

# Interleukin-6 Maintains Bone Marrow-Derived Mesenchymal Stem Cell Stemness by an ERK1/2-Dependent Mechanism

Katie L. Pricola, Nastaran Z. Kuhn, Hana Haleem-Smith, Yingjie Song, and Rocky S. Tuan\*

*Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland 20892*

## ABSTRACT

Adult human mesenchymal stem cells (MSCs) hold promise for an increasing list of therapeutic uses due to their ease of isolation, expansion, and multi-lineage differentiation potential. To maximize the clinical potential of MSCs, the underlying mechanisms by which MSC functionality is controlled must be understood. We have taken a deconstructive approach to understand the individual components in vitro, namely the role of candidate “stemness” genes. Our recent microarray gene expression profiling data suggest that interleukin-6 (IL-6) may contribute to the maintenance of MSCs in their undifferentiated state. In this study, we showed that *IL-6* gene expression is significantly higher in undifferentiated MSCs as compared to their chondrogenic, osteogenic, and adipogenic derivatives. Moreover, we found that MSCs secrete copious amounts of IL-6 protein, which decreases dramatically during osteogenic differentiation. We further evaluated the role of IL-6 for maintenance of MSC “stemness,” using a series of functional assays. The data showed that IL-6 is both necessary and sufficient for enhanced MSC proliferation, protects MSCs from apoptosis, inhibits adipogenic and chondrogenic differentiation of MSCs, and increases the rate of in vitro wound healing of MSCs. We further identified ERK1/2 activation as the key pathway through which IL-6 regulates both MSC proliferation and inhibition of differentiation. Taken together, these findings show for the first time that IL-6 maintains the proliferative and undifferentiated state of bone marrow-derived MSCs, an important parameter for the optimization of both in vitro and in vivo manipulation of MSCs. *J. Cell. Biochem.* 108: 577–588, 2009. © 2009 Wiley-Liss, Inc.

**KEY WORDS:** MESENCHYMAL STEM CELLS; DIFFERENTIATION; STEMNESS; INTERLEUKIN-6

Three components are required for successful tissue engineering and regeneration: cells with regenerative potential, a biocompatible scaffold or matrix, and environmental and endogenous influences to drive these cells toward desired functional tissue neogenesis. Adult human mesenchymal stem cells (MSCs) are a promising source of cells that can largely satisfy the first of our three necessary criteria. MSCs are tissue-resident stem cells [Pereira et al., 1995; Prockop, 1997]. They are commonly isolated from the bone marrow but can also be found in the perivascular regions of most tissues, such as adipose, skeletal muscle, and umbilical cord [Zuk et al., 2002; Sarugaser et al., 2005]. MSCs are capable of

differentiation into several cell lineages, including osteoblasts, chondrocytes, adipocytes, and myoblasts [Pittenger et al., 1999; Jiang et al., 2002]. Owing to their ease of isolation, expansion, and multi-lineage differentiation capability, MSCs are a promising cell source for both tissue engineering and in vivo stimulation of other tissue-resident stem cells. In addition, MSCs possess immunosuppressive activities and have been successfully used to treat graft versus host disease [Le Blanc et al., 2004] and as a source for gene therapy (osteogenesis imperfecta) [Le Blanc et al., 2005].

Although there is vast potential for MSCs, both future and realized, current limitations include the small number of cells that

Katie L. Pricola and Nastaran Z. Kuhn contributed equally to this work.

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Katie L. Pricola's present address is Stanford University School of Medicine, Palo Alto, CA.

Rocky S. Tuan's present address is Department of Orthopaedic Surgery, University of Pittsburgh, 3471 Fifth Avenue, Pittsburgh, PA 15213.

\*Correspondence to: Rocky S. Tuan, PhD, Cartilage Biology and Orthopaedics Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Department of Health and Human Services, 50 South Drive, Room 1140, MSC 8022, Bethesda, MD 20892-8022. E-mail: tuanr@mail.nih.gov

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can be harvested from a primary source and the inconsistent proliferation and differentiation capabilities across patients. A better understanding of the intrinsic gene regulation and the external environmental stimuli and cues governing MSC fate decisions will enhance both our understanding of MSC biology and our ability to promote MSC self-renewal and readiness to differentiate. We have recently taken a deconstructive approach into understanding the influence of individual cues in vitro, namely identification of candidate “stemness” genes. Our microarray data [Song et al., 2006] suggest that interleukin-6 (IL-6), a cytokine highly expressed in the bone marrow stroma [Ogasawara et al., 1996] and known for its role in bone homeostasis [Manolagas, 1995], may be responsible for maintenance of MSCs in the undifferentiated state. Specifically, we reported *IL-6* down-regulation in MSCs during differentiation and up-regulation during de-differentiation or the return to a more stem-like state [Song et al., 2006]. The additional role of IL-6 as a secreted signaling factor makes this an interesting gene to study both for its intrinsic and extrinsic effects. In this study, we have assessed the role of IL-6 in maintenance of MSC “stemness” by investigating its effects on proliferation, apoptosis, differentiation, and in vitro wound healing activity in human bone marrow-derived MSCs.

## MATERIALS AND METHODS

### MATERIALS

Cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise.

### ISOLATION AND IN VITRO EXPANSION OF MSCs

MSCs were isolated from femoral heads harvested during elective total hip arthroplasty in consenting patients (N = 8, ages 39–72) (IRB approval—University of Washington). All samples were from individuals with osteoarthritis that do not have rheumatoid arthritis, or other autoimmune or inflammatory diseases, or congenital deformities. For our studies, bone marrow-derived MSCs were expanded in vitro in basal medium (BM), consisting of high glucose Dulbecco’s modified Eagle’s medium, 10% lot-selected (Invitrogen, catalog # 12662029, lot # 1160485) MSC-qualified fetal bovine serum (FBS), and antibiotic/antimycotic. BM was replaced 72 h after initial plating to select for plastic-adherent cells and weekly thereafter. Confluent cultures ( $\sim 1.2 \times 10^4$  cells/cm<sup>2</sup>) were passaged at 1:3; all experiments were performed at passage 4. Previous phenotypical analysis of MSCs at passage 4 has been reported to show that they are acceptable as culture-expanded MSCs with multilineage potential [Song and Tuan, 2004].

### DIFFERENTIATION OF MSCs

MSCs were induced to undergo osteogenic, adipogenic, and chondrogenic differentiation as previously described [Song and Tuan, 2004]. *Osteogenesis*: To stimulate osteogenic differentiation, cells were plated at  $1.5 \times 10^4$  cells/cm<sup>2</sup> and BM was supplemented with 10 nM dexamethasone, 10 mM  $\beta$ -glycerophosphate, 50  $\mu$ g/ml ascorbate phosphate, and 10 nM 1,25-dihydroxyvitamin D<sub>3</sub>. *Adipogenesis*: To induce adipogenesis, cells were plated at

$4.0 \times 10^4$  cells/cm<sup>2</sup> and BM was supplemented with 1  $\mu$ M dexamethasone, 1  $\mu$ g/ml insulin, and 0.5 mM 3-isobutyl-1-methyl-xanthine. *Chondrogenesis*: In order to induce chondrogenesis, cells were cultured as a high-density pellet culture ( $2.5 \times 10^5$  cells/pellet) in serum-free BM supplemented with 0.1  $\mu$ M dexamethasone, 50  $\mu$ g/ml ascorbate phosphate, 40  $\mu$ g/ml L-proline, 100  $\mu$ g/ml sodium pyruvate, 1% ITS-premix, and 10 ng/ml transforming growth factor- $\beta$ 3 (TGF- $\beta$ 3) (R&D Systems, Minneapolis, MN). RNA was harvested at days 3, 7, 14, and 21 for all three differentiated lineages.

