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The Intermediate Filament Vimentin Regulates Chondrogenesis of Adult Human Bone Marrow-Derived Multipotent Progenitor Cells

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ABSTRACT

Cytoskeletal proteins play important regulatory roles in a variety of cellular processes, including proliferation, migration, and differentiation. However, whereas actin and tubulin have established roles regulating developmental chondrogenesis, there is no evidence supporting a function for the intermediate filament vimentin in embryonic cartilage formation. We hypothesized that vimentin may regulate the chondrogenic differentiation of adult multipotent progenitor cells (MPCs), such as those involved in cartilage formation during bone fracture repair. As our model of adult progenitor cell chondrogenesis, we employed high-density pellet cultures of human bone marrow-derived MPCs. siRNA-mediated knockdown of vimentin mRNA and protein triggered a reduction in the extent of MPC cartilage formation, as evidenced by depressed accumulation of mRNAs for the cartilage-specific marker genes aggrecan and collagen type II, as well as reduced levels of Alcian blue-stainable proteoglycan and collagen II protein in the extracellular matrix. Moreover, mRNA and protein levels for the chondro-regulatory transcription factors SOX5, SOX6, and SOX9 were diminished by vimentin knockdown. Depleted cellular vimentin also induced a drastic reduction in PKA phosphorylation levels but did not affect the phosphorylation of multiple other chondro-regulatory kinases and transcription factors, including ERK1/2, p38, Smad2, and Smad1/5/8. Importantly, siRNA-mediated knockdown of PKA C-α mRNA and protein mimicked the reduction in chondrogenesis caused by diminished cellular vimentin. Finally, overexpression of vimentin in MPCs significantly enhanced the activity of a transfected collagen II promoter-luciferase reporter gene. In conclusion, we describe a novel role for the intermediate filament vimentin as a positive regulator of adult human bone marrow-derived MPC chondrogenesis. J. Cell. Biochem. 109: 265–276, 2010. Published 2009 Wiley-Liss, Inc.[†]

KEY WORDS: CHONDROGENESIS; MULTIPOTENT PROGENITOR CELL; BONE MARROW STROMA; VIMENTIN; INTERMEDIATE FILAMENT; PROTEIN KINASE A

Chondrogenesis is a stringently regulated, multistep cellular differentiation program culminating in acquisition of the chondrocytic phenotype [reviewed in Hickok et al., 1998; Cancedda et al., 2000; DeLise et al., 2000; Shum et al., 2003]. An array of chondro-progenitor cell types can participate in the cartilage formation process in vivo. In the embryo, cells from three distinct mesenchymal lineages, sclerotome, somatopleure, and neural crest, embark upon chondrogenic differentiation. In the adult, multipotent progenitor cells (MPCs) exhibit the capacity to participate in a plethora of reparative and regenerative processes, including the cartilage formation component of bone fracture healing. Despite the occurrence of chondrogenesis in diverse cell types and at a variety of anatomical locations throughout the vertebrate embryo, each developmental chondrogenic event follows a fundamentally similar sequence of cellular and molecular steps. Furthermore, many of the mechanisms known to regulate embryonic cartilage formation are recapitulated during the chondrogenic differentiation phase of bone fracture repair [reviewed in Ferguson et al., 1998].

Many of the extracellular growth factors, cytoplasmic protein kinases, and nuclear transcription factors integral to chondrogenesis have been described. In the extracellular milieu, multiple members of the transforming growth factor-β (TGF-β) [Kulyk et al., 1989],

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bone morphogenetic protein (BMP) [Seghatoleslami et al., 2003], fibroblast growth factor (FGF) [Bobick et al., 2007], and wingless/Int (Wnt) secreted glycoprotein [reviewed in Tuan, 2003] families have been implicated in the regulation of chondrogenesis. Linking the extracellular chondro-regulatory molecules to the nuclear transcription factors, a variety of kinases, including protein kinase A (PKA) [Zakany et al., 2002], protein kinase C (PKC) [Lim et al., 2003], extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-jun N-terminal kinase (JNK) [reviewed in Bobick and Kulyk, 2008], are known to participate in chondrogenic differentiation. Finally, in the nucleus, the transcription factor Sox9 is required for chondrogenesis [Bi et al., 1999] and is known to function in cooperation with two other Sox family proteins, L-Sox5 and Sox6, in order to activate expression of structural extracellular matrix (ECM) components [Lefebvre et al., 2001]. Importantly, as chondro-progenitor cells progress through the chondrogenic differentiation program, they exchange their stellate, fibroblastoid shape for the spherical morphology of chondrocytes and commence synthesis of cartilage-specific ECM molecules like collagen types II, IX, and XI [reviewed in Hoffman et al., 2003; Mayne and Ala-Kokko, 2005] and the highly sulfated proteoglycan aggrecan [reviewed in Woods, 2005].

In addition to chondro-regulatory growth factors, kinases, and transcription factors, progression of the chondrogenic differentiation program can also be influenced by the cytoskeleton. Numerous studies, most of which employ cell culture systems comprised of undifferentiated chondro-progenitor mesenchyme or dedifferentiated chondrocytes, have been published describing roles for the actin and microtubule networks in the initiation of cartilage-specific gene expression [reviewed in Daniels and Solursh, 1991; Woods et al., 2007]. For example, Zanetti and Solursh [1984] found that embryonic chick wing bud mesenchymal progenitor cells cultured at subconfluent density acquire a fibroblastic morphology and fail to accumulate Alcian blue-positive cartilage ECM. However, treatment with cytochalasin D, a fungal metabolite that caps the growing ends of microfilaments [reviewed in Cooper, 1987], caused these progenitors to assume the spherical shape of chondrocytes and commence production of Alcian blue-stainable sulfated glycosaminoglycan (sGAG). More recently, Connelly et al. [2008] demonstrated that actin transduces the chondro-inhibitory effects of integrin adhesion in bovine bone marrow-derived mesenchymal stem cells (MSCs) cultured in an agarose hydrogel three-dimensional environment. Furthermore, Benya and Padilla [1993] and Loty et al. [1995] have shown that dedifferentiated chondrocytes can be stimulated to re-express genes encoding cartilage-specific ECM molecules via cytochalasin-induced actin disruption. Integrity of the microtubule network also seems to regulate in vitro chondrogenesis. Woods et al. [2005] found that accumulation of both *sox9* mRNA and Alcian blue-positive ECM was blocked in high-density cultures of mouse embryonic limb bud mesenchyme following treatment with colchicine, an inhibitor of microtubule polymerization.

Although roles for the actin and microtubule networks in the regulation of developmental chondrogenesis have been described, the intermediate filament vimentin does not appear to function as a mediator of normal embryonic cartilage formation. Vimentin-null mice were healthy and reproduced without an obvious abnormal phenotype [Colucci-Guyon et al., 1994]. More recent publications that have re-examined the vimentin knockout mouse [Henrion et al., 1997; Terzi et al., 1997; Eckes et al., 1998; Colucci-Guyon et al., 1999] have also failed to reveal any defects in developmental chondrogenesis. More specifically, Langa et al. [2000] generated embryonic stem (ES) cells from mice lacking vimentin. Teratocarcinomas formed by either *vim*−/− or wild-type ES cells were identical, including the presence of rounded chondrocytes housed in identifiable lacunae. Though currently no evidence exists supporting a regulatory role for vimentin in developmental chondrogenesis, it is possible that this intermediate filament helps mediate the cartilage differentiation program of adult MPCs, such as the mesenchymal progenitors involved in the chondrogenic component of bone fracture repair. Vimentin is known to regulate adult chondrocyte homeostasis [Blain et al., 2006] and mice lacking vimentin have been shown to exhibit significantly delayed wound healing [Eckes et al., 2000]. Therefore, we hypothesized that vimentin may be needed for the chondrogenic differentiation of adult human bone marrow-derived MPCs, a cell type that has the capacity to undergo chondrogenesis in vitro [Sekiya et al., 2002] and may be involved in bone fracture repair in vivo [Dimitriou et al., 2005; Taguchi et al., 2005].

