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Journal of Cellular Biochemistry 114:2032–2038 (2013)

Journal of Cellular Biochemistry

SOCS-1/3 Participation in FGF-2 Signaling to Modulate RANK Ligand Expression in Paget's Disease of Bone

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ABSTRACT

Paget's disease of bone (PDB) is a chronic focal skeletal disorder characterized by excessive bone resorption followed by disorganized new bone formation. Measles virus nucleocapsid (MVNP) is implicated in pathogenesis of PDB. RANK ligand (RANKL), a critical osteoclastogenic factor expressed on bone marrow stromal/preosteoblast cells is upregulated in PDB. We recently demonstrated that fibroblast growth factor-2 (FGF-2) which induces RANKL expression is elevated in PDB. In this study, we hypothesized that FGF-2 modulates suppressors of cytokine signaling (SOCS) to induce RANKL expression in PDB. We identified increased levels of SOCS-1/3 mRNA expression in bone marrow mononuclear cells derived from patients with PDB compared to normal subjects. Interestingly, conditioned media obtained from MVNP transduced osteoclast progenitor cells significantly increased SOCS-1/3 mRNA expression in stromal/preosteoblast cells. We next examined if SOCS participates in FGF-2 signaling to modulate RANKL gene expression. We showed that FGF-2 stimulation significantly increased SOCS-1/3 expression in human bone marrow stromal/preosteoblast cells. In addition, co-expression of SOCS-1/3 with hRANKL gene promoter-luciferase reporter plasmid in marrow stromal cells demonstrated a significant increase in promoter activity without FGF-2 stimulation. Furthermore, siRNA inhibition of STAT-1 suppresses FGF-2 increased SOCS-1/3 expression in these cells. Thus, our results suggest that SOCS participates in FGF-2 modulation of RANKL expression in PDB. J. Cell. Biochem. 114: 2032–2038, 2013.

KEY WORDS: PAGET's DISEASE OF BONE (PDB); MEASLES VIRUS NUCLEOCAPSID PROTEIN (MVNP); SUPPRESSORS OF CYTOKINE SIGNALING (SOCS); FGF-2; RANK LIGAND (RANKL)

Paget's disease of bone (PDB) is a chronic focal skeletal disorder which affects 2–3% of the population over the age of 60. PDB is an autosomal dominant trait with genetic heterogeneity and recurrent mutations have been identified in sequestome 1 (SQSTM1/p62) ubiquitin-associated (UBA) domain [Laurin et al., 2002]. The disease is characterized by abnormal osteoclast development and increased bone resorption activity followed by exaggerated osteoblast response resulting in poor quality new bone formation. Pagetic osteoclasts demonstrate the presence of paramyxoviral-like nuclear inclusions. Measles virus nucleocapsid (MVNP) is implicated in pathogenesis of PDB [Reddy et al., 2001]. MVNP has been shown to induce pagetic phenotype in osteoclasts [Kurihara et al., 2006]. However, others have been unable to detect paramyxoviral transcripts in patients with PDB [Helfrich et al., 2000; Matthews et al., 2008]. Osteotropic factors/cytokines such as 1,25-$(OH)_2D_3$, parathyroid hormone (PTH), interleukin-1β (IL-1β), IL-11, and prostaglandin E2 induce osteoclast differentiation through enhanced expression of RANK ligand (RANKL) in bone marrow stromal/preosteoblast cells [Nakashima et al., 2000; Lee et al., 2002], however the molecular mechanisms which regulate RANKL gene expression in PDB are unclear. It has been shown that IL-1β and TNF-α stimulate RANKL expression in human bone marrow stromal cells through activation of p38 MAP kinase signaling pathway [Rossa et al., 2006]. PTH stimulates RANKL expression through cAMP/protein kinase A/CREB cascade [Fu et al., 2006]. In addition, fibroblast growth factor-2 (FGF-2) induces RANKL production through COX-2-mediated prostaglandin synthesis in murine osteoblast cells [Nakagawa et al., 1999]. Lipopolysaccharide treatment increased the levels of RANKL expression through activation of Toll-like receptors in primary murine osteoblast cells [Kikuchi et al., 2001]. Furthermore, transforming growth factor β (TGF-β) has been shown to increase RANKL expression in activated T cells [Wang et al., 2002]. FGF-2 promotes human bone marrow stromal cell proliferation and maintenance of osteogenic precursors [Martin et al., 1997]. We

The authors have no conflict of interest.