### HUMAN IL-6 ELISA ASSAY

IL-6 protein levels in culture supernatants were measured using commercial ELISA kits (eBioscience, San Diego, CA). The ELISA assay was conducted as per the manufacturer’s instructions at room temperature unless indicated otherwise. Briefly, ELISA plates were coated with the capture antibody and incubated overnight at 4°C. Wells were washed extensively and then blocked for 1 h. The wells were incubated for 2 h with human IL-6 standards and supernatants from MSCs either in BM and osteogenic induction medium, which were diluted 1:100 in order to obtain measurements within the standard curve. After extensive wash steps, wells were incubated with detection antibody for 1 h and then washed several more times. Wells were incubated with avidin-HRP for 30 min, washed extensively, incubated with substrate solution for 15 min, followed by the addition of stop solution, and A<sub>450</sub> values were measured using a microplate reader.

### GROWTH AND PROLIFERATION ASSAY

MSCs were plated at  $1.5 \times 10^4$  cells/cm<sup>2</sup> in serum-free BM with or without 10 ng/ml human recombinant IL-6 (R&D Systems), 10  $\mu$ M of the MEK1/2 inhibitor, U0126, solubilized in 0.1% dimethylsulfoxide (DMSO) carrier, both U0126 and IL-6, or DMSO carrier alone. Growth and proliferation were assessed in parallel by hemacytometer counting and by thiazolyl blue tetrazolium bromide (MTT) proliferation assay at 1, 2, and 3 days post-plating. For counting, each condition was plated in triplicate and duplicate counts of trypan blue negative cells per well were averaged (mean  $\pm$  SD). In parallel plates, 1.25 mg/ml per well MTT reagent was added, incubated at 37°C for 2 h, solubilized with DMSO, and A<sub>540</sub> values of the eluant measured using a microplate reader.

### siRNA KNOCKDOWN

MSCs were transfected with 25  $\mu$ g of gene-specific siRNA or negative scramble control according to manufacturer specification using a BTX-ECM 830 Electroporator and siPORT Electroporation buffer (Ambion, Austin, TX). Following transfection, cells were plated for proliferation assays and RNA harvest at the same densities described elsewhere.

### IN SITU CELL DEATH ASSAY

Undifferentiated MSCs were seeded at  $1.28 \times 10^4$  cells/cm<sup>2</sup> and grown for 72 h in serum-free medium with or without IL-6

(10 ng/ml). After 72 h, cells were fixed in 4% buffered paraformaldehyde, permeabilized with 0.1% Triton X-100, incubated with the TUNEL reaction mixture (Roche, Indianapolis, IN) and nuclear-counterstained with DAPI. Apoptosis was evaluated by epifluorescence microscopy (475/35 nm excitation and 530/43 nm emission filters). Relative levels of apoptosis were quantified by calculating the percentage of TUNEL-positive cells at the center of each well. Field images with approximately equivalent numbers of positive cells in both control and sample populations were merged to assess both location and intensity of TUNEL staining as well as the size and appearance of nuclei (Leica DMRB microscope and Hamamatsu C4742-95 digital camera; IPLab software).

### REAL-TIME RT-PCR MICROARRAY ANALYSIS

Undifferentiated MSCs were seeded at  $1.5 \times 10^4$  cells/cm<sup>2</sup> and cultured under serum-free conditions with or without IL-6 (10 ng/ml) for 72 h (n = 3 each). RNA was harvested using TRIzol<sup>®</sup> reagent (Invitrogen) and purified with RNeasy Mini kit (Qiagen, Valencia, CA), and cDNA was synthesized using RT<sup>2</sup> First Strand Kit according to the RT<sup>2</sup> Profiler PCR Array System (SuperArray Bioscience, Frederick, MD). For each sample, cDNA synthesized from 0.5 µg starting RNA and RT<sup>2</sup> SYBR Green master mix were loaded into 96-well pathway-specific polymerase chain reaction (PCR) array plates containing probes for 84 genes in the apoptotic pathways and 12 controls. Plates were run in an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) using the manufacturer's protocol, and results were analyzed using the RT<sup>2</sup> Profiler PCR Array Data Analysis web portal. Using cut-off criteria, a 2.75-fold induction or repression of expression was considered to be significant.

### ANALYSIS OF MSC DIFFERENTIATION

Osteogenesis was detected at day 10 by alkaline phosphatase (ALP) histochemistry and ALP activity assay. Briefly, ALP yellow (pNPP) liquid substrate for ELISA was used, and enzyme activity was normalized to DNA content with Quanti-iT<sup>™</sup> Pico Green dsDNA Assay Kit (Invitrogen). At day 18, calcium deposition/matrix mineralization was assessed by Alizarin Red stain, and staining was quantified spectrophotometrically (A<sub>405</sub>) after solubilization with 5% SDS in 0.5 N HCl. Adipogenesis was detected at day 18 by the presence of Oil Red O stained cytoplasmic lipid droplets and assessed quantitatively by counting stained cells and grading stain intensity. To assess chondrogenic differentiation at day 21, high-density cell pellets were fixed with 4% paraformaldehyde and 8 µm cryosections were stained for sulfated proteoglycan with Alcian Blue (pH 1.0) as described previously [Song and Tuan, 2004]. Sulfated glycosaminoglycans (sGAG) were quantified using the Blyscan assay kit (BioColor, Carrickfergus, UK) after digestion with 300 µg/ml papain. Bound 1, 9-dimethyl-methylene blue was released and quantified spectrophotometrically (A<sub>656</sub>), and total sGAG was calculated from a chondroitin 4-sulfate standard curve. Total sGAG was normalized to DNA content as described above. Real-time reverse transcription-PCR (RT-PCR) was used to analyze expression levels of ALP and osteocalcin (OC) for osteogenesis, lipoprotein lipase (LPL), and fatty acid-binding protein-4 (FABP4) for adipogenesis, and aggrecan (AGC) and collagen type II (COL2A1) for chondrogenesis (see below for details).

### DIFFERENTIATION OF MSCs IN THE PRESENCE OF IL-6

MSCs were differentiated as described above with or without IL-6 (10 ng/ml). Secondly, to assess the effect of IL-6 pre-treatment, undifferentiated MSCs were grown with or without IL-6 (10 ng/ml), U0126 (10 µM) or both for 3 days prior to induction of differentiation. RNA was harvested at days 3, 7, 14, 21 and differentiation was analyzed as described above.

### GENE EXPRESSION ANALYSIS BY REAL-TIME RT-PCR

Total RNA was prepared from monolayer and pellet cultures using TRIzol<sup>®</sup> reagent (Invitrogen) and purified with RNeasy Mini-kit (Qiagen). cDNA was synthesized using SuperScriptIII first-strand synthesis system (Invitrogen). Ten nanograms of cDNA was amplified and detected using iQ-SYBR Green supermix and 10 µM F + R primer mix in an iCycler iQ real-time PCR detection system. For all real-time RT-PCR experiments, gene expression levels were normalized to an internal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control and averaged from triplicate samples. Primers used included: *IL-6*, 5'-cagttgctctccctctggg-3', 5'-tgagtggctgtctgtgtggg-3' [Seck et al., 2002]; *OC*, 5'-gccttgtgtccaagc-3', 5'-ggaccaccacatccatag-3'; *LPL*, 5'-gagatttctctgtatggcacc-3', 5'-ctgcaaatgagacacttctc-3'; *FABP4*, 5'-tgggcccaggaattgacgaagt-3', 5'-tcaacgtccttggcctatgct-3'; *ALP*, 5'-tcagaagctcaaccaacg-3', 5'-gtcaggacctgggcatt-3'; *AGC*, 5'-tgcgggtcaacagctatc-3', 5'-cacgatgccttccaccagac-3'; *COL2A1*, 5'-ggaaacttctgctcccagatg-3', 5'-tcaccaggttaccaggattgc-3'; *GAPDH*, 5'-caaggctgagaacgggaagc-3', 5'-agggggacagatgatgacc-3'. All primer sequences listed after IL-6 were previously published [Nesti et al., 2008].

### IN VITRO WOUND HEALING ASSAY

MSCs were plated at  $2.0 \times 10^4$  cells/cm<sup>2</sup> and grown in BM until near 100% confluence. At the time of initial wounding (defined here as 0 h), scratches were made in the cell monolayer with a 200 µl pipette tip. After wounding, each well was rinsed with low-serum BM (2% FBS). Control wells were replenished with low-serum BM; IL-6 treated wells were replenished with 10 ng/ml IL-6 in low-serum BM. A total of 30 defects (control, N = 12; IL-6, N = 18) in cultures derived from two different patients were followed over the course of 18 h. These time points were specifically chosen to be shorter than the doubling time reported for human bone marrow-derived MSCs [Conget and Minguell, 1999], such that any observed effects are due to cell migration and not cell proliferation. Images were captured using a Zeiss Axio Observer A.1 microscope and Axiovision Rel. 4.6 software, and blinded measurements were made at 0, 6, 12, and 18 h time points using the interactive measurement line tool scaled to the 5 × objective.

