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Journal of Cellular Biochemistry 115:1262–1268 (2014)

Journal of Cellular Biochemistry

LIF/STAT3/SOCS3 Signaling Pathway in Murine Bone Marrow Stromal Cells Suppresses Osteoblast Differentiation

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ABSTRACT

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine that belongs to the interleukin-6 family and is expressed by multiple tissue types. This study analyzed the effect of LIF on osteoblast differentiation using primary murine bone marrow stromal cells (BMSCs). Colony-forming unit-osteoblast formation by BMSCs was significantly suppressed by LIF treatment. To clarify the mechanism underlying the LIF suppressive effect on osteoblast differentiation, we analyzed the downstream signaling pathway of LIF. LIF/signal transducer and activator of transcription 3 (STAT3) signaling induces the expression of suppressor of cytokine signaling 3 (SOCS3). *SOCS3* knockdown experiments have previously demonstrated that short-hairpin SOCS3-BMSCs reversed the LIF suppressive effect. Our results demonstrated that LIF suppresses osteoblast differentiation through the LIF/STAT3/SOCS3 signaling pathway. J. Cell. Biochem. 115: 1262–1268, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: OSTEOBLASTS; BONE MARROW STROMAL CELLS; CELL DIFFERENTIATION; CYTOKINE; SIGNAL TRANSDUCTION

Mammalian bone marrow maintains two types of stem cells, hematopoietic stem cells and mesenchymal stem cells (MSCs), within an endosteal/osteoblastic niche and a vascular/sinusoidal niche. Bone marrow stromal cells (BMSCs), including MSCs, can differentiate in vitro into osteoblasts, chondrocytes, adipocytes, and myoblasts [Friedenstein et al., 1976; Piersma et al., 1985; Owen, 1988; Owen and Friedenstein, 1988; Prockop, 1997; Bianco et al., 2006; Itoh and Aubin, 2009; Itoh et al., 2012]. The endosteal/osteoblastic tissue is continuously remodeled according to physiological circumstances [Wagner and Karsenty, 2001]. Dysregulation of the balance between bone formation and resorption induces pathological conditions such as osteoporosis and osteosclerosis.

Bone remodeling is generally maintained by osteoblasts and osteoclasts, which in turn are modulated by cytokines, hormones, and growth factors [Boyle et al., 2003; Harada and Rodan, 2003]. Among the cytokines, those belonging to the interleukin-6 (IL-6) family are known to modulate osteoblast and osteoclast differentiation [Manolagas, 1995; Nishimura et al., 1998 Harada and Rodan, 2003; Itoh et al., 2006; Yoshitake et al., 2008]. Leukemia inhibitory factor (LIF) belongs to the IL-6 family of cytokines and its receptor consists of a ligand-binding subunit and a common signal-transducing subunit, LIF receptor-β (LIFRβ) and glycoprotein 130 (gp130), respectively. After ligand binding and receptor complex formation, Janus kinase (JAK) tyrosine kinases, which are associated with the cytoplasmic domains of gp130 and LIFRβ in an inactive state, are phosphorylated [Stahl et al., 1994]. Activated JAK molecules then phosphorylate several tyrosine residues in the cytoplasmic tails of gp130 and LIFRβ, which then act as phosphotyrosine docking sites for the Src homology 2 (SH2) domains of various cytoplasmic proteins such as the signal transducer and activator of transcription (STAT) proteins [Stahl and Yancopoulos, 1994; Stahl et al., 1995 Segal and Greenberg, 1996]. This reaction leads to signal transduction along the primary cytokine signaling pathway, the JAK/STAT pathway. Activated STAT molecules dimerize and translocate to the nucleus. Although there are at least six different STAT proteins (STAT1–STAT6), STAT3 tends to be the protein activated by cytokines that signal through the LIFRβ/gp130 complex [Tomida et al., 1999]. In the nucleus, the activated STAT proteins bind to specific DNA sequences and induce the expression of various genes.

