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Transforming Growth Factor-β-mediated Chondrogenesis of Human Mesenchymal Progenitor Cells Involves N-cadherin and Mitogen-activated Protein Kinase and Wnt Signaling Cross-talk*

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The multilineage differentiation potential of adult tissue-derived mesenchymal progenitor cells (MPCs), such as those from bone marrow and trabecular bone, makes them a useful model to investigate mechanisms regulating tissue development and regeneration, such as cartilage. Treatment with transforming growth factor-β (TGF-β) superfamily members is a key requirement for the *in vitro* chondrogenic differentiation of MPCs. Intracellular signaling cascades, particularly those involving the mitogen-activated protein (MAP) kinases, p38, ERK-1, and JNK, have been shown to be activated by TGF-βs in promoting cartilage-specific gene expression. MPC chondrogenesis *in vitro* also requires high cell seeding density, reminiscent of the cellular condensation requirements for embryonic mesenchymal chondrogenesis, suggesting common chondro-regulatory mechanisms. Prompted by recent findings of the crucial role of the cell adhesion protein, N-cadherin, and Wnt signaling in condensation and chondrogenesis, we have examined here their involvement, as well as MAP kinase signaling, in TGF-β1-induced chondrogenesis of trabecular bone-derived MPCs. Our results showed that TGF-β1 treatment initiates and maintains chondrogenesis of MPCs through the differential chondro-stimulatory activities of p38, ERK-1, and to a lesser extent, JNK. This regulation of MPC chondrogenic differentiation by the MAP kinases involves the modulation of N-cadherin expression levels, thereby likely controlling condensation-like cell-cell interaction and progression to chondrogenic differentiation, by the sequential up-regulation and progressive down-regulation of N-cadherin. TGF-β1-mediated MAP kinase activation also controls *WNT-7A* gene expression and Wnt-mediated signaling through the intracellular β-catenin-TCF pathway, which likely regulates N-cadherin expression and subsequent N-cadherin-mediated cell-adhesion complexes during the early steps of MPC chondrogenesis.

Adult-derived mesenchymal progenitor cells (MPCs)[1] have been considered a candidate cell source for tissue engineering and reparative medicine by virtue of their potential to differentiate into adipocytes, chondrocytes, fibroblasts, osteoblasts, marrow stromal cells, and other tissues of mesenchymal origin (1, 2). Numerous adult tissues have been identified that harbor MPCs, including bone marrow (3–5), muscle (6–8), adipose tissue (9, 10), periosteum (11, 12), and most recently in our laboratory, human trabecular bone (13, 14). We have also developed improved techniques for the isolation and culture of MPCs from trabecular bone to yield clinically significant numbers of such cells (15). That these cells retain their multilineage differentiation potential through long term culture expansion suggests they are a suitable cell source for potential therapeutic and clinical treatment at least in osteogenic, adipogenic, and chondrogenic applications.

The *in vitro* chondrogenic differentiation of MPCs requires the complex involvement of growth factors and cell-cell and cell-matrix interactions, similar to developmental chondrogenesis *in vivo* (16). Expression of members of the transforming growth factor-β (TGF-β) superfamily of growth factors has been localized to sites of bone repair as well as sites of embryonic bone and cartilage formation *in vivo* (17, 18), and the chondroinductive effects of the TGF-β superfamily members, particularly the bone morphogenetic proteins and the TGF-βs, have been well established in embryonic and adult mesenchymal cells (13, 19–24). Recent reports have demonstrated the critical roles of intracellular signaling cascades activated by TGF-β family members in promoting cartilage-specific gene expression (25–27), including the mitogen-activated protein (MAP) kinases, whose major subtypes include p38, extracellular signal-regulated kinase-1 (ERK-1), and c-Jun N-terminal kinase (JNK or stress-activated protein kinase). These major subtypes are activated by a variety of stimuli, and often are differentially regulated by a single stimulus. The roles of the specific MAP kinase subtypes, p38 and ERK-1, in regulating chondrogenesis were elucidated to some degree by Oh *et al.* (28) who found that p38 and ERK-1 have opposing roles during the chondrogenic induction of chick limb bud cells. Specifically, p38 was an

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[1] The abbreviations used are: MPCs, mesenchymal progenitor cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; COMP, cartilage oligomeric matrix protein; IGF I, insulin-like growth factor I; TGF-β, transforming growth factor-β; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; UTR, untranslated region; ERK-1, extracellular signal-regulated kinase-1; PBS, phosphate-buffered saline; TCF, T-cell factor; RT, reverse transcription; A-CAM, anti-A cell adhesion molecule; P, phosphorylated; ECM, extracellular matrix.

enhancer of chondrogenesis, whereas ERK-1 was a repressor of chondrogenesis, and control was exerted, at least in part, through the regulation of cell adhesion molecules, including N-cadherin, fibronectin, and its receptor $\alpha_5\beta_1$ integrin, during cellular condensation.

Precartilage mesenchymal condensation, a requisite for the initiation of chondrogenesis *in vivo*, has been shown to be equally important in *in vitro* cultures (29–31). The spatiotemporal expression pattern of the Ca^{2+}-dependent, homotypic cell adhesion molecule N-cadherin parallels its functional requirement for the initiation and subsequent progression of developmental chondrogenesis (32, 33). As a transmembrane glycoprotein, N-cadherin is composed of extracellular domains that mediate homophilic interactions between neighboring cells, predominantly via a peptide domain containing the His-Ala-Val (HAV) amino acid sequence, located near the N terminus of the protein within the interface of two molecules and shown to be critical for N-cadherin-mediated cell adhesion (34). Additionally, the cytoplasmic domain of N-cadherin is anchored to the intracellular actin cytoskeleton through interactions with the α-, β-, and γ-catenin complex. Besides its functional role, cytoplasmic β-catenin has also been found to interact with other proteins such as glycogen synthase kinase-3β, adenomatous polyposis coli, the scaffolding components, axin and conductin, as well as the transcriptional regulators, lymphoid enhancing factor-1 (LEF-1)/T-cell factor (TCF), all of which play critical roles in the canonical Wnt signal transduction pathway (35), recently implicated in regulating chondrocyte differentiation.

Wnts are a family of secreted glycoproteins that act in a paracrine fashion, thereby mediating cellular interactions during development (35, 36). Briefly, Wnt signaling proteins act by binding to Frizzled receptors, the activation of which leads to the stabilization of cytosolic β-catenin. Interaction of β-catenin with the high mobility group box transcription factors of the LEF-1/TCF family allows translocation of the complex into the nucleus to subsequently regulate the transcription of Wnt target genes (37). A number of *WNT* genes are expressed during development, including *WNT3A* (38) localized in mouse apical ectodermal ridge, *WNT4* localized in developing joints (39), *WNT5A* localized in distal mesenchyme (40), and WNT7A in dorsal ectoderm (41). Wnt-7a has been shown to be chondroinhibitory *in vitro* (42), and recently the misexpression of *WNT7A* in limb mesenchymal chondrogenic cultures directly led to the prolonged expression of N-cadherin, the stabilization of N-cadherin-mediated cell-cell adhesion, and the eventual inhibition of chondrogenesis (43, 44). The involvement of Wnt signaling has also been shown in the BMP-2 mediated chondrogenic effect on the mouse C3H10T1/2 mesenchymal cell line (45, 46).

Recent evidence has increasingly suggested that signaling cascades and pathways act in an interconnected manner within the cell. Here we attempt to define the early cellular processes and cross-regulatory signaling events that take place during mesenchymal chondrogenesis, specifically in adult tissue-derived, multipotential MPCs. A recent report (47) described the cross-regulation and subsequent inhibition of the Wnt signaling pathway by MAPKKK and the downstream MAP kinase, NLK. Here we report that TGF-β1-stimulated chondrogenesis of trabecular bone-derived MPCs initiates intracellular signaling via activation of the chondro-stimulatory MAP kinases, p38, ERK-1, and JNK, which differentially regulate cartilage-specific gene and protein expression in a lineage-specific manner. Additionally, our results suggest that mesenchymal cell condensation initiated by TGF-β1 within the pellet culture is mediated via N-cadherin and is critical for the progression of chondrogenesis, similar to developmental chondrogenesis *in vivo*. Moreover, N-cadherin expression appeared to be regulated at the cellular condensation phase by the tight control of *WNT7A* gene expression individually by the p38, ERK-1, and JNK MAP kinases.

