 cells were transfected with 20 nM siCTGF (Ac and Ad) or siCNTRL (Aa and Ab) and treated with TGF-β1 (1 ng/ml). Twenty-four hours later, the cells were cleared using a pipette tip (0 h, Aa and Ac), and incubated for an additional 8 h in the presence of TGF-β1 to allow for cell migration; 40× magnification. **B**: Cell migration was calculated as the area restored (difference between the cleared area at 0 h and the area that remained devoid of cells after 8 h) and expressed as mm^2, $\times 10^4$. There was a significant reduction in cell migration in the siCTGF transfected cultures ($^*P < 0.001$ compared to siCNTRL). **C**: C3H10T1/2 cells were transfected with 20 nM siCTGF or siCNTRL and allowed to proliferate for 24 h in the presence of 1 ng/ml TGF-β1 stimulation. Cell proliferation was significantly lower in the siCTGF transfected cultures ($^*P < 0.0001$ compared to siCNTRL). All experiments were repeated three times with similar results.

cultured for an additional 8 h in the presence of TGF-β1 (1 ng/ml) to allow for migration onto the cleared culture surface (8 h; Fig. 5A). Cell migration was determined by the difference between the cleared area prior to migration from the area that remained devoid of cells after 8 h of migration (area restored). Cell migration was significantly inhibited (approximately 60%) in cultures transfected with siCTGF compared to the siCNTRL (Fig. 5B; $P < 0.0009$). Cell migration was similar between the Lipofectamine transfection control and siCNTRL (data not shown). The effect of CTGF expression on TGF-β1 induced cell proliferation was also assessed. Cell proliferation was significantly decreased in C3H10T1/2 cells transfected with siCTGF compared to siCNTRL (Fig. 5C; $P < 0.0001$). Cell proliferation was similar between the Lipofectamine transfection control and the siCNTRL (data not shown). These results demonstrate that CTGF mediates mesenchymal stem cell condensation by increasing cell migration and proliferation.

TGF-β1 suppression of Sox9 expression is mediated by CTGF

Sry-type HMG box 9 (Sox9) is a transcription factor that is considered to be the master gene of cartilage differentiation (Bi et al., 1999). It is unclear whether Sox9 is required for MC condensation based on conflicting results and conclusions from previous studies (Bi et al., 1999; Yan et al., 2002). We were interested in examining the effects of TGF-β1 on Sox9 expression in our in vitro MC condensation model. Treatment of C3H10T1/2 micromass cultures with TGF-β1 resulted in a corresponding decrease of Sox9 protein expression (Fig. 6A). Sox9 mRNA expression was also significantly decreased following TGF-β1 treatment as seen in the positive control cultures transfected with the siCNTRL or Lipofectamine alone (Fig. 6B). In contrast, this inhibitory effect on Sox9 mRNA expression was abrogated in the CTGF silenced (siCTGF) cultures (Fig. 6B). Western blot analysis confirmed that expression of Sox9 protein correlated with that of its mRNA (Fig. 6C). These results demonstrate that Sox9 expression is suppressed by TGF-β1 in condensing micromass cultures of C3H10T1/2 cells, and that this effect is dependent on the concomitant up-regulation of CTGF.

Immunolocalization of CTGF, Sox9, FN, and TGF-β1 expression in developing vertebrae

Previous in situ hybridization studies have shown that CTGF is highly expressed in mesenchymal condensations of developing limbs, the axial skeleton, and Meckel's cartilage in mice (Friedrichsen et al., 2003; Ivkovic et al., 2003; Takigawa et al., 2003; Shimo et al., 2004). We were interested in examining the expression of CTGF along with the other factors pursued in this study, namely TGF-β1, FN and Sox9, during the stages of MC condensation and early chondrogenesis of developing vertebrae in mouse embryos. Longitudinal, midsagittal sections of developing mouse embryos stained with Safranin-O at E10.5 and E12.5 showed MC condensations (mc) at E10.5 (Fig. 7Aa,Ab) that differentiated into islands of chondrocytes (Ch) and cartilage matrix surrounded by a well-demarcated perichondrium (p) at E12.5 (Fig. 7Ac) in the developing vertebral column (arrowheads in Fig. 7Aa). Sections of mouse embryos at E10.5 and E12.5 were immunostained for CTGF, Sox9, and FN. At E10.5, CTGF and FN were highly expressed in the prechondrogenic condensations (Fig. 7Ad,Ah), while Sox9 expression was very low (Fig. 7Af). At E12.5, Sox9 expression was very high within chondrocytes of the developing vertebral cartilage (Fig. 7Ag), while CTGF expression was highest in the perichondrium and the surrounding mesenchyme (Fig. 7Ae). FN was expressed both in developing cartilage and in the perichondrium (Fig. 7Ai). CTGF and TGF-β1 were also found to be co-expressed at high