Suppressor of cytokine signaling (SOCS) proteins [Endo et al., 1997; Minamoto et al., 1997; Starr et al., 1997] are induced

Kenta Matsushita and Shousaku Itoh contributed equally to this work.

Grant sponsor: The Japan Society for the Promotion of Science Grant-in-aid for Scientific Research; Grant numbers: 24689070, 25670809.

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Manuscript Received: 14 November 2013; Manuscript Accepted: 22 January 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 25 January 2014

DOI 10.1002/jcb.24777 • © 2014 Wiley Periodicals, Inc.

by the JAK/STAT signaling pathway. They modulate signal transduction by inhibiting the components of the JAK/STAT pathway. Proteins belonging to the SOCS family consist of an SH2 domain and a segment called the SOCS box, which is located near the C terminal [Hilton et al., 1998]. These two domains are required for the appropriate functioning of the SOCS family proteins. These proteins function using two regulatory mechanisms of action. First, they can mark their targets for degradation through the ubiquitin pathway. SOCS proteins associate with the elongin B and C complex to recruit ubiquitin ligase complexes through the SOCS box [Kamura et al., 1998]. Second, SOCS proteins can inhibit the catalytic activities of enzymes by competing with substrates further downstream in the signaling pathway. The SH2 domain of SOCS3 binds to the Src homology phosphatase-2 (SHP-2)-binding domains of the gp130 receptor rather than to JAK [Nicholson et al., 2000; Sasaki et al., 2000; Schmitz et al., 2000]. It is thought that SOCS3 binds to receptors and inhibits JAK activity by accessing the activation loop of JAKs with its kinase inhibitor region domain. The variation in the expression and induction of SOCS proteins in different cell types may be another mechanism by which different cell types respond to the same cytokine in various ways. SOCS3 can inhibit the signaling cascade of several LIF/JAK/STAT-dependent cytokines [Masuhara et al., 1997; Minamoto et al., 1997; Naka et al., 1997; Starr et al., 1997; Auernhammer et al., 1998; Yasukawa et al., 1999]. SOCS3 overexpression inhibits LIF-induced gp130 and STAT3 phosphorylation, as well as STAT3-mediated downstream events.

In fetal rat calvarial cell experiments, LIF suppressed osteoblast differentiation [Falconi et al., 2007; Falconi and Aubin, 2007]. However, the effects of LIF on primary murine BMSCs remain unclear. Thus, we examined the effects of LIF from the perspective of signal transduction using murine BMSCs. We demonstrated that LIF suppresses osteoblast differentiation through the LIF/STAT3/SOCS3 signaling pathway.

MATERIALS AND METHODS

PRIMARY MURINE BMSC CULTURE

BMSCs were harvested by removing the femurs and tibias of C57BL/6J mice (age range: 4–6 weeks). Using a syringe, the bone cavity was flushed with α-minimum essential medium (α-MEM) (Life Technologies, Corp., CA, USA) supplemented with antibiotics and 10% fetal calf serum (FCS). Cell suspension was sieved to remove cell aggregates. Recovered cells were incubated in α-MEM supplemented with the same antibiotics and 10% FCS. After 3 days of incubation, nonadherent cells were removed by washing three times with phosphate-buffered saline. Approximately 2 weeks after seeding, when adherent cells reached 80% confluence, they were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.2% trypsin, 1 mM EDTA).

DIFFERENTIATION ASSAYS FOR OSTEOGENESIS

Cells were cultured in osteogenic induction medium consisting of α-MEM, 10% FCS, antibiotics, 50 μg/ml ascorbic acid (Sigma–Aldrich, MO, USA), 10 mM β-glycerophosphate (Sigma–Aldrich), and 10^{-8} M dexamethasone (Sigma–Aldrich). Some cultures were also treated with 50 ng/ml LIF (ESGRO, Millipore, Bedford, MA, USA). The medium was replaced every 3 days. After 3 weeks, the cells were fixed in 10% neutral formalin buffer for 30 min and double-stained for alkaline phosphatase (ALP) activity and mineral deposition (von Kossa staining). Colony-forming unit-osteoblasts (CFU-Os) were defined as colonies with ALP-positive cells associated with mineralized matrix (von Kossa-positive). CFU-Os were counted manually using a grid and a microscope.

