Human Circulating Fibrocytes Have The Capacity To Differentiate Osteoblasts And Chondrocytes

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Abstract

Fibrocytes are bone marrow-derived cells. Fibrocytes can differentiate into adipocyte- and myofibroblast-like cells. Since fibrocytes can behave like mesenchymal progenitor cells, we hypothesized that fibrocytes have the potential to differentiate into other mesenchymal lineage cells, such as osteoblasts and chondrocytes. In this study, we found that fibrocytes differentiated into osteoblast-like cells when cultured in osteogenic media in a manner similar to osteoblast precursor cells. Under these conditions, fibrocytes and osteoblast precursor cells displayed increased calcium deposition, and increased expression of specific osteogenic genes. In addition, dephosphorylation of cAMP-responsive element binding protein was associated with the increased ratio of Receptor activator of the NF-κB Ligand/osteoprotegerin gene expression and enhanced gene expression of osterix in these cells under these conditions. Both events are important in promoting osteogenesis. In contrast, fibrocytes and mesenchymal stem cells cultured in chondrogenic media in the presence of transforming growth factor-β3 were found to differentiate to chondrocyte-like cells. Fibrocytes and mesenchymal stem cells under these conditions were found to express increased levels of aggrecan and type II collagen genes. Transcription factor genes associated with chondrogenesis were also found to be induced in fibrocytes and mesenchymal stem cells under these conditions. In contrast, β–catenin protein and the core binding factor alpha1 subunit protein transcription factor were decreased in expression under these conditions. These data indicate that human fibrocytes have the capability to differentiate into osteoblast- and chondrocyte-like cells. These findings suggest that such cells could be used in cell-based tissue-regenerative therapy.

Keywords

fibrocytes; osteoblasts; chondrocytes; progenitor cells; stem cells

1. Introduction

Mesenchymal stem cells (MSCs) are emerging as attractive candidates for therapeutic purposes, including gene therapy and tissue regeneration. MSCs are primarily derived from bone marrow, although stem cells can reside in other adult tissues, such as adipose tissue (Picinich et al., 2007). MSCs exhibit characteristics of self-renewal, which have been demonstrated in vitro by serial propagation of these cells (Colter et al., 2000). MSCs

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differentiate into multiple cell types of mesenchymal lineage including adipocytes, myocytes, osteoblasts, and chondrocytes (Bruder et al., 1997; Dennis et al., 1999; Ferrari et al., 1998; Galmiche et al., 1993; Prockop, 1997; Yoo et al., 1998). In addition, bone marrow-derived cells have recently been shown to differentiate into non-mesenchymal lineages such as hepatic, renal, cardiac, and neural cells (Alhadlaq and Mao, 2004; Marion and Mao, 2006). MSCs have been identified in an increasing number of species including humans (Alhadlaq and Mao, 2004). Osteoblasts and chondrocytes, which are derived from a common mesenchymal precursor cell, are involved in bone formation and in mediating the articular cartilage formation, respectively (Zou et al., 2006). Disorders in articular cartilage affect many people; and are one of the leading causes of invalidity and decreased quality of life in adults (Magne et al., 2005).

Fibrocytes are a distinct population of bone marrow-derived fibroblast-like cells found in peripheral blood (Bellini and Mattoli, 2007; Keeley et al., 2009a; Hong et al., 2005, 2007; Mehrad et al., 2009; Picinich et al., 2007; Strieter et al., 2009a). They express the CD34 cell surface antigen, which is believed to be expressed on hematopoietic stem cells (Gomperts and Strieter, 2007; Keeley et al., 2009a, 2009b; Quan et al., 2004; Strieter et al., 2009b). They also express the common leukocyte antigen CD45 and mesenchymal markers such as collagen I and fibronectin (Andersson-Sjoeland et al., 2008; Gomperts and Strieter, 2007; Keeley et al., 2009a, 2009b; Strieter et al., 2009b). They also express the chemokine receptor CXCR4, and patients with idiopathic pulmonary fibrosis and fibrotic non-specific interstitial pneumonia have recently been found to have increased numbers of circulating fibrocytes (Andersson-Sjoeland et al., 2008; Mehrad et al., 2007), and the elevated presence of these cells in circulation in these patients appears to be associated with worse prognosis (Moeller et al., 2009). Fibrocytes constitutively produce extracellular matrix (ECM) components as well as ECM-modifying enzymes. Human fibrocytes produce large amounts of ECM components by stimulation with pro-fibrotic cytokines and growth factors, and further differentiate into contractile myofibroblast-like cells (Hong et al., 2005; Hong et al., 2007; Mehrad et al., 2009). They can also function as antigen presenting cells, participate in normal or aberrant wound-healing, and activate pathologic fibrosis in response to local inflammation (Chesney et al., 1997; Grab et al., 1999; Phillips et al., 2004).

Our laboratory has discovered that circulating fibrocytes can behave as mesenchymal progenitor cells with the capacity to differentiate into adipocyte- or myofibroblast-like cells (Hong et al., 2005; Hong et al., 2007). Transforming growth factor-β1 (TGF-β1) induces differentiation of fibrocytes to myofibroblasts by activation of Smad2/3 and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathways (Hong et al., 2007). Conversely, peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, troglitazone, promotes differentiation of fibrocytes to adipocytes with evidence of lipid accumulation and induction of adipocyte lipid-binding protein (aP2) (Hong et al., 2007). It has also been shown that circulating fibrocytes can differentiate into myofibroblast-like cells after recruitment into the bronchial tissue of asthmatic patients (Schmidt et al., 2003), in animal models of pulmonary fibrosis, and in patients with pulmonary fibrosis (Andersson-Sjoeland et al., 2008; Mehrad et al., 2009). These findings suggest that circulating fibrocytes have the ability to behave as progenitor cells and raise the notion that circulating fibrocytes can differentiate into other mesenchymal lineage cells, such as osteoblasts and chondrocytes.

To test this hypothesis, we isolated human fibrocytes and used commercially available osteoblast precursor cells (OBPC) as comparator cells. Fibrocytes and OBPC were exposed under the same conditions to osteogenic media supplemented with β-glycerophosphate, dexamethasone, and ascorbate. Both fibrocytes and OBPC that had undergone differentiation to osteoblasts displayed enhanced deposition of calcium. The increased deposition of calcium in these cells was also associated with enhanced expression of a number of osteogenic genes,

which involved the signaling pathway cAMP/cAMP-dependent protein kinase A (PKA) through dephosphorylation of cAMP-responsive element binding (CREB) protein and an increase in the ratio of Receptor activator of the NF-κB Ligand (RANKL)/osteoprotegerin (OPG) gene expression. In contrast, fibrocytes and commercially available human MSC (e.g., used as a comparator cell) exposed to chondrogenic media in the presence of TGF-β3 underwent differentiation to chondrocytes. TGF-β3 in chondrogenic media promoted the accumulation of proteoglycans and glycosaminoglycans by staining, and induced the expression of aggrecan and col2A1 genes in both fibrocytes and MSC, compatible with these cells undergoing differentiation to chondrocytes. Moreover, fibrocytes and MSC undergoing differentiation to chondrocytes were found to have augmented expression of the chondrogenic transcriptional factor genes, SOX5, SOX6, and SOX9. The expression of these chondrogenic transcriptional genes, especially SOX9, was associated with downregulation of expression of β-catenin and cbfa1 mRNA as early as 3 days post-exposure TGF-β3 in chondrogenic media. Our findings support the notion that human fibrocytes have the capacity to differentiate into osteoblast- and chondrocyte-like cells, compatible with the concept that fibrocytes represent a circulating pool of mesenchymal progenitor cells.

2. Materials and methods

2.1. Isolation, preparation, and purification of human fibrocytes

Fibrocytes were isolated, cultured and harvested from peripheral blood mononuclear cells (PBMCs) as previously described (Hong et al., 2005; Hong et al., 2007; Mehrad et al., 2007; Mehrad et al., 2009). Briefly, PBMCs were isolated from human leukophoresis packs by gradient centrifugation over Ficoll-Plaque (GE Healthcare) and stored at -150°C until used in the experiments. After thawing, PBMCs were cultured on fibronectin-coated flasks for 4 days in control media (Dulbecco's Modified Eagle's Medium with 20% fetal bovine serum, 10 mM HEPES, 1× Pen/Strep, and 4% L-glutamine; Invitrogen). After removal of non-adherent cells, the adherent cells were supplemented with new media and remained in culture for 7-10 days. For further isolation of fibrocytes, the cultured cells were rinsed with Hank's Balanced Salt Solution four times to remove any residual media, and then detached from flasks by incubation with Accutase (Innovative Cell Technologies) for 30 min at 37 °C. The fibrocytes were purified from contaminating monocytes, T cells, and B cells by negative selection using magnetic microbeads for CD14, CD2, and CD19 (Dynabeads, Invitrogen).