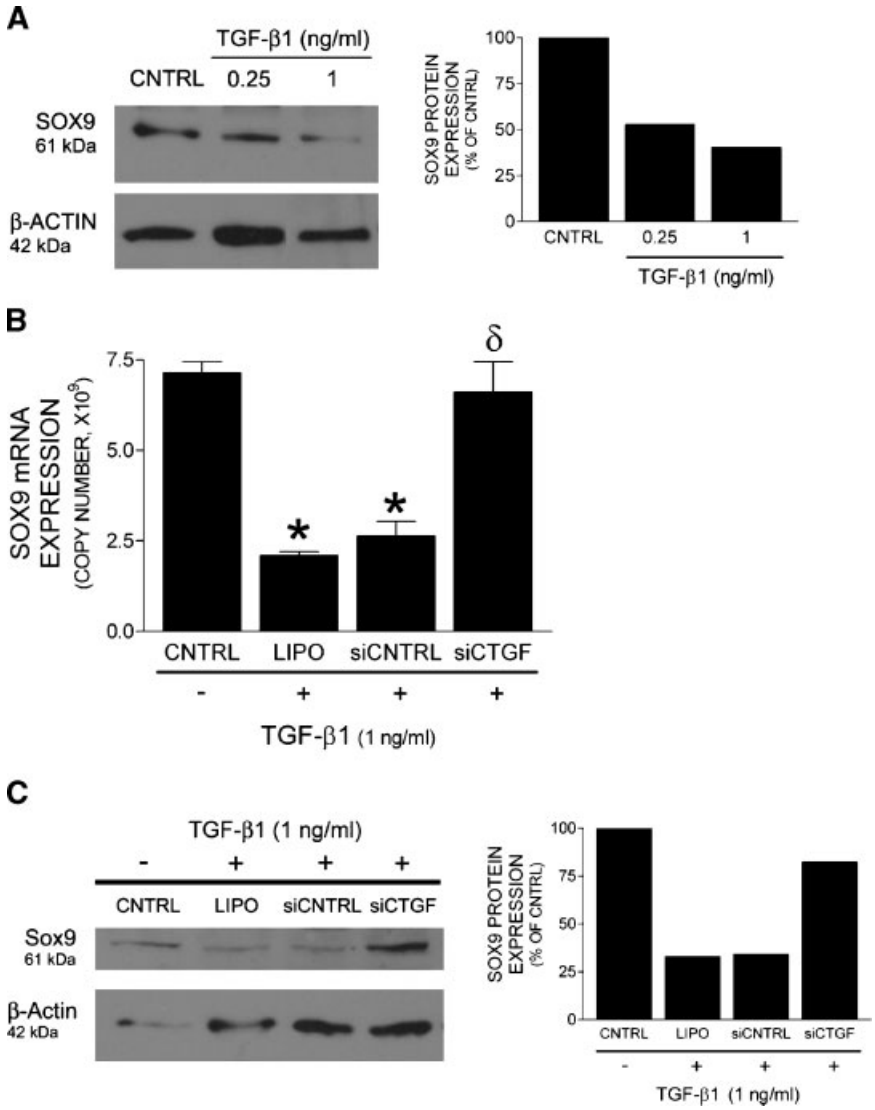

Fig. 6. Sox9 suppression by TGF-β1 is CTGF dependent. **A**: Micromass cultures were treated with 0.25 or 1 ng/ml of TGF-β1 for 48 h and Sox9 expression was determined by Western blot analysis using 40 μg total proteins. Compared to the untreated control (CNTRL), Sox 9 expression was down-regulated by TGF-β1 treatment. Sox9 expression was normalized to β-Actin and data are expressed as percent of CNTRL. **B**: Micromass cultures transfected with 20 nM CTGF siRNA (siCTGF) or non-silencing siRNA control (siCNTRL), or Lipofectamine (LIPO) were stimulated with 1 ng/ml TGF-β1 for 48 h and compared to negative control (CNTRL) cultures that were not treated with TGF-β1. All conditions were run in triplicate and Sox9 expression was normalized to GAPDH. Quantitative real-time PCR analysis showed that Sox9 expression was inhibited by TGF-β1 in LIPO and siCNTRL cultures ($^{*}P < 0.001$ compared to CNTRL), and effect was abrogated in the siCTGF transfected cultures ($^{\delta}P < 0.001$ compared to LIPO or siCNTRL). **C**: Western blot analysis confirmed that Sox9 down-regulation by TGF-β1 is CTGF dependent. Cultures were transfected and treated as in subpart (B); cell lysates were collected and Western blot analysis was performed using 40 μg total proteins. Sox9 expression was normalized to GAPDH and data are expressed as percent of CNTRL. The inhibition of Sox9 expression by TGF-β1 was reversed when up-regulation of CTGF expression was blocked by siCTGF. All experiments were repeated three times with similar results.

levels in the MC condensations at E10.5 (Fig. 7B). At E11.5, the expression of these two growth factors decreased from caudal to rostral in the vertebral condensations suggesting that the initiation of cartilage differentiation was coupled with decreased expression of CTGF and TGF-β1 (data not shown). These data demonstrate that there is a reciprocal relationship between the expression of TGF-β1/CTGF and Sox9 during the initial stages of vertebral development from MC condensation through early cartilage differentiation.

DISCUSSION

The development of the endochondral skeleton begins with a process of prechondrogenic condensation whereby mesenchymal stem cells aggregate via migration and proliferation at the sites where the cartilaginous templates of the endochondral bones will develop directly from the cells within the condensations. To study prechondrogenic condensation, we first established an in vitro model system adapted from Denker et al. (1995) using micromass cultures of C3H10T1/2 cells. In these cultures, TGF-β1 was used to initiate condensation, as cultures treated with 1 ng/ml TGF-β1 condensed to form large nodules within the first 48 h of treatment. Since TGF-β1 up-regulates CTGF expression and CTGF has been shown to be an important downstream effector of TGF-β1 in certain cell types, such as fibroblasts and endothelial cells (Shimo et al., 1998; Blalock et al., 2004), we were interested in