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Manuscript Received: 28 january 2013; Manuscript Accepted: 12 March 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 28 March 2013

DOI 10.1002/jcb.24554 • © 2013 Wiley Periodicals, Inc.

recently demonstrated elevated levels of FGF-2 are associated with PDB and that FGF-2 signaling induces RANKL expression in bone marrow stromal/preosteoblast cells [Roccisana et al., 2004; Sundaram et al., 2009].

Suppressors of cytokine signaling (SOCS) family consists of eight members (SOCS-1 to 7 and CIS) that contains a central SH2 domain and C-terminal SOCS box [Masuhara et al., 1997]. Several studies have reported that SOCS participate in a negative feedback loop modulating cytokine mediated signaling pathways [Yoshimura, 1998; Greenhalgh and Hilton, 2001]. SOCS protein plays an important role in the regulation of several tyrosine kinase receptor signaling pathways and has been shown that SOCS binds to the epidermal growth factor receptor (EGFR), insulin receptor, c-kit, and EPO and can facilitate proteosomal degradation [Xia et al., 2002]. SOCS-1/3 have been shown to interact and modulate FGF receptor signaling [Ben-Zvi et al., 2006]. However, SOCS participation in FGF-2 regulation of RANKL expression in bone marrow stromal/preosteoblast cells is unknown. In this study, we demonstrated that SOCS-1/3 participates in FGF-2 modulation of RANKL expression in PDB.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Normal human bone marrow monocytes were obtained from Stem Cell Technologies, Inc. (Vancouver, Canada). Cell culture and DNA transfection reagents were purchased from Invitrogen (Carlsbad, CA). Human recombinant FGF-2 and anti-RANKL antibody were purchased from R&D systems, Inc. (Minneapolis, MN). Anti-rabbit-SOCS-1/3, siRNAs and peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Super-signal enhanced chemiluminescence (ECL) reagent was obtained from Amersham Bio-Science (Piscataway, NJ), and nitrocellulose membranes were purchased from Millipore (Bedford, MA). A luciferase reporter assay system was obtained from Promega (Madison, WI).

RETROVIRAL EXPRESSION OF MVNP

We have previously developed a retroviral plasmid construct, pILXAN#1 that transcribes MVNP mRNA expression under the control of 5 LTR viral promoter elements [Kurihara et al., 2000]. The recombinant plasmid construct was transfected into the PT67 amphotropic packaging cell line using the lipofectamine reagent (Invitrogen, Corp.). Stable clonal cell lines producing MVNP recombinant retrovirus at high titer (1×10^6 virus particles/ml) were established by selecting for resistance to neomycin (600 μg/ml). Similarly, a control retrovirus producer cell line was established by transfecting the cells with the pLXSN empty vector (EV). Producer cell lines were maintained in DMEM containing 10% fetal calf serum (FCS), 100 U/ml each of streptomycin and penicillin, 4 mM L-glutamine, and high glucose (4.5 g/L). Normal human bone marrow-derived non-adherent mononuclear cells were transduced with EV or MVNP retroviral supernatants (20%) from the producer cell lines in the presence of polybrene (4 μg/ml) and serum free conditioned media (CM) collected after 48 h was used for further experiments. All human samples were obtained following the Institutional Review Board approved protocol at the Medical University of South Carolina.

LUCIFERASE REPORTER GENE ASSAY

Normal human bone marrow derived SAKA-T stromal cells were cultured in α-minimum essential medium (MEM) supplemented with 10% FCS, 100 units/ml penicillin/streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. DNA transfections were performed using lipofectamine transfection reagent. We have previously developed hRANKL gene promoter (+1 to −2 kb relative to the transcription start site)-luciferase reporter plasmid construct (hRANKL P#3) as described [Roccisana et al., 2004]. Cells were transiently co-transfected with the hRANKL P#3 reporter construct and SOCS-1/3 expression plasmids or siRNA. Cells were cultured in the presence or absence of FGF-2 (4 ng/ml) for 48 h. A 20 μl of total cell lysates of each sample was mixed with 100 μl of the luciferase assay reagent. The light emission was measured for 10 s of integrated time using Sirius Luminometer. The transfection efficiency was normalized by co-transfection with pRSV β-gal plasmid and measuring the β-galactosidase activity in these cells.