Our goal was to ascertain the regulatory role(s) of vimentin in the early stages of adult human bone marrow-derived MPC chondrogenesis. To accomplish this, we first established a succinct, temporally defined model of growth factor-enhanced MPC chondrogenic differentiation. We selected two members of the TGF-β superfamily, growth and differentiation factor 5 (GDF5) and TGF-β3, known promoters of both developmental chondrogenesis in vitro [Coleman and Tuan, 2003; Jin et al., 2006] and the chondrogenic component of bone fracture repair in vivo [Cho et al., 2002; Chhabra et al., 2005], as the chondro-stimulatory molecules responsible for enhancing cartilage-specific gene expression in high-density MPC pellet cultures. We profiled the temporal expression of vimentin protein during MPC pellet chondrogenesis and discovered that cellular vimentin levels parallel the progression of MPC cartilage formation. Subsequently, we found that siRNA-mediated knockdown of vimentin mRNA and protein in adult human MPCs drastically reduces the capacity of these cells to form cartilage. Moreover, we discovered that depleted cellular vimentin is associated with significantly depressed PKA phosphorylation levels and correspondingly that siRNA-mediated knockdown of PKA C-α mRNA and protein mimics the chondro-inhibitory effects of vimentin depletion. Finally, we found that vimentin overexpression markedly enhances adult human MPC pellet culture chondrogenesis. Taken together, these findings suggest a novel, hitherto unreported role for the intermediate filament vimentin as an important positive regulator of adult human bone marrow-derived MPC chondrogenesis.

MATERIALS AND METHODS

ISOLATION AND EXPANSION OF ADULT HUMAN MPCs

With approval from the Institutional Review Board of the University of Washington (Seattle, WA), MPCs were isolated from the proximal

femora of one male and two female patients (aged 60–62 years) undergoing total hip arthroplasty and processed as previously described [Mueller and Tuan, 2008]. Briefly, bone marrow was curetted from the exposed cutting plane of the femoral neck, washed with high-glucose Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine and sodium pyruvate (Gibco/Invitrogen), and separated from trabecular bone fragments and other tissue debris by passing the eluate through a 70-μm cell strainer (BD Biosciences). Mononuclear cells were enriched via a Ficoll-Paque density gradient (GE Healthcare) and plated in expansion medium (EM) consisting of high-glucose DMEM, 10% MSC qualified fetal bovine serum (FBS; Gibco/Invitrogen), and 100 μg/ml primocin (InvivoGen). Non-adherent cells were discarded following a 24-h incubation at 37°C in a 5% CO_2/95% air atmosphere. When adherent monolayers reached near confluence, cells were detached with 0.25% trypsin/0.38 g/L EDTA (Gibco/Invitrogen) and re-plated at a density of $\sim 3 \times 10^3$ cells/cm^2.

IN VITRO CHONDROGENESIS OF MPCs

To establish high-density chondrogenic pellet cultures, passage 3 MPCs in monolayer were trypsinized and re-suspended in chondrogenic phenotype maintenance medium [CPMM; high-glucose DMEM supplemented with 50 μg/ml ascorbate (Sigma–Aldrich), 40 μg/ml L-proline (Sigma–Aldrich), 1:100 (v/v) ITS + universal culture medium supplement (BD Biosciences), and 100 μg/ml primocin]. 2.5×10^5 MPCs were pipetted into individual wells of conical bottom 96-well plates (Nalge Nunc International/Thermo Fisher Scientific) and pelleted by centrifugation (300*g*, 5 min). Pellets were maintained in CPMM for 24 h prior to replacement with CPMM containing growth factor (see Fig. 1A). All pellets were incubated at 37°C in a 5% CO_2/95% air atmosphere. Recombinant mouse GDF5 and recombinant human TGF-β3 were purchased from R&D Systems.

ELECTROPORATION-MEDIATED siRNA TRANSFECTION OF MPCs

Passage 3 MPCs in monolayer were trypsinized, washed once with Dulbecco's PBS (D-PBS; Gibco/Invitrogen), and re-suspended in siPORT siRNA Electroporation Buffer (Ambion/Applied Biosystems) at a density of 2.5×10^6 cells/ml. Three hundred microliters of cell suspension containing 22.5 μg of siRNA was transferred to a 2-mm gap BTX electroporation cuvette (Thermo Fisher Scientific). A Harvard Apparatus BTX ECM 830 Electro Square Porator was used to deliver two identical square wave pulses (parameters: 1,075 V, 105-μs pulse length, 5-s duration between pulses). Transfected MPCs were incubated for 15 min at 37°C in a 5% CO_2/95% air atmosphere prior to pelleting (see Fig. 3A) or seeding as high-density monolayers. Monolayers were generated by seeding 4.0×10^3 MPCs in individual wells of eight-well Lab-Tek Permanox chamber slides (Nalge Nunc International/Thermo Fisher Scientific). Monolayers were maintained in EM for 48 h before replacement with CPMM containing growth factor. Silencer pre-designed siRNA sequences 138995, 13111, 138994, 13019 (targeted against vimentin mRNA),