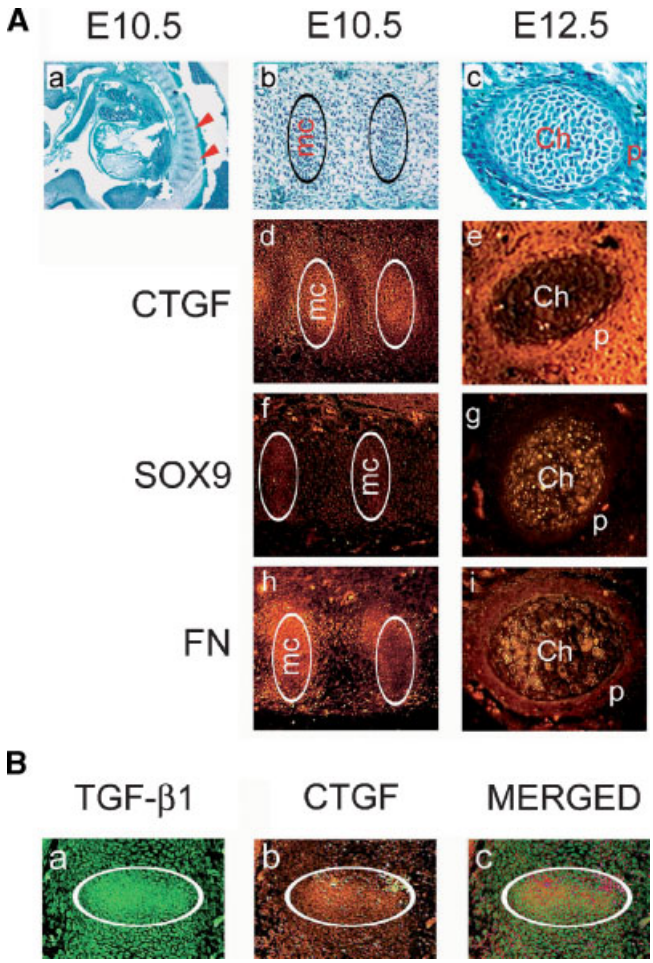

Fig. 7. Immunofluorescent localization of CTGF, Sox9, FN, and TGF-β1 expression during vertebral development. **A**: Whole embryonic day (E) 10.5 (Aa, Ab, Ad, Af, and Ah) and E12.5 (Ac, Ae, Ag, and Ai) embryos were sectioned and analyzed by safranin-O staining (Aa–Ac) or immunofluorescent staining (Ad–Ai) for CTGF (Ad and Ae), Sox9 (Af and Ag) and FN (Ah and Ai). Safranin-O staining of E10.5 whole embryos shows the location of the developing vertebrae at low power (arrowheads in Aa). At higher magnifications (Ab and Ac), the condensations of MCs (outlined and labeled mc) present at E10.5 (Ab) differentiated into chondrocytes (Ch) surrounded by a perichondrium (p) at E12.5 (Ac). CTGF expression was high in the MC condensations of the developing vertebrae at E10.5 (Ad), and in the perichondrium, but not in the chondrocytes of developing cartilage at E12.5 (Ae). The pattern of Sox 9 expression was opposite that of CTGF; Sox 9 expression was low in MC condensations at E10.5 (Af), but highly expressed in chondrocytes of developing cartilage while being absent in the perichondrium at E12.5 (Ag). FN expression was observed in both MC condensations at E10.5 (Ah) and in the developing cartilage at E12.5 (Ai). **B**: Co-localization of TGF-β1 and CTGF in MC condensations. TGF-β1 (Ba) and CTGF (Bb) were highly co-expressed (Bc) in the MC condensations of developing vertebrae at E10.5. Magnifications: Aa—15×; Ab, d, f, and h—100×; Ac, e, g and i—400×; Ba, b and c—150×.

determining whether CTGF played a role in TGF-β1 induction of prechondrogenic condensation. CTGF mRNA and protein levels were markedly increased in micromass cultures of C3H10T1/2 cells treated with TGF-β1 (1 ng/ml) and this up-regulation occurred in parallel with condensation. Both siRNA and antisense oligonucleotide approaches were used to block the up-regulation of CTGF induced by TGF-β1 treatment, with the siRNA approach providing a more robust inhibition (>95%) of CTGF mRNA and protein up-regulation. Silencing of CTGF expression prevented the cells from condensing and forming nodules, and this effect was related to the extent to which CTGF up-regulation was blocked. Our results establish CTGF as an essential downstream mediator of TGF-β1 in prechondrogenic condensation of C3H10T1/2 cells. Immunofluorescent localization demonstrated that CTGF was highly expressed in prechondrogenic condensations of developing vertebrae at E10.5, and was co-expressed with TGF-β1 in these mesenchymal condensations. These data support a role for TGF-β1 and CTGF during mesenchymal stem cell condensation in vivo.