2.2. Osteogenic differentiation

Purified fibrocytes were seeded at the concentration of 1×10^5 cells/well of fibronectin-coated 12 well plate (Corning), and treated with osteogenic basal media, which was supplemented with dexamethasone, ascorbate, mesenchymal cell growth supplement (MCGS), L-glutamine, 1× Pen/Strep, and β-glycerophosphate (Lonza catalog number PT-3002; defined as "osteogenic media"). Dexamethasone is a glucocorticoid steroid capable of stimulating osteogenic differentiation of MSCs at its higher concentration (Marion and Mao, 2006). In addition to its stimulatory effects on cell proliferation, ascorbate further facilitates osteogenic differentiation, including collagen biosynthesis. β–glycerophosphate is critical to stimulate calcified matrix formation in combination with the effects of dexamethasone and ascorbate. Media was replaced every 3 days. Fibrocytes treated with control instead of osteogenic media were used for negative control comparison. OBPC, at 80-90% confluent (Lonza, Walkersville, MD, USA), were harvested, seeded, and treated with osteogenic media in the same manner as fibrocytes, and served the purpose as a positive comparator cell for osteogenesis. To exclude the potential of non-fibrocytes (e.g., CD45 negative circulating progenitor cells) contributing to fibrocyte osteogenic differentiation, in separate experiments isolated fibrocytes were further enriched to only a CD45-positive population using CD45 magnetic microbeads.

2.3. Chondrogenic differentiation

Purified fibrocytes were seeded at the concentration of 5×10^4 cells/tube in 15 ml sterile polypropylene tubes, followed by centrifugation at ~300 ×g for 10 min to form micromass pellets using a modification as previously described for human MSC differentiation to chondrocytes (Mackay et al., 1998). Supernatant was carefully removed in order not to disrupt the fibrocyte micromass pellet, followed by the addition of chondrogenic differentiation (i.e., basal media supplemented with dexamethasone, ascorbate, L-glutamine, Pen/Strep, sodium pyruvate, proline, and ITS-supplement (Lonza catalog number PT-3003; defined as "chondrogenic media"). For further differentiation, chondrogenic media was supplemented with 10 ng/ml of TGF-β3 (PeproTech) as previously described (Mackay et al., 1998). Chondrogenic media with and without TGF-β3 was changed every 3 days. Human MSC, at 80-90% confluent (Lonza, Walkersville, MD, USA), were harvested, seeded, and treated with chondrogenic media with or without TGF-β3 in the same manner as fibrocytes, and served the purpose as a positive comparator cell for chondrogenesis. To exclude the potential of non-fibrocytes (e.g., CD45 negative circulating progenitor cells) contributing to fibrocyte chondrogenic differentiation, in separate experiments isolated fibrocytes were further enriched to only a CD45-positive population using CD45 magnetic microbeads.

2.4. Von Kossa staining

Von Kossa staining was used to detect calcium in fibrocytes and OBPC undergoing differentiation to osteoblasts, and was performed as previously described (Bonewald et al., 2003). Briefly, fibrocytes and OBPC that had undergone differentiation to osteoblasts, as compared to appropriate control cells, were washed twice with phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde for 15 min at room temperature. After rinsing with distilled water, the cells were incubated with 1% silver nitrate solution, and placed under ultraviolet light for 20 min. The cells were rinsed in several changes of distilled water, and then incubated with 5% sodium thiosulfate for 5 min to remove unreacted silver. After rinsing in distilled water, the cells were dehydrated through successive treatment of 70%, 95% and 100% alcohol. Pictures of the cells were taken by Axiovert 40CFL microscope (Carl Zeiss, Germany).

2.5. Alizarin red S staining

Alizarin red S staining was used to detect calcium in fibrocytes and OBPC, and was performed as previously described (Hinoi et al., 2006). Briefly, cells were rinsed with PBS twice and fixed in 10%-buffered formalin for 30 min at room temperature. Fixed cells were washed twice with distilled water, and then stained with 2% alizarin Red S (Sigma) in water for 10 min. After rinsing in several changes of distilled water and air-drying, pictures of stained cells were taken by Axiovert 40CFL microscope (Carl Zeiss, Germany).

2.6. Safranin-O staining

Safranin-O staining was used to detect proteoglycans and glycosaminoglycans found in chondrocytes, and was performed as previously described (Rosemberg, 1971). Briefly the fibrocyte and MSC micromass pellets were gently disrupted in 1 ml of chondrogenic media by repetitive pipetting up-and-down. The cells were centrifuged onto microscopic slide (Cytospin3, Shandon), washed 2 times with PBS, and then fixed with 4% paraformaldehyde for 15 min at room temperature. After rinsing with distilled water, the cells were incubated with 1% Safranin-O solution (Sigma) for 1 h, washed in plenty of distilled water, and then dehydrated through successive treatment of 70%, 95% and 100% ethanol. Pictures of stained cells were taken by Axiovert 40CFL microscope (Carl Zeiss, Germany).

2.7. Alcian blue staining

Alcian blue staining, which stains acid mycosubstances and acetic mucins found in chondrocytes was performed as previously described (Rahman and Tsuchiya, 2001). Briefly, disrupted chondrogenic pellets were deposited into TissueTechs slide (BD Falcon) in the presence of 0.5 ml of chondrogenic media supplemented with 10 ng/ml TGF-β3 followed by incubation for 24 h at 37°C. Cells were gently washed twice with PBS and then fixed with 4% paraformaldehyde for 15 min at room temperature. After rinsing with distilled water cells were incubated with 1% alcian blue solution (Sigma) for 30 min at room temperature. After rinsing in several changes of distilled water and air-drying, pictures of stained cells were taken by Axiovert 40CFL microscope (Carl Zeiss, Germany).

2.8. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared from cells that were either undergoing differentiation or had differentiated to a mesenchymal phenotype using TRIzol (Invitrogen) reagent according to manufacturer's instructions, and then quantified with NanoDrop machine (NanoDrop Technologies). RT-PCR analyses were done with Access RT-PCR kit (Promega) according to manufacturer's instructions, using DNA Engine Thermal Cycler (Bio-Rad). Electrophoresis with 2% agarose gel was used for detection of amplified bands. Nucleotide sequences of RT-PCR primers used are shown in Table 1 (Frank et al., 2002;Yang et al., 2008).

2.9. Western blotting

Total cellular protein was prepared using whole cell extraction buffer [20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP-40, 1× protease inhibitor cocktail (Roche) and 1× phosphatase inhibitor cocktail 2 (Sigma)]. Total protein was quantified with BCA Protein Assay Kit (Pierce). The appropriate amount of protein was diluted with SDS-PAGE sample buffer, boiled at 100°C for 5 min and clarified by centrifugation. Equal amounts of protein (30 μg) were then loaded onto 10% Tris-Glycine gels and electrophoresed at 100 V for 90 min. The gels were transferred to PVDF membrane using the manufacturer's recommended protocol. The membranes are blocked using 5% (w/v) non-fat dried milk in Tris-buffered saline containing 0.001% (v/v) Tween 20. The membranes were incubated with primary antibody overnight using antibodies against the targets listed: osteonectin, aggrecan (R&D Systems), CREB, phosphor-CREB (Cell Signaling Technology), SOX9 (Abcam), or β-tubulin (Novus Biologicals). After removing unbound primary antibody by washing, anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad) was used at 1:4,000 dilution. Final chemiluminescence detection is based on the ECL+ kit (GE Healthcare) per the manufacturer's protocol.

2.10. Immunocytochemistry

Immunostaining experiments were performed using a modification of a previously described method (Hong et al., 2007). Briefly, fibrocytes that had undergone differentiation to osteoblasts were washed twice with PBS. The cells were fixed in 10%-buffered formalin for 30 min at room temperature, and then blocked with 1× PowerBlock (Biogenex) for 1 h. After washing twice with PBS, the anti-human osteonectin antibody (R&D Systems) was incubated with the cells (1:200 dilution, overnight at 4°C). Unbound antibodies were removed by washing with PBS. The cells were then incubated with Alexa Fluor 594 anti-mouse IgG (Invitrogen; 1:2,000) for 1 h at room temperature. After washing with PBS to remove unbound secondary antibodies, the cells were visualized with a Zeiss fluorescence microscope equipped with the Axiovert 40CFL imaging system (Carl Zeiss, Germany).