WESTERN BLOT ANALYSIS

SAKA-T cells and homogeneous population of normal human bone marrow derived primary stromal/preosteoblast cells isolated as described [Roccisana et al., 2004] were seeded (5×10^5 cells/well) in six well plates and supplemented with α-MEM containing 10% FCS. After 24 h, cells were stimulated with FGF-2 (4 ng/ml) for 48 h and lysed in a buffer containing 20 mM Tris–HCl at pH 7.4, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 150 mM NaCl, 0.1 mM Na_3VO_4, and 1× protease inhibitor cocktail. The protein content of the samples was measured using the BCA protein assay reagent (Pierce, Rockford, IL). Protein (20 μg) samples were then subjected to SDS–PAGE using 12% Tris–HCl gels and blot transferred on to a nitrocellulose membrane, immunoblotted with anti-RANKL, anti-SOCS-1/3 antibodies. The bands were detected using the ECL detection system. The band intensity was quantified by densitometric analysis using the NIH ImageJ Program.

siRNA Interference

Bone marrow stromal/preosteoblast cells were seeded (5×10^5 cells/well) in six well plates and supplemented with α-MEM containing 10% FCS. After 24 h, cells were co-transfected with hRANKL promoter-luciferase reporter plasmid (hRANKL P#3; 2 μg) and in the presence or absence of siRNA (20 nM) against SOCS-1 and SOCS-3 by lipofectamine. Cells were cultured with or without FGF-2 (4 ng/ml) for 48 h period. Luciferase activity levels were measured in the total cell lysates and siRNA suppression of SOCS expression was confirmed by Western blot analysis.

REAL-TIME RT-PCR

SOCS-1/3 and RANKL mRNA expression levels were determined by quantitative real-time reverse transcription-PCR as described previously [Sundaram et al., 2007]. Briefly, total RNA was isolated from bone marrow derived monocytes from normal ($n = 10$) and patients with PDB ($n = 6$) and human bone marrow derived stromal/preosteoblast cells stimulated with and without FGF-2 (4 ng/ml) for 48 h, using RNAzol reagent (Biotecx labs, TX). Reverse transcription reaction was performed using poly-dT primer and Moloney murine

leukemia virus reverse transcriptase in 25 µl reaction volume containing total RNA (2 µg), 1× PCR buffer and 2 mM $MgCl_2$, at 42°C for 15 min followed by 95°C for 5 min. Real-time PCR was performed using IQ™ SYBR Green Supermix in an iCycler (iCycler iQ Single-color Real Time PCR detection system; Bio-Rad, Hercules, CA). The primer sequences used to amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were 5′-CCTACCCC-CAATGTATCCGTTGTG-3′ (sense) and 5′-GGAGGAATGG-GAGTTGCTGTTGAA-3′ (anti-sense); hRANKL mRNA were 5′-ACCAGCATCAAAATCCCAAG-3′ (sense) and 5′-TAAGGAGTTG-GAGACCT-3′ (anti-sense); SOCS-1, mRNA were 5′-AGACCCCTTCT-CACCTCTTG-3′ (sense) and 5′-GCACAGCAGAAAAATAAAGC-3′ (anti-sense); SOCS-3 mRNA were 5′-CCCGCCGGCACCTTTCTG-3′ (sense) and 5′-AGGGGCCGCCTCAACACC-3′ (anti-sense). Thermal cycling parameters were 94°C for 3 min, followed by 40 cycles of amplifications at 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and 72°C for 5 min as the final elongation step. Relative levels of mRNA expression were normalized in all the samples analyzed with respect to the levels of GAPDH amplification.

STATISTICAL ANALYSIS

Results are presented as mean ± SD for three independent experiments and compared by Student *t*-test. Values were considered significant at $^{*}P < 0.05$.