QUANTITATIVE ANALYSIS OF GENE EXPRESSION

RNA was extracted from BMSCs that had been incubated for 2 weeks with or without 50 ng/ml LIF using Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan) and reverse transcribed. The expression levels of each marker were assessed by real-time polymerase chain reaction (PCR; 7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) using the following primers: *ALP* forward, 5′-CCAACTCTTTTGTGCCAGAGA-3′; *ALP* reverse, 5′-GGCTACATTGGTGTTGAGCTTTT-3′; *Type I collagen* (*ColI*) *αI* forward, 5′- GCTCCTCTTAGGGGCCACT-3′; *ColIαI* reverse, 5′-CCACGTCTCACCATTGGGG-3′; *Bone sialoprotein* (*BSP*) forward, 5′-CAGGGAGGCAGTGACTCTTC-3′; *BSP* reverse, 5′-AGTGTGGAAAGTGTGGCGTT-3′; *Osteocalcin* (*OCN*) forward, 5′-CTGACCTCACAGATGCCAAGC-3′; *OCN* reverse, 5′-TGGTCTGATAGCTCGTCACAAG-3′; *Runt-related transcription factor 2* (*Runx2*) forward, 5′-TGTTCTCTGATCGCCTCAGTG-3′; *Runx2* reverse, 5′-CCTGGGATCTGTAATCTGACTCT-3′; *Osterix* (*OSX*) forward, 5′-ATGGCGTCCTCTCTGCTTG-3′; *OSX* reverse, 5′-TGAAAGGTCAGCGTATGGCTT-3′; *L32* forward, 5′-CACAATGTCAAGGAGCTGGAAGT-3′; and *L32* reverse, 5′-TCTACAATGGCTTTTCGGTTCT-3′. The relative amounts of transcripts were normalized to the amount of the L32 transcript.

MICROARRAY ANALYSIS

A comparison of GeneChip array data was obtained using the KURABO custom analysis services [KURABO Industries, Osaka, Japan, an authorized service provider for Affymetrix Japan K.K. (Tokyo)]. Total RNA was isolated from BMSCs incubated for 24 h with or without 50 ng/ml LIF using Sepasol-RNA I and was reverse transcribed to cDNA using a T7 oligo d(T) primer (Affymetrix). The cDNA products were used in an in vitro transcription reaction containing T7 RNA polymerase and biotinylated nucleotide analogs (pseudouridine base). Next, the labeled cDNA products were fragmented, loaded onto GeneChip(R) Mouse Genome 430 A2.0 arrays (Affymetrix), and hybridized according to the manufacturer's protocols. Streptavidin–phycoerythrin (Molecular Probes, Eugene, OR, USA) was used as a fluorescent conjugate to detect the hybridized target sequences. Raw intensity data from the GeneChip array were analyzed using GeneChip Operating Software (Affymetrix).

LENTIVIRUS TRANSFECTION

BMSCs were grown to 90% confluence and infected with a lentivirus for SOCS3 or the control (pLKO.1-puro-SOCS3-sh or pLKO.1-puro-Control-sh) (Sigma–Aldrich) at a multiplicity of infection of 1 for 24 h before adding fresh medium. Total proteins were then extracted for western blotting using anti-SOCS3 antibody and rabbit

anti-β-actin antibody (Cell Signaling, Beverly, MA, USA). shSOCS3-BMSCs were cultured in osteogenic induction medium with or without LIF (50 ng/ml). The cells were fixed at day 21 and double stained for ALP activity and mineral deposition (von Kossa staining).