### WESTERN BLOT ANALYSIS

MSCs plated at  $1.5 \times 10^4$  cells/cm<sup>2</sup> and grown to 80% confluence in BM were switched to serum-free medium for 12–15 h prior to treatment and protein harvest. After treatment with IL-6 and/or U0126, cells were rinsed with cold PBS and lysed in RIPA buffer, 0.1% SDS containing 1% IGEPAL Ca-630, and a cocktail of protease inhibitors—Complete, EDTA-free protease inhibitor cocktail (Roche) and Halt-Phosphatase Inhibitor cocktail (Pierce, Rockford, IL). Fifteen micrograms of sample protein, nonphosphorylated, and

phosphorylated STAT3 and STAT1 control cell extracts were run on 10% SDS–Tris–HCl Ready Gels (Bio-Rad), transferred onto PVDF membrane (Bio-Rad) and probed for  $\beta$ -actin (Sigma), STAT3, phospho-STAT3, STAT1, phospho-STAT1, ERK1/2, phospho-ERK1/2 (all antibodies from Cell Signaling, Danvers, MA). Expression was detected with the Amersham ECL system (Piscataway, NJ) and SuperSignal (Pierce).

### STATISTICAL ANALYSIS

All data are presented as mean  $\pm$  SD unless otherwise specified. Statistical significance calculated by Student's *t*-test was used for pairwise comparisons and one-way ANOVA was used for multiple comparisons (Software used was SPSS 16.0; specific parameters used were the general linear model, univariate analysis: one-way ANOVA with Tukey post hoc analysis). Statistical significance was analyzed on data from at least three independent experiments on three different patient samples performed in triplicate. *P*-values <0.05 were defined as significant (\**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001).

## RESULTS

### IL-6 EXPRESSION IS DOWN-REGULATED DURING MSC DIFFERENTIATION

Previously, our laboratory reported that MSC expression of *IL-6* decreases during lineage-specific differentiation [Song et al., 2006]. To validate our previous microarray data, we compared the levels of *IL-6* mRNA by real-time RT-PCR in undifferentiated MSCs and their differentiated counterparts—osteoblasts, adipocytes, and chondrocytes (Fig. 1A). *IL-6* message levels were significantly greater in undifferentiated MSCs compared to osteoblasts, adipocytes, and chondrocytes at days 7 and 14 of differentiation. Additionally, *IL-6* protein secretion levels measured by ELISA were greatly elevated in 7-day cultures of MSCs compared to MSCs that had been differentiated into osteoblasts (Fig. 1B), showing that *IL-6* mRNA transcript levels correlate with protein expression levels in MSCs.

### IL-6 INDUCES BIOLOGIC RESPONSES IN MSCs

**IL-6 enhances proliferation of undifferentiated MSCs.** To assess the role of *IL-6* in undifferentiated MSC proliferation, we examined the effect of exogenous *IL-6* on proliferation under serum-free conditions by MTT assay (data not shown) and direct cell counting. By both measures and across three patients, *IL-6* significantly enhanced MSC proliferation after 1, 2, and 3 days in culture compared to untreated serum-free controls (Fig. 2A).

**Timing of *IL-6*-induced proliferation.** In vivo, MSCs in the bone marrow reside in an *IL-6*-rich environment. Moreover, bone marrow-derived MSCs proliferate in relatively low levels of exogenous *IL-6* [Ruster et al., 2006]. Therefore, we wished to determine whether pre-treatment of MSCs with *IL-6* could improve later proliferative capacity in *IL-6*-free conditions. MSCs were pre-treated for 48 h with 10 ng/ml *IL-6* in serum-free medium, then

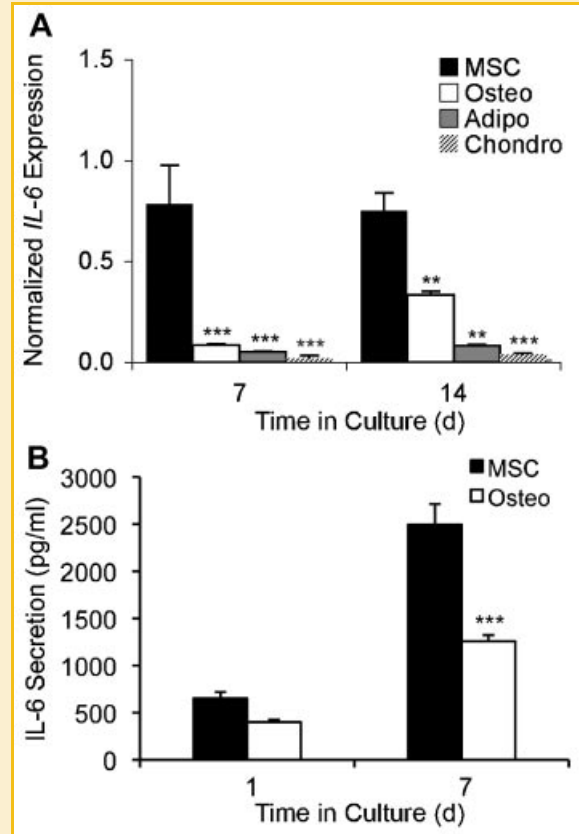


Fig. 1. *IL-6* gene expression is significantly lower in differentiated versus undifferentiated MSCs. A: Graph showing *IL-6* gene expression of undifferentiated MSCs (MSC) and MSCs that have undergone osteogenic (Osteo), adipogenic (Adipo), and chondrogenic (Chondro) differentiation. Data are normalized against *GAPDH*. Compared to undifferentiated MSCs, *IL-6* mRNA levels were 5- and 2-fold lower in osteogenic cells at days 7 and 14 of differentiation; 9- and 3-fold lower in adipogenic cells at days 7 and 14 of differentiation; and 18- and 20-fold lower in chondrogenic cells at days 7 and 14 of differentiation (ANOVA, all compared to MSC: \*\**P* < 0.01, \*\*\**P* < 0.001). B: Graph showing *IL-6* protein secretion levels as measured by ELISA for undifferentiated MSCs and osteogenic differentiated MSCs. After 7 days of osteogenic differentiation, there was a significant twofold drop in *IL-6* secretion levels compared to undifferentiated MSC controls (*t*-test: \*\*\**P* < 0.001).

placed in an *IL-6*-free environment and assessed for proliferation using the MTT assay. MSCs pre-treated with *IL-6* showed a significant increase in proliferation compared to untreated controls at both 1 and 2 days following *IL-6* removal (Fig. 2B). This finding suggests that early *IL-6* sensitization improves MSC proliferation and may play an important role in regulating their response to proliferative signals.