RESULTS

SOCS-1/3 EXPRESSION IN PAGET'S DISEASE OF BONE (PDB)

SOCS modulates cytokine signaling. The SOCS-1/3 expression has been shown to increase during osteoclast differentiation [Hayashi et al., 2002; Fox et al., 2003]. However, the SOCS expression and a functional role in RANKL regulation in PDB is unknown. Therefore, we examined the mRNA expression of SOCS-1/3 in bone marrow mononuclear cells derived from normal and patients with PDB. Real-time PCR analysis of total RNA isolated from bone marrow mononuclear cells demonstrated that SOCS-1/3 mRNA expression levels were significantly increased in patients with PDB (n = 6) compared to normal subjects (n = 10; Fig. 1A). We have previously identified expression of MVNP transcript in bone marrow cells and peripheral blood-derived monocytes from patients with PDB [Reddy et al., 2001]. We further examined whether CM obtained from MVNP transduced normal human bone marrow derived non-adherent cells regulates SOCS mRNA expression in stromal/preosteoblast cells. Normal human bone marrow derived SAKA-T stromal/preosteoblast cells were stimulated with EV and MVNP CM (20%) for 48 h. Total RNA isolated from these cells was subjected to real-time PCR analysis for SOCS-1/3 mRNA expression. As shown in Fig. 1B, MVNP CM significantly increased SOCS-1 (5.8-fold) and SOCS-3 (4.2-fold) mRNA expression compared to EV CM treated cells. These results suggest that SOCS-1/3 expression is upregulated in PDB.

FGF-2 ENHANCES SOCS-1/3 EXPRESSION IN STROMAL/PREOSTEOBLAST CELLS

We recently identified elevated levels of FGF-2 are associated with PDB and that MVNP transduced human bone marrow mononuclear cells demonstrated increased FGF-2 levels [Sundaram et al., 2009]. It has been shown that SOCS-1/3 modulates FGF receptor (FGFR) signaling in chondrocytes [Ben-Zvi et al., 2006]. We therefore examined whether FGF-2 regulates SOCS expression in stromal/preosteoblast cells. Primary human bone marrow derived stromal/preosteoblast cells were stimulated with and without FGF-2 (4 ng/ml) for 0–48 h. Total cell lysates obtained were subjected to Western blot analysis for SOCS-1/3 and RANKL expression. As shown in Figure 2A. FGF-2 induced SOCS-1/3 and RANKL expression in a time-dependent manner. Real-time PCR analysis of total RNA isolated from these cells further demonstrated that FGF-2 stimulation significantly increased RANKL (6.2-fold), SOCS-1 (5.3-fold) and SOCS-3 (4.8-fold) mRNA expression (Fig. 2B). Confocal microscopy analysis further confirmed that FGF-2 increased SOCS-1/3 protein

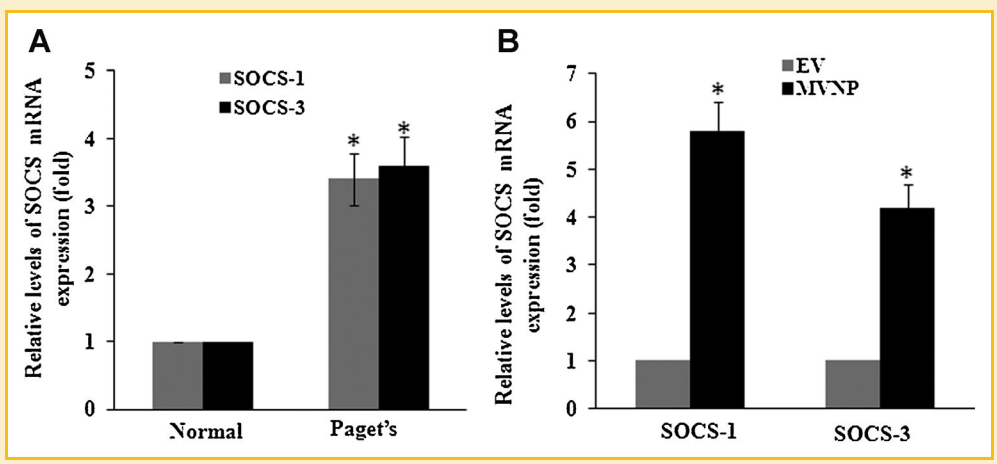

Fig. 1. SOCS-1/3 mRNA expression in PDB. Real-time PCR analysis of total RNA isolated from (A) normal human bone marrow mononuclear cells and patients with PDB. (B) Human bone marrow derived stromal/preosteoblast SAKA-T cells were stimulated with 20% of EV and MVNP conditioned media (CM) for 48 h and total RNA isolated was subjected to real-time PCR for SOCS-1/3 mRNA expression as described in methods. The data are shown as mean ± SD for three independent experiments ($^{*}P < 0.05$).

