




Volume 12, issue 2, June 2001 | Recherches

# Negative regulation of onconstatin M signaling by suppressor of cytokine signaling (SOCS-3)

## Abstract

Oncostatin M (OSM) is known to inhibit the growth of melanocytes and early-stage melanomas, but this ability is lost with melanoma progression. The biological effects of OSM involve the activation of Janus kinases (Jak) and signal transducer and activator of transcription (STAT) factors. Since SOCS (suppressor of cytokine signaling) a recently described family of regulatory proteins, has been shown to act through down-regulation of Jak-STAT signaling, we investigated their putative role in the inhibition of OSM signaling in the

human melanoma cell line A375. We observed that, among the SOCS family members examined, only SOCS-3 mRNA was strongly and rapidly induced by OSM. SOCS-3 protein was present within 1h and rapidly declined thereafter. Constitutive expression of SOCS-3 protein completely abolished the activation of the Jak-STAT signaling pathway as well as the Ras-MAP kinase pathway. As a result, A375 cells acquired an OSM-resistant phenotype. Our findings demonstrate that SOCS-3 is a potent regulator of OSM response and suggest that dysregulation of SOCS-3 expression could provide a mechanism for OSM resistance acquisition during tumour progression.

## Full text

### INTRODUCTION

OSM was originally identified by its ability to inhibit the growth of human A375 melanoma cells [1, 2]. OSM can exert opposite biological effects depending on cell types. For example, OSM stimulates the proliferation of Kaposi's sarcoma [3], muscle cells [4] and endothelial cells [5] while it inhibits the growth of melanoma [1, 2], breast carcinoma [6] and lung carcinoma [7]. OSM is a cytokine structurally and functionally related to the IL-6 family members that use gp130 as common signaling receptor subunit. This family includes IL-6, ciliary neurotrophic factor (CNTF), IL-11, cardiotrophin-1 (CT-1), leukaemia inhibitory factor (LIF) and a new member named novel neurotrophin-1/B cell stimulating factor-3 (NNT-1/BSF-3). The biological effects of OSM are mediated through two different types of high affinity receptor complexes. Type I OSM receptor is composed of the gp130-transducing protein associated with the LIF receptor chain (gp190). The use of this receptor complex is shared by LIF [8, 9], CT-1 [10], and CNTF [11]. The type II OSM receptor consists of the OSM specific receptor subunit (OSMRbeta) and the gp130 [12]. Stimulation of either OSM receptors leads to activation of cytokine receptor-associated Janus kinases family, Jak1, Jak2 and TYK2 [13], subsequent recruitment and activation of STAT1, STAT3 and STAT5 as well as the activation of extracellular-signaled-regulated protein kinase (ERK) mitogen-activated protein (MAP) kinase pathway [14-17]. However, the two OSM receptors appear to be functionally different since only type II receptor recruits STAT5b [18], and it is a more potent MAP kinase activator than type I receptor [14]. Expression of either receptors is cell type-dependent. HepG2 and Hela cell lines express type I and type II OSM receptor, while SKNMC and JAR cell lines only express type I OSM receptor and A375 cells only express

type II OSM receptor [12, 14, 18]. OSM has been described as a growth inhibitor for early-stage human melanomas and strikingly, this inhibitory effect is not found in the advanced stages of the disease [19]. Since a novel family of proteins referred to as SOCS/CIS/JAB/SSI-1 [20-24] has been shown to be involved in the down-regulation of cytokine signaling, we investigated their potential role in the suppression of OSM-induced growth inhibition in human melanoma cell line A375.

We found that SOCS-3 mRNA but not CIS, SOCS-1, SOCS-2 and CIS6 mRNA was strongly induced in response to OSM in A375 cells. Constitutive expression of SOCS-3 in A375 cells led to the suppression of activation of the Jak-STAT signaling pathway as well as the Ras-MAP kinase pathway. Consequently, these cells acquired an OSM-resistant phenotype. Our data demonstrate that SOCS-3 plays a key role in the control of OSM receptor type II signaling.