EXPERIMENTAL PROCEDURES

Reagents—All reagents were purchased from Sigma unless otherwise stated.

Isolation and Culture of Human Trabecular Bone-derived Cells—Normal human trabecular bone was obtained from the femoral heads of patients undergoing total hip arthroplasty and processed using a high efficiency and high yield protocol established previously in our laboratory (15) and approved by the Institutional Review Boards of Thomas Jefferson University and George Washington University. Processed trabecular bone fragments were subsequently cultured in Dulbecco's modified Eagle's medium (high glucose and L-glutamine; Mediatech, Inc., Herndon, VA) supplemented with 10% fetal bovine serum (Premium Select, Atlanta Biologicals, Atlanta, GA), from selected lots (48), and 50 μg/ml penicillin/streptomycin. Subconfluent cell monolayers were dissociated and removed using 0.25% trypsin containing 1 mM EDTA (Invitrogen) and either passaged at a ratio of 1:3 or utilized for study.

In Vitro Chondrogenic Differentiation of MPCs—The chondrogenic induction of trabecular bone-derived MPCs was initiated using high density pellet cultures (2×10^5 cells/pellet, $500 \times g$ for 5 min) in a chemically defined medium containing Dulbecco's modified Eagle's medium supplemented with 50 μg/ml ascorbate, 0.1 μM dexamethasone, 40 μg/ml L-proline, 100 μg/ml sodium pyruvate, and ITS-plus (Collaborative Biomedical Products, Cambridge, MA) (4, 13, 19, 23, 49). Recombinant human transforming growth factor-β1 (R&D Systems, Minneapolis, MN) was added to the pellet cultures at a final concentration of 10 ng/ml. For MAP kinase inhibition studies, specific chemical inhibitors of p38 (SB20350), ERK-1-specific MAP kinase kinase (MEK1) inhibitor (PD98059), and JNK (SP600125, Calbiochem-Novabiochem) were used at final concentrations of 5, 10, and 100 nM, respectively, representing concentrations well within the range of IC_{50} values determined for similar cell types.

Reverse Transcription (RT)-PCR Analysis—Total cellular RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Chondrogenic pellet cultures were first briefly homogenized in Trizol reagent to increase yield efficiency. Equal amounts of RNA samples were reverse-transcribed by using random hexamers and the SuperScript First Strand Synthesis System (Invitrogen). PCR amplification of cDNA was carried out using AmpliTaq DNA polymerase (PerkinElmer Life Sciences) and the gene-specific primer sets listed in Table I. Amplification cycles consisted of 1-min denaturation at 95 °C, 1-min annealing, 1-min polymerization at 72 °C, and finally a 10-min extension at 72 °C. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was used as a control for RNA loading of samples. PCR products were analyzed electrophoretically using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Reporter Gene Assays—Human trabecular bone-derived MPCs were harvested at 60–80% confluence for electroporation using the Human Mesenchymal Stem Cell Nucleofector kit (Amaxa Biosystems, Cologne, Germany) according to a modified protocol established in our laboratory for the efficient transfection of human MPCs.[2] Promoter reporter constructs include the following: 1) TOPFLASH, containing multimeric TCF-binding sites (Upstate Cell Signaling Solutions, Waltham, MA); 2) FOPFLASH, containing multimeric mutated TCF-binding sites (Upstate Cell Signaling Solutions, Waltham, MA); 3) a pGL3 basic vector (Promega, Madison, WI) containing 4.0 kb of the 5′-flanking sequences of the human collagen type II α1 procollagen (*COL2A1*, −577/+3428) gene, which encompasses the promoter, exon 1, and the putative enhancer sequence in the first intron, linked to a luciferase reporter (a kind gift from Dr. M. Goldring); and 4) a plasmid pAGC1(−2368)/5′-UTR containing 2368 bp of the human aggrecan promoter region along with the entire exon 1 and 5′-UTR, linked to a luciferase reporter (a kind gift from Dr. W. B. Valhmu). Luciferase activity was determined using the Luciferase Assay System kit (Promega, Madison, WI). A green fluorescent protein expression vector under the control of the SV40 promoter (pCMS-EGFP; Clontech, Palo Alto, CA) was used to normalize transfection efficiencies. Results were analyzed using Student's t test, $p \leq 0.05$.

[2] H. Haleem-Smith, A. Derfoul, C. Okafor, R. Tuli, D. Olsen, D. J. Hall, and R. S. Tuan, manuscript in preparation.

TABLE I
RT-PCR primers for differentiation-specific gene expression analysis, sequence and expected product size

Gene	Primer sequences (5′–3′)	Expected product size
		bp
GAPDH[a]	GGGCTGCTTTTAACTCTGGT TGGCAGGTTTTTCTAGACGG	702
Aggrecan[b]	TGAGGAGGGCTGGAACAAGTACCGGAGGTGGTAATTGCAGGGAACA	350
Col II	CAGGTCAAGATGGTCTTCAGCACCTGTCTCACCA	377
Col IX	GAAAATGAAGACCTGCTGGGAAAAGGCTGCTGTTTGGAGAC	516
Col X	GCCCAAGAGGTGCCCCTGGAATACCCTGAGAAAGAGGAGTGGACATAC	703
COMP	CAACTGTCCCCAGAAGAGCAATGGTAGCCAAAGATGAAGCCC	588
DMPN	TGGACCTCAGTCTTCTCTGGGTCCTAGCTAGCTTCAGAGCCG	547
IGF 1	TGTCCTCCTCGCATCTCTTCTGTACTTCCTTCTGGTCTTGGG	310
Sox 9	ATCTGAAGAAGGAGAGCGAGTCAGAAGTCTCCAGAGCTTG	264
WNT-3A[c]	CAGGAACTACGTGGAGATCATGCCATCCCACCAAACTCGATGTC	326
WNT-5A	ACACCTCTTTCCAAACAGGCCGATTGTTAAACTCAACTCTC	392
WNT-7A	GCCGTTCACGTGGAGCCTGTGCGTGCAGCATCCTGCCAGGGAGCCCCGCAGCT	438
WNT-11	GTGAAGGACTCGGAACTCGTAGCGCTATGTCTGCAAGTGA	364

[a] Control.
[b] Cartilage-specific genes.
[c] WNT-specific genes.

Protein Isolation and Western Analysis—Cell pellets were washed twice with ice-cold phosphate-buffered saline (PBS), lysed with immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) and protease and phosphatase inhibitor mixture, incubated on ice for 30 min, homogenized, and centrifuged at 14,000 × *g* for 15 min. The supernatant was collected, and protein concentrations were determined by using the BCA assay (Pierce). Equal amounts of protein extracts were fractionated by 10% SDS-PAGE, electroblotted onto Hybond-P membrane (Amersham Biosciences), probed with antibodies to p38, P-p38, ERK1, P-ERK1/2, JNK1, P-JNK, N-cadherin, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), and β-catenin (Cell Signaling Technology, Beverly, MA), immunoblotted using the ECF Western blotting kit according to the manufacturer's protocol (Amersham Biosciences), and visualized using the Typhoon 9410 Imager (Amersham Biosciences).

Metabolic Sulfate Incorporation—Chondrogenic pellet cultures received 1.0 μCi/ml sodium [^{35}S]sulfate and 1.0 μCi/ml [^{3}H]leucine (PerkinElmer Life Sciences) 24 h prior to the time point chosen for measurement of newly synthesized proteoglycans and protein, respectively. Incorporation of radioactivity was measured by liquid scintillation counting (51) and statistically analyzed by using Student's *t* test ($p \leq 0.05$).

Histological and Immunohistochemical Analysis—Cell pellet cultures, rinsed twice with PBS, were fixed for 2 h in 2% PBS-buffered paraformaldehyde, dehydrated through a graded ethanol series, infiltrated with isoamyl alcohol, embedded in paraffin, and sectioned at 8 μm thickness for analysis. Histological staining with Alcian blue (pH 1.0) or hematoxylin and eosin was performed as described previously (20, 31). Immunohistochemical localization of collagen type II (II-II6B3, 15 μg/ml) and aggrecan (1-C-6, 10 μg/ml, Developmental Studies Hybridoma Bank, Iowa City, IA) was performed by pre-digesting sections with 300 units/ml hyaluronidase or 1.5 units/ml chondroitinase for 15 min at 37 °C, respectively. Colorimetric detection of staining was performed using Histostain-SP kit for DAB (Zymed Laboratories Inc., San Francisco, CA). Cell pellets treated with the N-cadherin antibody, A-CAM (see below), were fixed in 2% PBS-buffered paraformaldehyde and stained directly using Alcian blue, pH 1.0.