2.11. Statistical analysis

Comparisons were evaluated by Student's unpaired t-test. Data were considered statistically significant if $p < 0.05$.

3. Results

3.1. Fibrocytes display the ability to differentiate into osteoblasts

For initial studies to determine whether cultured fibrocytes and OBPC could differentiate to osteoblasts, we isolated fibrocytes from PBMCs and exposed the cells to osteogenic media for up to 21 days. For negative control, isolated fibrocytes were treated in parallel with only conventional media. Von Kossa staining showed that at day 7 after initiation of osteogenesis of fibrocytes, the cells revealed detectable calcium deposition (Fig. 1A). At day 14 there was a significant increase of calcium deposition in differentiated fibrocytes as compared to day 7, and the increased staining of calcium was maintained up to 21 days (Fig. 1A). Fibrocytes undergoing differentiation to osteoblasts showed detectable calcium deposition by von Kossa staining after exposure to osteogenic media for 21 days (Fig. 1B). OBPC were used as a positive control for osteogenic differentiation. OBPC, which were seeded 1×10^5 cells/well in 12-well plates and exposed to osteogenic media for 21 days also showed calcium deposition by von Kossa staining (Fig. 1B). In contrast, neither fibrocytes nor OBPC exposed to control media for 21 days showed any calcium deposition (Fig. 1B). The deposition of calcium by fibrocytes undergoing differentiation to osteoblasts in osteogenic media was further evaluated using alizarin red S staining. Both fibrocytes and OBPC that had undergone differentiation to osteoblasts in osteogenic media for 21 days demonstrated positive alizarin red S staining which represented deposition of calcium (Fig. 1C). Neither fibrocytes nor OBPC exposed to control media for 21 days showed any detectable calcium deposition by alizarin red S staining (Fig. 1C). These results suggest that fibrocytes exposed to osteogenic media differentiated into cells that induce calcium deposition similar to OBPC, which is compatible with differentiation to osteoblasts.

3.2. Fibrocytes undergoing osteogenesis express genes for osteogenic markers

In order to confirm whether the fibrocytes exposed to osteogenic media were differentiated into osteoblasts, we next evaluated whether these cells expressed specific osteogenic gene markers during osteogenesis. Osteogenic differentiation of progenitor cells in vitro is characterized by formation of mineralized bone-like nodules containing osteonectin, osteopontin, and osteocalcin protein (Frank et al., 2002). On this basis, osteonectin, osteopontin, and osteocalcin mRNA were chosen as gene markers for evidence of osteogenesis as assessed by RT-PCR. As shown in Fig. 2A, all three osteogenic genes were upregulated in both fibrocytes or OBPC exposed to osteogenic media as early as day 7. Each osteogenic gene marker showed a slightly different expression profile during the time-course of osteogenic differentiation of fibrocytes. Fibrocytes exposed to osteogenic media showed that osteonectin mRNA was highly expressed as early as day 7, with persistence of osteonectin expression at days 14 and 21. In contrast, fibrocytes undergoing osteogenic differentiation showed that osteopontin mRNA expression was minimally expressed by day 7, but was maximal by day 21. Osteocalcin gene expression in fibrocytes undergoing osteogenic differentiation was induced as early as day 7 with persistence of maximal expression at days 14 and 21 similar to osteonectin (Fig. 2A). The kinetics of osteonectin, osteopontin, and osteocalcin gene expression by OBPC were similar to fibrocytes under the same osteogenic culture conditions, with the exception that osteopontin gene expression appeared to peak earlier in OBPC at day 14 (Fig. 2A).

To exclude the hypothesis that non-fibrocytes (e.g., CD45-negative circulating progenitor cells) were contributing to osteogenesis, in separate experiments isolated fibrocytes were

further enriched to only a CD45-positive population using CD45 magnetic microbeads. CD45 positive fibrocytes initially expressed collagen type I, which is a fibrocyte gene marker; however, CD45 positive fibrocytes that were exposed to osteogenic media undergoing differentiation to osteoblasts demonstrated loss of collagen type I and concomitant gain of osteonectin gene expression at day 21 (Supplemental Fig. 1B). These findings support the notion that fibrocytes, and not the potential contamination of non-fibrocytes, are the cells that are undergoing osteogenic differentiation when exposed to osteogenic media.

We next determined whether fibrocytes and OBPC undergoing osteogenic differentiation not only expressed mRNA for the osteogenic genes as above, but also the relevant osteogenic protein. Total cellular protein was processed from both fibrocytes and OBPC undergoing osteogenic differentiation at days 7, 14, and 21 in response to osteogenic media, as compared to cells that were exposed to control media alone. Western blot analysis demonstrated that both fibrocytes and OBPC exposed to osteogenic media displayed markedly elevated levels of osteonectin as early as 7 days (e.g., for fibrocytes) (Fig. 2B). In contrast, both fibrocytes and OBPC exposed to control media failed to express significant levels of osteonectin protein (Fig. 2B). We next assessed whether osteonectin protein was cellularly associated with fibrocytes undergoing osteogenesis using immunofluorescence microscopy. As shown in Fig. 2C, both fibrocytes and OBPC that had been exposed to osteogenic media for 21 days demonstrated positive immunofluorescence for osteonectin protein, as compared to absence of immunofluorescence for osteonectin protein in cells exposed to control media alone. These results indicated that there was increased expression of osteogenic gene markers in the fibrocytes and OBPC undergoing osteogenic differentiation.

3.3. Fibrocytes undergoing osteogenic differentiation demonstrate dephosphorylation of CREB protein, enhanced ratio of RANKL/OPG gene expression, and induction of osterix gene expression

Since it was apparent that fibrocytes similar to OBPC had the ability to undergo osteogenic differentiation, we next assessed whether signaling pathway(s) known to be involved in osteogenic differentiation were found in fibrocytes under these conditions. Recently, it has been shown that the cAMP/PKA signaling pathway is important in regulating bone homeostasis via controlling cyto-differentiation of MSCs and RANKL/OPG gene expression (Yang et al., 2008). Cyclic AMP is an important intracellular second messenger molecule, and its main role is to activate the PKA. We evaluated whether the initiation of osteogenic differentiation of fibrocytes altered the activated (e.g., phosphorylated CREB) cellular expression of CREB protein, which is a key regulator of cAMP signaling pathway in cells. As shown in Fig. 3A, the magnitude of total CREB protein expression did not significantly differ in fibrocytes and OBPC exposed to osteogenic media for 21 days; however, activated CREB (e.g., phosphorylated CREB) was markedly reduced under these same conditions. These findings imply that the cAMP/PKA signaling pathway is involved in the regulation of osteogenesis of fibrocytes and OBPC.

The ratio of RANKL/OPG gene expression was evaluated in the fibrocytes exposed to osteogenic or control media for 21 days (Fig. 3B). Fibrocytes exposed to osteogenic media for 21 days demonstrated increased RANKL gene expression, while OPG gene expression remained at steady levels, as compared to fibrocytes exposed to control media (Fig. 3B). When the ratio of RANKL/OPG was quantified by densitometer, fibrocytes exposed to osteogenic media for 21 days showed a 9-fold increase of RANKL/OPG gene expression, as compared to fibrocytes exposed to control media (Fig. 3B). These data indicated that the cAMP/PKA signaling pathway may contribute to the osteogenesis of cultured fibrocytes through dephosphorylation of CREB and enhanced ratio of RANKL/OPG gene expression. In further support of this notion, fibrocytes exposed to osteogenic or control media were cultured in the

presence or absence of H89 (10 μM), a PKA inhibitor for 21 days (Kato et al., 2006). The presence of H89 in the culture markedly reduced the ratio of RANKL/OPG gene expression in fibrocytes exposed to osteogenic media to a ratio comparable to control media conditions (Supplemental Fig. 2). These results support the importance of the cAMP/PKA signaling pathway during osteogenic differentiation of fibrocytes.