## MATERIALS AND METHODS

### *Cell culture and transfection*

A375 cells were grown in DMEM, 5% foetal calf serum, 1% glutamine. Full length human SOCS-3 was amplified by RT-PCR using the following primers; SOCS-3: sense 5'-GCGGATCCATGGTCACCCACAGCAAG, antisense 5'-GCGAATTCAAGCGGGGCATCGTACTGGT. The resulting fragments were cloned into the BamH1 and EcoR1 sites of the pcDNA3.1/His vector (Invitrogen Corp, San Diego, California, USA). DNA fragments were verified by double strand DNA sequence analysis. A375 cells ($5 \times 10^6$) were transfected by electroporation (at 900 muF and 300 V) with 20 mug of SOCS-3 cDNA. Transfected cells (SOCS-3) were maintained for 24 hours in a culture medium containing DMEM, 5% foetal calf serum, 1% glutamine, then cultured in the same medium with 0.8 mg/ml G418 (Sigma Chemical Co, St Louis, Missouri, USA).

### *RT-PCR experiments*

The first cDNA strand was synthesised using total RNA (5 mug) at 37° C for 1 hour in a 50 mul reaction mixture containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol (DTT), 3 mM $MgCl_2$, 5 U of RNase inhibitor (Gibco BRL, Gaithersburg, MD, USA), 0.5 mM of each deoxynucleotide triphosphate (dNTP), 400 U of MoMuLV reverse transcriptase (Gibco BRL) and 0.5 mug of oligo(dT) 15

mer. Five mul of the reaction mixture was made up to 50 mul using Taq polymerase buffer (10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$) containing 25 pmol of each primer, 1 mM of each deoxynucleotide triphosphate (dNTP), and 1 U of AmpliTaq DNA polymerase (Amersham Pharmacia Biotech). Amplifications were performed using a thermal cycler for 30 cycles under the following conditions: denaturation for 1 min at 94° C, annealing for 1 min at 55° C and elongation for 1 min at 72° C, with the exception of SOCS-1 amplification which was done in 5% DMSO. CIS was amplified using the following primers sense primer: 5'-GCGGATCCTTCTCCTACCTTCGGGAATCTG; and antisense primer: 5'-GCGAATTCGAGCTGGAAGGGGTACTGTC; SOCS-1 amplification with sense primer: 5'-ATGGTAGCACGCAACCAGGTG; and antisense primer: 5'-GTCAGATCTGGAAGGGGAAGG; SOCS-2 amplification with sense primer: 5'-CCACCATTTCGGACACCCTGC and antisense primer: 5'-CTTGTTGGTAAAGGCAGTCCC; SOCS-3 amplification with sense primer: 5'-GGTCACCACAGCAAGTTTCC; and antisense primer: 5'-ATCCAGGAACTCCCGAATGGG; CIS6 amplification with sense primer: 5'-CGTTCCAACCAGCGAACTCTAAC and antisense primer: 5'-GCCTGATTTGCTTCAAATTACAG [25]. PCR products were separated in a 1% agarose gel and directly visualised after ethidium bromide staining. Expression of beta-actin using primers: 5'-CGTGCTGCTGACCGAGGCC and antisense: 5'-TTCGTGGATGCCACAGGAC was assessed to ensure uniformity of amplification.

### *Reagents*

Human *E. coli* recombinant OSM was purchased from R&D Systems Inc, Minneapolis, Minnesota, USA. Anti-phosphoSTAT5(Tyr$^{694}$) antibody was purchased from Zymed Laboratories. Anti-phosphoSTAT3(Tyr$^{705}$), anti-phosphoSTAT3(Ser$^{727}$), anti-phosphoSTAT1(Tyr$^{701}$), anti-ERK1/2 and anti-phospho-ERK1/2(Thr$^{202}$/Tyr$^{204}$) antibodies were from New England Biolabs Inc, Beverly MA, USA. Anti-STAT5 and anti-STAT3 antibodies were from Transduction Laboratories, Lexington, Kentucky, USA. Anti-SOCS-3 (sc-7009) and anti-His (sc-803) antibodies were from Santa Cruz Biotechnology Inc. Santa Cruz, California, USA. Anti-tubulin (Amersham Pharmacia Biotech Europe GmbH, Orsay, France).

### *Apoptosis assays*

Parental A375 and A375 cells expressing SOCS-3 were seeded in 24-well microtiter plates at a density of 20 x $10^3$ cells in 500 mul per well. Cells were incubated (in a final volume of 600 mul) with serial dilutions of OSM (100 ng/ml). Assays were performed in duplicate. After incubation at 37° C for 96 hours, cellular proliferation was assessed by a (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based assay (Sigma Chemical Co.).

### *Western blot analysis*

Cells were washed in PBS and cultured overnight in DMEM, 1% glutamine, before stimulation with 100 ng/ml OSM. Cells were washed with PBS and whole cell or nuclear extracts were prepared as described elsewhere [25].