### siRNA KNOCKDOWN OF *IL-6* DECREASES PROLIFERATION IN MSCs

Given that exogenous *IL-6* enhanced MSC proliferation, we next assessed whether endogenous *IL-6* was also needed for MSC proliferation. *IL-6* gene expression was significantly inhibited by transfection with *IL-6* siRNA relative to controls transfected with negative control siRNA (Fig. 2C). Following transfection with siRNA



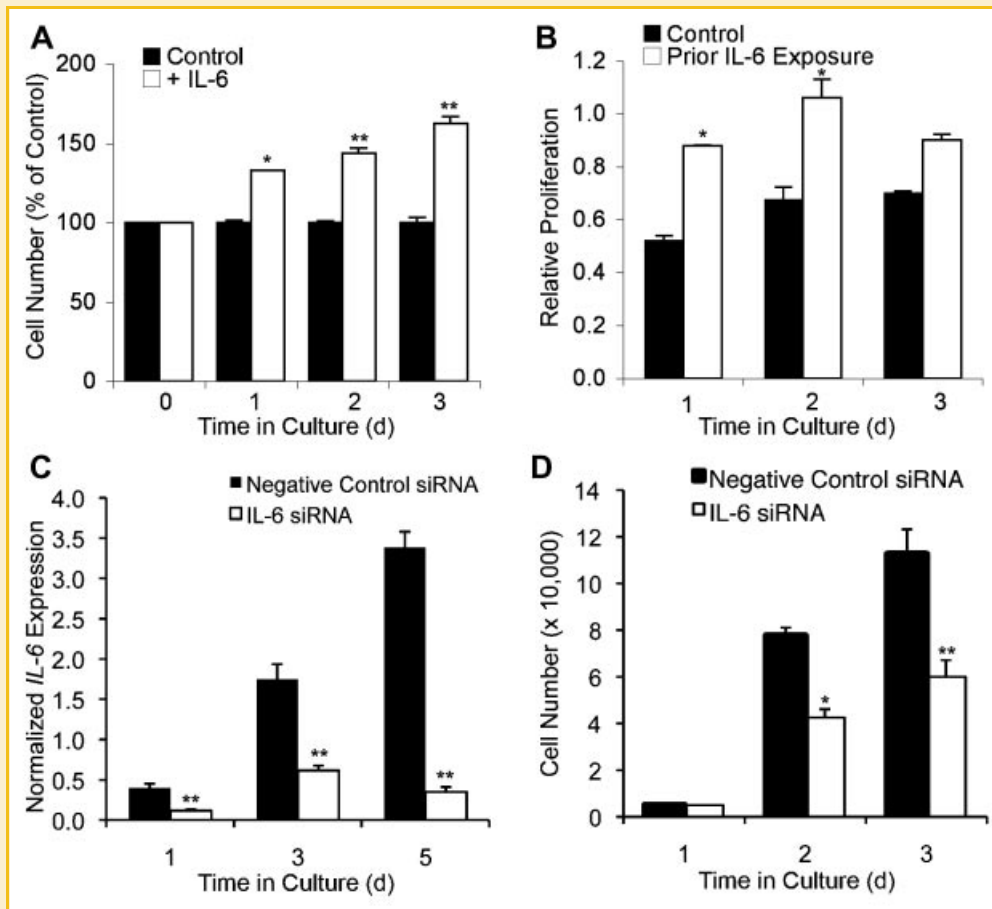


Fig. 2. IL-6 enhances proliferation of undifferentiated MSCs. A: Compared to nonstimulated controls, undifferentiated MSCs grown in the presence of IL-6 (10 ng/ml) had a  $32 \pm 3\%$ ,  $44 \pm 8\%$ , and  $62 \pm 14\%$  increase in cell number after 1, 2, and 3 days of growth, respectively. B: Graph showing results from the MTT assay. Compared to nonstimulated controls, undifferentiated MSCs previously maintained in an IL-6 rich environment proliferated at a higher rate up to 2 days following removal of IL-6 stimulation. C: Following siRNA knockdown, real-time RT-PCR analysis of *IL-6* transcript levels revealed an average 60–90% decrease in *IL-6* expression compared to transfected controls up to 5 days post-transfection. D: Loss of IL-6 expression decreased MSC proliferation by almost 50% at both 2 and 3 days post-transfection. A–D: Student's *t*-test analysis, \* $P < 0.05$ , \*\* $P < 0.01$ .

targeting IL-6 mRNA, growth assessment revealed that loss of IL-6 expression was accompanied by significantly decreased MSC proliferation at both 2 and 3 days post-transfection, strongly suggesting that IL-6 plays a marked role in MSC proliferation (Fig. 2D).

#### IL-6 PROTECTS MSCs FROM SERUM STARVATION-INDUCED APOPTOSIS

To assess the effect of IL-6 on serum starvation-induced apoptosis of MSCs, undifferentiated MSCs were cultured under serum-free conditions for 72 h with or without IL-6 (10 ng/ml). Control cells had smaller, more pyknotic nuclei and brighter, more distinct TUNEL staining than MSCs serum-starved in the presence of IL-6 (Fig. 3A). The average ratio of apoptotic cells in the absence versus presence of IL-6 was 3.5:1 (Fig. 3B), indicating that IL-6 protects MSCs from apoptosis induced by serum starvation. Additionally, IL-6 increased the overall cell density per well, consistent with the proliferation data described above.

To further analyze the mechanism by which IL-6 treatment delayed and/or decreased apoptosis after 72 h of serum starvation, we used pathway-based microarray tools to profile gene expression. IL-6-treated MSCs from three separate patients were compared to patient matched, serum-free controls. Using cut-off criteria, a 2.75-fold induction or repression of expression was considered to be of biologic importance. Fourteen genes were identified with greater than 2.75-fold repression of expression, consistent across all three patients (Fig. 3C). Of these genes, *CD27* and *TRAF2* were significantly down-regulated.

#### IL-6 AND DIFFERENTIATION

**IL-6 inhibits adipogenesis in MSCs.** To assess the role of IL-6 in MSC differentiation down the adipogenic lineage, MSCs were induced to differentiate in adipogenesis induction medium with or without 10 ng/ml IL-6, and mRNA levels of the adipogenic markers *LPL* and *FABP4* were determined by real-time RT-PCR analysis. *LPL* gene expression was significantly reduced by IL-6 treatment