Runx2/core binding factor alpha 1 (Cbfa1) and osterix are osteoblast-specific transcription factors essential for the development of mature osteoblasts, and these genes are thought to activate osteoblast gene markers in vivo to produce a bone-specific matrix (Igarashi et al., 2004). Cbfa1 is necessary for an early step in the osteoblasts/chondrocyte differentiation pathway, whereas osterix (e.g, a zinc finger-containing protein) is required for the differentiation of pre-osteoblasts into fully functioning osteoblasts. Osterix appears to act downstream of Cbfa1 during osteogenic differentiation (Tai et al., 2004). As shown in Fig. 3C, osterix gene expression was markedly elevated in both fibrocytes and OBPC exposed to osteogenic media for 21 days, as compared with cells exposed to control media alone. There was no significant change in expression of Cbfa1 in fibrocytes or OBPC under these conditions (Fig. 3C). These results showed that osterix transcription factor may be related to the osteogenesis of cultured fibrocytes and OBPC downstream of Cbfa1.

3.4. Fibrocytes display the ability to differentiate into chondrocytes

Since we found that fibrocytes could differentiate into osteoblasts, we next determined whether fibrocytes could differentiate into chondrocytes. Previously it has been shown that TGF-β3 in combination with dexamethasone in defined media can induce MSC chondrogenesis when MSC are in micromass pellets (Mackay et al., 1998). We determined that both fibrocytes and human MSC in the presence TGF-β3 required culture in a micromass pellet in order to undergo chondrogenesis, whereas culture of these cells as a monolayer failed to induce chondrogenesis (data not shown). In terms of number of fibrocytes placed into a micromass pellet, we found that 5×10^4 cells/pellet was the lower limit of the number of fibrocytes that could yield successful detection of fibrocytes undergoing chondrogenesis. On this basis, we cultured fibrocytes and human MSC in the form of micromass pellets and in the presence of chondrogenic media in the presence or absence of TGF-β3 in a kinetic manner for 28 days. Fibrocytes and MSC exposed to chondrogenic media alone without TGF-β3 were used as negative controls. At specific time points, fibrocytes and MSC were isolated and subjected to alcian blue staining, which stains acid mycosubstances and acetic mucins in chondrocytes. As shown in Fig. 4A, fibrocytes that had been exposed to chondrogenic media in the presence of TGF-β3 stained positive for alcian blue as early as 9 days, with an increase in alcian blue staining out to 28 days, as compared to the absence of staining of cells exposed to control media. Human MSC cultured under the same conditions demonstrated similar kinetics for alcian blue staining as fibrocytes (data not shown). These findings suggest that TGF-β3 is essential to induce chondrogenesis of fibrocytes under these conditions. To further confirm that fibrocytes can undergo differentiation to chondrocytes, we performed staining with safranin-O. Safranin-O staining detects proteoglycans and glycosaminoglycans, which are deposited in the differentiated chondrocytes (Rosenberg, 1971). As shown in Fig. 4B, we found that fibrocytes cultured in chondrogenic media in the presence of TGF-β3 for 28 days were positive for safranin-O staining, as compared to fibrocytes cultured in chondrogenic media alone. These findings demonstrate fibrocytes exposed to both chondrogenic media in the presence of TGF-β3 develop a chondrocyte phenotype with increased ability to express proteoglycans and glycosaminoglycans within the cells. In addition fibrocytes undergoing chondrogenesis also showed a more compact morphology as compared to fibrocytes exposed to chondrogenic media alone.

3.5. Fibrocytes undergoing chondrogenesis express specific chondrogenic genes

Since fibrocytes undergoing differentiation to chondrocytes were found to produce more chondrocyte-specific molecules, we next assessed the expression profile of chondrogenic gene markers [e.g., aggrecan and type II collagen (Col2A1) genes (Marion and Mao, 2006)] in fibrocytes and MSC undergoing differentiation to chondrocytes. Fibrocytes and MSC were first cultured in a kinetic manner up to 28 days in the presence of chondrogenic media with or without TGF-β3 and assessed for the expression of aggrecan and Col2A1. As shown in Fig. 5A, fibrocytes were found to express aggrecan and Col2A1 mRNA as early as 9 days, and with maximal expression by 28 days in response to chondrogenic media and TGF-β3. MSC were found to express similar levels of aggrecan and Col2A1 mRNA at 28 days (Fig. 5A). Western blot analysis of total cellular proteins from fibrocytes and MSC under these conditions demonstrated that fibrocytes and MSC exposed to chondrogenic media supplemented with TGF-β3 expressed aggrecan protein, as compared to lack of expression of aggrecan when the cells were cultured in chondrogenic media alone (Fig. 5B). To exclude that CD45-negative progenitor cells were contributing to the above findings, we specifically isolated only CD45 positive fibrocytes and subjected them to similar conditions to induce chondrogenesis. We found that CD45-positive fibrocytes exposed to chondrogenic media in the presence of TGF-β3, as compared to chondrogenic media alone, demonstrated gain in expression of aggrecan and loss of expression of collagen type I gene expression (Supplemental Fig. 1C). These findings suggest that fibrocytes exposed to chondrogenic media in the presence of TGF-β3 undergo chondrogenesis with associated expression of specific chondrogenic genes.

3.6. Chondrogenic transcription factor gene expression are upregulated during fibrocyte differentiation to chondrocytes

SOX, SOX6, and SOX9 are transcription factors that promote the biosynthesis of Col2A1and aggrecan protein during chondrogenesis (Marion and Mao, 2006). Previous studies have supported that SOX9 is activated at an early stage of chondrogenesis during mouse embryonic development (Lefebvre and de Crombrugghe, 1998). SOX5 and SOX6 have a high degree of sequence identity with each other, and are coexpressed with SOX9 during chondrogenic differentiation (Lefebvre et al., 2001). On this basis, we assessed the SOX5, SOX6, and SOX9 gene expression during fibrocyte differentiation to chondrocytes. As shown in Fig. 6A, both fibrocytes and MSC exposed to chondrogenic media in the presence of TGF-β3 for 3 days expressed significant levels of SOX9>SOX6>SOX5 mRNA, as compared to the absence of gene expression in cells exposed to chondrogenic media alone. These findings support the notion that SOX5, SOX6, and SOX9 mRNA expression is initiated during the early stage of fibrocyte and MSC chondrogenesis. To extend these studies, we determined the kinetics of SOX9 gene expression in fibrocytes undergoing chondrocyte differentiation out to 28 days. As shown in Fig. 6B, SOX6 gene expression was markedly upregulated at days 9, 18, and 28 in fibrocytes exposed to chondrogenic media with TGF-β3, as compared to chondrogenic media alone. Similar findings for SOX gene expression were shown from MSC cultured under the same conditions for 28 days (Fig. 6B).

Beta-catenin has been shown to be an important molecular switch that appears to be involved in modulating differentiation of MSC to chondrocytes (Zou et al., 2006). During chondrogenesis low levels of β-catenin have been found to suppress cbfa1 expression and at the same time induce the gene expression of SOX9, which leads to increased chondrogenic gene expression, such as aggrecan and Co12A1 (Zou et al., 2006). To determine whether these mechanisms may be operative in fibrocytes undergoing differentiation to chondrocytes, we assessed β-catenin, Cbfa1, and SOX9 gene expression at day 3. As shown in Fig. 6C, β-catenin and Cbfa1 mRNA was found to be elevated in fibrocytes in the presence of chondrogenic media alone, but was found to be markedly reduced in fibrocytes that were exposed to chondrogenic media and TGF-β3. In conjunction with the reduction in the expression of β-catenin and Cbfa1

mRNA, SOX9 gene expression was found to be induced when fibrocytes were undergoing chondrogenesis (Fig. 6C). These findings suggest that β-catenin-Cbfa1 molecular axis may inversely regulate SOX9 signaling, and may contribute to the initiation of differentiation of circulating fibrocytes into chondrocytes. Future studies will access this link.

4. Discussion

Fibrocytes have previously been shown to differentiate to other mesenchymal lineage cells, such as adipocyte- and myofibroblast-like cells (Hong et al., 2005; Hong et al., 2007). Differentiation of fibrocytes to myofibroblasts is induced by TGF-β1. TGF-β1 induces the activation of Smad2/3 and SAPK/JNK signaling pathways that leads to fibrocyte differentiation to myofibroblast-like cells (Hong et al., 2007). The differentiation of fibrocytes to adipocytes has been found to be induced by PPAR-γ that leads to lipid accumulation and induction of aP2 gene expression (Hong et al., 2007). Troglitazone, a PPAR-γ agonist, was found to inhibit fibrocyte differentiation to myofibroblast-like cells via decreased Smad 2/3 transactivation, which was a result of disruption of the SAPK/JNK signaling pathway (Hong et al., 2007). In contrast, TGF-β1 was found to inhibit PPAR-γ-dependent transactivation and induction of aP2 through SAPK/JNK pathway activation (Hong et al., 2007). These findings suggest that circulating fibrocytes have the ability to behave as a circulating multipotent mesenchymal progenitor cell.