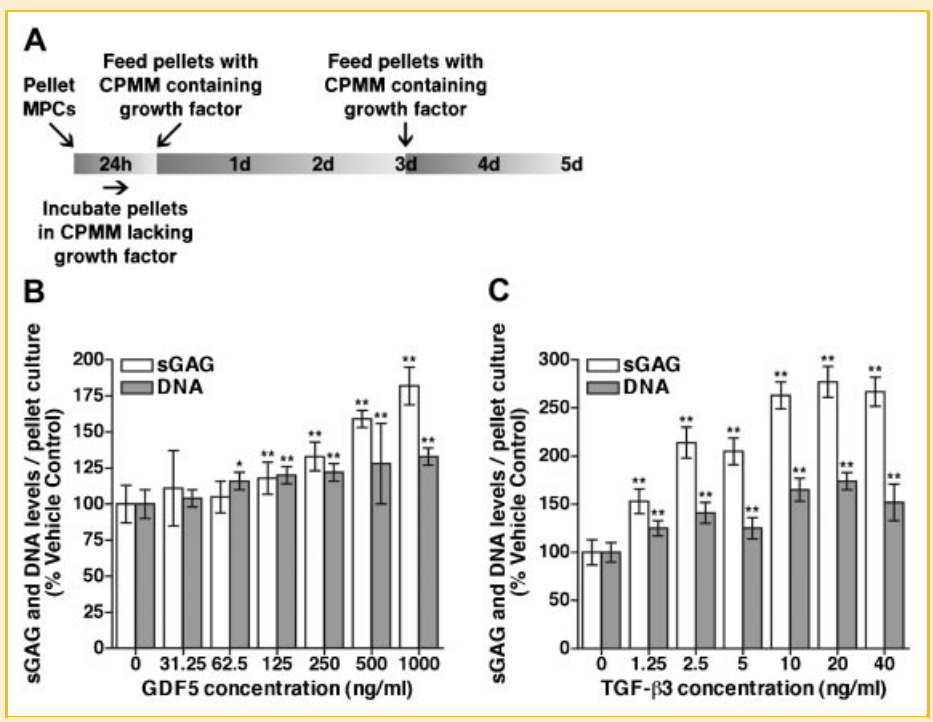

Fig. 1. A: Schema of the 5-day MPC pellet culture chondrogenic time course employed in this study. B,C: GDF5 and TGF-β3 promote dose-dependent increases in MPC pellet culture sGAG and DNA accumulation. Graphed values are the mean ± SD of measurements from nine cultures (three replicate cultures of MPCs from a single donor × three donors). * and ** indicate values different from controls at $P < 0.05$ and $P < 0.01$, respectively.

292, and 293 (targeted against PKA C-α mRNA), as well as Silencer Negative Control #1 siRNA, were purchased from Ambion/Applied Biosystems.

AMAXA NUCLEOFECTOR-MEDIATED PLASMID TRANSFECTION OF MPCs

MPCs were transiently transfected with plasmid DNA using the Amaxa Human MSC Nucleofector Kit (Lonza) as previously described [Haleem-Smith et al., 2005]. Passage 3 MPCs in monolayer were trypsinized, washed once with D-PBS, and re-suspended at a density of 7.5×10^6 cells/ml in Human MSC Nucleofector Solution (Lonza). Eight micrograms of vimentin expression plasmid or the corresponding empty vector pCMV6-XL5 (OriGene Technologies), 7 μg of collagen type II promoter-firefly luciferase reporter plasmid, and 1 μg of herpes simplex virus thymidine kinase (TK) promoter-*Renilla* luciferase construct (pRL-TK; Promega) were added to 100 μl of MPC suspension. Nucleofection was performed using program U-23. Immediately following electroporation, 1 ml of CPMM was added to the Nucleofection mixture and the cells pelleted. Pellets were maintained in the 10:1 (v/v) CPMM/Human MSC Nucleofector Solution mixture for 24 h prior to replacement with and incubation in CPMM containing growth factor for an additional 48 h. Determination of firefly and *Renilla* luciferase activities was performed using the Dual Luciferase Reporter Assay System (Promega) and a Turner BioSystems 20/20n Single Tube Luminometer. The collagen type II promoter-firefly luciferase reporter plasmid contained a ~4 kb segment of the human *COL2A1* gene (−577 to +3428), encompassing the promoter, first exon, and intron 1 enhancer, cloned in a pGL3 basic vector (provided by Dr. M.B. Goldring, Hospital for Special Surgery).

BLYSCAN DYE sGAG ASSAY

sGAG content of MPC pellets was determined using the Blyscan dye assay (Biocolor), according to the manufacturer's protocol. DNA contents of MPC pellets were determined using the Quant-iT PicoGreen dsDNA assay kit (Molecular Probes/Invitrogen).

WESTERN BLOT ANALYSIS

For each experimental time point, three replicate cultures of MPCs isolated from a single donor were homogenized in radioimmunoprecipitation assay (RIPA) buffer (Sigma–Aldrich) supplemented with protease and phosphatase inhibitors (Sigma–Aldrich). Proteins were separated on 10% SDS–polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane (Santa Cruz Biotechnology) using the Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories). Primary antibodies employed for detection of vimentin, actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), SOX5, SOX6, SOX9, ERK5, and p-ERK5 were purchased from Santa Cruz Biotechnology. Total and phosphosite-specific antibodies directed against PKA C-α, p-PKA C, Smad2, p-Smad2, Smad5, p-Smad1/5/8, Smad4, MEK1/2, p-MEK1/2, ERK1/2, p-ERK1/2, p38, p-p38, JNK, and p-JNK were purchased from Cell Signaling Technology. The anti-p-SOX9 antibody was purchased from Abcam. Primary antibodies were detected using appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) and Pierce SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology/Thermo Fisher Scientific).

RNA ISOLATION AND GENE EXPRESSION ANALYSIS

Total RNA was isolated from MPC pellet cultures using the RNeasy Mini Kit (Qiagen) and quantified spectrophotometrically based on A_{260}. Following first strand cDNA synthesis using the SuperScript First-Strand Synthesis System (Invitrogen), levels of specific mRNA transcripts were determined by real-time polymerase chain reaction (PCR) utilizing SYBR Green PCR Master Mix and an iCycler real-time PCR detection system (Bio-Rad Laboratories). Relative levels of cartilage-specific gene transcripts, as well as transcripts encoded by the *VIM* and *PKA C*-α genes, were calculated using individual standard curves and normalized to relative mRNA levels of the constitutively expressed *GAPDH* and β-actin genes. All gene-specific primer sequences employed in this study were described previously and purchased from Integrated DNA Technologies. The following primer sets were used: *VIM*, CAC GAA GAG GAA ATC CGG AGC and CAG GGC GTC ATT GTT CCG [Chen et al., 2005]; *AGC*, TGC GGG TCA ACA GTG CCT ATC and CAC GAT GCC TTT CAC CAC GAC [Nesti et al., 2008]; *COL2A1*, GGA AAC TTT GCT GCC CAG ATG and TCA CCA GGT TCA CCA GGA TTG C [Nesti et al., 2008]; *SOX9*, GCA GGC GGA GGC AGA GGA G and GGA GGA GGA GTG TGG CGA GTC [Mueller and Tuan, 2008]; *L-SOX5*, ATC CCA ACT ACC ATG GCA GCT and TGC AGT TGG AGT GGG CCT A [Tew et al., 2005]; *SOX6*, ACT GTG GCT GAA GCA CGA GTC and TCC GCC ATC TGT CTT CAT ACC [Sekiya et al., 2002]; *PKA C*-α, ACC AGC AGG GCT ACA TTC AG and AAT CTC AGG GGC CAG GTA CT [Chang et al., 2003]; β-actin, ATT GCC GAC AGG ATG CAG AA and GCT GAT CCA CAT CTG CTG GAA [Jinquan et al., 2000]; and *GAPDH*, CCA GGC TGA GAA CGG GAA GC and AGG GGG CAG AGA TGA TGA CC [Nesti et al., 2008].

HISTOCHEMICAL STAINING AND IMMUNOHISTOCHEMISTRY

For histological analysis, pellets were rinsed twice with PBS, fixed for 20 min in 4% paraformaldehyde (Sigma–Aldrich), dehydrated through a graded ethanol series, infiltrated with xylene, embedded in paraffin, and sectioned at a thickness of 7 μm. Some histological sections were stained with Alcian blue, pH 1.0 (Rowley Biochemical) or hematoxylin and eosin (H&E; Sigma–Aldrich). Immunohistochemical localization of collagen type II was performed following antigen retrieval via 1 mg/ml hyaluronidase (Sigma–Aldrich) digestion for 1 h at 37°C. Incubation with primary antibody (Developmental Studies Hybridoma Bank) was carried out at 4°C overnight. Detection of primary antibody was performed with the Zymed SuperPicTure Polymer Detection Kit (Zymed Laboratories) employing an HRP polymer-conjugated secondary antibody and diaminobenzidine (DAB) as a chromogen.