### *Flow cytometric analysis*

Parental A375 and A375 cells expressing SOCS-3 were incubated for 4 days with or without OSM. DNA fragmentation in apoptotic cells was vizualized by detection of biotinylated nucleotides incorporated onto the free 3'-ends of these DNA fragments using FlowTACS Apoptosis Detection Kit R&D Systems Inc. The samples were analysed on a FACSCAN from Becton Dickinson (Mountain View, California, USA).

## RESULTS

### *Rapid induction of SOCS-3 upon OSM stimulation in human A375 melanoma cells*

In order to examine the induction of transcription of five members of the SOCS gene family, A375 melanoma cells were deprived of serum for 12 hours and subsequently stimulated for the indicated time with OSM. RT-PCR was then performed to evaluate the expression of CIS, SOCS-1, SOCS-2, SOCS-3 and CIS6 mRNAs in human A375 melanoma cells (Figure 1A). In the absence of stimulation, little expression of the SOCS genes, with the exception of SOCS-2, was detectable in A375 cells. Upon stimulation with OSM, the SOCS-3 gene was strongly and rapidly induced, appearing at the first time point (30 min), peaking at 1 hour and decreasing thereafter. In contrast, CIS and SOCS-1 genes were only weakly induced and with delayed kinetics as compared to the SOCS-3 gene. Conversely, SOCS-2 gene expression was diminished upon OSM stimulation

suggesting that this protein plays a different biological role than other SOCS family members [26]. In the case of the CIS6 gene, no significant induction could be observed. Next, we examined the effect of OSM on the expression of SOCS-3 protein. Western blot using anti-SOCS-3 antibody showed that SOCS-3 protein reached a peak expression level at 1 hour after OSM stimulation, and declined rapidly thereafter (Figure 1B). The apparent size of the protein detected was consistent with the predicted molecular mass of SOCS-3 *i.e.* 32 kDa. Among the five SOCS family members studied, SOCS-3 protein appeared to be the major target for OSM stimulation in A375 cells.

### *Abrogation of OSM-induced growth inhibition in A375 cells expressing SOCS-3*

To further investigate the functional consequence of OSM-induced SOCS-3 expression, A375 cells were stably transfected with a cDNA encoding a N-terminally polyhistidine tagged version of SOCS-3. The level of expression of SOCS-3 protein was assessed using either anti-SOCS-3 or anti-His antibodies (Figure 2). We then examined the effect of this transfection on the growth inhibitory effect of OSM. As shown in Figure 3A, while OSM inhibited the proliferation of parental A375 cells, it was without effect on SOCS-3 transfected cells. Furthermore OSM-induced apoptosis observed in parental A375 cells, was not observed in transfectants expressing SOCS-3 (Figure 3B).

### *Suppression of OSM-induced STAT activation in A375 cells expressing SOCS-3*

The mechanism by which SOCS-3 inhibited OSM effects on A375 cells was further explored by analyzing the OSM-signaling pathways. Since it has been shown that STAT3 plays an essential role in gp130-mediated growth arrest of A375 cells [17], we studied the effect of SOCS-3 on OSM-induced STAT activation. As shown in Figure 4A, constitutive expression of SOCS-3 suppressed tyrosine-phosphorylation of STAT3 induced by OSM. Because tyrosine-phosphorylation of STAT3 is required for its dimerization, translocation to the nucleus and participation in transcriptional regulation by binding to specific DNA sites [27], we examined STAT3 levels in the nucleus of parental A375 cells or A375 cells expressing SOCS-3. Upon OSM-induction, nuclear STAT3 level in parental A375 cells was strongly increased, whereas it remained unchanged in A375 cells expressing SOCS-3 (Figure 4B). Since serine-

phosphorylation of STAT3 is required for its maximal transcriptional activity [28], we also investigated the effect of SOCS-3 on this process. As shown in Figure 4C, SOCS-3 transfection completely prevented OSM-induced serine-phosphorylation of STAT3. In addition to STAT3 inhibition, SOCS-3 strongly diminished tyrosine-phosphorylation of STAT1 and STAT5 induced by OSM (Figure 4D). These results together indicate that SOCS-3 is a key inhibitor of the Jak-STAT signaling pathway.