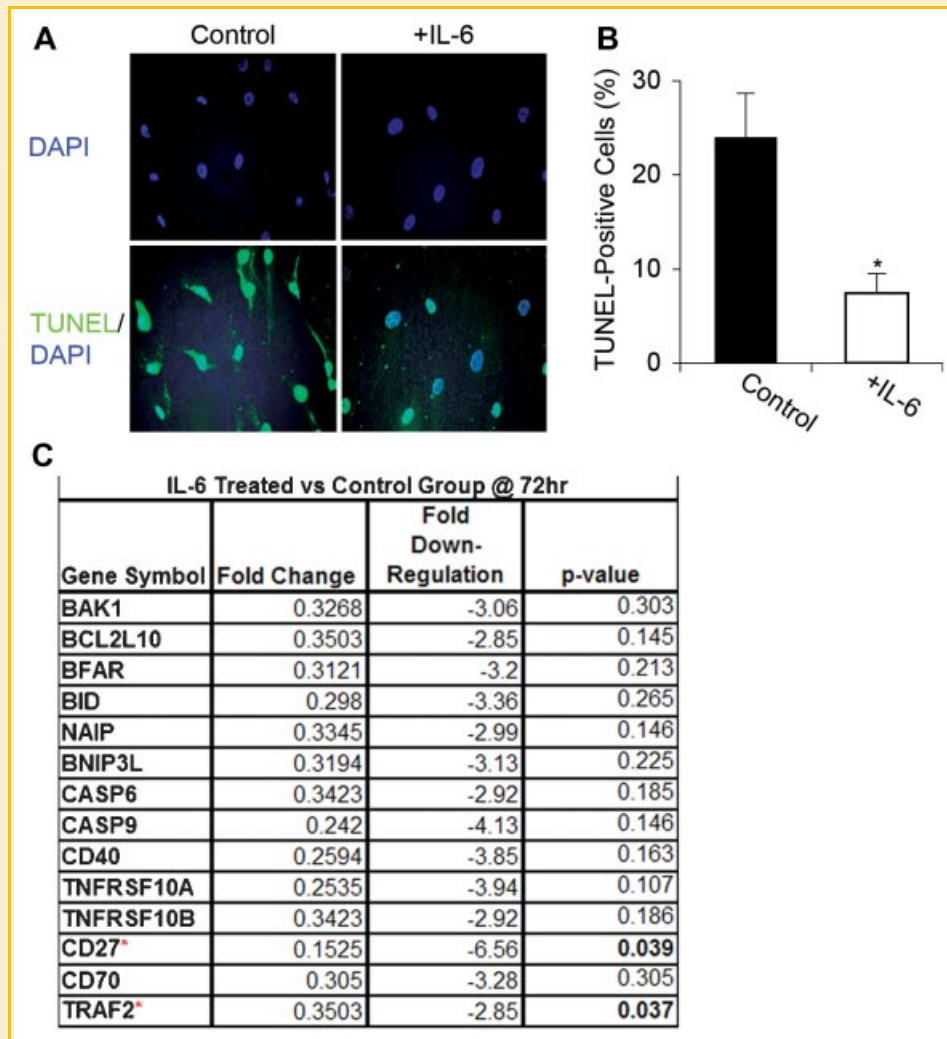


Fig. 3. IL-6 protects MSCs from serum starvation-induced apoptosis. A: *TUNEL*: Apoptosis was evaluated using TUNEL staining in undifferentiated MSCs grown in serum-free media for 72 h with or without IL-6. IL-6-treated populations exhibited fewer apoptotic cells compared to untreated controls. DAPI staining revealed that serum-starved MSCs (Control) had smaller, more pyknotic nuclei and more intense TUNEL staining than MSCs treated with IL-6 (+IL-6). Magnification: 20 $\times$ . B: *Cell counts*: For each condition, total numbers of TUNEL-positive cells were counted and a ratio of the means was calculated. The mean ratio of apoptotic cells in serum-free conditions to apoptotic cells in IL-6 supplemented conditions was 3.5:1 (*t*-test: \**P* < 0.05). C: *Microarray gene expression analysis*: Expression levels of pro-apoptotic genes decreased upon treatment with IL-6 for 72 h, only significantly for *CD27* and *TRAF2*. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

(Fig. 4A). Moreover, a similar trend was observed in *FABP4* gene expression (Fig. 4B). Similarly, Oil Red O staining revealed both a qualitative and quantitative decrease in lipid droplet formation in MSCs differentiated in the presence of IL-6 (Fig. 4C,D).

**Time course of IL-6 suppression of adipogenesis.** The anti-adipogenic effects of IL-6 were next characterized by assessing whether IL-6 pre-treatment could effectively decrease MSC adipogenic differentiation. It should be noted that adipogenic differentiation, as described above, was carried out in differentiation medium for 21 days with serum, whereas the proliferation and apoptosis studies were done under serum-free conditions. The 3-week time course of differentiation precluded the use of serum-free culture conditions. The experimental design to assess early, rather than continuous IL-6 exposure allowed us to explore the effects of IL-6 both with and without serum. Early exposure was defined as

48 h of stimulation with IL-6, after which cells were allowed to differentiate in adipogenic medium in the absence of IL-6 for 18 days.

Early IL-6 exposure in serum-free medium prior to differentiation decreased mRNA transcript levels of *LPL* and *FABP4* (Fig. 4E,F) at day 14. Early exposure to IL-6 in the presence of serum prior to differentiation did not alter *LPL* or *FABP4* expression across patients, possibly due to IL-6 binding and sequestration by serum proteins.

**Effects of IL-6 on osteogenic differentiation.** Cells were induced to differentiate in osteogenic medium in the absence or presence of 10  $\mu$ g/ml IL-6. A statistically significant increase in *ALP* gene expression was observed at day 7 by real-time RT-PCR (Fig. 5A). However, ALP histochemistry on day 10 showed equivalent staining intensity in the two groups (Fig. 5C). Similarly, day 10 ALP activity

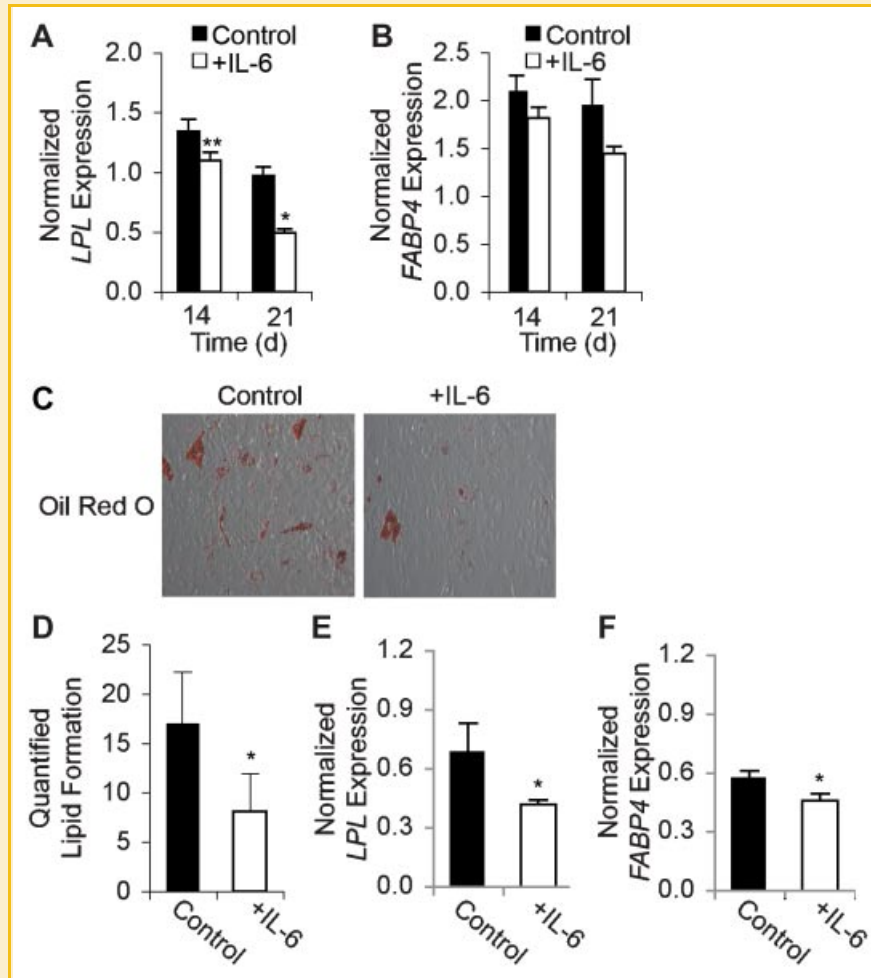


Fig. 4. IL-6 inhibits adipogenic differentiation of MSCs. A: Graph showing real-time RT-PCR results of *LPL* and (B) *FABP4* gene expression normalized to *GAPDH* in MSCs induced to undergo adipogenesis without (Control) or with IL-6 treatment for 14 or 21 days. In adipocytes differentiated in the presence of IL-6, *LPL* expression decreased by 20% at day 14 and 50% by day 21, and *FABP4* gene expression decreased by 20% at day 21 ( $N = 3$ ). C: Oil Red O staining is shown to reveal the presence of lipid droplets, indicating adipogenic differentiation. Images were taken at 20 $\times$  magnification. D: Graph showing quantification of Oil Red O staining, which reveals that IL-6 treatment significantly decreased lipidization. E: Graph showing real-time RT-PCR results of *LPL* and (F) *FABP4* gene expression normalized to *GAPDH* in MSCs pre-treated with IL-6 for 48 h prior to adipogenic differentiation for 18 days. A,D–F: Student's *t*-test analysis, \* $P < 0.05$ , \*\* $P < 0.01$ . [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

assay revealed IL-6 exposure did not result in any reproducible trend across multiple patients (Fig. 5D).

Day 18 Alizarin Red staining for calcium deposition revealed equivalent staining intensity in IL-6-treated cells (Fig. 5C), and there was no significant quantitative difference in dye retention (Fig. 5E). Real-time RT-PCR analysis of *OC* gene expression did not corroborate with the calcium deposition as indicated by Alizarin Red staining. By day 21 of differentiation, *OC* mRNA transcript levels were significantly decreased in IL-6-treated populations (Fig. 5B).

**Chondrogenesis.** In a sample population ( $N = 4$  patients), MSC chondrogenic differentiation in the presence of IL-6 exhibited a statistically significant trend toward decreased *COL2A1* mRNA transcripts at day 21 (Fig. 6A). Alcian Blue staining for sulfated sGAG confirmed the chondro-inhibitory effect of IL-6 (Fig. 6B). Concordantly, sGAG levels, as determined by the Blyscan assay,

were significantly decreased by IL-6 treatment (Fig. 6C). Furthermore, the larger size of IL-6-treated pellets (Fig. 6B; quantification data not shown) was consistent with the mitogenic activity of IL-6 on MSCs.