DETECTION OF VIMENTIN IN MPC MONOLAYER CULTURE

To visualize cellular vimentin, MPCs maintained in monolayer culture for 5 days were fixed with Cytoskelfix reagent (Cytoskeleton, Inc.) for 4 min at −20°C, permeabilized with 0.2% Triton X-100 (Sigma–Aldrich) in PBS, and incubated with a Cy3-conjugated monoclonal antibody directed against vimentin (Sigma–Aldrich) for 1 h at room temperature. Nuclei were stained via a 3-min incubation

with 10 μg/ml Hoechst 33342 (Molecular Probes/Invitrogen) in PBS containing 0.1% Tween-20 (Sigma–Aldrich).

STATISTICAL ANALYSIS

Quantitative data regarding sGAG, DNA, and mRNA transcript accumulation, as well as luciferase activity, were analyzed by two-tailed unpaired *t*-test or one-way analysis of variance with the Dunnett or Tukey post-test using InStat 3 software (GraphPad Software).

RESULTS

GDF5 AND TGF-β3 ENHANCE CARTILAGE ECM PRODUCTION IN MPC PELLET CULTURES

To examine the regulatory role(s) of vimentin in the early stages of adult progenitor cell chondrogenesis, we first devised a succinct, temporally defined model of growth factor-enhanced MPC cartilage formation (see Fig. 1A). GDF5 and TGF-β3 were selected as the growth factors responsible for enhancement of MPC pellet culture chondrogenesis. In order to determine the optimal concentrations of GDF5 and TGF-β3 required to significantly enhance MPC synthesis of sGAG, pellets were incubated in control CPMM containing growth factor vehicle (0 ng/ml growth factor) or in CPMM supplemented with increasing concentrations of GDF5 (Fig. 1B) or TGF-β3 (Fig. 1C) for 5 days. GDF5 and TGF-β3 treatments promoted dose-dependent increases in pellet culture sGAG and DNA accumulation relative to parallel controls (Fig. 1B,C). At concentrations of 1 μg/ml and 20 ng/ml, respectively, GDF5 and TGF-β3 were most effective at enhancing the sGAG/DNA ratio, with TGF-β3 increasing this ratio to a greater extent than GDF5. Therefore, these concentrations were employed to increase cartilage ECM production in MPC pellet cultures for the remainder of the study.

ENDOGENOUS VIMENTIN PROTEIN LEVELS PARALLEL THE PROGRESSION OF CHONDROGENESIS IN MPC PELLET CULTURES

Western blotting was used to examine whether the progression of chondrogenesis in MPC pellet cultures was accompanied by temporal alterations in endogenous levels of total vimentin protein. MPC pellets were cultured for 1–5 days in CPMM lacking growth factor (Fig. 2A) or in CPMM supplemented with GDF5 (Fig. 2B) or TGF-β3 (Fig. 2C). Electroblots of cell lysates containing equivalent amounts of total GAPDH protein were immunoprobed with antibodies specific for total vimentin and actin, as well as total and p-SOX9. In pellets maintained in the absence of growth factor, total and p-SOX9 levels remained relatively constant throughout the 5-day culture period. In GDF5- and TGF-β3-treated pellets, levels of both total and p-SOX9 increased steadily throughout the 5-day time course, confirming an enhancement of chondrogenesis triggered by these growth factors. TGF-β3-induced increases in total and p-SOX9 protein levels were greater than those stimulated by GDF5 treatment, in agreement with the higher levels of pellet sGAG accumulation elicited by TGF-β3 application (see Fig. 1B,C). Collectively, these data indicate that TGF-β3 is a more potent enhancer of MPC chondrogenesis than GDF5 in our culture system. Importantly, under all experimental conditions, vimentin protein levels paralleled expression of both total and p-SOX9 (Fig. 2A–C).

Film exposure periods employed to achieve visualization of bands for vimentin, SOX9, and p-SOX9 were significantly longer for cell lysates derived from pellets incubated in the absence of growth factor (Fig. 2A) than for growth factor-treated pellet lysates (Fig. 2B,C). At chondrogenic culture day 5, levels of vimentin, SOX9, and p-SOX9 were greater in GDF5- and TGF-β3-treated MPC cultures than in pellets maintained in the absence of chondrostimulatory growth factor (Fig. 2D).

Similar to previous studies [Lambrecht et al., 2008; Tilleman et al., 2008], we also observed that lysates derived from different human donor cell populations display visually discernible "vimentin cluster" banding patterns. Specifically, lysates derived from MPC populations isolated independently from the bone marrow of a 62-year-old female donor and a 60-year-old male donor yielded identical "vimentin cluster" banding patterns, whereas lysates derived from another 62-year-old female donor displayed a different pattern of "vimentin cluster" banding (Fig. 2E).

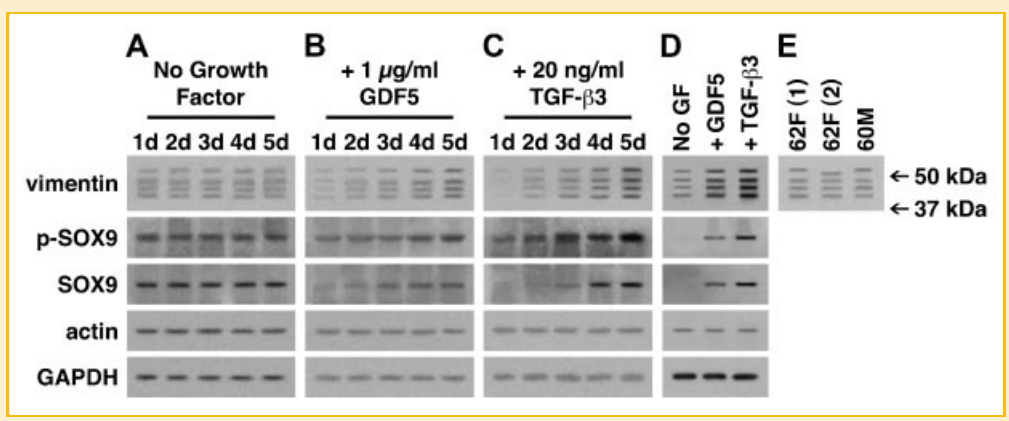

Fig. 2. A–C: Temporal changes in endogenous levels of vimentin, SOX9, and p-SOX9 protein during MPC pellet culture chondrogenesis, as determined by Western blotting. D: Five-day lysates illustrate relative levels of vimentin, SOX9, and p-SOX9 between chondrogenic conditions. E: Band migration distances within the "vimentin cluster" differ between donor MPC lysates. Arrows correspond to molecular mass markers.