Inhibition of N-cadherin-mediated Cell Adhesion—A monoclonal antibody to N-cadherin, anti-A cell adhesion molecule (A-CAM, Sigma), was used to functionally inhibit homotypic interactions between N-cadherin molecules during precartilage condensation. Trabecular bone-derived MPCs were prepared for pellet culture as described above, and A-CAM was added as a single dose at the beginning of culture at concentrations varying from 0 to 240 μg/ml. A nonspecific mouse antibody was used as a negative control. Chondrogenesis was assayed at day 3 using the pAGC1(−2368)/5′-UTR promoter reporter construct, and at day 21 by Alcian blue staining, as described above.

RESULTS

Activation of MAP Kinase Subtypes, p38, ERK-1, and JNK, upon TGF-β1 Stimulation—Trabecular bone-derived cell pellets treated with and without TGF-β1 were assayed for MAP kinase activities and kinetics of activation. The addition of TGF-β1 led to the rapid transient phosphorylation of p38, ERK-1, and JNK, as determined by Western analysis (Fig. 1). Phosphorylated p38 (P-p38) levels increased dramatically at 0.5 h, peaked at 1 h, and returned to basal levels by 2 h, remaining constant through day 5 of chondrogenic culture. This transient increase in protein levels upon TGF-β1 treatment was kinetically mimicked by phosphorylated ERK-1 (P-ERK-1), the major ERK isoform, as well as phosphorylated JNK (P-JNK), which similarly increased at 0.5 h relative to time 0 h, peaked at 1 h, and returned to basal levels by 2 h. Unlike P-p38, however, P-ERK protein levels peaked again at day 3, and P-JNK levels peaked again at day 1 as chondrogenesis proceeded. Reprobing the same blots with antibodies against the unphosphorylated forms of p38, ERK-1, and JNK MAP kinases revealed no change in total p38, ERK-1, or JNK levels, respectively, during the chondrogenic culture period. Activations of p38, ERK-1, and JNK were absent in untreated control pellet cultures (data not shown).

p38, ERK-1, and JNK MAP Kinases Differentially Regulate Chondrogenesis-associated Activities Stimulated by TGF-β1—RT-PCR analysis of MPC pellets maintained in TGF-β1-supplemented chondrogenic medium for 21 days showed significant up-regulation of cartilage-specific gene expression (Fig. 2*C*), as compared with the untreated control (Fig. 2*A*). The addition of 5 μM p38 specific inhibitor (SB203580), 10 μM MEK-1 inhibitor (PD98059), or 100 nM JNK-specific chemical inhibitor (SP600125), to TGF-β1-treated pellet cultures led to the lineage-specific down-regulation or complete abrogation of chondrogenic gene expression levels (Fig. 2*D*). Inhibition of p38 with SB203580 completely abrogated TGF-β1-induced collagen type II (*COL2A1*) and *SOX9* gene expression, with significant down-regulation of aggrecan expression, and reduction of collagen type X (*COL10*), dermatopontin, and insulin-like growth factor I (IGF I) expression. Relative to the housekeeping gene, GAPDH, collagen type IX (*COL9*) and cartilage oligomeric matrix protein (COMP) expression levels were unaffected by addition of the p38 inhibitor. ERK inhibition with PD98059 also completely inhibited the TGF-β1-induced gene expression of aggrecan, as well as collagen types II and IX and *SOX9*. Down-regulated expression of collagen type X, dermatopontin, and IGF I was also seen, whereas similar to the p38 inhibited cultures, mRNA levels of GAPDH and COMP remained unaltered. Finally, JNK inhibition with SP600125 abolished TGF-β1-induced collagen type IX and Sox 9 mRNA expression, while

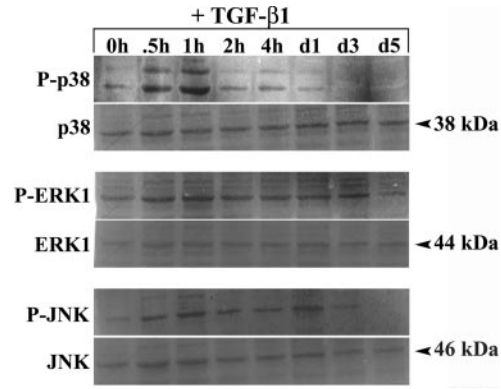

FIG. 1. **Temporal profiles of TGF-β1 activation of p38, ERK-1, and JNK MAP kinases.** Western analysis of fractionated lysates from trabecular bone-derived MPC pellets treated with TGF-β1 and probed for the phosphorylated forms of p38 (*P-p38*), ERK-1 (*P-ERK-1*), and JNK (*P-JNK*). Blots were stripped and reprobed for total amounts of p38, ERK-1, and JNK, included as controls. *d*, day.

diminishing mRNA levels of aggrecan, collagen types II and X, dermatopontin, and IGF I. GAPDH and COMP gene expression were unaffected by addition of the JNK inhibitor, results that were comparable with the p38 and ERK-1 MAP kinase studies, suggesting their regulation by TGF-β1 through signaling cascades other than p38, ERK-1, or JNK MAP kinase. Cell pellets treated with 5 μM SB203580, 10 μM PD98059, or 100 nM SP600125 alone (Fig. 2*B*) were unaffected similar to the negative control (Fig. 2*A*). Experiments were repeated as described above using additional inhibitors specific for the p38, ERK-1, or JNK MAP kinase pathways; results obtained were similar to those above (data not shown).

MPCs transiently transfected with either the pAGC1(−2368)/5′-UTR-Luc promoter reporter (Fig. 2*E*) or the COL2a1-Luc promoter reporter (Fig. 2*F*) were cultured as pellets in TGF-β1 containing chondrogenic medium for 3 days, with or without cotreatment with 5 μM SB203580, 10 μM PD98059, or 100 nM SP600125. Culture in medium without TGF-β1 was used as the untreated control. TGF-β1 treatment of the pAGC1(−2368)/5′-UTR-Luc transfected cell pellets resulted in an almost 2-fold significant increase in relative luciferase activity as compared with the control. Inhibition of p38 activation in TGF-β1-treated cultures led to a significant 133% decrease in aggrecan promoter activation as compared with TGF-β1 treated alone, and a 71% decrease in activity as compared with the control. Similarly, cotreatment of pellets with PD98059 resulted in a 94 and 32% decrease in TGF-β1-mediated transcriptional activation of aggrecan as compared with TGF-β1 alone and the control, respectively. Finally, the addition of JNK inhibitor, SP600125, also suppressed TGF-β1-induced transcriptional activation by 127%, which was also 65% less than the control. These data clearly indicate the lineage-specific positive control of aggrecan promoter activation by the MAP kinases.

Analysis using the *COL2A1*-Luc reporter yielded similar results (Fig. 2*F*). As seen in the RT-PCR study, treatment of pellets with TGF-β1 led to a 3.5-fold significant increase in *COL2a1* transcriptional activation as compared with the untreated control. Addition of 5 μM SB203580 to TGF-β1-treated cultures resulted in a 172% decrease in relative luciferase activity when compared with TGF-β1 treatment alone. The addition of 10 μM PD98059 and 100 nM SP600125 to TGF-β1-treated cultures also repressed *COL2A1* transcriptional activation by 264 and 203%, respectively. There were no significant differences in *COL2a1* transcriptional activation between TGF-β1-treated MAP kinase inhibited cultures and the untreated control or pellets treated with inhibitors alone. The pCMS-EGFP expression plasmid was used to normalize transfection efficiencies in each experiment.