Previous studies have shown that micromass cultures of C3H10T1/2 cells stimulated to condense with TGF-β1 treatment progress along the chondrocytic lineage when maintained in culture for longer periods (5–7 days) (Denker et al., 1995). Following condensation, the nodules displayed a cartilage-like histology with increased type II collagen (Col2α1) expression, increased Alcian blue staining, and increased synthesis of sulfated proteoglycans, demonstrating that the cells were producing a cartilage-like matrix. Although TGF-β1 is essential for condensation, the role of this factor in promoting chondrocyte differentiation is unclear since most studies utilize other factors, such as BMP-2, demineralized bone proteins and/or a chondrogenic cocktail including insulin and transferring, to promote chondrogenic differentiation (Atkinson et al., 1997; Denker et al., 1999; Haas and Tuan, 1999). In micromass cultures of primary chicken mesenchymal cells, both TGF-β1 and BMP-2 have been shown to promote the accumulation of cartilage extracellular matrix, though TGF-β1 is most effective on cells which have not yet undergone cell condensation, a critical event in early cartilage differentiation, whereas BMP-2 is most effective after the cells condensed (Roark and Greer, 1994). Ex vivo micromass cultures of primary mouse limb bud MCs have also been shown to aggregate and differentiate in the presence of TGF-β1 and other factors including insulin and transferrin, as evidenced by an increase in Col2α1 and aggrecan (Agc) expression and a cellular morphology typical of differentiated chondrocytes (Zhang et al., 2004). Although there are clear differences between C3H10T1/2 cells and primary limb bud MCs, it is important to note that studies using primary limb bud MCs harvest the cells at a stage when they are already committed and can spontaneously aggregate and differentiate toward the chondrocytic lineage (Ahrens et al., 1977; Zhang et al., 2004). On the other hand, C3H10T1/2 cells are uncommitted MCs that do not condense without TGF-β1 stimulation, thereby providing a unique opportunity to study the importance of TGF-β1 during MC condensation and early commitment of MCs toward the chondrocytic lineage.