#### IN VITRO WOUND HEALING IS ACCELERATED IN THE PRESENCE OF IL-6

As tissue-resident stem cells that can home to injury sites in vivo, MSCs are postulated to play an important role in wound healing [Francois et al., 2006; Ringden et al., 2007]. To assess the role of IL-6 in MSC-mediated acute wound healing, undifferentiated MSCs were injured in an in vitro model and defect closure following injury (cell migration) was monitored over 18 h in a total of 30 defects (Control,  $N = 12$ ; IL-6,  $N = 18$ ) using cells from two different patients. Cells treated with 10 ng/ml of IL-6 immediately following wounding

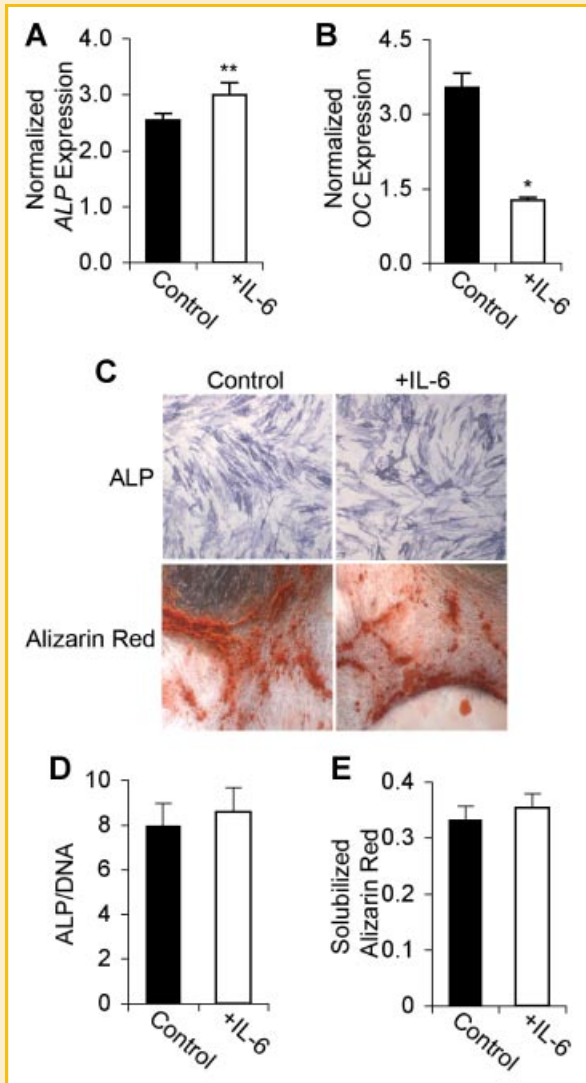


Fig. 5. IL-6 does not affect osteogenic differentiation of MSCs. A: Graph showing real-time RT-PCR results of alkaline phosphatase (ALP) gene expression at day 7 and (B) osteocalcin (OC) gene expression at day 21, both normalized to *GAPDH* in MSCs induced to undergo osteogenesis without (control) or with IL-6 treatment. A,B: Student's *t*-test analysis, \* $P < 0.05$ , \*\* $P < 0.01$ . C: Immunohistochemical staining of ALP and Alizarin Red, both showing no difference between control and +IL-6 treated cells. Images were taken at 4 $\times$  magnification. D: ALP activity assay results of osteogenic differentiated MSCs in the absence (control) or presence of IL-6. Data are normalized to total DNA content. E: Graph showing quantification of Alizarin Red staining. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

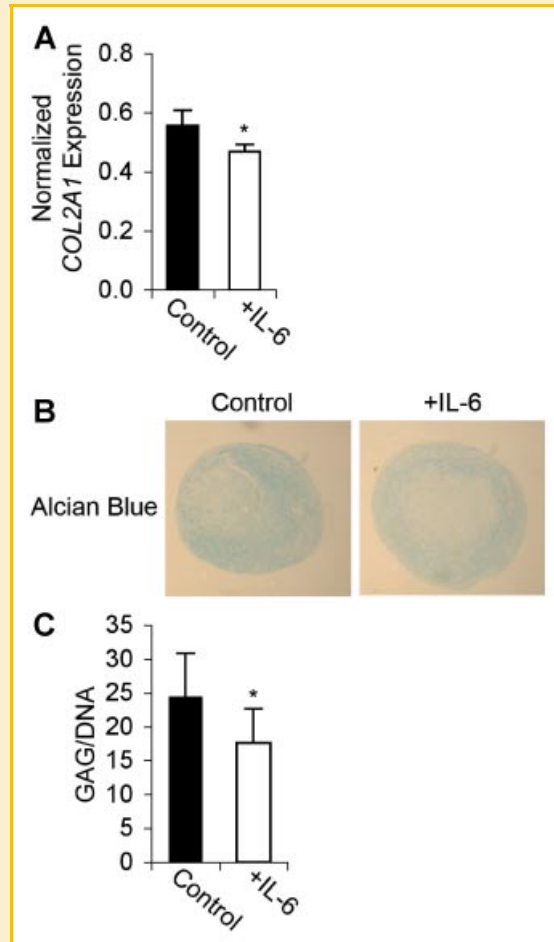


Fig. 6. IL-6 treatment suppresses chondrogenic differentiation of MSCs. A: Graph showing real-time RT-PCR results of collagen II (*COL2A1*) gene expression normalized to *GAPDH* at day 21 of differentiation in MSCs induced to undergo chondrogenesis without (control) or with IL-6 treatment (N = 4). B: Alcian Blue immunohistochemical staining showing decreased glycosaminoglycan expression in chondrogenic-differentiated pellets treated with IL-6. Images were taken at 4 $\times$  magnification. C: Consistent with the qualitative observations above, the sGAG assay results show a significant decrease in sulfated glycosaminoglycan formation in chondrogenic-differentiated MSCs treated with IL-6 for 21 days. Data are normalized to total DNA content. A,C: Student's *t*-test analysis, \* $P < 0.05$ . [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

#### IL-6 ACTIVATES ERK1/2, BUT NOT STAT3 IN MSCs

To assess the signaling pathway(s) through which IL-6 exerts its effects on human MSCs, we first explored whether IL-6 induces activation of the JAK/STAT pathway through phosphorylation of STAT3 at Tyr705 or Ser727, the mechanism commonly implicated in growth and proliferation of stem cell populations [Yoshida et al., 1994]. Stimulation with 10 ng/ml IL-6 for 60 min did not induce activation of STAT3 by phosphorylation at Tyr705 (Fig. 8A) or Ser727 (data not shown). STAT3 was detected in all protein lysates, both experimental and control; however, only positive control cell lysates exhibited phospho-STAT3 expression at both Tyr705 (Fig. 8A) and Ser727 (data not shown). STAT1 activation levels were also not affected by IL-6 treatment (data not shown).

showed an accelerated rate of wound closure compared to controls (Fig. 7A). Quantitative analysis of these data revealed that IL-6 treatment results in a significant difference in the defect size at 6, 12, and 18 h post-injury compared to untreated controls (Fig. 7B). The increased capacity for in vitro wound healing of IL-6-treated MSCs as early as 6 h in low-serum conditions indicates that IL-6 can induce MSC migration.