MAP Kinase Subtypes Individually Mediate Chondrogenic Induction by TGF-β1—To elucidate further the functional involvement of p38, ERK-1, and JNK MAP kinases in the TGF-β1-stimulated chondrogenic differentiation of trabecular bone-derived MPCs, the effects of SB203580, PD98059, and SP600125 added individually to TGF-β1-treated and untreated pellet cultures were assessed by [^{35}S]sulfate incorporation, as an estimate of sulfated proteoglycan synthesis as a function of time (Fig. 3). In cell pellets treated with TGF-β1, a significant increase in the levels of [^{35}S]sulfate incorporation was seen at days 1, 14, and 21 as compared with all other cultures. The level of sulfate incorporation in TGF-β1 cultures peaked at day 14 and remained stable thereafter, indicating a relatively steady rate of incorporation from days 14 to 21. Inhibition of MAP kinase activation in TGF-β1-stimulated pellet cultures led to a significant decrease in the rates of sulfate incorporation beginning at day 7 and continuing through day 21 of culture. Levels of inhibition ranged from 35–44, 57–58, and 39 to 25%, from days 14 to 21 in p38, ERK-1, and JNK-inhibited cultures, respectively. This inhibition was considered significant because in the absence of TGF-β1 treatment, cell pellets treated with individual MAP kinase inhibitors alone exhibited no differences as compared with the untreated control (data not shown).

Following 21 days of chondrogenic culture, the extent of chondrogenesis was also assessed histologically using Alcian blue and hematoxylin and eosin staining, as well as by immunostaining for collagen type II and aggrecan (Fig. 4). Alcian blue staining of cultures confirmed the [^{35}S]sulfate incorporation results, showing an abundant sulfated proteoglycan-rich cartilage-like matrix in the TGF-β1-treated cultures (Fig. 4*A2*), which also contributed significantly to the dramatic increase in pellet size as compared with control untreated cultures (Fig. 4*A1*). Inhibition of p38 MAP kinase in TGF-β1-treated cultures using 5 μM SB203580 led to a marked down-regulation in the level of sulfated proteoglycan staining intensity (Fig. 4*A3*), with resulting pellet size comparable with that of the untreated control. Cotreatment with PD98059 cultures also resulted in reduced staining intensity and pellet size (Fig. 4*A4*), with an apparently distinct pattern of inhibition and staining relative to other treatments. JNK-inhibited, TGF-β1-treated cultures also exhibited lower levels of Alcian blue staining, with a less elaborate proteoglycan matrix (Fig. 4*A5*); levels of staining intensity in JNK-inhibited cultures, similar to the sulfate incorporation data of 21 days, were most comparable with TGF-β1-treated cultures, indicative of their less crucial role in regulating cartilage-specific proteoglycan production. Higher magnification of Alcian blue-stained, TGF-β1-treated cultures (Fig. 4*B2*) revealed a considerably more organized and structured matrix as compared with control (*B1*) and inhibitor cotreated cultures (*B3–B5*). Hematoxylin and eosin-stained pellets showed morphologically distinct, chondrocyte-like round cells evenly distributed throughout pellets treated with TGF-β1 (*C2*), in contrast to the more fibroblast-like, elongated cells in control (Fig. 4*C1*) and in MAP kinase inhibitor-treated cultures. Additionally, eosin staining of acidophilic collagen fibers demonstrates the abundant ECM elaborated by TGF-β1 cultures (Fig. 4*C2*) as compared with the untreated (*C1*) and MAP kinase-inhibited cultures (*C3–C5*).

Immunocytochemical detection of the cartilage-specific collagen type II and aggrecan ECM molecules (Fig. 4, *D1–D5* and *E1–E5*) supported the RT-PCR gene expression data of 21 days (Fig. 2, *A–D*). Collagen type II and aggrecan staining appear more intense and extensively distributed throughout TGF-β1-treated cultures (Fig. 4, *D2* and *E2*), as compared with their respective controls (*D1* and *E1*). Cotreatment with 5 μM

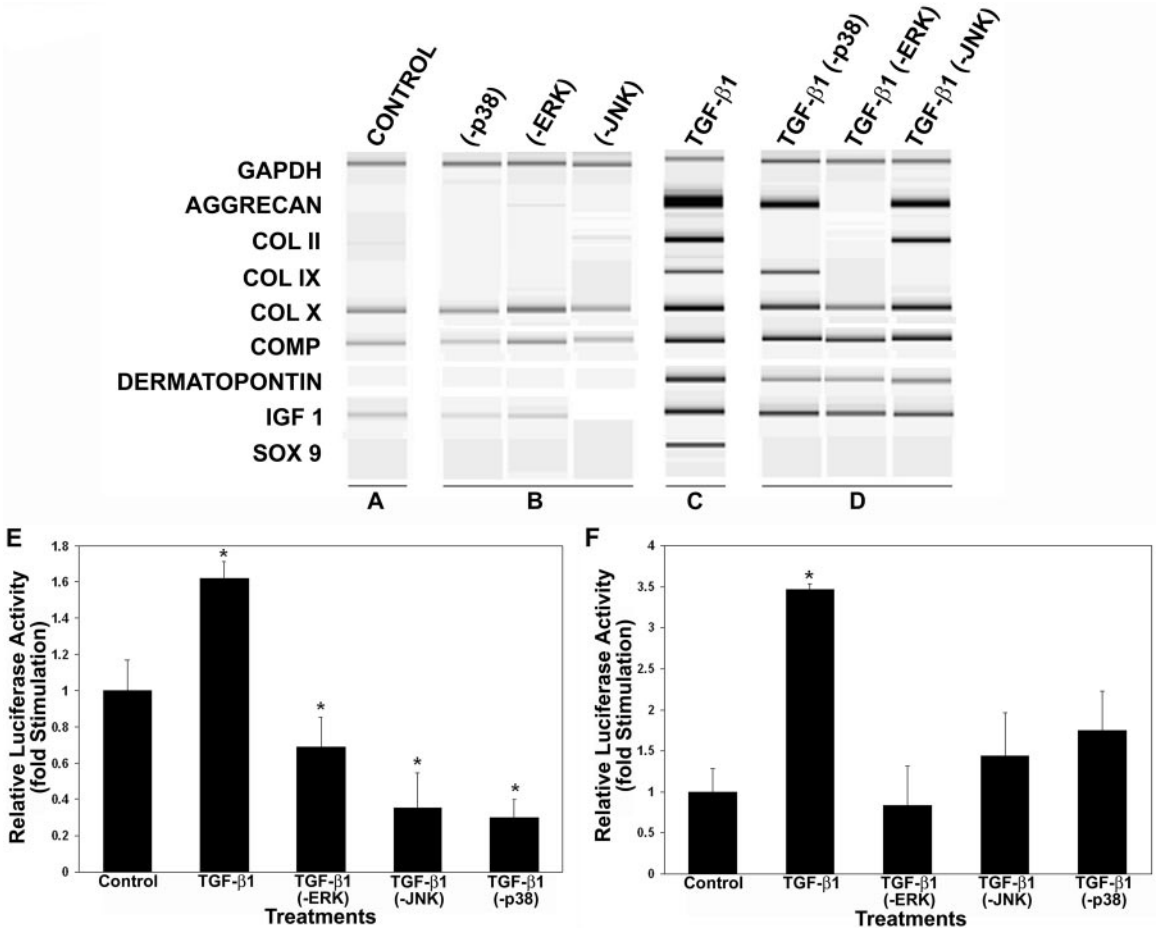

FIG. 2. **Effect of MAP kinase inhibitors on cartilage-specific gene expression.** RT-PCR analysis of genes associated with chondrogenesis (see Table I) and GAPDH was performed on day 21 cell pellets that were either left untreated (*A*), treated with MAP kinase inhibitors specific for p38 (*−p38*), ERK (*−ERK*), and JNK (*−JNK*), respectively (*B*), treated with TGF-β1 (*C*), or exposed to concurrent administration of TGF-β1 and individual MAP kinase inhibitors (*D*). The addition of TGF-β1 significantly up-regulated the expression of cartilage-specific genes as compared with the control and inhibitor treatments alone, whereas simultaneous treatment with TGF-β1 and MAP kinase inhibitors either significantly down-regulated or completely abrogated TGF-β1-induced gene expression in a lineage-specific manner. Regulation of aggrecan (*E*) and collagen type II (*Col II*) (*F*) gene expression in control, TGF-β1, and TGF-β1 plus MAP kinase inhibitor-treated cell pellets analyzed using promoter-luciferase constructs. Significant up-regulation of aggrecan and collagen type II promoter activity was seen upon treatment with TGF-β1 as compared with respective controls, which was significantly inhibited upon cotreatment with MAP kinase inhibitors. *, $p \leq 0.05$, relative to control cultures. *COMP*, cartilage oligomeric matrix protein.