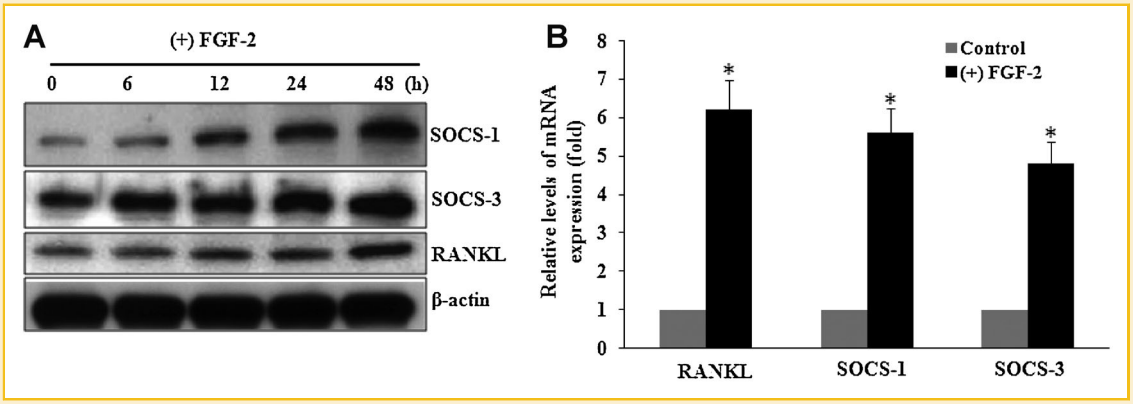

Fig. 2. FGF-2 upregulates SOCS-1/3 expression in stromal/preosteoblast cells. (A). Primary normal human bone marrow derived stromal/preosteoblast cells were stimulated with FGF-2 (4 ng/ml) for variable time point (0–48 h). Total cell lysates were subjected to Western blot analysis for SOCS-1/3 and RANKL expression. β-Actin expression served as loading control. (B) Total RNA isolated from these cells was subjected to real-time PCR analysis for SOCS-1/3 and RANKL mRNA expression. Values are expressed as mean ± SD for three independent experiments ($^{*}P < 0.05$).

expression in stromal/preosteoblast cells (Fig. 3). These results indicate that FGF-2 modulates SOCS-1/3 expression in human bone marrow stromal/preosteoblast cells.

SOCS REGULATES RANKL EXPRESSION

We previously reported that FGF-2 stimulates hRANKL gene promoter activity in stromal/preosteoblast cells [Roccisana et al., 2004]. Therefore, we further tested if SOCS play a functional role in FGF-2 modulation of RANKL gene promoter activity. Human bone marrow derived SAKA-T stromal cells were transiently transfected with hRANKL promoter–luciferase reporter plasmid (hRANKL P#3) and co-transfected with SOCS-1/3 expression vectors or siRNA against SOCS-1 and 3. Cells were stimulated with and without FGF-2 for 48 h and total cell lysates obtained were analyzed for luciferase activity. As shown in Figure 4A, SOCS-1/3 over-expression significantly increased the hRANKL gene promoter activity in the absence of FGF-2 stimulation. Furthermore, siRNA suppression of SOCS-1 and 3 expression inhibits FGF-2 increased hRANKL gene promoter activity in these cells (Fig. 4B). These results suggest that SOCS-1/3 participates in FGF-2 induced RANKL gene expression in bone marrow stromal/preosteoblast cells.

STAT-1 CONTROL OF SOCS-1/3 EXPRESSION

We recently demonstrated that STAT-1 is a downstream effector of FGF-2 signaling to stimulate RANKL expression in PDB [Sundaram et al., 2009]. Therefore, we further determined if STAT play a role in