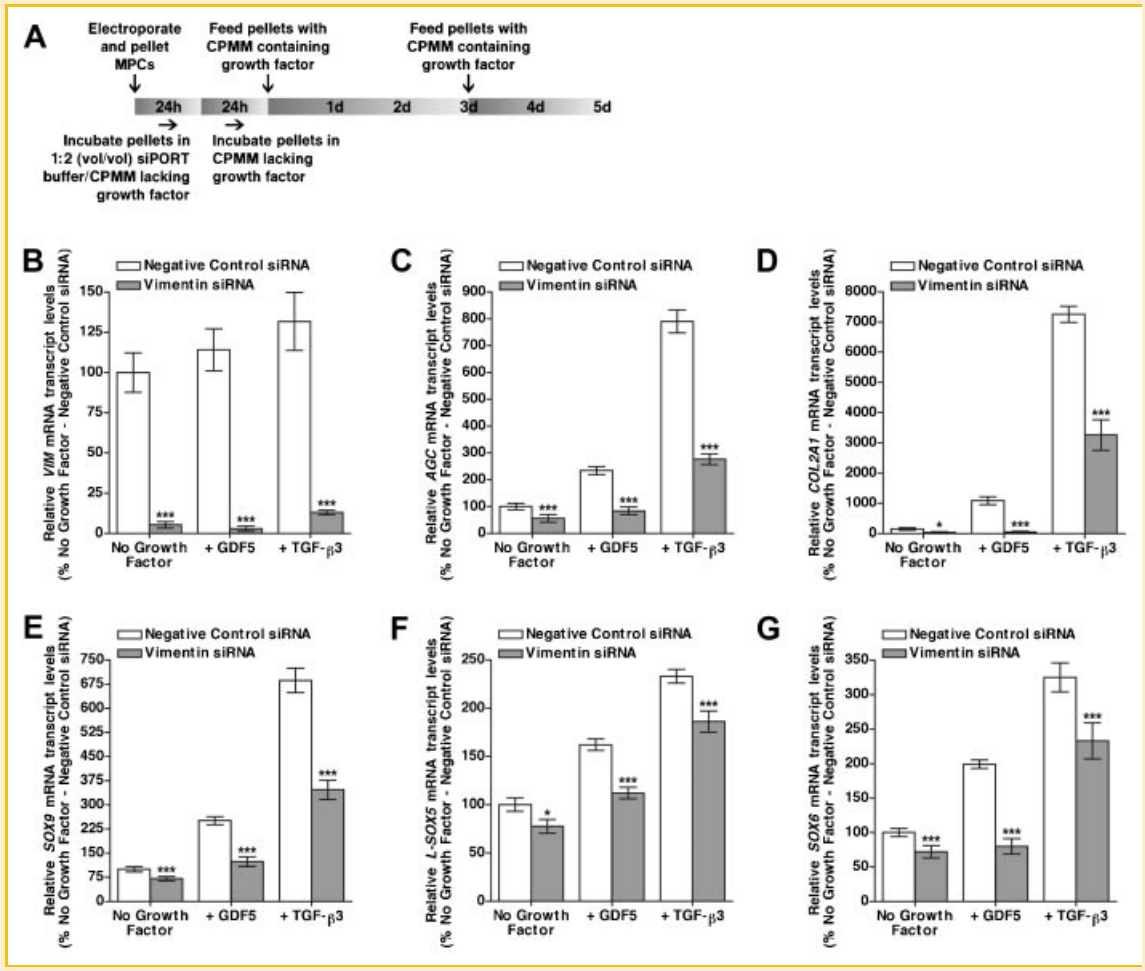

Fig. 3. A: Schema depicting transfection of MPCs with siRNA and the ensuing 5-day chondrogenic time course employed in this study. B–G: Transfection of MPCs with siRNA targeting the *VIM* transcript depresses accumulation of cartilage-characteristic mRNAs, as determined by real-time PCR. Graphed values are the mean ± SD of measurements from three pellet sets (one set of replicate pellets comprised of MPCs from a single donor × three donors). * and *** indicate values different than controls at $P < 0.05$ and $P < 0.001$, respectively.

siRNA-MEDIATED VIMENTIN KNOCKDOWN DEPRESSES CARTILAGE-SPECIFIC MARKER GENE mRNA ACCUMULATION DURING MPC PELLET CULTURE CHONDROGENESIS

Following establishment of a short-term cartilage formation model for adult human MPCs and characterization of vimentin protein levels during the chondrogenic progression of this model, we wished to assess the involvement of vimentin in the expression of cartilage-specific mRNAs by MPCs in pellet culture. In order to accomplish this, MPCs were transfected with siRNA targeting the *VIM* transcript prior to induction of chondrogenic differentiation (see Fig. 3A). Non-targeting scramble sequence siRNA was used as a negative control. Quantitative real-time PCR analysis was employed to determine the relative levels of *VIM* mRNA (Fig. 3B), as well as mRNA levels for the cartilage-specific marker genes aggrecan (*AGC*; Fig. 3C), collagen type II (*COL2A1*; Fig. 3D), *SOX9* (Fig. 3E), *L-SOX5* (Fig. 3F), and *SOX6* (Fig. 3G). MPCs transfected with Ambion vimentin siRNA sequences 138995, 13111, 138994, or 13019, cultured as pellets in the presence of 20 ng/ml TGF-β3 for 5 days, showed *VIM* mRNA levels that were reduced to ~10% ($P < 0.001$, n = 3; Fig. 3B), ~43%, ~45%, and ~83% ($P < 0.001$, $P < 0.001$, and $P < 0.01$, all n = 3; data not shown) of negative controls, respectively. Importantly, MPC pellets transfected with any of the Ambion vimentin siRNAs were characterized by significantly depressed accumulation of *AGC*, *COL2A1*, and *SOX9* mRNAs relative to scramble sequence-transfected controls (Fig. 3C–E for siRNA sequence 138995 and data not shown). Vimentin siRNA 138995 was most effective at depressing MPC pellet culture levels of *VIM*, *AGC*, *COL2A1*, and *SOX9* gene transcripts and thus was employed to knockdown *VIM* mRNA levels in all subsequent experiments reported in this study. Under all chondrogenic conditions (No Growth Factor, +GDF5, +TGF-β3), pellet cultures comprised of vimentin siRNA-transfected MPCs accumulated significantly lower levels of mRNAs encoding the cartilage ECM components aggrecan and collagen type II, as well as the chondro-regulatory transcription factors SOX9, L-SOX5, and SOX6 (Fig. 3C–G).

VIMENTIN siRNA TRANSFECTION DEPRESSES LEVELS OF TOTAL VIMENTIN PROTEIN, AS WELL AS LEVELS OF THE SOX TRANSCRIPTION FACTORS, IN MPC PELLET CULTURES

In order to confirm that vimentin siRNA transfection reduced the amount of total vimentin protein in MPCs, in addition to depressing

accumulation of *VIM* gene mRNA transcripts (see Fig. 3B), we performed Western blotting with an antibody specific for total vimentin. Pellets of MPCs transfected with negative control or vimentin siRNA were maintained as chondrogenic cultures (No Growth Factor, +GDF5, +TGF-β3) for 2 or 5 days. Cell lysate samples were immunoblotted and loads adjusted so as to contain equivalent amounts of GAPDH protein. Under all culture conditions (Fig. 4A–C) and in accordance with the real-time PCR data, vimentin siRNA transfection caused a marked reduction in total vimentin protein levels relative to negative controls. Immunofluorescence with a Cy3-conjugated monoclonal antibody directed against vimentin further confirmed that transfection of MPCs with siRNA targeting the *VIM* gene transcript drastically reduces the amount of total cellular vimentin protein present (Fig. 4D). Western blotting also revealed reduced levels of total and p-SOX9 (Fig. 4A–C), as well as diminished SOX5 and SOX6 protein levels (data not shown), in pellet cultures characterized by depleted vimentin.