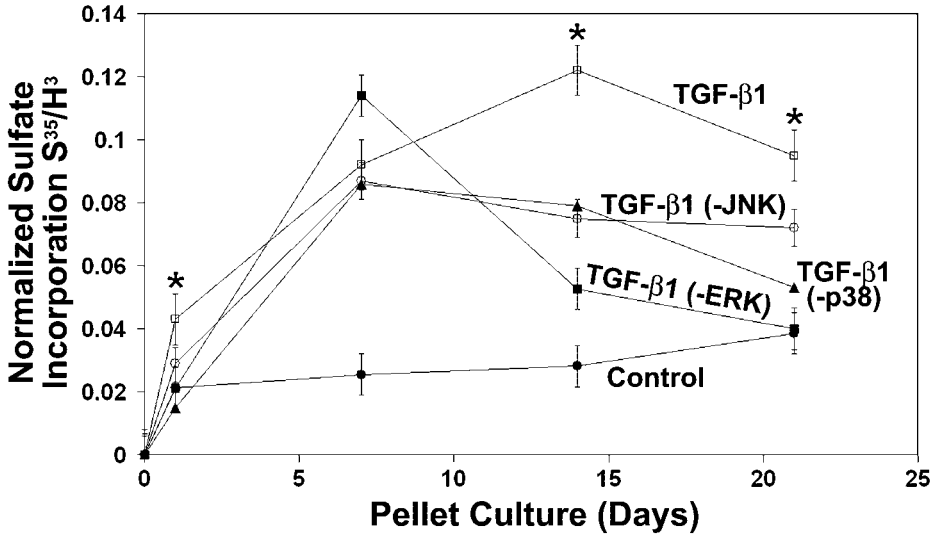

FIG. 3. **Effect of MAP kinase inhibitors on MPC pellet proteoglycan synthesis as a function of time.** Cultures are designated as in Fig. 2. Chondrogenesis was assayed on days 1, 7, 14, and 21 for [^{35}S]sulfate incorporation. Treatment of cell pellets with TGF-β1 led to a significant increase in levels of sulfate incorporation at days 1, 14, and 21. Inhibition of individual MAP kinases in the TGF-β1-stimulated cultures resulted in a significant decrease in the rates of sulfate incorporation beginning at day 7 and continuing through day 21. *, $p \leq 0.05$, relative to control and cotreated cultures.

SB203580, 10 μM PD98059, or 100 nM SP600125 in TGF-β1-treated pellets led to a reduction of both collagen type II (Fig. 4, *D3–D5*) and aggrecan (*E3–E5*) staining, indicative of the control exerted by the individual p38, ERK-1, and JNK MAP kinase pathways in regulating TGF-β1-induced cartilage-specific ECM molecule production.

Effect of N-cadherin Inhibition on TGF-β1-stimulated Chondrogenesis—A-CAM, a monoclonal antibody reactive with the

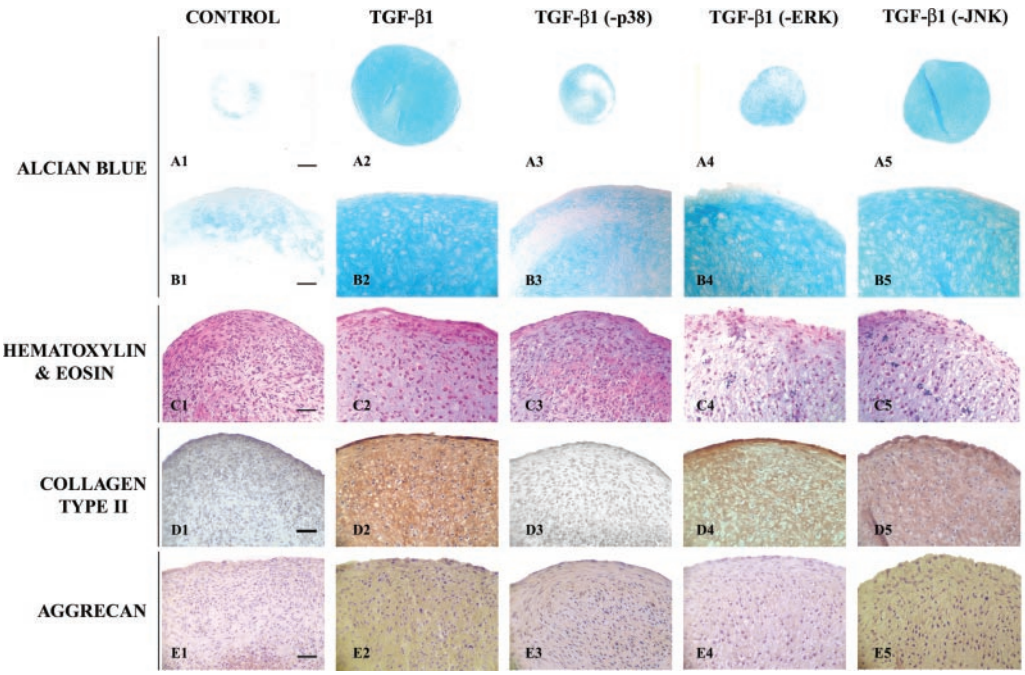

FIG. 4. **Inhibition of MAP kinases disrupts TGF-β1-induced chondrogenic phenotype.** All cultures were treated as described and examined histologically on day 21. Cultures are designated as in Fig. 2. *A* and *B*, Alcian blue staining; *C*, hematoxylin and eosin staining; *D*, collagen type II immunostaining; and *E*, aggrecan immunostaining. *A*, Alcian blue staining reveals an abundant proteoglycan-rich cartilage-like matrix in TGF-β1-treated pellets (*A2*), which contributes significantly to the dramatic increase in size as compared with control cultures (*A1*). Cotreatment with MAP kinase inhibitors (*A3–A5*) drastically reduces the quantity of matrix proteoglycans as evidenced by the decrease in staining intensity, as well as overall pellet sizes. Higher magnification of Alcian blue-stained, TGF-β1-treated cultures (*B2*) reveals a much more organized and ordered matrix, with higher staining intensity as compared with control and cotreated cultures (*B1* and *B3–B5*). Hematoxylin and eosin-stained pellets show morphologically distinct, chondrocyte-like round cells throughout TGF-β1-treated cultures (*C2*), as compared with more fibroblast-like, elongated cells in control (*C1*) and MAP kinase-treated cultures (*C3–C5*). Immunohistochemistry of cartilage matrix components showed that collagen type II staining is significantly higher in cell pellets cultured with TGF-β1 (*D2*) as compared with the respective control (*D1*). Cotreatment of pellet cultures with MAP kinase inhibitors leads to a differential decrease in level of staining (*D3–D5*). Immunocytochemical detection of aggrecan follows a similar pattern of staining (*E1–E5*). *A*, *bar* = 200 μm; *B–E*, *bar* = 75 μm.

N-terminal extracellular domain of N-cadherin, was used to test the functional involvement of N-cadherin-mediated cell-cell junction formation in TGF-β1-stimulated chondrogenesis of MPCs (Fig. 5). pAGC1(−2368)/5′-UTR-transfected cells were cultured as a cell pellet under chondrogenic conditions for 3 days with and without TGF-β1 and treated with a single dose of A-CAM, at concentrations varying from 80 to 240 μg/ml. The results showed that TGF-β1-induced transcriptional activation of the aggrecan promoter was significantly down-regulated by the addition of 80 μg/ml A-CAM (Fig. 5*A*). Similar levels of inhibition of luciferase activity were seen using 160 and 240 μg/ml (Fig. 5*A*), suggesting that TGF-β1 activation of the cartilage-specific aggrecan promoter and subsequent chondrogenesis is dependent on cell adhesion activities mediated by N-cadherin. Histologically, as seen in Fig. 5*B*, day 21 pellets maintained in the presence of TGF-β1 and treated with 80 μg/ml A-CAM revealed a significantly smaller pellet size as well as less intense Alcian blue staining as compared with culture treated with TGF-β1 alone, suggesting their inability to elaborate a proteoglycan-rich matrix. At 240 μg/ml A-CAM, high density cell pellet formation was unsuccessful, presumably as a result of extensive inhibition of cell-cell adhesion and inability to form pre-cartilage condensation. Controls of TGF-β1-treated pellets incubated with nonspecific antibodies showed no effect on chondrocytic phenotype following 21 days of culture, strongly suggesting a specific role for N-cadherin-mediated cell-cell interactions during precartilage condensation for the chondrogenic differentiation of MPCs.