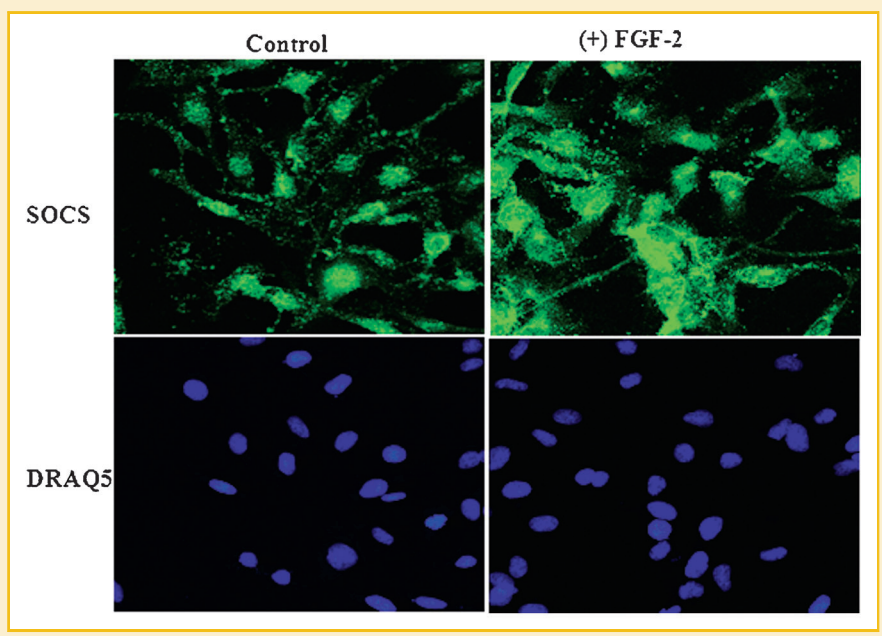

Fig. 3. Confocal microscopy analysis for SOCS expression in SAKA-T stromal cells. Normal human bone marrow derived stromal cells (SAKA-T) were stimulated with FGF-2 (4 ng/ml) for 24 h. Immunostaining for SOCS-1/3 was performed using Alexa 488 conjugated anti-rabbit IgG and nuclear staining by DRAQ5.

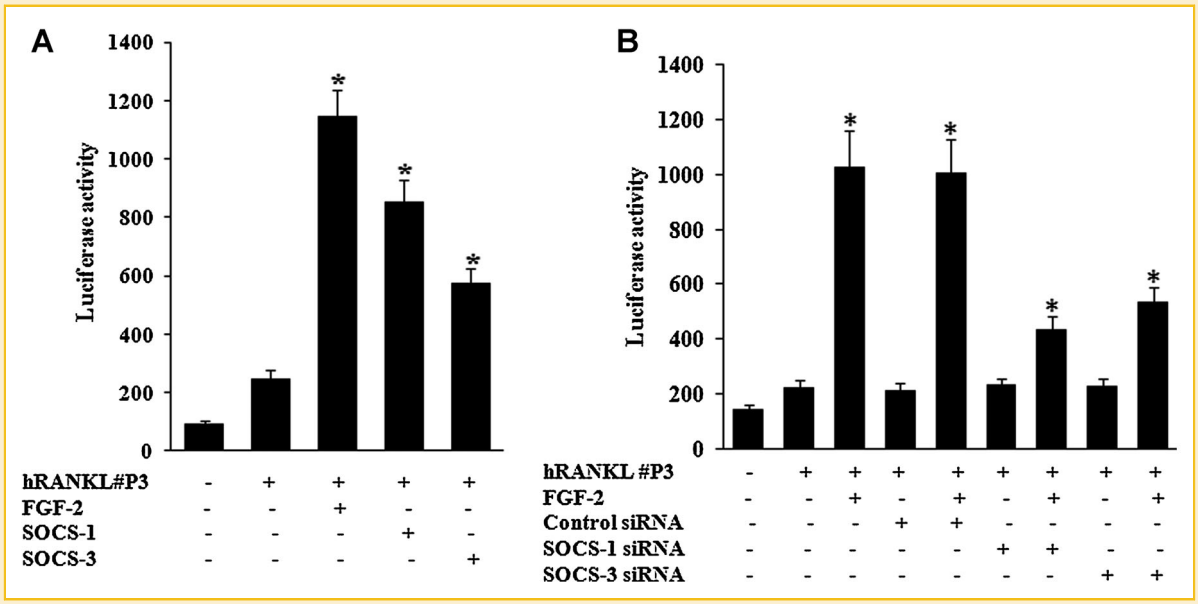

Fig. 4. Participation of SOCS in FGF-2 modulation of hRANKL gene promoter activity. (A). SAKA-T cells were co-transfected with hRANKL promoter-luciferase reporter plasmid (hRANKL P#3) with SOCS-1/3 expression plasmids. (B). SAKA-T cells were co-transfected with hRANKL P#3 with siRNA against SOCS-1/3. Cells were stimulated with and without FGF-2 (4 ng/ml) for 48 h and total cell lysates were assayed for luciferase activity. The transfection efficiency was normalized by β-galactosidase activity co-expressed in these cells. Values are expressed as mean ± SD for three independent experiments ($^{*}P < 0.05$).