VIMENTIN KNOCKDOWN DEPRESSES CARTILAGE ECM PRODUCTION IN MPC PELLET CULTURES

We subsequently examined whether vimentin knockdown reduced the amount of sGAG and collagen type II protein present in the ECM of MPC pellets. MPCs were transfected with either negative control or vimentin siRNA and subjected to chondrogenic induction as pellet cultures. Histological sections of MPC pellets were stained with Alcian blue, a cationic dye that detects negatively charged sGAG (Fig. 5A; large photomicrographs), or probed with a monoclonal antibody specific for collagen type II (Fig. 5A; smaller inset photomicrographs). Under all chondrogenic conditions (No Growth Factor, +GDF5, +TGF-β3), vimentin knockdown reduced accumulation of both Alcian blue-positive ECM and total collagen type II protein relative to controls (Fig. 5A).

To ensure that the inhibitory effects of vimentin knockdown on sGAG and collagen type II accumulation were not due to reduced culture cell number, PicoGreen analysis was performed to determine DNA levels of 5-day pellets. Our results showed that the mean DNA contents of *VIM* siRNA pellet cultures were ~1.2-, ~1.1-, and ~1.2-times those of negative controls maintained under the three chondrogenic culture conditions, respectively ($P < 0.001$, $P < 0.05$, and $P < 0.001$, $n = 9$; data not shown).

H&E staining (Fig. 5B) was employed to analyze the effect(s) of vimentin knockdown on the shape of MPCs in chondrogenic pellets. Under culture conditions that resulted in lower levels of chondrogenesis (No Growth Factor, +GDF5), vimentin knockdown yielded cells characterized by an elongated, fibroblastic shape, whereas cells transfected with negative control siRNA displayed the round morphology typical of chondrocytes. Under culture conditions that generated higher levels of cartilage-specific gene expression (+TGF-β3), all siRNA-transfected MPCs were characterized by the same spherical shape (Fig. 5B; smaller inset photomicrographs), even though MPCs lacking vimentin underwent significantly lower levels of chondrogenesis. This observation is consistent with our finding that in the presence of TGF-β3, vimentin-deficient MPCs undergo lower levels of chondrogenic differentiation than scramble sequence siRNA-transfected controls, yet still display cartilage-characteristic mRNA and protein levels that are significantly greater than those of negative control MPCs cultured in the absence of growth factor or in the presence of GDF5 (Figs. 3–5A).

VIMENTIN KNOCKDOWN DEPRESSES PKA PHOSPHORYLATION IN MPC PELLET CULTURES

We wished to identify the intracellular signaling mechanism responsible for linking the disrupted vimentin network to the depressed chondrogenic transcriptional activity in MPCs. Western blotting with antibodies specific for total and phosphorylated PKA (Fig. 6A), ERK1/2, MEK1/2, p38, JNK, ERK5, Smad2, and Smad1/5/8 (data not shown) was employed to determine whether phosphorylation levels of these known chondro-regulatory kinases and transcription factors were affected by vimentin knockdown. Pellets

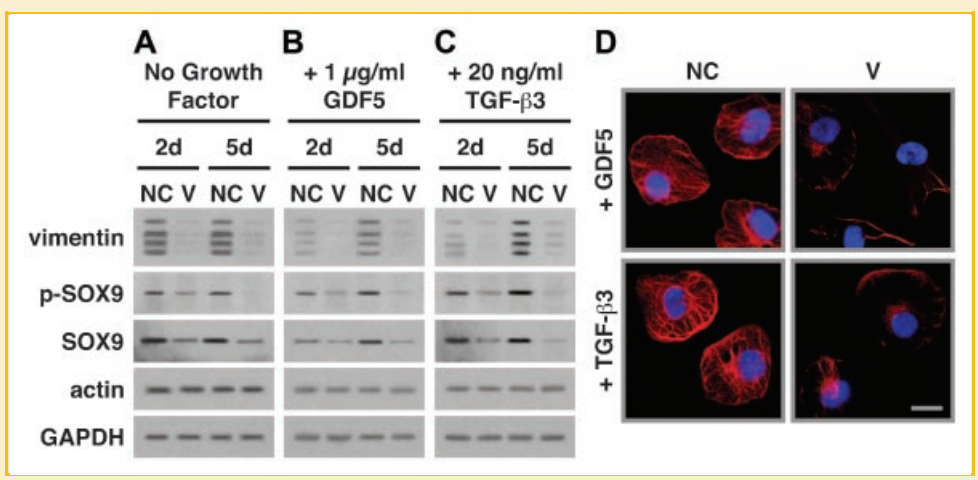

Fig. 4. A–C: Transfection of MPCs with siRNA targeting the *VIM* transcript (V) depresses accumulation of vimentin, SOX9, and p-SOX9 protein relative to negative controls (NC), as determined by Western blotting. D: Immunofluorescence with a Cy3-conjugated antibody demonstrates that MPCs transfected with siRNA targeting the *VIM* transcript exhibit lower levels of total cellular vimentin than MPCs transfected with negative control siRNA. Scale bar: 10 μm.

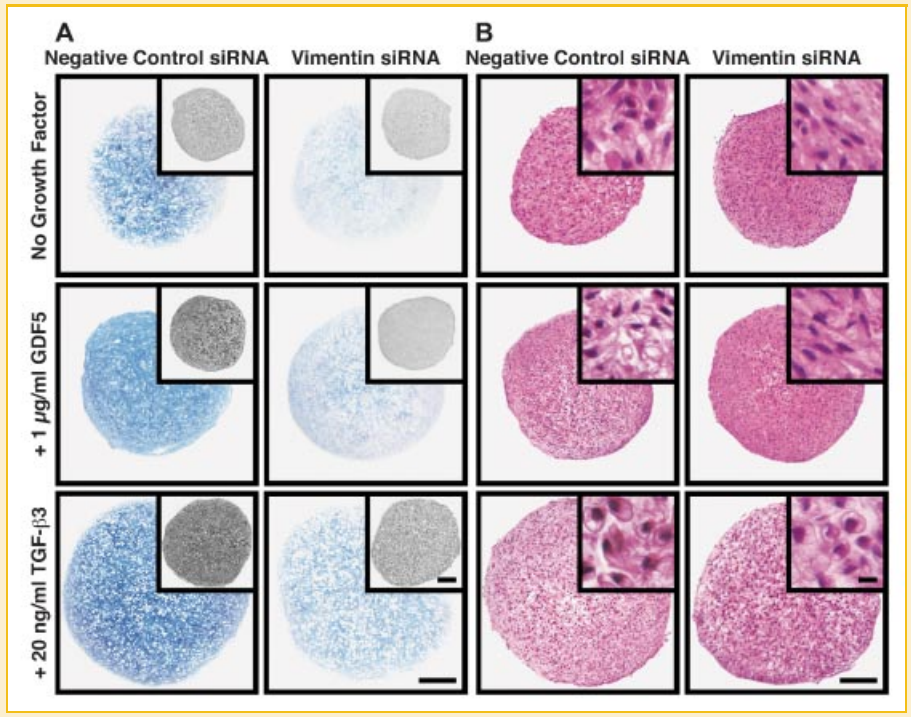

Fig. 5. A: siRNA-mediated vimentin knockdown depresses MPC pellet culture accumulation of sGAG (large photomicrographs) and collagen type II (smaller inset photomicrographs), as visualized in histological sections via staining with Alcian blue dye and immunohistochemistry with a collagen type II monoclonal antibody, respectively. B: H&E staining demonstrates the effects of vimentin knockdown on pellet cellular organization and morphology. Scale bars: 250 μm for (A) (all photomicrographs) and (B) (large photomicrographs); 10 μm for (B) (smaller inset photomicrographs).