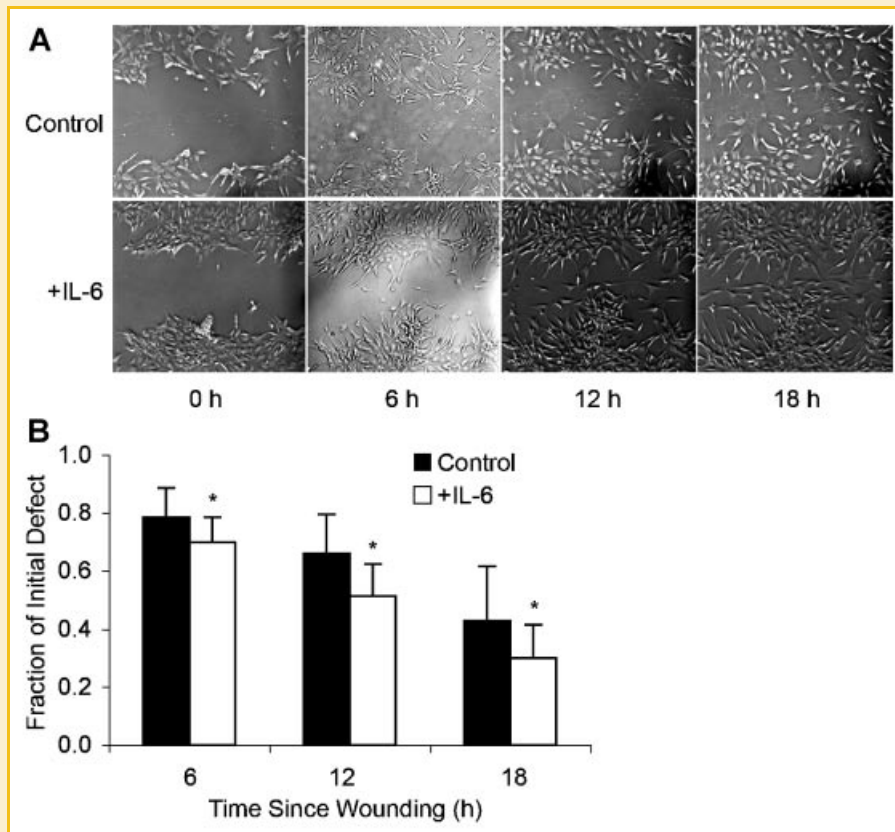


Fig. 7. IL-6 accelerates in vitro wound healing in MSCs. **A: Wound healing:** Confluent, undifferentiated MSCs were wounded and defect closure was observed over 18 h either in the absence (Control) or presence of IL-6. Defects were imaged at the time of wounding (0 h) and then at 6 h intervals for 18 h. MSCs exposed to IL-6 after initial wounding showed increased capacity for in vitro healing. Images were taken at 5 $\times$  magnification. **B: Quantitative analysis:** The remaining wound area in the presence of IL-6 at 6 h post-injury was approximately 15% less than that of untreated MSCs, at 12 h post-injury was approximately 33% less than untreated MSCs, and at 18 h was approximately 24% less than untreated MSCs (*t*-test: \**P* < 0.05).

To assess whether the ERK1/2 pathway is activated by IL-6 in MSCs, we stimulated MSCs with IL-6 and probed the cell lysates for total and phospho-ERK1/2. After 5 min of stimulation with IL-6, ERK1/2 phosphorylation could be detected. IL-6-mediated ERK1/2 activation peaked 30 min post-IL-6-treatment before returning to baseline levels (Fig. 8B). Therefore, in undifferentiated MSCs, IL-6 treatment activates ERK1/2 rather than STAT3.

#### SUPPRESSION OF ERK1/2 ACTIVATION INHIBITS IL-6-MEDIATED INCREASE IN MSC PROLIFERATION

To determine the importance of the ERK1/2 pathway, we blocked the activation of ERK1/2 with the MEK1/2 inhibitor U0126 prior to stimulation with IL-6 (Fig. 8C). Following U0126-induced MEK1/2 inhibition, growth analysis revealed that reduced ERK1/2 signaling effectively inhibited IL-6-mediated enhancement of MSC proliferation (Fig. 8D). These data suggest that signaling via the ERK1/2 pathway is critical for IL-6-mediated proliferation of MSCs.

#### IL-6 INHIBITION OF MSC DIFFERENTIATION IS REVERSED IN THE PRESENCE OF U0126

We next examined the involvement of ERK1/2 signaling in IL-6-induced inhibition of adipogenic differentiation by using U0126. Prior to differentiation, MSCs were expanded for 72 h with or

without IL-6 and/or U0126, and then grown in adipogenic medium for 18 days.

The IL-6-mediated decrease in adipogenic mRNA transcripts previously observed at day 14 for *LPL* and *FABP4* was abrogated by exposure to U0126 (Fig. 8E,F). The IL-6 effect on chondrogenesis was also dependent on ERK1/2 activation. Specifically, the IL-6-mediated decrease in *COL2A1* expression was abrogated by co-treatment with U0126 (Fig. 8G). These data suggest that signaling via the ERK1/2 pathway is critical for IL-6-mediated stem cell maintenance.

## DISCUSSION

We have recently shown that *IL-6* expression is down-regulated as MSCs undergo mesenchymal differentiation, and that this expression resumes its undifferentiated levels upon dedifferentiation from adipogenic and chondrogenic lineages [Song et al., 2006]. We chose to pursue IL-6 as a candidate "stemness" gene for a number of reasons. IL-6 and IL-6 type cytokines have been shown to activate the JAK/STAT, MAPK, and AKT pathways and have been implicated as potent mediators of numerous important biological processes, including differentiation, proliferation, and apoptosis [Heinrich et al., 1998]. In stem cell biology, IL-6 family cytokines