FGF-2 modulation of SOCS expression in bone marrow stromal/preosteoblast cells. Human bone marrow derived SAKA-T stromal cells were transfected with siRNA against STAT-1 and stimulated with FGF-2 (4 ng/ml) for 48 h. Total cell lysates obtained were subjected to Western blot analysis for SOCS-1/3 expression. As shown in Fig. 5, siRNA suppression of STAT-1 expression inhibits FGF-2 enhanced SOCS-1/3 expression in these cells. These results suggest that STAT-1 is a downstream effector of FGF-2 signaling to modulate SOCS-1/3 expression in bone marrow stromal/preosteoblast cells. Taken together, our results suggest that SOCS participates in the FGF-2 modulation of RANKL expression in PDB.

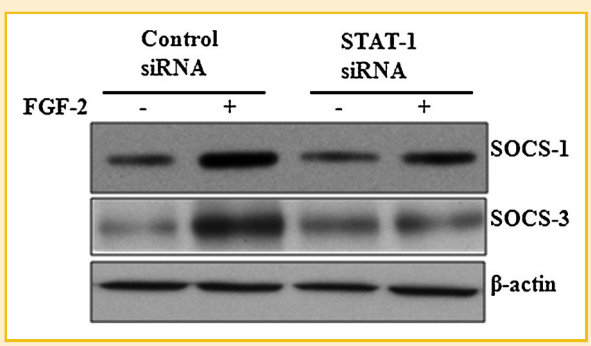

Fig. 5. STAT-1 is a downstream effector of FGF-2 signaling to increase SOCS-1/3 expression. SAKA-T cells were transfected with control and STAT-1 siRNA and stimulated with FGF-2 (4 ng/ml) for 48 h. Total cell lysates obtained were subjected to western blot analysis for SOCS-1/3 expression. β-Actin expression served as loading control.

DISCUSSION

This study demonstrates increased levels of SOCS-1/3 expression in pagetic bone marrow mononuclear cells, which implicate a functional role in cytokine signaling in PDB. Recently, we have identified elevated levels of FGF-2 which stimulates RANKL expression in bone marrow stromal/preosteoblast cells in PDB [Sundaram et al., 2009]. Our results that FGF-2 upregulate SOCS-1/3 expression suggest that SOCS may play a functional role in FGF-2 signaling to stimulate RANKL expression in human bone marrow stromal/preosteoblast cells. MVNP expression has been shown to induce pagetic osteoclast development [Kurihara et al., 2011]. We recently showed MVNP increases FGF-2 production in preosteoclast cells [Sundaram et al., 2009]. This study further demonstrate that MVNP CM induces SOCS-1/3 expression in human bone marrow stromal/preosteoblast cells suggesting that SOCS signaling may play an important role in FGF-2 modulation of RANKL expression in these cells. SOCS family proteins have previously identified as negative regulators of cytokine signaling to induce gene expression [Greenhalgh and Hilton, 2001]. It has also been shown that FGF inducible osteocalcin expression was elevated in rat osteosarcoma cells transfected with SOCS-1/3 [Ben-Zvi et al., 2006]. Therefore, it is more likely that SOCS modulates FGF-2 signaling in bone marrow stromal/preosteoblast cells. SOCS proteins could modulate gene expression in a cell specific manner [Cacalano et al., 2001]. FGF-2 has been shown to stimulate osteoclast formation in mouse bone marrow cultures through prostaglandin synthesis [Hurley et al., 1998]. However, our results do not delineate whether a prostaglandin pathway is involved in FGF-2 elevated SOCS expression in bone marrow stromal/preosteoblast cells. SOCS-3 expression in RANKL positive T cells has been correlated with