of MPCs transfected with negative control or vimentin siRNA were maintained under the chondrogenic conditions (No Growth Factor, +GDF5, +TGF-β3) and analyzed at culture days 2 and 5. Under all conditions, vimentin knockdown depressed levels of PKA phosphorylation at both culture days relative to negative controls (Fig. 6A). In contrast, phosphorylation levels of ERK1/2, MEK1/2, p38, JNK, ERK5, Smad2, and Smad1/5/8 were unaffected by vimentin knockdown (data not shown). Total levels of Smad4, the common partner Smad, were also unaltered by vimentin depletion (data not shown).

PKA C-α KNOCKDOWN DEPRESSES CHONDROGENESIS IN MPC PELLET CULTURES

We employed siRNA-mediated knockdown of PKA C-α mRNA and protein (see Fig. 6B,C) in order to elucidate the role(s) of PKA pathway signaling in MPC chondrogenesis. Real-time PCR analysis revealed that, following 5 days of TGF-β3 treatment, PKA C-α knockdown decreased *AGC*, *COL2A1*, *SOX9*, *L-SOX5*, and *SOX6* mRNA transcript levels relative to negative controls (Fig. 6B). In accordance with the mRNA transcript data, levels of total and p-SOX9 protein (Fig. 6C), as well as levels of Alcian blue-stainable sGAG and collagen type II protein in the cartilage ECM (Fig. 6D), were diminished by PKA C-α knockdown. Interestingly, depleted PKA C-α levels also caused a significant reduction in the amount of vimentin mRNA (Fig. 6B) and protein (Fig. 6C) present in MPC pellets following 5 days of TGF-β3-induced chondrogenesis.

VIMENTIN OVEREXPRESSION ENHANCES ADULT HUMAN MPC CHONDROGENESIS

To examine the effect(s) of vimentin overexpression on adult progenitor cell chondrogenesis, MPCs were transfected with an expression plasmid encoding full-length human vimentin prior to chondrogenic induction via pelleting. Control pellets were composed of MPCs transfected with the corresponding empty vector. Effects of the transfected plasmids on chondrogenic differentiation were assayed by measuring the activity of a co-transfected collagen type II promoter-luciferase reporter gene. Following 2 days of incubation under the three chondrogenic conditions (No Growth Factor, +GDF5, +TGF-β3), pellets comprised of MPCs transfected with the vimentin expression plasmid were characterized by levels of collagen II promoter-luciferase reporter activity that were ~2.6-, ~1.9-, and ~1.8-times those of control pellets, respectively (all $P < 0.001$, $n = 9$; data not shown).

DISCUSSION

The functional role of the cytoskeleton in chondrogenic differentiation has long been a topic of interest in mesenchymal developmental biology. Published studies have typically employed cultures of undifferentiated mesenchymal progenitor cells derived from chick or mouse limb buds to recapitulate embryonic cartilage formation in vitro. Using these cell culture model systems, actin [Woods et al., 2007] and tubulin [Woods et al., 2005] have been shown to play vital

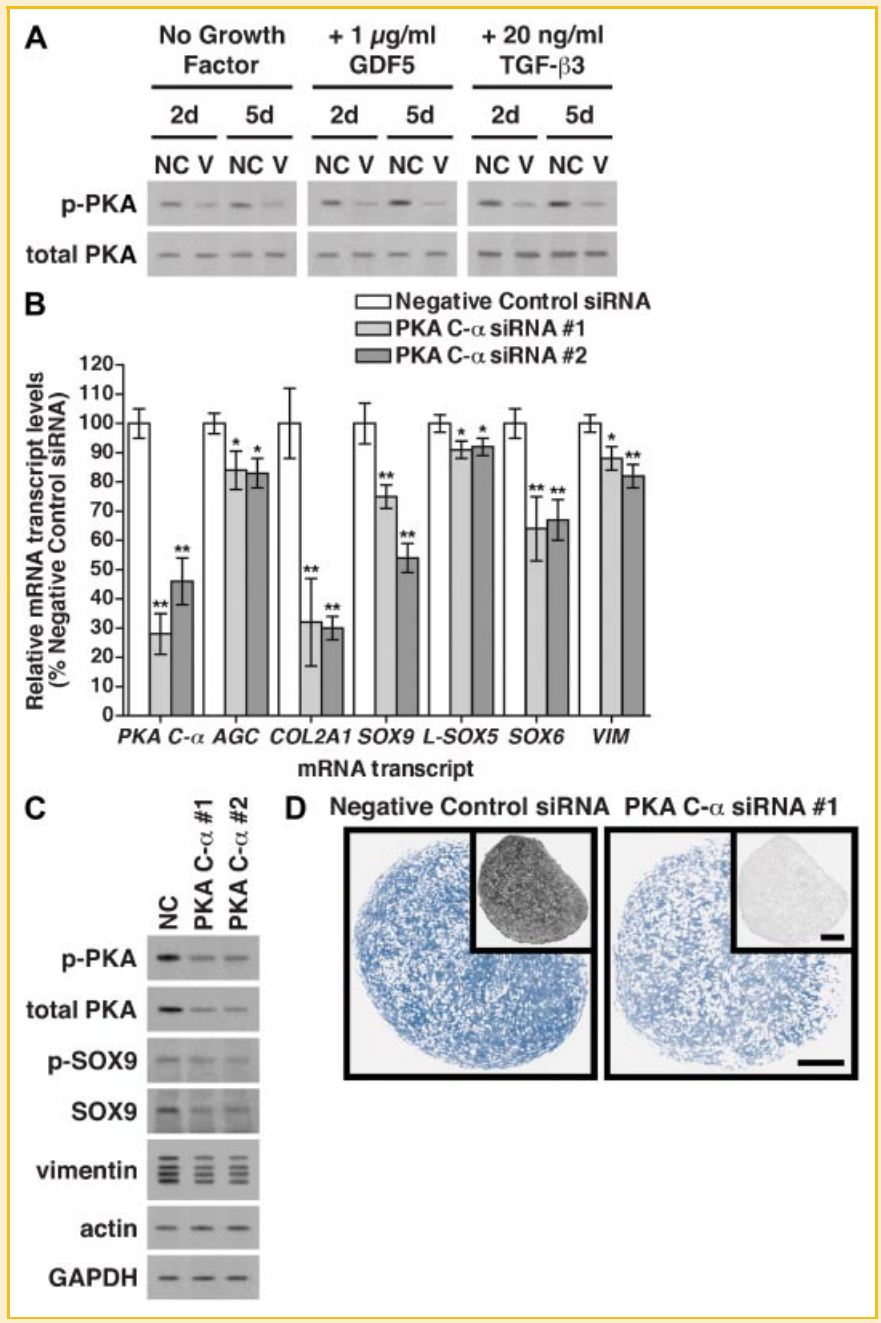

Fig. 6. A: Vimentin knockdown (V) decreases PKA phosphorylation relative to negative control conditions (NC) in MPC pellets undergoing chondrogenesis, as determined by Western blotting. Transfection of MPCs with two different siRNA sequences targeting the *PKA C-α* transcript depresses expression of cartilage-characteristic mRNAs, as determined by real-time PCR (B), and protein, as determined by Western blotting (C) and immunohistochemistry with a collagen type II antibody (D; smaller inset photomicrographs). PKA C-α knockdown also reduces MPC pellet culture accumulation of Alcian blue-stainable sGAG (D; large photomicrographs). Graphed values are the mean ± SD of measurements from three pellet sets (one set of replicate pellets comprised of MPCs from a single donor × three donors). * and ** indicate values different from controls at $P < 0.05$ and $P < 0.01$, respectively. Scale bars: 250 μm.