WESTERN BLOTTING

Following LIF stimulation, BMSC lysates were prepared in lysis buffer [1 M Tris-HCl (pH 7.5), 1.5 M NaCl, 1% Nonidet P-40, 500 μM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride]. Proteins were diluted in 20 μl of 3× Laemmli sodium dodecyl sulfate loading buffer, separated on a 5% gradient polyacrylamide gel (Wako Pure Chemical Industries, Osaka, Japan), and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with Block Ace (DS Pharma Biomedical, Osaka, Japan) and incubated with anti-phospho-STAT3, anti-STAT3, and anti-SOCS3 antibodies (Cell Signaling) for 1 h at room temperature. The membranes were then washed three times (5 min each) with TBS-T [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween-20] and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. They were then washed three times with TBS-T. Immune complexes were visualized using a chemiluminescene system (GE Healthcare, Buckinghamshire, UK).

IMMUNOPRECIPITATION

The cells were lysed in lysis buffer and precipitated with anti-β-catenin antibody conjugated to Sepharose beads (ADAR Biotech, Rehovot, Israel) at 4 °C overnight. The precipitate was immuno-blotted with anti-SOCS3 antibody.

STATISTICAL ANALYSIS

All results were representative of these independent experiments. All data were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made by one-way analysis of variance or Student's *t* test. $P < 0.05$ was considered significant.

RESULTS

LIF SUPPRESSES OSTEOBLAST DIFFERENTIATION

BMSCs cultured in osteogenic induction medium developed CFU-Os (Fig. 1A), whereas BMSCs cultured in osteogenic induction medium with LIF (50 ng/ml) did not. These cells developed only CFU-ALP (Fig. 1A). LIF almost completely suppressed CFU-O development (Fig. 1A and B). Furthermore, we confirmed the LIF suppressive effect by studying the expression of osteoblast differentiation markers. BMSCs cultured with LIF showed a suppressed expression of all osteoblast differentiation markers (Fig. 2). These results demonstrated that LIF has a strong suppressive effect on osteoblast differentiation.

LIF INDUCES SOCS3 EXPRESSION

STAT3 phosphorylation was observed 15 min after LIF stimulation and almost disappeared at 90 min (Fig. 3A). To clarify the mechanism underlying the LIF suppressive effect on osteoblast differentiation, we compared the gene expression profiles of BMSCs incubated for 24 h with or without LIF, and *SOCS3* was identified (Table I). SOCS3

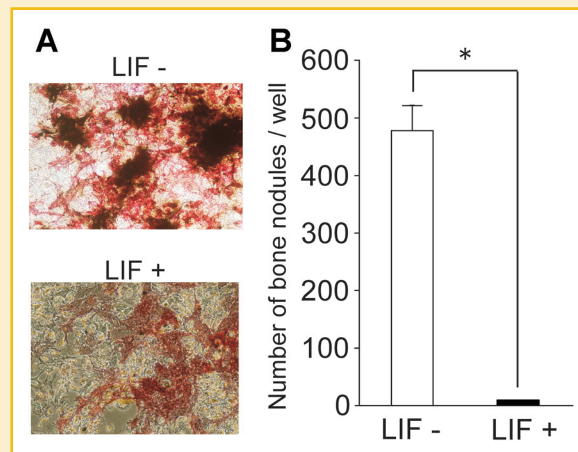

Fig. 1. Number of CFU-O in BMSCs cultured with LIF. (A) BMSCs were cultured in osteogenic induction medium with or without LIF (50 ng/ml). Cultured cells were fixed at day 21 and double stained for ALP activity and mineral deposition (von Kossa staining), ×100. (B) CFU-Os were counted. Similar results were obtained from three independent experiments. Data are expressed as mean ± SEM. Asterisks indicate significant differences: $P < 0.005$ (*).