In this study we showed that fibrocytes from human peripheral blood are circulating progenitor cells that possess the ability to differentiate into osteoblast- and chondrocyte-like cells. We showed that fibrocytes exposed to osteogenic media can differentiate into osteoblast-like cells. Differentiation of fibrocytes to osteoblast-like cells was confirmed by histochemical staining of calcium deposition by Alizarin Red S and von Kossa staining, and by upregulated gene expression of osteonectin, osteopontin and osteocalcin, which are markers of osteogenesis. In addition, we showed that the cAMP/PKA signaling pathway was involved in regulation of osteogenesis of circulating fibrocytes by finding that dephosphorylation of CREB protein and enhanced ratio of RANKL/OPG gene expression occurred in both fibrocytes and OBPC undergoing osteogenesis. The osteogenic transcription factor gene expression, osterix, was also shown to be upregulated during fibrocyte and OBPC osteogenesis. In contrast, we found that fibrocytes exposed to chondrogenic media in the presence of TGF-β3 resulted in fibrocyte differentiation to chondrocyte-like cells in a similar manner as MSC. The presence of TGF-β3 in chondrogenic media led to enhanced production of proteoglycans and glycosaminoglycans. TGF-β3 also induced upregulation of col2A1 and aggrecan gene expression in fibrocytes undergoing differentiation to chondrocyte-like cells. These findings are compatible with the development of a chondrocyte-like cell.

Previous studies have found that TGF-β3 can induce the expression of chondrocyte specific genes in MSC, and that these cells can form cartilaginous matrix in vitro in a number of species (Hao et al., 2008; Moioli and Mao, 2006; Park et al., 2008; Mrugala et al., 2008; Jin et al., 2006). The differentiation of both fibrocytes and MSC were found to be dependent on culture as micromass pellets in the presence of TGF-β3. These findings are compatible with previous studies that determined that MSC cultured in monolayers in the presence of TGF-β3 expressed undetectable or very low levels of chondrocyte specific genes, as compared to these cells expressing high levels of these genes when cultured as micromass pellets with TGF-β3 (Wang et al., 2005). These findings suggest that a 3-dimensional environment is more conducive for fibrocytes and MSC to undergo differentiation to chondrocyte-like cells in vitro that may simulate their microenvironment in vivo.

The finding of co-expression of SOX5, SOX6, and SOX9 in both fibrocytes and MSC undergoing chondrogenesis that correlated with their ability to differentiate into chondrocyte-

like cells is consistent with previous studies that have determined that these specific transcriptional factors are required for specific chrondrocyte gene expression (Lefebvre and de Crombrugghe, 1998; Lefebvre et al., 2001). The SOX-series gene family consists of transcription factors, which are characterized by a high-mobility-group-box DNA binding domain, whose sequence is at least 50% identical with that of the sex determining factor SRY (Ikeda et al., 2005; Smits et al., 2001). The SOX family is composed of several subgroups based on sequence homologies both inside and outside of HMG-box domain (Ikeda et al., 2005). SOX9 is expressed in all chondrocyte progenitor cells undergoing chondrogenesis and in differentiated chondrocytes (Ng et al., 1997). Studies have shown that chondrocyte-specific promoters for col2a1, col11a2, and CDRAP genes contain SOX9 binding sites (Ikeda et al., 2005).

Our findings that fibrocytes have the capacity to undergo differentiation to osteoblast- and chondrocyte-like cells raises the possibility that isolated fibrocytes could be used for regenerative therapy related to bone or articular cartilage repair. Previous studies have demonstrated that MSC can induce bone formation in a transgenic mouse model of Osteogenesis Imperfecta (OI) (Picinich et al., 2007). Infusion of wild-type MSCs into OI mice resulted in a significant increase in collagen and mineral content in their bone (Pereira et al., 1998). Based on findings of this preclinical study, a clinical trial was performed to test the efficacy of allogeneic bone marrow transplantation for the treatment of children with severe OI (Horwitz et al., 2001). Patients that received this intervention demonstrated improvement in growth, bone structure and function (Horwitz et al., 2001). However, the concern from this study was that they used allogeneic bone marrow transplantation, and not treatment with isolated allogeneic MSC that may have had less immunological impact on this patient population. Thus, one could speculate that isolated fibrocytes could also serve as a source of alternative progenitor cells for regeneration of bone in this type of patient.

The repair of articular cartilage remains an unresolved and challenging problem (Mierisch et al., 2003). Limited potential exists for the repair of critical-sized defects in mature articular cartilage, related to the relative avascular nature of the cartilage and absence of local stem cells (Mierisch et al., 2003). Partial-thickness defects show only minimal healing response (Mierisch et al., 2003). Both partial- and full-thickness defects of articular cartilage in the knee may progress to osteoarthritis (Brittberg et al., 1994). Studies have had only limited success when they have transplanted autologous fully differentiated chondrocytes into the knee to repair articular defects (Brittberg et al., 1994; Horas et al., 2003). However, a recent preclinical study has demonstrated in full-thickness articular defects, treatment with MSC can induce reconstitution of the articular cartilage surface (Mierisch et al., 2003). Based on our experimental results in vitro, fibrocytes could be considered as another attractive candidate for a source of autologous progenitor cells, which could be enriched in vitro, followed by use as therapy for articular cartilage defects.

In summary, our experimental results support the notion that human circulating fibrocytes can differentiate into osteoblast- or chondrocyte-like cells when exposed to specific defined media and cytokines in a similar manner as OBPC and MSC, respectively. We and others have shown that fibrocytes have the capacity to differentiate into other mesenchymal lineage cells, such as adipocyte- and myofibroblast-like cells (Hong et al., 2005; Hong et al., 2007). Taken together, human circulating fibrocytes have the capability to differentiate into diverse mesenchymal lineage cells. These findings also suggest that human circulating fibrocytes could potentially be used in cell-based tissue-regenerative therapy. This could open new possibilities for the novel repair of bone and articular cartilage damage. Circulating fibrocytes could be isolated from the patient's own blood, processed for differentiation into osteoblasts or chondrocytes, followed by transplantation into the damaged tissue. This notion can be assessed with future

studies that will directly focus on the role of fibrocytes in regenerative therapy in pre-clinical models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