The importance of CTGF in skeletal development was demonstrated with the generation of CTGF knockout mice (Ivkovic et al., 2003). CTGF gene ablation resulted in mice with misshapen skeleton, craniofacial abnormalities and defects associated with endochondral ossification. The defects were associated with the growth plate cartilage and the zone of ossification in CTGF deficient mice, but the full extent of these defects was difficult to assess since the knock out mice died shortly after birth from respiratory distress resulting from failure of the misshapen rib cage to expand. The phenotype observed in CTGF deficient mice was attributed to defects in cell proliferation, matrix formation and remodeling during endochondral ossification (Ivkovic et al., 2003). Since CTGF is highly expressed during

prechondrogenic condensation of MCs, as demonstrated in this study and by others (Friedrichsen et al., 2003; Shimo et al., 2004), we speculate that a disruption in the formation of mesenchymal condensation at specific skeletal sites may also have contributed to the phenotype observed in CTGF deficient mice. There are numerous skeletal malformations that have been attributed to disturbances in prechondrogenic condensation (Hall and Miyake, 2000). Normal skeletal formation is so sensitive to disruptions in prechondrogenic condensation that a delay of 24 h in the initiation of this process is sufficient to cause skeletal malformations in mice (Hall and Miyake, 1992). The fact that the skeletal malformations were not more severe in CTGF deficient mice is likely due to functional redundancy of other closely related members of the CCN family of proteins, which has been shown to have some similar, overlapping functions with CTGF (Brigstock, 2003). In particular, Cyr61, a CCN family member shown to have the highest homology to CTGF, has been shown to be expressed in limb bud mesenchyme during chondrogenesis and recombinant Cyr61 has been shown to enhance cell–cell aggregation and chondrocyte differentiation in mouse limb bud cultures (Wong et al., 1997; Brigstock, 2003). This strongly supports the notion of functional redundancy between CTGF and Cyr61 thereby masking an overt defect in condensation in CTGF knockout mice.

CTGF has been shown to increase proliferation and migration in several cell types, including fibroblasts, endothelial cells and cartilage progenitor cells (Moussad and Brigstock, 2000). Since both of these events play an important role in the condensation of MCs, we were interested in determining whether the up-regulation of CTGF induced by TGF-β1 promotes proliferation and/or migration of C3H10T1/2 cells. Our results clearly demonstrated that when TGF-β1 induction of CTGF expression was blocked by siRNA there was a significant decrease in cell proliferation and migration of C3H10T1/2 cells. These data help explain, at least in part, the mechanism whereby CTGF regulates cellular processes that are necessary for condensation to occur. Our data are also supported by previous studies in other cell types/systems. For example, CTGF down-regulation by a Hammerhead ribozyme inhibited proliferation in TGF-β treated fibroblasts (Blalock et al., 2004). In vascular endothelial cells, when CTGF expression was down-regulated by antisense oligonucleotide, this led to decreased proliferation and migration (Shimo et al., 1998).

FN is an extracellular matrix protein that plays an important role in cellular adhesion, another event that has been shown to be important in mesenchymal stem cell condensation. Previous studies have shown that FN expression is up-regulated by TGF-β1 in several cell types, including mesenchymal stem cells (Ignotz and Massague, 1986; Ignotz et al., 1987; Kulyk et al., 1989b; Kawai et al., 1999). Furthermore, FN mRNA has been shown to be increased during prechondrogenic condensation and blocking antibodies to FN inhibited condensation, thereby establishing FN as a functional protein involved in condensation (Frenz et al., 1989; Kulyk et al., 1989a). In our cultures of condensing C3H10T1/2 cells, FN mRNA increased with TGF-β1 stimulation, and this up-regulation of FN was nullified upon CTGF silencing. These results indicate that FN mRNA up-regulation by TGF-β1 was CTGF dependent, and are consistent with earlier work reported in other cell types (Chen et al., 2000; Shi-wen et al., 2000). Thus, the up-regulation of FN that is mediated by CTGF in TGF-β1-treated cultures is another potential mechanism involving the regulation of cellular adhesion. Previous studies have suggested that the process of chondrogenesis proceeds through a sequence of events in which cell adhesion molecules, such as FN, mediate cell–cell interactions during cell condensation and commitment, and then extracellular matrices mediate cell-matrix interactions during overt cartilage differentiation (Kawai et al., 1999). As expected, our immunofluorescent data demonstrated that FN was strongly expressed in the prechondrogenic condensations of developing vertebrae at E10.5 (Fig. 7Ah). However, at day E12.5, FN was expressed in the developing cartilage as well as in the perichondrium. Although FN plays an important role during condensation, it is also an important extracellular matrix protein in various other tissue types. In cartilage, a specific splice variant has been shown to be expressed by chondrocytes (Gehris et al., 1996).