roles in the regulation of cartilage-specific gene expression during developmental chondrogenesis. On the other hand, vimentin does not seem to regulate the chondrogenic differentiation mechanism that occurs during normal development of the vertebrate embryo [Colucci-Guyon et al., 1994]. Vimentin is required, however, for both embryonic and adult wound healing to proceed normally [Eckes et al., 2000]. This, along with the recent finding that an intact vimentin network is necessary for adult chondrocyte homeostasis [Blain et al., 2006], stirred us to formulate the hypothesis that vimentin may function to regulate the chondrogenic differentiation program of adult progenitor cells, such as that which is initiated during the endochondral ossification phase of bone fracture repair.

We employed high-density pellet cultures of adult human bone marrow stroma-derived MPCs, a cell type that is likely to contribute

to reparative chondrogenesis in vivo [reviewed in Dimitriou et al., 2005], as our model of adult progenitor cell chondrogenic differentiation. As we desired to study the regulatory role(s) of vimentin in the early stages of adult chondrogenesis, we developed a short-term, temporally defined model of growth factor-enhanced MPC chondrogenic differentiation. Following a 5-day incubation period in the absence of growth factor or in the presence of GDF5 or TGF-β3, adult human MPCs maintained in pellet culture expressed multiple markers of chondrogenic differentiation and acquired morphological characteristics indicative of the chondrocytic phenotype. Specifically, MPCs maintained as pellets expressed a variety of cartilage-specific marker genes, including transcripts for the chondro-regulatory SOX transcription factors, as well as mRNAs for the ECM constituents aggrecan and collagen type II. In addition, following 5 days of culture as pellets, MPCs acquired the spherical shape typical of chondrocytes and generated a robust cartilage ECM, highlighted by the presence of sGAG and collagen type II protein. Importantly, pellets maintained in the presence of growth factor consistently underwent chondrogenesis to a greater extent than those lacking growth factor treatment. And, at the concentrations we employed, TGF-β3 was always a more potent inducer of MPC chondrogenesis than GDF5.

Within this experimental model, we found that levels of the ''vimentin protein cluster'' paralleled the progression of MPC chondrogenesis, a result indicative of a positive regulatory role for the intermediate filament in this differentiation process. Successful siRNA-mediated knockdown of vimentin mRNA and protein subsequently confirmed this positive chondro-regulatory role. Under all chondrogenic conditions employed, vimentin knockdown significantly depressed accumulation of mRNAs for the cartilage-specific marker genes *AGC*, *COL2A1*, *L-SOX5*, *SOX6*, and *SOX9*, in addition to reducing levels of collagen type II, SOX5, SOX6, and SOX9 protein along with Alcian blue-stainable sGAG. Finally, in support of these findings, we discovered that transfection of adult human MPCs with a vimentin expression plasmid significantly enhanced activity of a co-transfected collagen type II promoter-luciferase reporter gene.

Our results are the first to implicate vimentin as a regulator of mesenchymal chondrogenesis. However, a role for this intermediate filament in the regulation of chondrocyte ECM production has previously been reported. Blain et al. [2006] showed that acrylamide-induced collapse of the vimentin network in bovine articular chondrocytes diminishes total collagen type II synthesis, in addition to depressing accumulation of both *agc* and *col2a1* transcripts. Similarly, we have found that a 6-h acrylamide treatment, delivered at the beginning of a 5-day MPC chondrogenic time course, reduces accumulation of cartilage ECM, as indicated by decreased levels of 1,9-dimethylmethylene blue (DMMB)-stainable sGAG (Chen, Bobick, Tuan, unpublished work). Importantly, the results of Blain et al. [2006] and those reported here strongly suggest that a properly assembled vimentin cytoskeleton is required for normal cartilage-specific gene expression in both chondrocytes and adult MPCs undergoing chondrogenesis. Consistent with this supposition, osteoarthritic chondrocytes have been shown to express reduced levels of vimentin [Capin-Gutierrez et al., 2004] and display a disorganized vimentin architecture [Fioravanti et al., 2003].

A plethora of intracellular signaling pathways have been implicated in the regulation of cartilage formation in embryonic chondro-progenitor cells, chondrogenic cell lines, and adult MSCs, including the Smad1/5/8 [Hatakeyama et al., 2003] and 2/3 [Ito et al., 2002] pathways, as well as the PKA [Lee and Chuong, 1997], ERK1/2, p38, and JNK cascades [reviewed in Bobick and Kulyk, 2008]. We have shown here that depleted cytoplasmic vimentin in MPCs maintained in pellet culture does not affect the activation levels of transcription factors and protein kinases belonging to the Smad1/5/8, Smad2/3, ERK1/2, p38, JNK, or ERK5 signaling pathways. However, vimentin knockdown did drastically reduce PKA phosphorylation levels. Moreover, successful siRNA-mediated knockdown of PKA C-α mRNA and protein mimicked the inhibitory effects of vimentin depletion on MPC chondrogenesis. In accordance with our findings, PKA has been shown previously to function as a positive regulator of cartilage formation. In micromass cultures of embryonic chick limb bud chondro-progenitor mesenchyme, Lee and Chuong [1997] demonstrated that the chondro-stimulatory effects of cyclic adenosine 3′,5′-monophosphate (cAMP) and BMP2 can be suppressed by the PKA inhibitor H8. Moreover, the catalytic subunit of PKA is able to interact with and phosphorylate Sox9. Phosphorylation of Sox9 by PKA enhances both Sox9 binding to a *col2a1* enhancer element and Sox9 transcriptional activity [Huang et al., 2000].

In this study, we have both demonstrated a novel role for vimentin in the regulation of adult human MPC chondrogenesis and provided a possible protein kinase-mediated signaling mechanism by which alterations in vimentin protein levels affect expression of cartilage-specific genes. It has been postulated that MPCs are the chondro-progenitor cell type participating in adult cartilage regenerative activities, such as the cartilage formation phase of bone fracture healing and the reparative chondrogenesis that occurs in osteoarthritic articular cartilage lesions. Our in vitro findings suggest that vimentin may have in vivo functional importance regulating the chondrogenic differentiation of adult mesenchymal chondro-progenitor cells. However, the extent of vimentin's involvement in these in vivo adult regenerative processes remains to be explored.

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