|	
aP2	adipocyte lipid-binding protein
Cbfa1	core binding factor alpha1 subunit protein
CREB	cAMP-responsive element binding
ECM	extracellular matrix
JNK	c-Jun N-terminal kinase
MCGS	mesenchymal cell growth supplement
MSC	mesenchymal stem cells
OBPC	osteoblast precursor cell
OI	Osteogenesis imperfecta
OPG	osteoprotegerin
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PKA	cAMP-dependent protein kinase A
PPAR-γ	peroxisome proliferator-activated receptor-γ
RANKL	Receptor activator of the NF-κB Ligand
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAPK	stress-activated protein kinase
SOX	SRY (sex determining region Y)–box
TGF	transforming growth factor
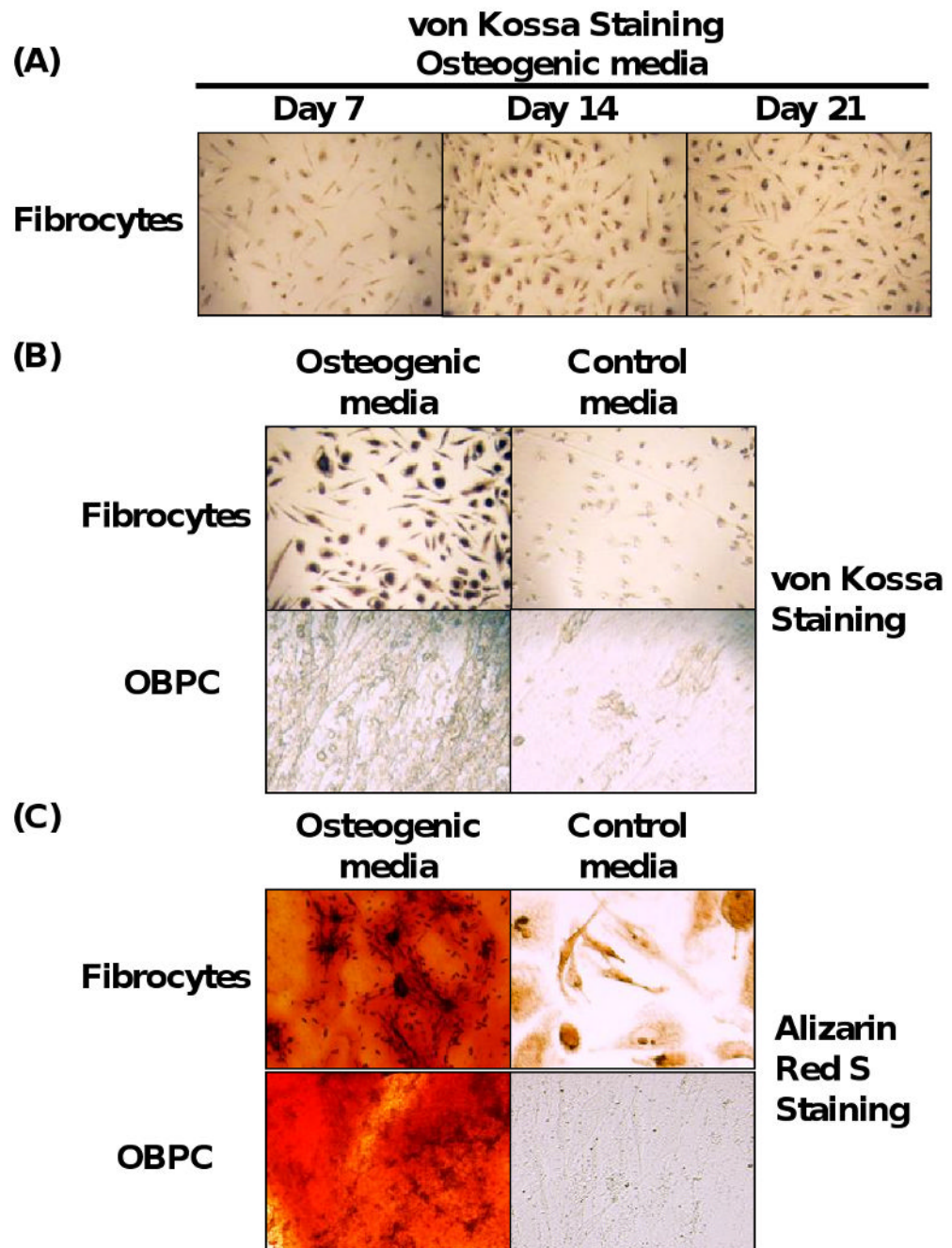
Fig. 1.
Fibrocytes display the ability to differentiate into osteoblasts. **(A)** Time course of calcium deposition from fibrocytes undergoing differentiation to osteoblast-like cells in the presence of osteogenic media, as assessed by von Kossa staining. **(B)** von Kossa staining and **(C)**, Alizarin Red S staining of fibrocytes and OBPC exposed to osteogenic media for 21 days. Magnification 400×. Representative picture from at least 5 experiments for each panel is shown.
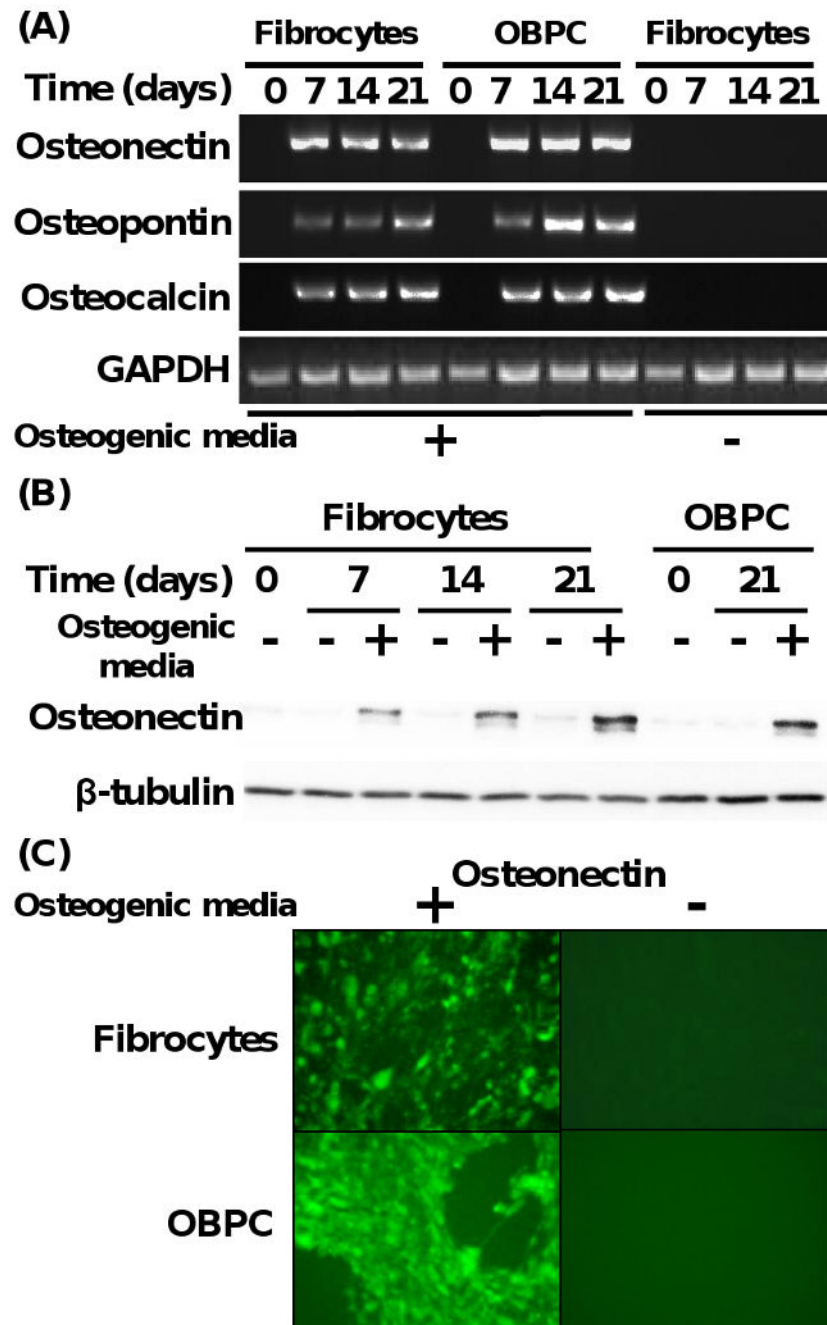
Fig. 2.
Fibrocytes undergoing osteogenesis express genes for osteogenic markers. (**A**) Time course of gene expression of osteonectin, osteopontin, and osteocalcin from fibrocytes and OBPC undergoing differentiation to osteoblast-like cells in the presence or absence of osteogenic media. GAPDH served as a control housekeeping gene. (**B**) Time course of osteonectin protein expression from fibrocytes and OBPC exposed to the presence or absence of osteogenic media. β-tubulin was used as a control for loading equivalent amounts of protein per well. Results are representative of 3 experiments. (**C**) Immunofluorescent image of osteonectin protein from fibrocytes and OBPC exposed to the presence or absence of osteogenic media. Magnification 400×. Results are representative of 2 experiments.

(A)

(B)