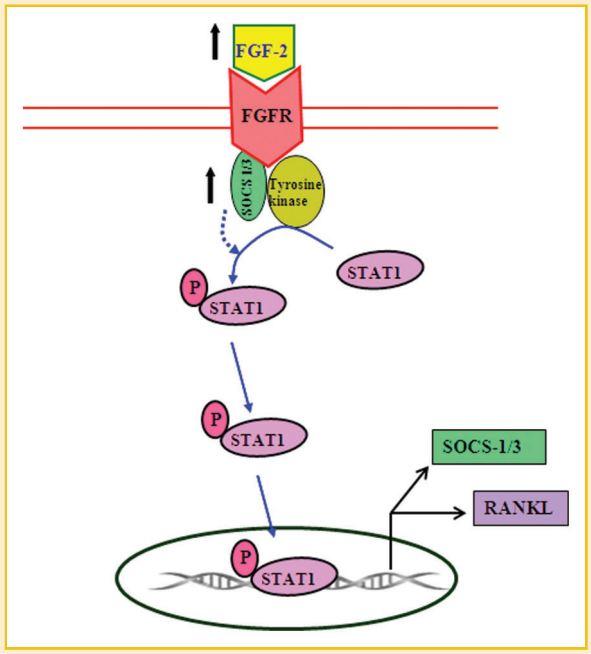

Fig. 6. Schematic illustration of SOCS-1/3 participation in FGF-2 modulation of RANKL expression in PDB. Patients with PDB demonstrate increased levels of FGF-2 and SOCS-1/3 expression. STAT-1 is a downstream effector of FGF-2 signaling to enhance SOCS and RANKL expression in bone marrow stromal/preosteoblast cells.

increased osteoclastogenesis and bone resorption in vivo [Zhang et al., 2009]. Reactive oxygen species have been shown to increase RANKL gene expression [Bai et al., 2005]. Also, Sp1 and Sp3 transcription factors are involved in basal RANKL gene expression in stromal/preosteoblast cells [Liu et al., 2005]. As evident from confocal microscopy, SOCS expression in naïve stromal/preosteoblast cells may play a role in basal level RANKL expression. Furthermore, SOCS inhibition significantly decreased FGF-2 stimulated hRANKL promoter activity which indicated participation of SOCS in FGF-2 control of RANKL gene transcription. Mouse RANKL expression has also been shown to be up-regulated by calcemic hormones such as 1,25-dihydroxyvitamin D_3 and PTH; however, these transcriptional regulatory regions are located long range (−76 kb in mouse and −96 kb in human) relative to the transcription start site [Fu et al., 2006; Kim et al., 2006]. We showed that FGF-2 signaling through STAT-1 induce RANKL expression in stromal/preosteoblast cells [Sundaram et al., 2009]. Furthermore, STAT-5 has been shown to regulate the proximal mouse RANKL gene promoter [Srivastava et al., 2003]. STAT transcription factors also modulate SOCS gene expression [Yoshimura, 1998]. Consistently, we identified that STAT-1 inhibition significantly decreased FGF-2 enhanced SOCS-1/3 expression in bone marrow stromal/preosteoblast cells. In addition to the genetic control of the mouse RANKL gene promoter, epigenetic control has also been demonstrated [Kitazawa and Kitazawa, 2002]. It has been reported that SOCS-1/3 are induced by CpG-DNA and modulates cytokine responses in macrophage cells [Dalpke et al., 2001]. Therefore, SOCS may play a role in epigenetic control of RANKL expression in stromal/preosteoblast cells.

Enhanced level of RANKL mRNA expression has been identified in bone marrow derived stromal/preosteoblast cells from patients with PDB [Menaa et al., 2000]. Given the pros and cons of paramyxovirus etiology for PDB, evidence is strong that CDV and MVNP protein expression play an important role in pathogenesis of PDB [Ralston and Layfield, 2012]. Recently, it has been shown that MVNP decreases Fox03/Sirt1 signaling to enhance the levels of IL-6 which in part contribute to pagetic osteoclast development [Wang et al., 2013]. However, IL-6 has been shown to stimulate RANKL expression in murine systems but not in human osteoblast cells [Hofbauer et al., 2000]. Thus, RANKL expression could be modulated by complex cytokine-mediated transcriptional regulatory mechanisms in pagetic lesions. In summary, our results suggest that SOCS is upregulated in PDB and that SOCS participates in FGF-2 stimulation of RANKL expression in stromal/preosteoblast cells (Fig. 6). These studies may provide molecular insights into new therapeutic targets to control excessive bone turnover in PDB.

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