MAP Kinase Regulation of N-cadherin in TGF-β1-stimulated Chondrogenesis—We next investigated whether N-cadherin is involved in the TGF-β1-mediated regulation of chondrogenesis. After 1 day of pellet culture, N-cadherin protein levels were markedly up-regulated in TGF-β1-treated cultures as determined by Western analysis (Fig. 6). In a temporal profile suggestive of active involvement in precartilage cellular condensation, N-cadherin levels decreased slightly in these TGF-β1-treated cultures by day 3, and returned to basal levels by day 5, at the onset of overt chondrogenic differentiation. Inhibition of p38, ERK-1, or JNK MAP kinases in TGF-β1-treated cultures did not affect the elevated N-cadherin protein levels on day 1. Interestingly, the N-cadherin levels in these MAP kinase-inhibited cultures remained high throughout the entire culture period, suggesting the cell adhesion junctions were stabilized in these cultures. β-Actin protein levels, included as internal controls, remained constant through 5 days of pellet culture.

TGF-β1-stimulated Chondrogenesis Involves Regulation of Wnt Signaling by MAP Kinases—We next investigated whether TGF-β1-stimulated endogenous MAP kinase activation and signaling involves Wnt signal transduction. We analyzed the effects of 5 μM SB203580, 10 μM PD98059, and 100 nM SP600125 MAP kinase inhibitors on the transcriptional activation of a β-catenin-TCF-responsive luciferase reporter construct (TOPFLASH) following 3 days of MPC pellet culture (Fig. 7*A*). Treatment of cell pellets with TGF-β1 resulted in a significant 2-fold increase in luciferase activity relative to untreated, TOPFLASH-transfected pellet cultures. Cotreatment with p38 inhibitor enhanced the TGF-β1-induced TCF-dependent transcriptional activation by 3-fold relative to the untreated control. Similarly, the individual addition of ERK and JNK inhibitors to TGF-β1 treated cultures led to a 4.5- and 4.6-fold increase, respectively, in luciferase activity relative to the untreated control, levels that were also significantly higher than TGF-β1-treated cultures alone. As expected, cells transfected with the mutated reporter construct FOPFLASH showed

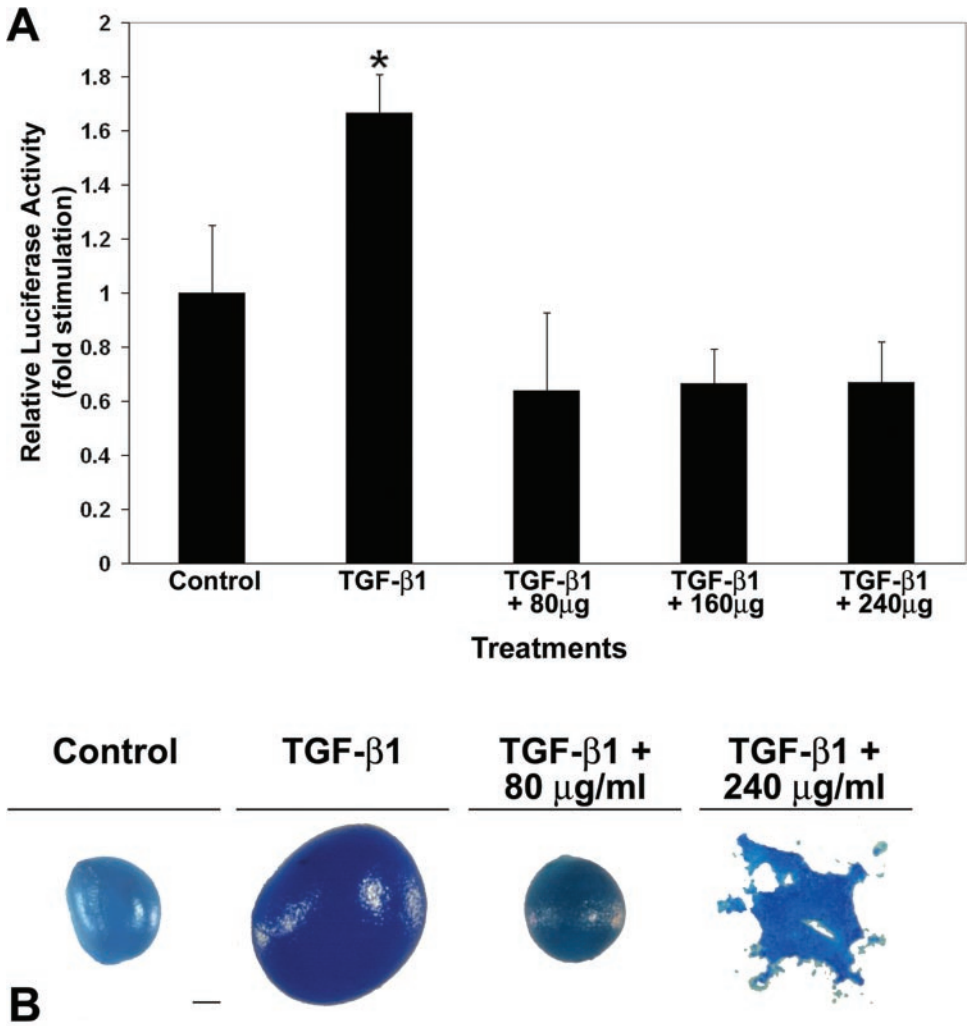

FIG. 5. **Effects of N-cadherin function-blocking antibody (A-CAM) on TGF-β1-induced chondrogenesis as analyzed by aggrecan promoter luciferase activity (*A*) and Alcian blue staining (*B*).** A-CAM was added at varying concentrations to pellets of MPCs transfected with an aggrecan promoter-luciferase construct with or without TGF-β1 treatment. *A,* treatment of cell pellets with 80 μg/ml A-CAM for 3 days significantly down-regulated TGF-β1-induced transcriptional activation of the aggrecan promoter. Similar inhibition was seen with both 160 and 240 μg/ml A-CAM. *B,* Alcian blue staining of day 21 cell pellets maintained in the presence of TGF-β1 and treated with A-CAM led to significant inhibition of proteoglycan production and reduction of pellet size at 80 μg/ml A-CAM and complete inhibition of pellet formation and abrogation of the chondrocytic phenotype at 240 μg/ml. *B*, *bar* = 300 μm.

no differences in luciferase activity between control and treated cultures (Fig. 7*B*). Western analysis of β-catenin protein levels following 3 days of pellet culture (Fig. 7*C*) revealed a corresponding increase in the nuclear pool of β-catenin in TGF-β1 cultures relative to the untreated control, which was consistent with TOPFLASH activation. When both cytoplasmic and nuclear pools are considered, an even greater increase of both β-catenin levels was seen upon individual inhibition of p38, ERK-1, or JNK MAP kinase in TGF-β1-induced cultures, consistent with the significant increase in β-catenin-dependent TCF activation seen in Fig. 7*A*. These results suggest that TGF-β1-stimulated chondrogenesis of MPCs is likely to involve the negative regulation of Wnt signaling by the p38, ERK-1, and JNK MAP kinases.

TGF-β1-stimulated Chondrogenesis Involves MAP Kinase Regulation of WNT7A Gene Expression—To assess further the involvement of Wnt, the effect of TGF-β1 on *WNT* expression was examined. RT-PCR analysis of MPC pellets maintained in chondrogenic culture showed the up-regulated gene expression of *WNT7A* following 1 day of TGF-β1 treatment (Fig. 8*B*), as compared with the untreated control (Fig. 8*A*). Specifically, the increase in Wnt-7a mRNA levels was transient and was temporally coincident with that of N-cadherin protein levels; by day 3, *WNT7A* expression returned to basal levels where they remained until at least day 5. The addition of MAP kinase inhibitors (5 μM SB203580, 10 μM PD98059, or 100 nM SP600125) individually enhanced TGF-β1-stimulated *WNT7A* gene expression levels at day 1, and sustained these elevated levels through day 5 of pellet culture (Fig. 8, *C–E*, respectively). Similar analysis showed that *WNT3A* and *WNT11* were not expressed in the chondrogenic cultures and that *WNT5A* gene expression, although present, was not regulated by the addition of TGF-β1 or MAP kinase inhibitors. Expression of *WNT5A* was also unchanged upon treatment of cell pellets with SB203580, PD98059, or SP600125 (data not shown). GAPDH mRNA levels, included as internal controls, remained constant through the 5-day culture period.