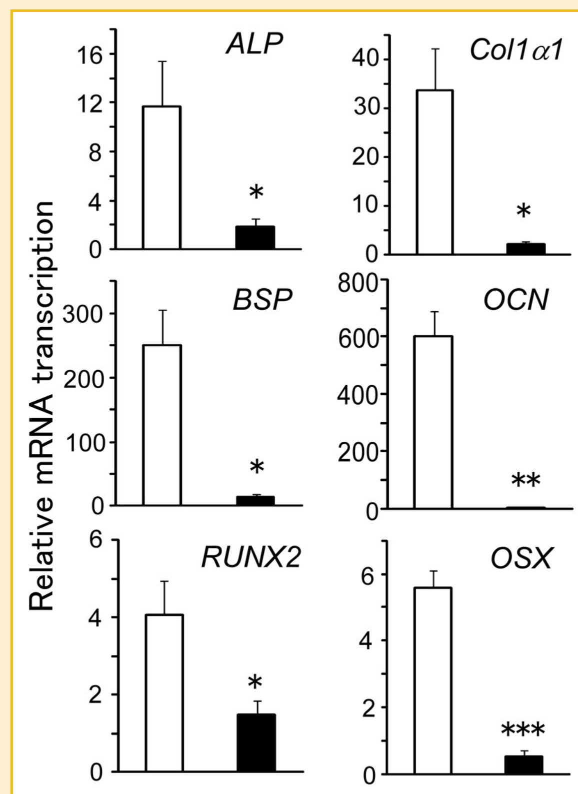

Fig. 2. LIF suppressed the expression of osteoblast differentiation markers. mRNA extracted from BMSCs cultured in osteogenic induction medium with LIF (50 ng/ml: black bar) or without LIF (white bar) was reverse transcribed in three independent experiments. Samples were subjected to quantitative real-time PCR using specific primers for *OCN*, *BSP*, *ALP*, *Col1α1*, *Runx2*, and *OSX*. mRNA expression levels were normalized to L32 expression levels. Data are expressed as mean ± SEM. Asterisks indicate significant differences: $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.005$ (***).

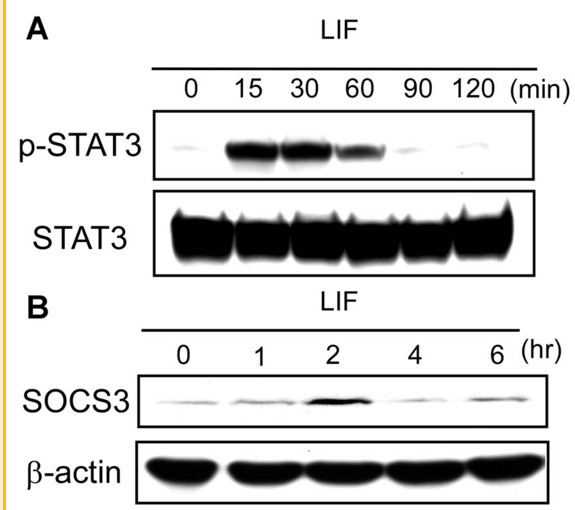

Fig. 3. LIF induces STAT3 phosphorylation and SOCS3 expression. BMSCs were stimulated with LIF (50 ng/ml) at the indicated time points. (A) STAT3 phosphorylation. (B) SOCS3 expression levels were determined by western blotting. Data are representative of three different experiments.

is recognized as the downstream signaling molecule of LIF/STAT3 signaling. To confirm the results of GeneChip analysis, we analyzed the SOCS3 protein expression level. SOCS3 expression was observed 2 h after LIF stimulation (Fig. 3B). Thus, the LIF/STST3 signaling pathway induced SOCS3 expression in BMSCs.

LIF EFFECTS ARE ABROGATED BY SOCS3 KNOCKDOWN

To evaluate the function of SOCS3 induced by the LIF/STAT3 signaling pathway, we performed a *SOCS3* knockdown experiment. First, a reduction in the SOCS3 protein level was confirmed by Western blotting (Fig. 4A). Although LIF suppressed CFU-O development, shSOCS3-BMSCs cultured with LIF showed a capacity to develop CFU-Os (Fig. 4B). The number of CFU-Os from shSOCS3-BMSCs cultured with LIF was almost the same as that from BMSCs cultured without LIF (Fig. 4C). These results suggested that SOCS3 transmits the LIF suppressive effect.