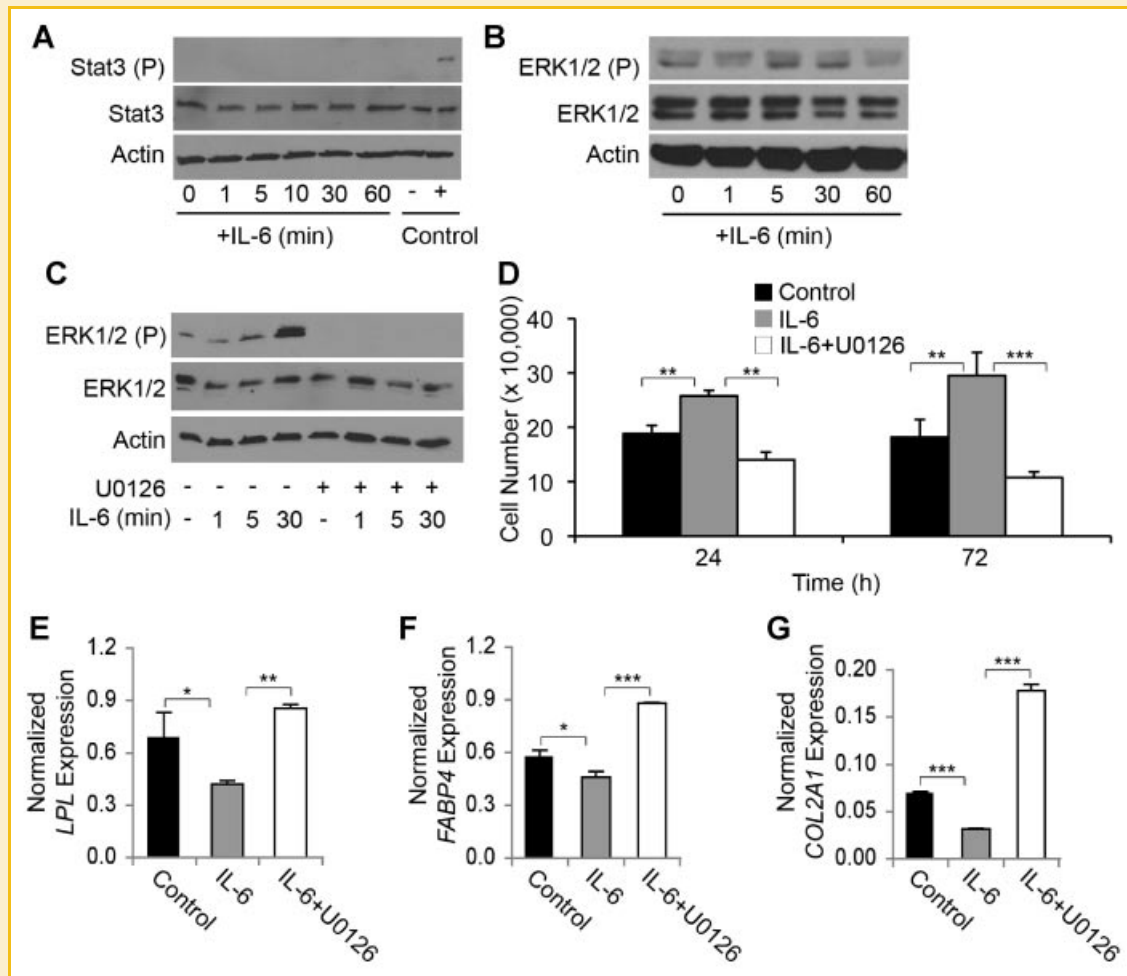


Fig. 8. IL-6 mediated effects in MSCs are dependent upon ERK1/2 activation. Undifferentiated MSCs were serum-starved for 12 h and then stimulated with IL-6 (10 ng/ml) for 0–60 min. A: *STAT3*: IL-6 stimulation did not induce activation of STAT3 in undifferentiated MSCs. Activated STAT3 (STAT3 (P)) was only observed in the positive control lysates, while total STAT3 levels remained constant with IL-6 treatment. Actin is shown as a loading control. B: *ERK1/2*: Serum-starved MSCs have baseline activation of ERK1/2 (ERK1/2 (P)), which increased after 5 min of IL-6 stimulation, before returning to baseline levels at 60 min. Actin is shown as a loading control. C: *U0126 inhibition*: IL-6-induced ERK1/2 activation (ERK1/2 (P)) in undifferentiated MSCs was inhibited by U0126. Actin is shown as a loading control. D–G: *Effect of ERK1/2 inhibition*: (D) IL-6 alone significantly increased proliferation over controls, while IL-6 stimulation in the presence of U0126 failed to induce an increase in proliferation (N = 3). E, F: *LPL* and *FABP4* mRNA levels were decreased by day 14 in cells differentiated after exposure to IL-6; *LPL* and *FABP4* expression was increased in cells differentiated after exposure to IL-6 + U0126 (N = 3). G: *COL2A1* gene expression in MSCs pre-treated with vehicle (Control), IL-6, or IL-6 + U0126. All real-time RT-PCR data shown are normalized against *GAPDH* (ANOVA: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

have been implemented in the maintenance of both embryonic and adult stem cells. IL-6 increases proliferation in placenta-derived MSCs [Li et al., 2007] and is highly expressed in cord blood [Liu and Hwang, 2005] and bone marrow-derived MSCs [Rougier et al., 1998]. Oncostatin M, an IL-6 type cytokine, induces proliferation [Song et al., 2005], increases osteogenesis, and inhibits adipogenesis [Gimble et al., 1994; Song et al., 2007] in adipose-derived MSCs. IL-6 is also integral to normal stromal cell function and osteoclast activation [Manolagas and Jilka, 1995] and both hematopoiesis [Ikebuchi et al., 1987; Ishibashi et al., 1989; Bernad et al., 1994]. Moreover, liver regeneration in vivo involves IL-6, as IL-6 knockout mice have impaired liver regeneration and fail to repair ischemia/reperfusion injury, a defect that is correctable by addition of exogenous IL-6 [Cressman et al., 1996; Camargo et al., 1997]. Our data reported here show, for the first time, that IL-6

maintains the “stemness” of bone marrow-derived MSCs. Specifically, IL-6 enhances proliferation and protects from serum starvation-induced apoptosis of undifferentiated MSCs. Furthermore, IL-6 inhibits adipogenic and chondrogenic differentiation, and increases the rate of in vitro wound healing of MSCs.

In this study, we show that IL-6 induces ERK1/2 activation in undifferentiated MSCs but not STAT3, the downstream regulator of the gp130/IL-6 family signaling pathway. Furthermore, ERK1/2 pathway inhibition mitigates the IL-6 effects, resulting in decreased proliferation and increased differentiation along both the adipogenic and chondrogenic lineages. Osteogenesis was not affected by IL-6 exposure, suggesting different transcriptional regulation. A balance between ERK1/2 and STAT3 activation, in terms of signal transduction, has been described in many cell types. Mouse ES cells express IL-6, gp130 receptors, and other members of the IL-6

cytokine family, and the “stemness” of these cells can be maintained by activation of the JAK/STAT pathway [Nichols et al., 1994; Yoshida et al., 1994]. Human ES cells express these same cytokines and receptors; however, loss of the pluripotency markers Nanog and Oct-4 occurs despite signaling through gp130 pathways [Humphrey et al., 2004]. In fact, despite similar marked activation of both ERK1/2 and STAT3, different effects on “stemness” were observed in human and mouse ES cells. Our results show that, although IL-6 maintains MSC “stemness,” it does not activate the JAK/STAT pathway. This is distinct from the balance between STAT3 (pluripotent) and ERK1/2 (loss of pluripotency) activity previously described in ES cells, and suggests a novel signaling mechanism in MSCs [Smith, 2001; Burdon et al., 2002].

It has been demonstrated that p38 MAPK is a critical regulator of IL-6 production in human thymic epithelial cells [Mainiero et al., 2003]. Accordingly, p38 may be involved in the gene expression of IL-6; however, it is unknown if p38, or JNK for that matter, are critical for IL-6-mediated cell proliferation and survival, particularly in MSCs. We did not test if JNK or p38 are activated in response to IL-6 in MSCs. It is possible that p38 may be important for MSC proliferation since it has been shown to play an important role in IL-6-mediated proliferation of B9 hybridoma cells [Zauberman et al., 1999]. However, in contrast to our findings in MSCs, that study did not find induction of ERK1/2 in response to IL-6 treatment. So it appears that the involvement of particular MAP kinases in IL-6-mediated proliferation and survival is highly cell type specific. The unique role of IL-6-induced ERK1/2 activation in MSCs may be due to a signature profile of the ligand-specific cell surface receptors. This signature has yet to be defined for MSCs.

Of further interest is the mechanism by which IL-6 appears to mitigate apoptotic pathways in MSCs. The down-regulated gene profile in our PCR array includes genes involved in NF $\kappa$ B-mediated apoptosis, certain immunoregulatory factors, as well as elements of the intrinsic apoptotic pathway. For example, of the down-regulated genes in our experimental system TRAF2 is known to interact with TNFR1, CD40 [Hu et al., 1994] as well as CD27 [Akiba et al., 1998] amongst other regulators of apoptosis, and its over-expression can induce NF $\kappa$ B activation [Rothe et al., 1995]. Similarly, CD27 signaling activates NF $\kappa$ B and JNK/SAPK through a TRAF2-dependent mechanism [Akiba et al., 1998]. Additionally, the down-regulation of caspases 9 and 6 is of interest because caspase 9 is an important initiator of intrinsic apoptosis [Li et al., 1997], and caspase 6 is an effector caspase shown to inhibit caspase 9 signaling [Guerrero et al., 2008]. The significance of other down-regulated genes is more ambiguous, such as anti-apoptotic BCL2L10 and NAIP and pro-apoptotic BID and BAK1, mediators of the intrinsic apoptotic pathway [Cory et al., 2003].

In the adult stem cell field, it is unknown whether MSCs regenerate tissue directly or act via their byproducts to re-invigorate other adult tissue-resident stem cells. Preliminary data from our laboratory suggest that IL-6 is one of the MSC products functioning to re-invigorate tissue. Specifically, the addition of exogenous, recombinant IL-6 to MSC cultures stimulated endogenous *IL-6* gene expression in MSCs (data not shown). Based on these initial data, we speculate that exogenous IL-6 may form a positive autocrine feedback loop in MSCs, perhaps functioning to augment the

proliferative signal to neighboring MSCs. Our results show an important role for IL-6 in MSC maintenance and functionality. We believe IL-6 is necessary not only for maintenance of MSCs in their stem state and niche, but also for their support of the hematopoietic stem cells with which they interact in the bone marrow microenvironment. IL-6 has long been known to play an integral role in normal hematopoiesis and myelopoiesis, and it is thus particularly interesting that IL-6 would also play a prominent role in the maintenance of MSCs. The inhibition of stromal adipogenesis by IL-6 is directly relevant for homeostasis of the bone marrow niche and is most likely a key mechanism by which co-infusion of MSC and HSCs results in more efficient reconstitution of the bone marrow following bone marrow transplantation [Koc et al., 2000].

In summary, we have shown here, for the first time, that IL-6 is a critical factor for maintaining the stemness of adult human bone marrow-derived MSCs. This finding is important for understanding the regulation of MSCs by their microenvironment, as well as optimizing the manipulation of these cells in vitro and in vivo.

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