DISCUSSION

MPCs derived from human trabecular bone serve as a useful model for the investigation of mechanisms responsible for the generation, maintenance, and particularly the regeneration of cartilage tissue (13, 15). By using this model system, in the present study we have examined the mechanisms of TGF-β1-mediated MPC chondrogenesis, specifically the involvement of MAP kinase and Wnt signaling cascades. Our results show the

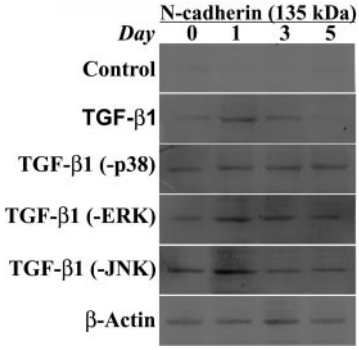

FIG. 6. **Temporal profile of the effects of TGF-β1 and MAP kinase inhibitor treatment on N-cadherin protein levels in MPC pellet cultures as determined by Western analysis.** Cultures are designated as in Fig. 2. Compared with untreated controls, TGF-β1 treatment elicited a rapid transient increase in N-cadherin protein expression with 24 h of TGF-β1 treatment, peaking at day 1, and returning to basal levels by day 5 of pellet culture. Upon cotreatment with inhibitors to p38, ERK-1, and JNK MAP kinases, the elevated level of N-cadherin protein was sustained continuously through 5 days of culture, as compared with TGF-β1 treatment alone. Loading was normalized on the basis of β-actin level.

requisite involvement of the p38, ERK-1, and JNK MAP kinase cascades and their positive regulation of mesenchymal chondrogenesis induced by TGF-β1. Additionally, we demonstrate the functional role of TGF-β1-stimulated N-cadherin expression in the chondrogenic differentiation of MPCs. Interestingly, this control of N-cadherin expression by TGF-β1 occurs via MAP kinase regulation with coincident *WNT7A* gene expression and signaling, reminiscent of our recent findings in which Wnt-7a signaling has been shown to inhibit chondrogenesis in embryonic limb mesenchymal cultures by modulating N-cadherin expression and cell adhesion complexes (43).

MAP kinase signaling activities have been implicated in many forms of cellular differentiation, including chondrogenesis of mesenchymal cells (28, 52). Our results indicate that the transient activations of p38, ERK-1, and JNK are independently essential for the chondrogenic differentiation of adult multipotent MPCs. The requirement of each of these MAP kinase subtypes for chondro-stimulation is most clearly evident by using inhibition studies. In the presence of individual MAP kinase inhibitors, cartilage-specific gene expression induced by TGF-β1 is differentially down-regulated or completely abrogated in a manner specific to each signaling pathway. Thus, our results suggest that a more complete inhibition of TGF-β1-induced chondrogenesis and gene expression in MPC pellet cultures results from inhibition of p38 or ERK-1, compared with JNK. In this manner, activation of each MAP kinase pathway by TGF-β1 is required for and, as such, contributes significantly to the thorough induction of chondrogenic differentiation. It is also interesting to note that although the three subtypes are activated by a single ligand (TGF-β1), their downstream transcriptional effects are markedly different. However, that the gene expression of the transcription factor Sox 9, a critically important transcriptional regulator of cartilage-specific genes and a potent inducer of the chondrocytic phenotype (53), is exclusively regulated by the p38, ERK-1, and JNK subtypes suggests the importance of MAP kinase signaling in chondrogenesis and the regulation of expression of chondroregulatory and chondrocyte-specific genes.

The regulated expression of cell adhesion molecules such as N-cadherin, which functions during precartilage mesenchymal condensation leading to subsequent progression to overt chondrogenic differentiation, has been well established in the chick limb bud (29, 30, 32, 33) and murine C3H10T1/2 micromass systems (31). We report here that the chondrogenic induction of adult MPCs also requires the precise control of N-cadherin expression, dependent on TGF-β1-initiated MAP kinase signaling cascades, most likely to mediate the appropriate cell-cell adhesion required for precartilage mesenchymal condensation and ensuing differentiation. Thus, the rapid transient up-regulation of N-cadherin protein levels by day 1 in TGF-β1-treated cultures initiates the requisite cell-cell interactions in precartilage condensation. The subsequent down-regulation in N-cadherin expression by day 5 is consistent with the increase in production of ECM components and changes in cellular morphology from fibroblast-like to round, morphologically distinct chondrocytes, and a concomitant termination of N-cadherin-mediated cell-cell interaction. The functional involvement of N-cadherin-mediated activities in TGF-β1-induced chondrogenesis is verified by the effect of treating the pellet cultures with the N-cadherin-specific A-CAM antibody, resulting in a significant reduction in aggrecan promoter activation following 3 days of culture, and decreased Alcian blue positive staining and pellet size of 21 day cell pellets, as compared with pellets treated with TGF-β1 alone or cotreated with a nonspecific antibody.

Of the possible signaling mechanisms activated by TGF-β1 ligand binding that regulate N-cadherin expression during precartilage mesenchymal condensation, the p38, ERK-1, and JNK MAP kinases were individually shown to be involved. Based upon our results, we conclude that inhibition of MAP kinase signaling in TGF-β1-treated cultures retarded the progression from precartilage condensation to overt chondrogenic differentiation by sustaining N-cadherin expression and presumably stabilizing the cell-cell adhesion complexes. This was evident upon microscopic examination of TGF-β1-treated, MAP kinase-inhibited pellet cultures, which appeared to condense normally from days 1 through 3, similar to pellets treated with TGF-β1 alone. However, the cotreated pellets remained at this condensed size and appeared not to proceed further along the differentiation pathway, whereas the TGF-β1 cultures began to elaborate a cartilage-specific ECM (data not shown). The addition of p38, ERK-1, and JNK inhibitors individually to TGF-β1-treated pellet cultures led to persistently high levels of N-cadherin protein that failed to return to basal levels even following 5 days of chondrogenic culture, thereby blocking chondrogenic differentiation. Interestingly, De Lise and Tuan (29) have found that transfection-mediated overexpression of wild-type N-cadherin in primary chick limb mesenchymal cultures allows cells to condense normally but inhibits subsequent differentiation due to the persistence of increased cell-cell interaction. Thus, our results here strongly implicate TGF-β1-induced MAP kinase signaling through the p38, ERK-1, and JNK cascades in the regulation of N-cadherin during the progression of prechondrogenic mesenchymal cells to differentiated cartilage. However, the mechanisms by which this is accomplished are not understood.