### *Suppression of OSM-induced ERK1/2 activation in A375 cells expressing SOCS-3*

Since OSM has been shown to be a strong activator of the Ras-MAP kinase pathway in cells expressing type II OSM receptor [14], we also explored the role of SOCS-3 in the regulation of OSM-induced MAP kinase activation in A375 cells. Western blot using total lysates purified from parental or SOCS-3 transfected A375 cells was performed with antibodies that specifically recognise the activated forms of ERK1/2 that are downstream effectors of the Ras-MAP kinase signaling pathway [29]. Our results showed that activation of ERK1/2 in parental A375 cells induced by OSM was completely suppressed in A375 cells overexpressing SOCS-3 (Figure 5). These data demonstrate that SOCS-3 is also a potent modulator of the Ras-MAP kinase pathway.

## DISCUSSION

The present study was conducted in order to confirm the molecular mechanisms that could control the growth-arresting action of OSM in the melanoma-derived cell line A375. We show that, OSM, through binding to its specific type II receptors, is a potent activator of STAT1, STAT3 and STAT5. In addition to Jak-STAT pathway activation, OSM also induced the Ras-MAP kinase signaling pathway. The recent characterisation of a family of cytokine-signaling inhibitors referred as SOCS/CIS, prompted us to investigate their potential role in the feedback regulation of OSM signaling in A375 cells. Among the five SOCS/CIS family members examined, only SOCS-3 was rapidly and strongly induced upon OSM stimulation. Constitutive expression of SOCS-3 strongly decreased STAT1, STAT3 and STAT5 activation as well as ERK1/2 activation. To our knowledge, these studies represent the first example of inhibition of both Jak-STAT and Ras-MAP kinase signaling pathways by SOCS-3 protein. The strong inhibitory effect of SOCS-3 on these two major transduction pathways

correlated with the acquisition of resistance to growth-inhibitory and pro-apoptotic effects. Our results clearly demonstrate that SOCS-3 plays a major role as negative feedback regulator of OSM signaling in A375 cells. We and others have recently shown that SOCS-3, through inhibition of the Jak-STAT pathway, is involved in the suppression of IL-11 signaling in the murine proB cell line, Ba/F3 [30], and in the inhibition of IL-6- and LIF-induced differentiation in the murine monocytic leukaemic cell line, M1 [31]. The mechanism of action of SOCS-3 is not clearly understood, although both its pre-SH2 and SH2 domains are suggested to be required [31]. Recently, it has been described that SOCS-3 binds to phosphotyrosine residue 1,007 within the activation loop of Jak2 and inhibits the kinase activity through a N-terminal kinase inhibitory region located in its pre-SH2 domain [32]. This observation is consistent with results of Haque *et al.* [33] who established that the pre-SH2 domain of SOCS-3 is necessary for inhibition of IL-4 signaling. Furthermore, recent studies have revealed that SOCS-3 binds with a high affinity to a phosphopeptide encompassing phosphotyrosine-759 in gp130, but not to phosphopeptides corresponding to regions of other gp130 cytoplasmic tyrosine residues or of known tyrosine phosphorylation sites of STAT1 and STAT3, or to phosphopeptides derived from the activation loop of Jaks 1-3 [34]. Phosphotyrosine-759 is required for inhibition of IL-6 signaling by SOCS-3 [34, 35], and is also essential for SHP2 recruitment and activation of the Ras-MAP kinase pathway [36]. These results together suggest that SOCS-3 suppresses IL-6 signaling by binding to gp130 through its SH2 domain and by inhibiting Jak kinase activity through its N-terminal kinase inhibitory region. This dual activity of SOCS-3 could explain the inhibition of STAT1, STAT3 and STAT5 activity as well as the inhibition of the Ras-MAP kinase pathway observed in A375 cells (Figures 4 and 5).

SOCS-3 plays a critical role in the inhibition of gp130-mediated IL-6-type cytokine signaling. Thus abnormal production of SOCS-3 could be one way to escape from IL-6- or OSM-induced growth arrest during melanoma progression. Further experiments are in progress to address this possibility.

## CONCLUSION

**Acknowledgements.** This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Centre National de la Recherche Scientifique (CNRS), La Ligue contre le Cancer (FM), the European

Union Biotechnology Research Programme; BIOT CT 972010 (OB), Roche Diagnostics (SD).