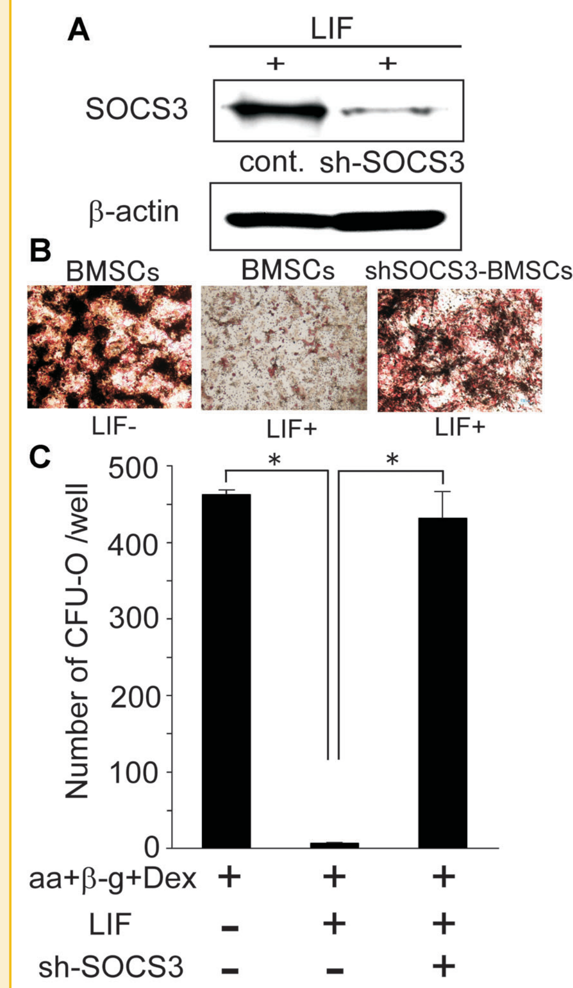

Fig. 4. shRNA-mediated SOCS3 knockdown experiment. (A) BMSCs were infected with lentivirus shSOCS3 and stimulated with LIF (50 ng/ml) for 2 h. The cells were lysed and western blotting was performed. (B) BMSCs and shSOCS3-BMSCs were cultured in osteogenic induction medium with or without LIF (50 ng/ml). The cells were fixed at day 21 and double stained for ALP activity and mineral deposition (von Kossa staining), ×100. (C) CFU-Os were counted. Similar results were obtained from three independent experiments. Data are expressed as mean ± SEM. Asterisks indicate significant differences: $P < 0.05$ (*).

TABLE I. Genes With Significantly Upregulated Transcription Levels

Gene symbol	Gene title	Accession	Fold change
Ccl12	Chemokine (C-C motif) ligand 12	U50712	3.6
Ccl8	Chemokine (C-C motif) ligand 8	NM_021443	3.2
Tnfsf11	Tumor necrosis factor (ligand) superfamily, member 11	NM_011613	3.2
Ccl7	Chemokine (C-C motif) ligand 7	AF128193	2.8
Socs3	Suppressor of cytokine signaling 3	BB241535	2.7
Il4ra	Interleukin 4 receptor, alpha	AF000304	2.6
Socs3	Suppressor of cytokine signaling 3	NM_007707	2.4
Gda	Guanine deaminase	AW911807	2.4
Ednrb	Endothelin receptor type B	BF100813	2.4
Csgalnact1	Chondroitin sulfate N-acetyl galactosaminyltransferase 1	AV371987	2.3
Fgl2	Fibrinogen-like protein 2	BF136544	2.3
Ccl2	Chemokine (C-C motif) ligand 2	AF065933	2.2
Cd244	CD244 natural killer cell receptor 2B4	NM_018729	2.2