We therefore investigated the possible regulation of the Wnt signaling pathway by the MAP kinases. TOPFLASH-transfected cell pellets treated with TGF-β1 showed significantly increased transcriptional activation of the β-catenin-TCF-responsive reporter, as well as corresponding nuclear β-catenin protein levels, indicating the activation of the canonical Wnt signaling pathway in response to TGF-β1 ligand binding, and suggesting a probable role for the Wnt cascade in mediating chondrogenesis. Cotreatment with MAP kinase-specific inhibitors significantly enhanced the TGF-β1 stimulation of TCF-dependent transcriptional activation, at least in part by further elevating total β-catenin protein levels, whereas no significant effects were noted using the control, mutated reporter construct, FOPFLASH. These results indicate that the individual

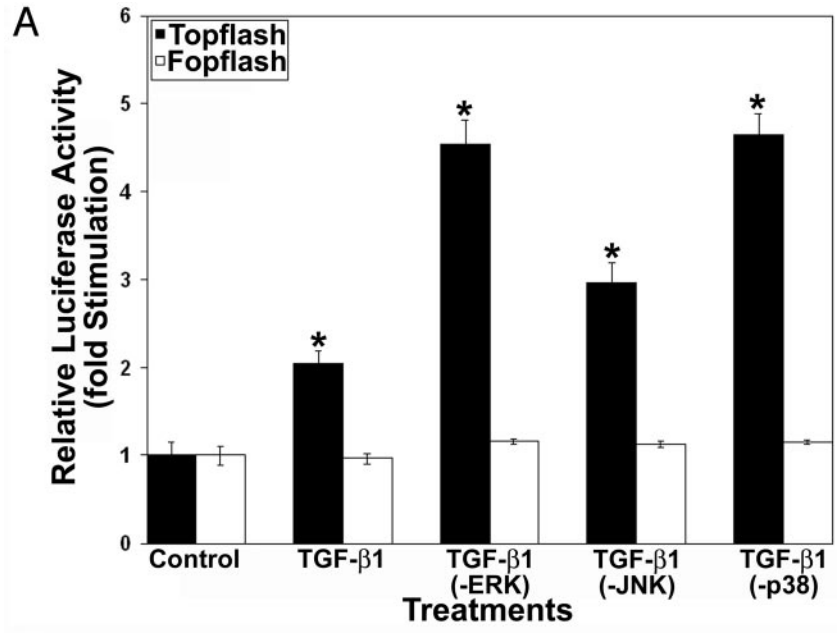

FIG. 7. **Regulation of TGF-β1-mediated Wnt signaling by MAP kinases on the basis of β-catenin-TCF promoter activity and β-catenin stabilization and nuclearization.** Cultures are designated as in Fig. 2. *A*, pellet cultures treated with and without TGF-β1 for 3 days were assayed for Wnt signal-mediated β-catenin-TCF-regulated transcription using luciferase reporter plasmids containing intact (TOPFLASH) and mutated (FOPFLASH) multimeric TCF-binding sites. TGF-β1-induced transcriptional activation of TOPFLASH is further enhanced by inhibition of individual MAP kinase signaling pathways, whereas no effects are seen using the mutated response element. Concomitant with TOPFLASH activation, an increase in the nuclear localization of β-catenin protein levels is seen in TGF-β1-treated pellet cultured for 3 days as compared with control cultures (*B*). Similarly, inhibition of MAP kinases in TGF-β1-stimulated cultures results in even higher levels of cytoplasmic and nuclear β-catenin protein.

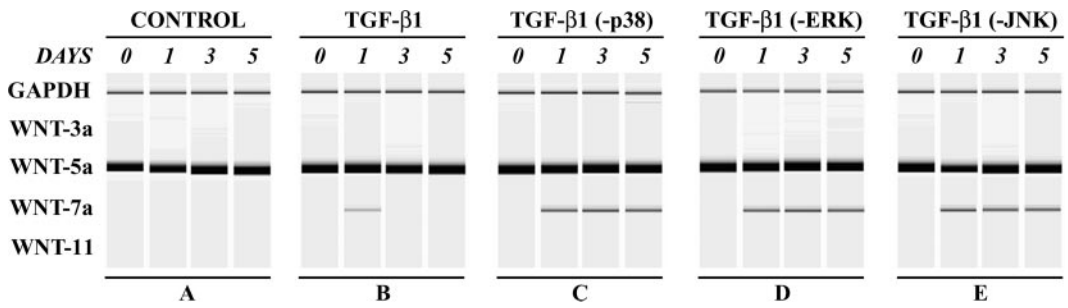

FIG. 8. **Effects of p38, ERK, and JNK MAP kinase inhibitors on TGF-β1-mediated regulation of *WNT* gene expression.** Cultures are designated as in Fig. 2. RT-PCR analysis was performed on cell pellets maintained with and without TGF-β1 for 5 days. Complementary DNA was primed for Wnt-3a, -5a, -7a, and -11, as well as the internal control GAPDH. A transient increase in *WNT-7A* gene expression is seen following 1 day of culture in TGF-β1-treated cultures (*B*) returning to basal levels by day 3, as compared with the untreated control (*A*). Following inhibition of individual MAP kinases in TGF-β1-stimulated cultures (*C–E*), Wnt-7a mRNA expression levels are further up-regulated at day 1 and sustained through 5 days of pellet culture. *WNT-5A* appears to be constitutively expressed in a manner unaffected by TGF-β1 treatment or MAP kinase inhibition.

activation of the p38, ERK-1, and JNK pathways by TGF-β1 in chondrogenic pellet cultures differentially represses the β-catenin-mediated canonical Wnt signal to levels that allow the pathway to remain active under TGF-β1 induction to promote mesenchymal chondrogenesis.

Interestingly, our recent studies (43, 54) have shown that Wnt-7a, a member of the Wnt signaling glycoprotein family, is capable of interfering with the progression of limb mesenchymal cells from precartilage condensation to overt chondrogenic differentiation *in vitro* by modulating the expression of N-cadherin mRNA and protein levels; this effect likely prolongs the stabilization of N-cadherin-dependent intercellular junctions, also seen when N-cadherin is overexpressed (29), and misexpressed, similar to the effects of MAP kinase inhibition reported here (Fig. 6). We therefore investigated the potential regulation of gene expression of representative *WNT* family members from the two major functional groups, the *WNT1* and *WNT5A* class, by TGF-β1 and MAP kinases. Fortuitously, we found that *WNT7A* gene expression was up-regulated in TGF-β1-treated cell pellets following 24 h of culture, diminishing to basal levels by day 3, and remaining there through day 5 (Fig. 8); this transient up-regulation parallels N-cadherin expression levels in similarly treated cultures and, taken together, suggest the regulation of N-cadherin by Wnt-7a. In this manner, the process of cell-cell adhesion mediated by N-cadherin is likely to be regulated by the action of Wnt-7a. This is consistent with the role of Wnt-1 class signaling molecules shown to induce the stabilization of β-and γ-catenin, proteins critical to

the formation of N-cadherin intercellular junctions, thereby prolonging cell-cell adhesion (50). Moreover, the inhibition of MAP kinases in TGF-β1-treated cultures significantly up-regulated *WNT7A* gene expression levels through day 5 of culture as compared with TGF-β1-induced levels (Fig. 8); in addition, the effect of MAP kinases on *WNT* gene expression is totally dependent on the presence of TGF-β1. These sustained levels of *WNT7A* expression in MAP kinase inhibited TGF-β1-treated pellet cultures, again analogous to N-cadherin expression levels in similarly treated cultures, suggest the involvement of MAP kinase signaling in the Wnt-7a regulation of N-cadherin-mediated cell-cell junctions, required for cells to progress into overt chondrogenic differentiation. These findings suggest that a strictly regulated, tonically inhibited level of Wnt-7a, controlled by the individual activation of the p38, ERK-1, and JNK MAP kinase cascades by TGF-β1, is required for the progression of cells from mesenchymal condensation to overt differentiation. Although Wnt-5a mRNA was expressed in all treatment groups throughout the chondrogenic culture period, the level of gene expression did not appear to be regulated by TGF-β1 and/or the MAP kinases. Moreover, it is unlikely that Wnt-5a contributed to the increase in β-catenin-TCF-dependent Wnt signaling or the regulation of N-cadherin expression, especially because the TOPFLASH response elements were differentially regulated by both TGF-β1 as well as the MAP kinases, unlike *WNT5A* gene expression, and our previous studies (43) have shown misexpression of *WNT5A* to have no effect on N-cadherin expression during mesenchymal chondrogenesis in chick limb bud micromass cultures.

In conclusion, we have demonstrated in this study that TGF-β1 initiates and maintains chondrogenesis of trabecular bone-derived MPCs through the differential yet well coordinated chondro-stimulatory activities of p38, ERK-1/2, and to a lesser extent JNK. This regulation of MPC differentiation by the MAP kinases involves the indirect modulation of N-cadherin expression levels to control precartilage condensation events and the progression to chondrogenic differentiation. A target of MAP kinase activation in regulating the events of cell-cell adhesion via N-cadherin is the control of *WNT7A* gene expression levels as well as subsequent Wnt-mediated signaling through the intracellular β-catenin-TCF pathway, which is likely to translate into the strict regulation of N-cadherin expression during condensation. Efforts are currently underway to assess further the specificity of such a mechanistic pathway by means of targeted perturbation of gene expression using small interfering RNA technology, as well as to identify additional candidate regulatory genes by means of microarray-based gene expression profiling.

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