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## Figures

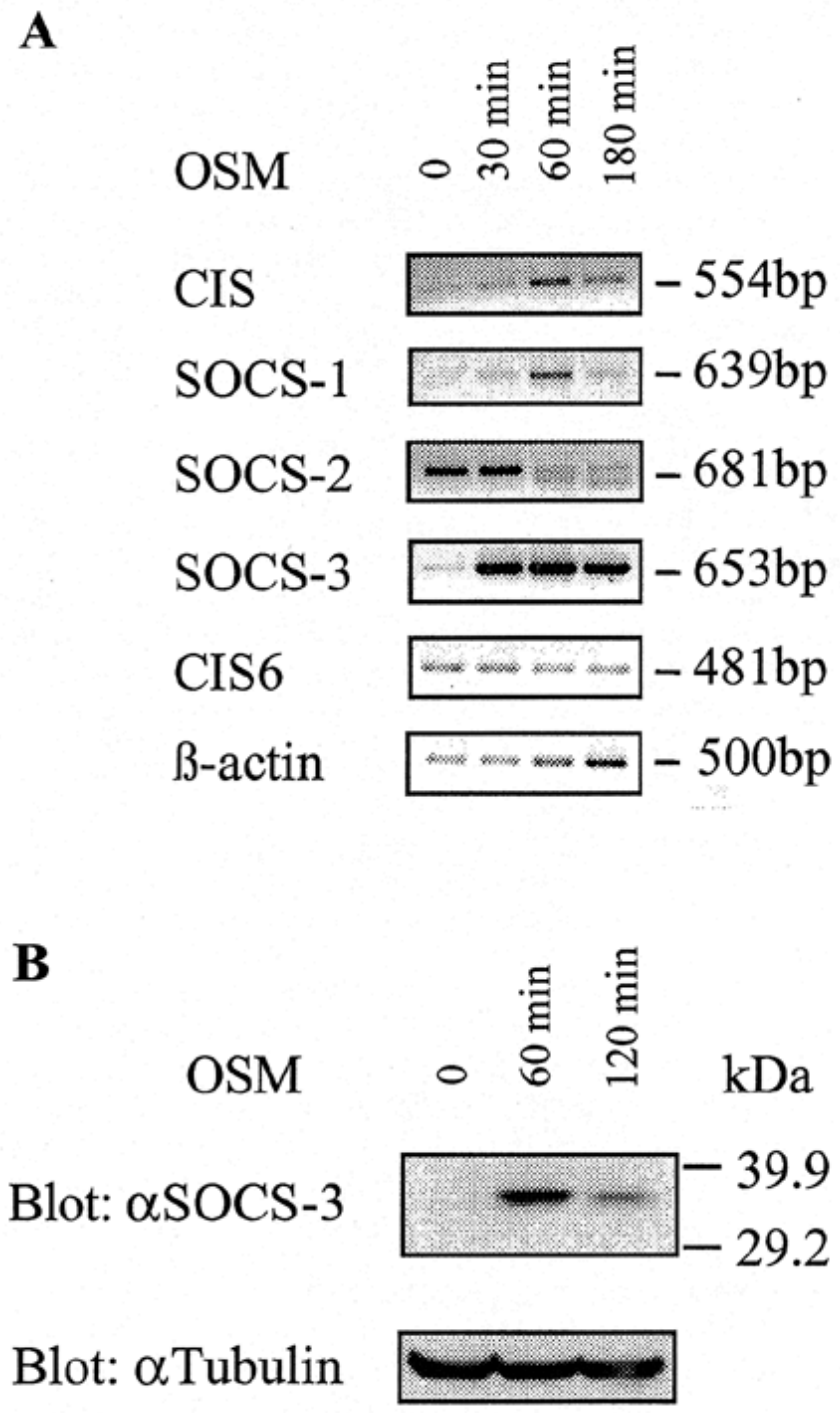



**Figure 1**

**Expression of SOCS family members in response to OSM.**

**A:** Induction of expression of SOCS family members by OSM using reverse transcriptase PCR of mRNA from A375 cells. **B:** Time course of SOCS-3 protein expression in response to OSM. Whole cell lysates were loaded on gel,

and immunoblots were performed using anti-SOCS-3 antibody. Subsequent reblotting with anti-tubulin antibody was done to verify equal protein loading in each lane. Molecular masses are indicated on the right.