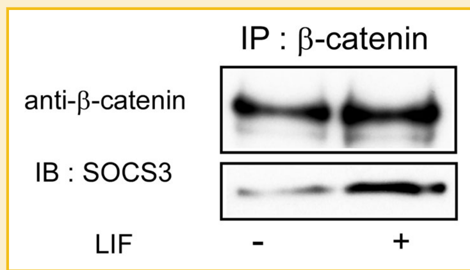

Fig. 5. Interaction between SOCS3 and β-catenin. BMSCs were stimulated with LIF (50 ng/ml) for 2 h, and whole cell lysates were immunoprecipitated with anti-β-catenin antibody conjugated to Sepharose beads. The precipitate was immunoblotted with anti-SOCS3 antibody. Data are representative of three different experiments.

SOCS3 INTERACTS WITH β-CATENIN

The Wnt/β-catenin signaling pathway is known to play a critical role in osteoblast differentiation. We investigated whether LIF stimulation promoted an interaction between SOCS3 and β-catenin. Immunoprecipitation experiments demonstrated that LIF induced a SOCS3—β-catenin interaction (Fig. 5).

LIF SUPPRESSES β-CATENIN PROTEIN EXPRESSION

Although Wnt3a stimulation alone upregulated the β-catenin protein expression level, stimulation with both Wnt3a and LIF reduced β-catenin expression (Fig. 6). These results suggested that LIF stimulation-induced SOCS3 interacts with β-catenin and degrades it.

DISCUSSION

LIF is a pleiotropic cytokine expressed by multiple tissue types. The functional analysis of LIF for osteoblast differentiation has been performed using fetal rat calvarial cells, which showed that LIF suppressed osteoblast differentiation and induced adipocyte differentiation [Falconi et al., 2007; Falconi and Aubin, 2007]. On the other hand, previous in vivo studies showed that LIF induces bone formation [Cornish et al., 1993; Poulton et al., 2012]. Although the precise reasons for the discrepancy between the in vivo and in vitro effects of LIF on osteoblast differentiation are currently unknown, one possibility is that, in in vitro studies, cells isolated from the bone marrow were grown in a simple, supplemented culture medium (e.g., osteogenic induction medium) despite the existence of numerous actions and interactions of cells and proteins (e.g., cytokines in an in vivo microenvironment). Although all of the analyses in this study were performed in vitro, we have clearly demonstrated that LIF directly suppressed osteoblast differentiation. Because the function of LIF in murine BMSCs remains unclear, we analyzed its role in osteoblast differentiation in murine BMSCs. LIF almost completely suppressed CFU-O development, similar to that observed previously in the fetal rat calvarial cell experiments (Fig. 1). In this report, we evaluated mineralization not only by the formation of CFU-O, which was observed by double staining the cells with ALP and von Kossa, but also by the expression levels of mineralization markers such as *ALP* and *OCN*, because the previous study showed that von Kossa-positive colonies of MC3T3-E1 did not display a significant amount of minerals [Bonewald et al., 2003]. LIF almost completely suppressed not only ALP/von Kossa double-positive colony development but also *ALP* and *OCN* expression. Previous reports had only demonstrated the LIF phenotype for osteoblast differentiation; however, they had not clarified the mechanism underlying the LIF suppressive effect. Thus, to determine the mechanism underlying the LIF suppressive effect on osteoblast differentiation, we analyzed the LIF signaling pathway in murine BMSCs. When LIF binds to its receptors, gp130 and LIFRβ, activated JAK tyrosine kinases phosphorylate STAT3. We first checked the activation status of STAT3 in BMSCs after LIF stimulation and confirmed STAT3 phosphorylation· The LIF/STAT3 signaling pathway has multiple roles in several endocrine functions such as bone cell metabolism [Auernhammer and Melmed, 2000]. Because LIF/STAT3 signaling can induce the expression of several target genes, we were prompted to narrow down our search to critical target genes that impart suppressive effects on osteoblast differentiation after LIF stimulation. GeneChip analysis was performed to narrow down the search for target genes of the LIF/STAT3 signaling pathway in BMSCs, resulting in the identification of *SOCS3* (Table I). SOCS3 is known to inhibit the JAK/STAT3 signaling pathway by disrupting JAK kinase activity. We performed the *SOCS3* knockdown experiment to determine the exact function of SOCS3 in the LIF/STAT3 signaling pathway. When BMSCs were cultured in osteogenic induction medium with LIF, only CFU-ALPs and not CFU-Os were observed. In contrast, the number of CFU-Os from shSOCS3-BMSCs cultured with LIF was almost the same as that from BMSCs cultured with only osteogenic induction medium. These results thus suggest that SOCS3 plays a critical role in the LIF suppressive effect.