(C)
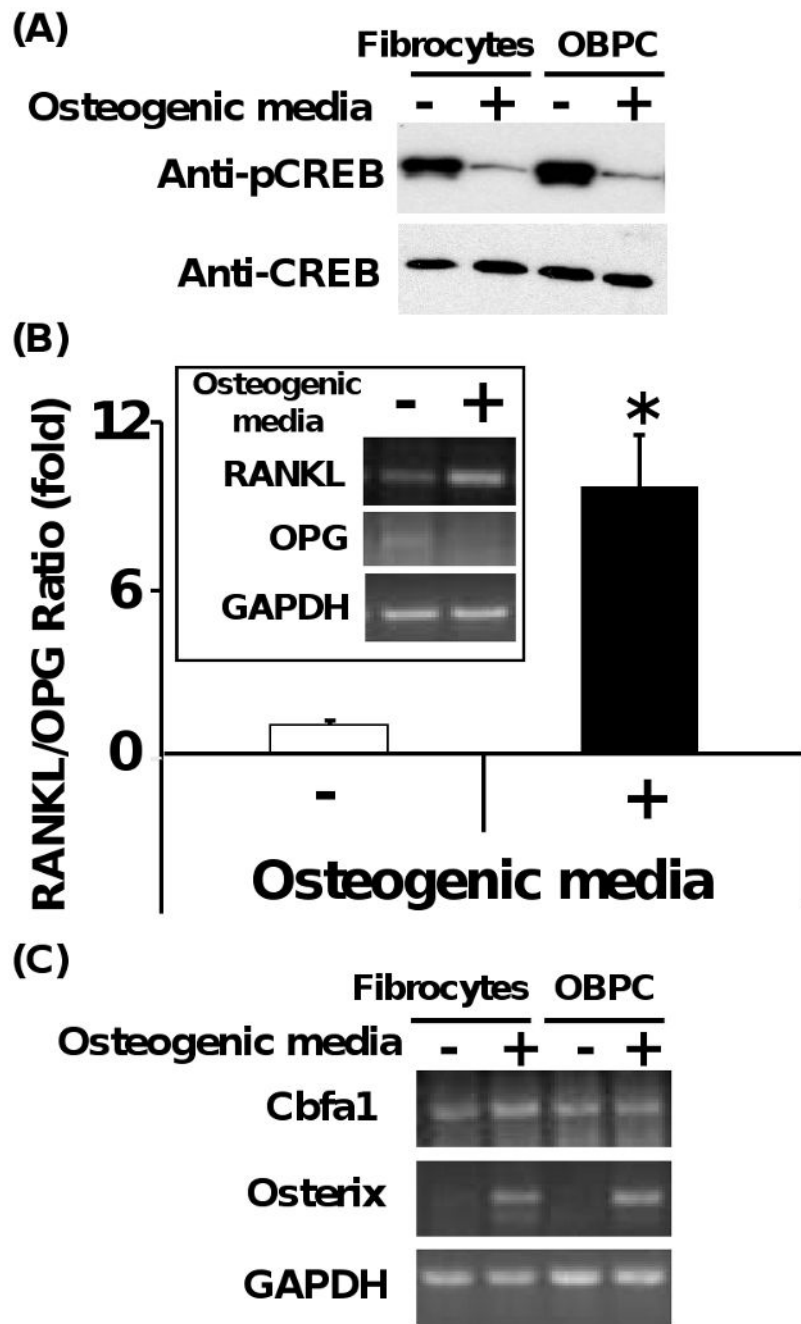
Fig. 3.
Fibrocytes undergoing osteogenic differentiation demonstrate dephosphorylation of CREB protein, enhanced ratio of RANKL/OPG gene expression, and induction of osterix gene expression. **(A)** Dephosphorylation of CREB is seen in fibrocytes and OBPC undergoing differentiation to osteoblast-like cell in the presence of osteogenic media for 21 days. **(B)** Ratio of RANKL/OPG gene expression from fibrocytes exposed to the presence or absence of osteogenic media for 21 days. Intensity of the bands from RT-PCR with the corresponding genes was quantified by densitometer (Quantity-1 software, Bio-Rad). Representative image from 3 different experiments of gel electrophoresis is shown. N=6 for each data for quantification of band intensity using densitometer software (*p<0.01). **(C)** Osterix and Cbfa1
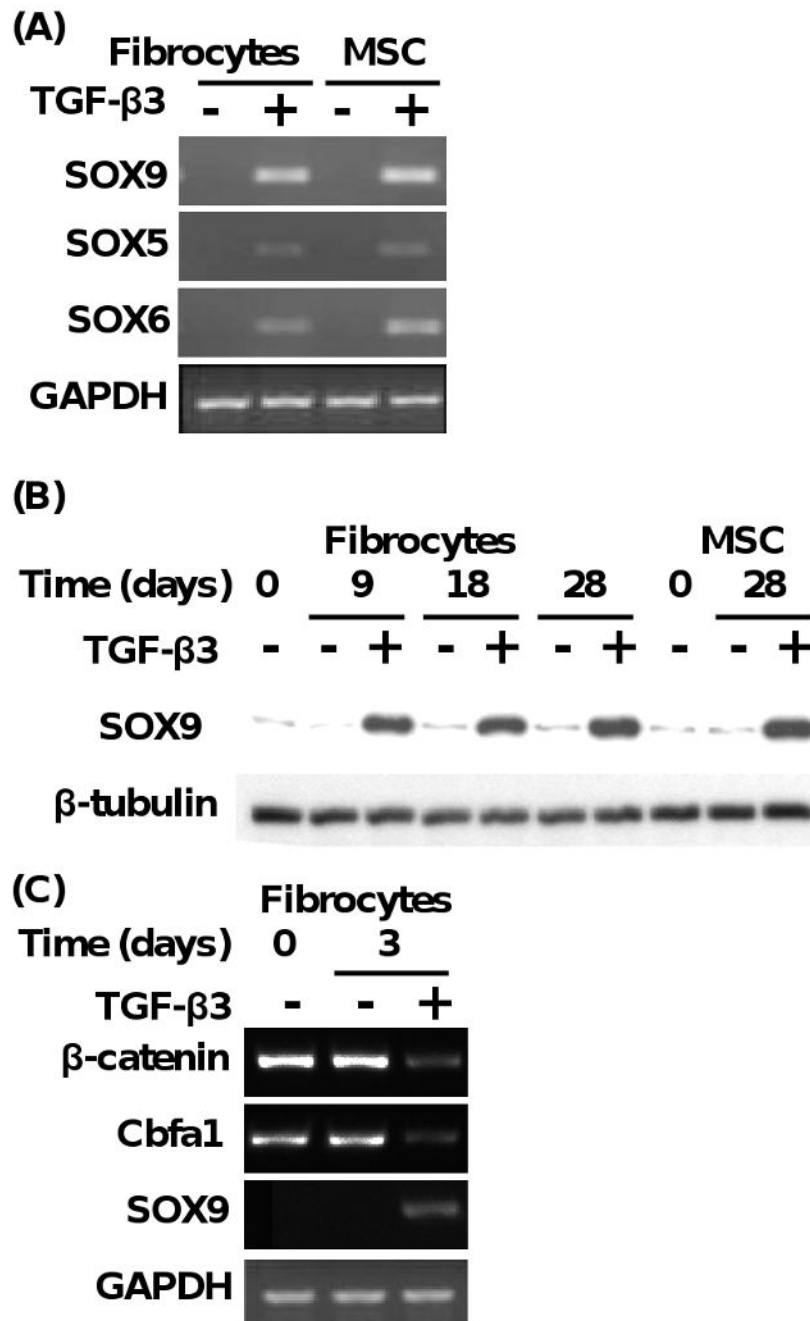
gene expression from fibrocytes and OBPC exposed to the presence or absence of osteogenic media for 21 days. Representative image from 3 different experiments of gel electrophoresis was shown.
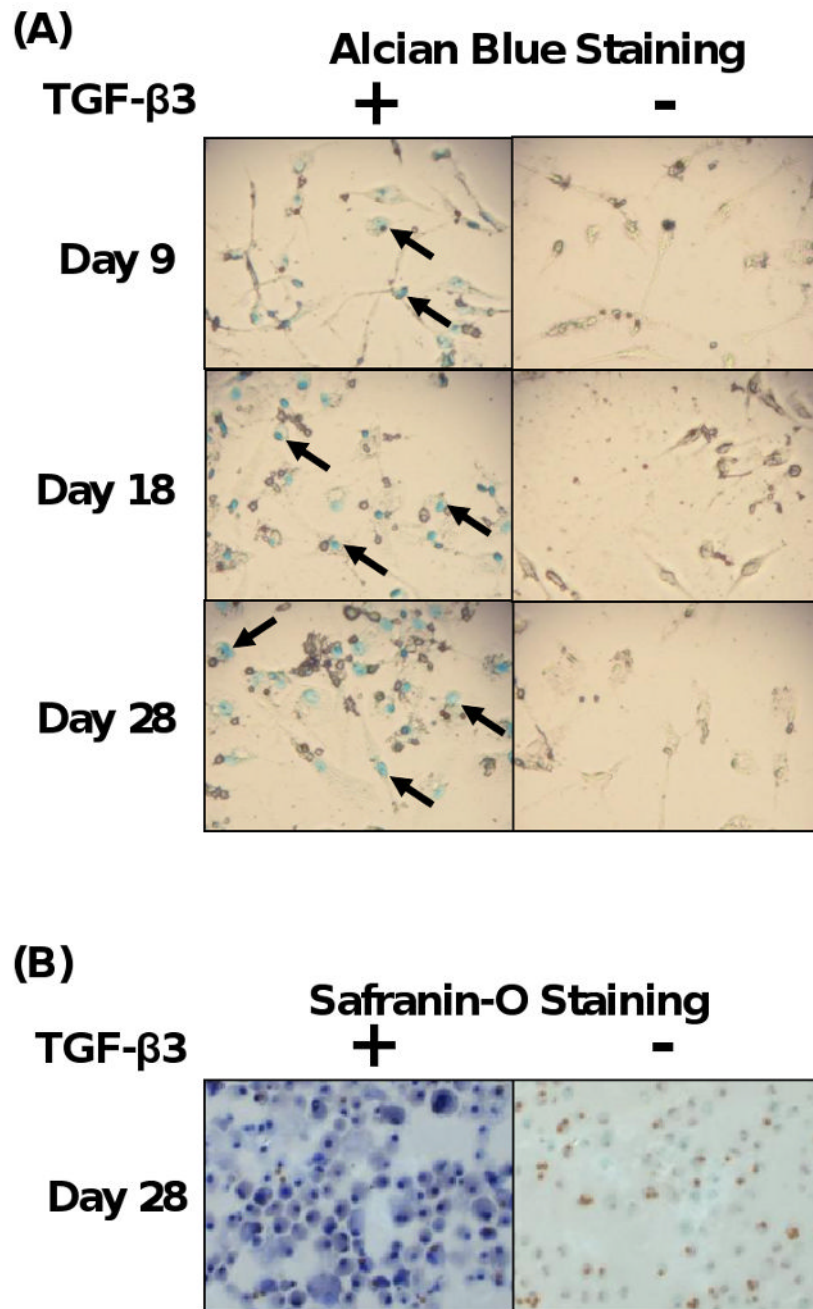
Fig. 4.
Fibrocytes display the ability to differentiate into chondrocytes. **(A)** Time course of alcian blue staining of fibrocytes exposed to chondrogenic media in the presence or absence of TGF-β3 (10 ng/ml). **(B)** Safranin-O staining of fibrocytes exposed to chondrogenic media with or without TGF-β3. Magnification 400×. Representative images from at least 3 experiments.