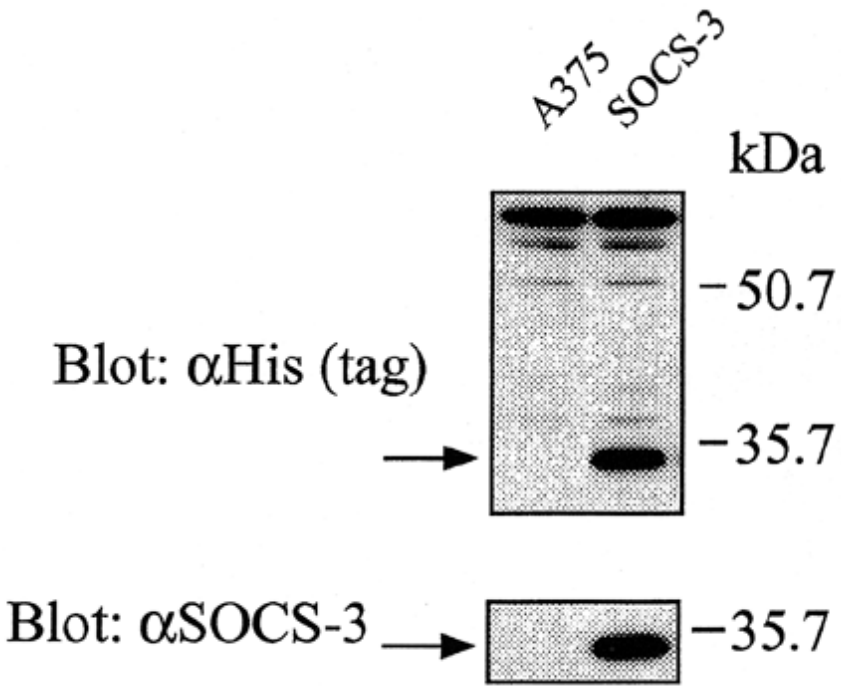



**Figure 2**

**SOCS-3 expression level.**

Whole cell extracts from parental A375 cells and transformants expressing SOCS-3 were immunoblotted using anti-His or anti-SOCS-3 antibodies. Arrows indicate the migration of the His-tagged SOCS-3 protein. Molecular masses are indicated in the right.