The results of the *SOCS3* knockdown experiment suggested that LIF transmitted the LIF suppressive effect to signaling pathway molecules regulating osteoblast differentiation, such as the Wnt/β-catenin signaling pathway. The Wnt/β-catenin signaling pathway has a critical role in osteoblast differentiation [Hoeppner et al., 2009; Kubota et al., 2009]. Binding of Wnt proteins to their receptors, frizzled proteins, and essential co-receptors of the low-density lipoprotein receptor-related protein family activates a conserved "canonical" signaling pathway that results in the stabilization of cytoplasmic β-catenin, its translocation to the nucleus, and the

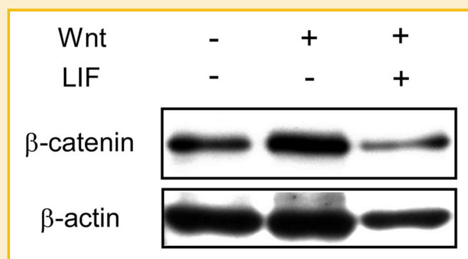

Fig. 6. LIF decreased the β-catenin protein expression level. BMSCs were stimulated with Wnt3a with or without LIF (50 ng/ml) for 2 h. Whole cell lysates were immunoblotted with anti-β-catenin and anti-β-actin antibodies. Data are representative of three different experiments.

formation of active transcription complexes between β-catenin and members of the LEF/TCF family of DNA-binding factors [Wodarz and Nusse, 1998; Bejsovec, 2000]. *SOCS3* harbors a segment called the SOCS box, which is located near the C terminal. Through the SOCS box, SOCS proteins associate with the elongin B and C complex to recruit ubiquitin ligase complexes [Kamura et al., 1998] and subsequently mark their targets for degradation through the ubiquitin pathway. We hypothesized that SOCS3 could bind to β-catenin. The immunoprecipitation experiment demonstrated that LIF stimulation-induced SOCS3 interacted with β-catenin. Furthermore, the β-catenin protein level was reduced in BMSCs after stimulation with both Wnt3a and LIF. Thus, these results suggest that LIF stimulation-induced SOCS3 interacts with β-catenin and degrades it, thus suppressing osteoblast differentiation. On the other hand, it has also been reported that β-catenin functions as an adaptor molecule by connecting E-cadherin and cytoskeletal proteins [Xu and Kimelman, 2007; Nelson, 2008; Schmalhofer et al., 2009; Fu and Jiang, 2010]. The inhibition of cell–cell contacts has a suppressive effect on differentiation [Soncin et al., 2009]. The reduction in the β-catenin protein level caused by SOCS3 could have inhibited cell–cell communication, and thus suppressed osteoblast differentiation. Although we clearly demonstrated that LIF has a suppressive effect on osteoblast differentiation, we should carefully evaluate the effects of LIF and conduct further analysis because it has been recently reported that culture conditions can also influence gene expression in BMSCs [Lee et al., 2013].

The results of our study demonstrated that LIF/STAT3/SOCS3 signaling regulates osteoblast differentiation by degrading β-catenin. This finding not only provides a new perspective for understanding the mechanism underlying osteoblast differentiation but also for regenerative medicine applications, including those in the fields of orthopedics or dentistry.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-aid for Scientific Research (24689070 and 25670809) from the Japan Society for the Promotion of Science.

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