(A)

(B)
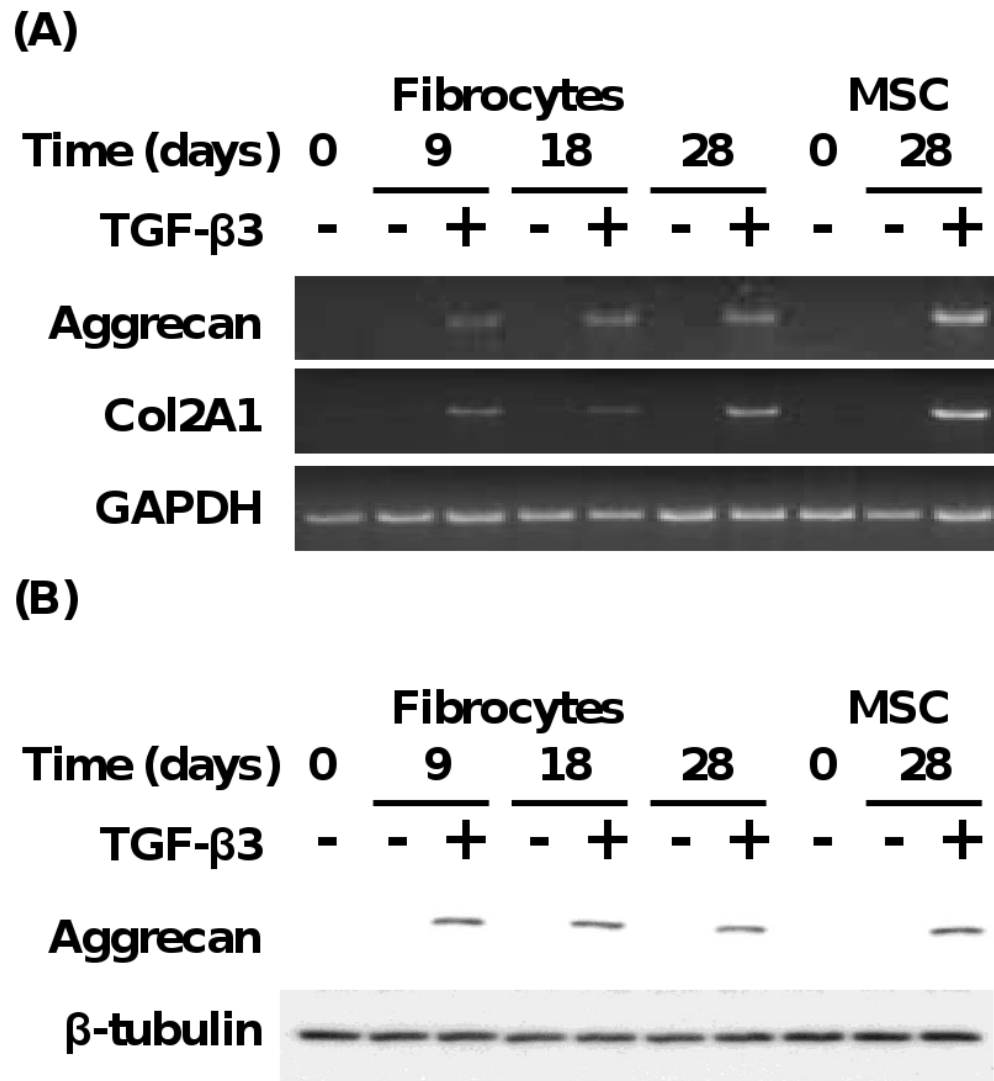
Fig. 5.
Fibrocytes undergoing chondrogenesis express specific chondrogenic genes. **(A)** Time course of gene expression of aggrecan and Col2A1 from fibrocytes and MSC exposed to chondrogenic media with or without TGF-β3 (10 ng/ml). **(B)** Time course of aggrecan protein expression from fibrocytes and MSC exposed to chondrogenic media with or without TGF-β3 (10 ng/ml). Results are representative of 3 experiments.

Fig. 6.
Chondrogenic transcription factor gene expression (i.e., SOX5, SOX6, and SOX9) are upregulated during fibrocyte differentiation to chondrocytes. **(A)** Expression of SOX9, SOX5, and SOX6 transcription factor genes from fibrocytes and MSC exposed to chondrogenic media with or without TGF-β3 (10 ng/ml) for 3 days. **(B)** Time course of the expression of SOX9 protein from fibrocytes and MSC exposed to chondrogenic media in the presence or absence of TGF-β3 (10 ng/ml). Results are representative of 3 experiments. **(C)** Expression of β-catenin, Cbfa1, and SOX9 genes from fibrocytes exposed to chondrogenic media for 3 days in the presence or absence of TGF-β3 (10 ng/ml). Results are representative of 3 experiments.

Table 1

Nucleotide sequences of RT-PCR primers.

Gene	Forward primer	Reverse primer
Osteocalcin	5′-TGA GAG CCC TCA CAC TCC TC-3′	5′-CGC CTG GGT CTC TTC ACT AC-3′
Osteonectin	5′-GAG GGC CTG GAT CTT CTT TC-3′	5′-TCC ACC TGG ACA GGA TTA GC-3′
Osteopontin	5′-ATG ATG GCC GAG GTG ATA GT-3′	5′-GAT GGC CTT GTA TGC ACC AT-3′
RANKL	5′-AAT AGA ATA TCA GAA GAT GGC ACT C-3′	5′-TAA GGA GGG GTT GGA GAC CTC G-3′
OPG	5′-GCT AAC CTC ACC TTC GAG-3′	5′-TGA TTG GAC CTG GTT ACC-3′
COL2A1	5′-TGG TGA ACC TGG TGT CTC TG-3′	5′-GTT CCT GGG AAA CCA CGA G-3′
Aggrecan	5′-CCA GGT GTG TGG GAC TGA A-3′	5′-ACA CTC AGC GAG TTG TCA TGG-3′
SOX5	5′-AGC CAG AGT TAG CAC AAT AGG-3′	5′-CAT GAT TGC CTT GTA TTC-3′
SOX6	5′-ACT GTG GCT GAA GCA CGA GTC-3′	5′-TCC GCC ATC TGT CTT CAT ACC-3′
SOX9	5′-GAA CGC ACA TCA AGA CGG AG-3′	5′-TCT CGT TGA TTT CGC TGC TC-3′
GAPDH	5′-AGC CAC ATC GCT CAG AAC AC-3′	5′-GAG GCA TTG CTG ATG ATC TTG-3′