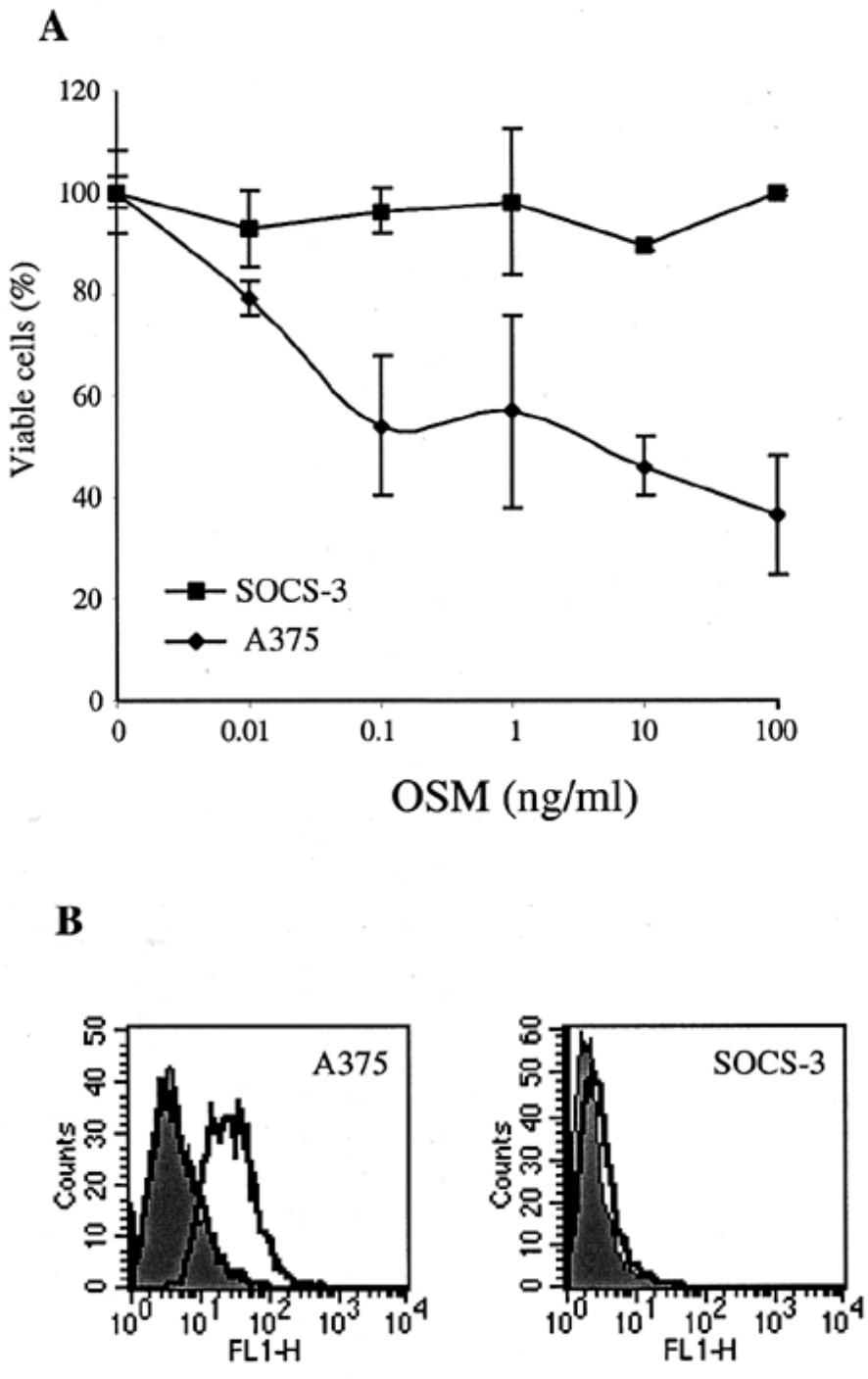



**Figure 3**

**Effect of SOCS-3 protein on OSM-induced growth arrest and apoptosis on A375 melanoma cells.**

**A:** Parental A375 cells and A375 cells expressing SOCS-3, were cultured in a medium supplemented with various

concentration of OSM for 4 days and viable cells were scored. viable cells in medium alone were used as 100%. Error bars represent deviation of triplicate sample. **B:** Flow cytometric analysis of apoptotic cells. DNa fragmentation was assessed in parental A375 cells and A375 cells expressing SOCS-3 treated or not with 100 ng/ml OSM for 4 days (open area) or left untreated (closed area).