Sox9 is a transcription factor that is essential for cartilage differentiation (Bi et al., 1999). Studies have shown that Sox9 is expressed in early differentiating chondrocytes, while being absent in late differentiated or hypertrophic chondrocytes (Wright et al., 1995; Ng et al., 1997; Zhao et al., 1997). Based on previous studies it is unclear whether Sox9 plays a role in prechondrogenic condensation of MCs; some have concluded that Sox9 is important in prechondrogenic condensation (Bi et al., 1999), while others have concluded that it is not required for mesenchymal condensation (Yan et al., 2002). To study the role of Sox9 during condensation, we examined Sox9 expression and demonstrated that it decreased in TGF-β1-treated micromass cultures of C3H10T1/2 cells, and that this suppression of Sox9 expression was dependent upon CTGF levels, demonstrating a reciprocal relationship in the expression of TGF-β1/CTGF and Sox9.

A close look at the literature indicates that regulation of Sox9 by TGF-β is complex. TGF-β1 has been reported to have both stimulatory and inhibitory effects depending on the origin and stage of cells being studied (Kulyk et al., 1989a; Chen et al., 1991; Leonard et al., 1991; Roark and Greer, 1994). Furthermore, Chen et al. reported that the ECM may also regulate chondrogenesis in TGF-β1 stimulated mesenchymal progenitor cells. The addition of TGF-β1 and Col2α1 increased Sox9 expression in mesenchymal progenitor cells, while treatment with TGF-β1 and type I collagen (Col1α2) reduced Sox9 and Agc expression (Chen et al., 2005). Several studies have demonstrated that CTGF stimulation alone or in conjunction with TGF-β1 cause a significant increase in Col1α2 expression both in vivo (Chujo et al., 2005) and in vitro (Inagaki et al., 1994; Nishida et al., 2000; Shi-wen et al., 2000; Yokoi et al., 2001; Uchio et al., 2004). In this study, we found that during prechondrogenic condensation there was an increase in the expression of type I collagen concomitant with a decrease in the expression of type II collagen (data not shown), providing an explanation for the inhibitory effect of CTGF on Sox9 expression being mediated through modulation of Col1α2/Col2α1 expression. Based on the current literature and our results from this study, we believe that CTGF expression precedes that of Sox9 to regulate the migration, proliferation, and aggregation of mesenchymal stem cells during condensation, while Sox9 expression subsequently increases as CTGF declines, to regulate the commitment and differentiation into chondrocytes during early chondrogenesis. This switch from CTGF to Sox9 expression may also explain the transition from

the predominantly Col1α2 extracellular matrix of prechondrogenic condensations to the Col2α1 extracellular matrix characteristic of early differentiating cartilage.

Immunofluorescent analysis of developing vertebrae demonstrated that Sox9 expression was very low during prechondrogenic condensation at E10.5, at a time when both TGF-β1 and CTGF expression was at their highest levels. TGF-β1 and CTGF expression levels gradually declined as the mesenchymal condensations matured being absent from the early differentiated chondrocytes at E12.5, when Sox9 expression was at its highest levels. With the appearance of chondrocytes at E12.5, CTGF expression resided only in the perichondrium where mesenchymal stem cells are still present. The temporal and spatial patterns of expression of TGF-β1/CTGF and Sox9 in vivo are consistent with our functional observations in micromass cultures. Although there are clear differences between C3H10T1/2 cells and primary MCs, the immunofluorescent data warrant further analysis of the functional relationship between TGF-β1/CTGF and Sox9/type II collagen during cartilage development in vivo.

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