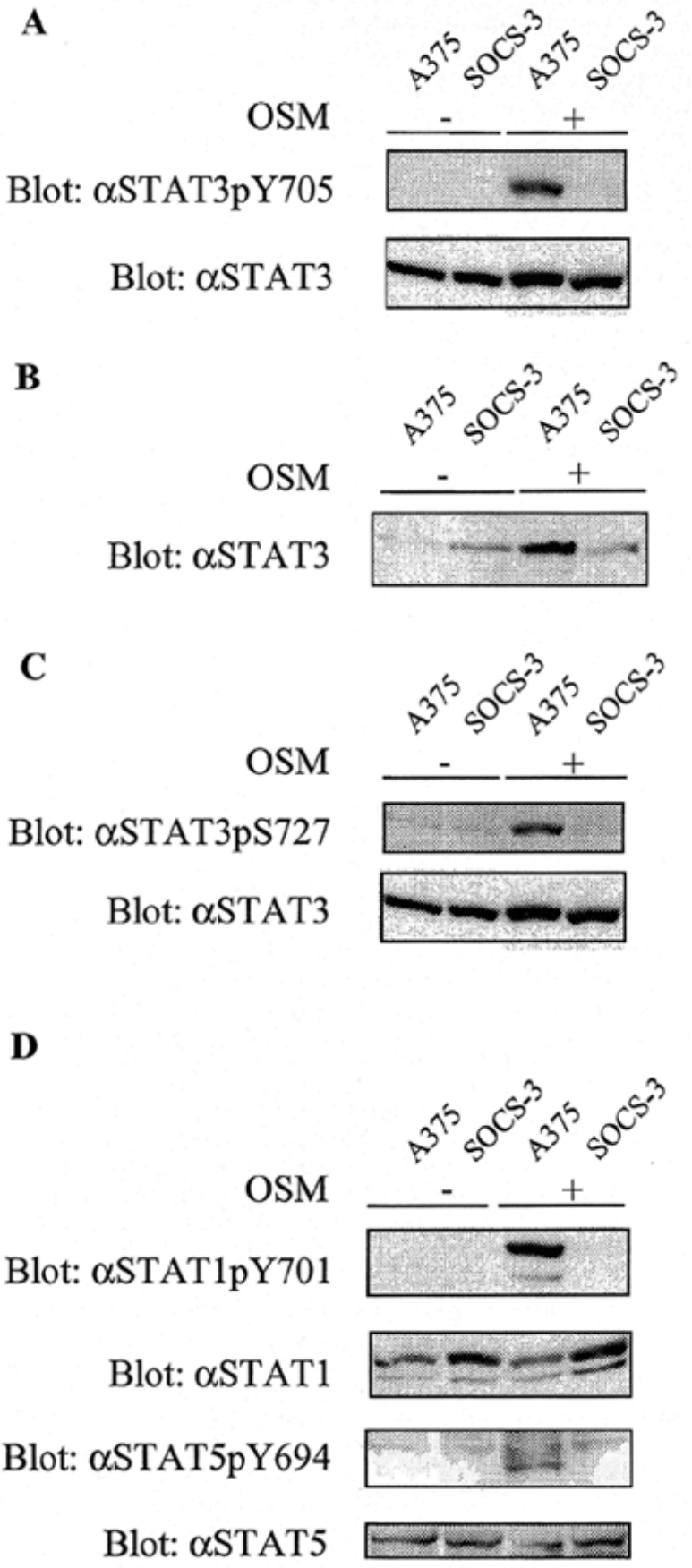

A
A375
SOCS-3
A375
SOCS-3
OSM
-
+
Blot: αSTAT3pY705
Blot: αSTAT3
B
A375
SOCS-3
A375
SOCS-3
OSM
-
+
Blot: αSTAT3
C
A375
SOCS-3
A375
SOCS-3
OSM
-
+
Blot: αSTAT3pS727
Blot: αSTAT3
D
A375
SOCS-3
A375
SOCS-3
OSM
-
+
Blot: αSTAT1pY701
Blot: αSTAT1
Blot: αSTAT5pY694
Blot: αSTAT5

Figure 4

**Inhibition of OSM-induced STAT1, STAT3 and STAT5 activation in A375 cells expressing SOCS-3.**

Whole cell lysates were prepared from serum-deprived parental A375 cells or A375 cells expressing SOCS-3, after stimulation for 10 min with 100 ng/ml OSM. **A:** Cell extracts were loaded on gel, and immunoblot was performed using antibody that specifically recognised tyrosine phosphorylated STAT3. Subsequent reblotting with anti-STAT3 antibody was done to verify equal protein loading in each lane. **B:** Nuclear extracts were immunoblotted with anti-STAT3 antibody. **C:** Cell extracts were immunoblotted with anti-serine phosphorylated STAT3 and reblotted with anti-STAT3. **D:** Cell extracts were loaded on gel, and immunoblots were performed using antibodies that specifically recognised tyrosine phosphorylated STAT1 and STAT5. Subsequent reblotting with anti-STAT1 and STAT5 antibodies were done to verify equal protein loading in each lane.

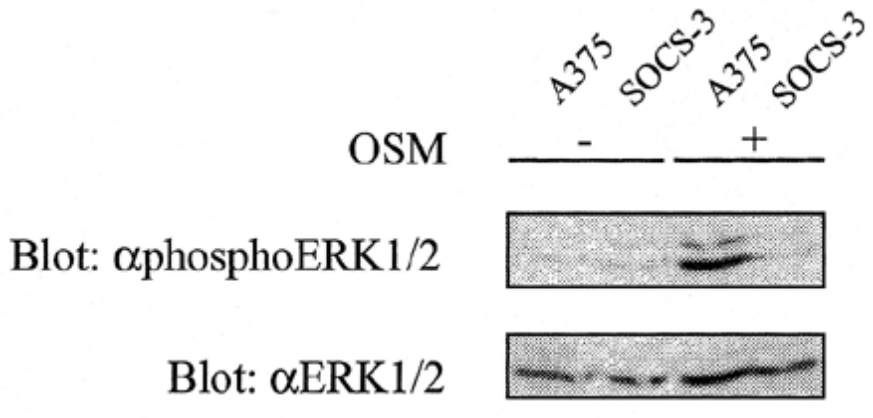



Figure 5

**Inhibition of OSM-induced ERK1/2 activation in A375 cells expressing SOCS-3.**

Whole cell lysates were prepared from serum-deprived parental A375 cells or A375 cells expressing SOCS-3 after stimulation for 10 min with 100 ng/ml OSM. Cell extracts were loaded on the gel, and immunoblots were performed using antibodies that specifically recognised threonine and tyrosine phosphorylated ERK1/2. Subsequent reblotting with anti-ERK1/2 was done to verify equal protein loading in each lane.

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THP-1 cells, macrophages, estrogen receptors, estrogens, monocytes.

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