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Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase-Dependent *SOCS-3* Gene Induction Requires c-Jun, Signal Transducer and Activator of Transcription 3, and Specificity Protein 3 Transcription Factors

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ABSTRACT

SOCS-3 gene induction by cAMP-elevating agents or the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), in primary HUVECs was found to require PKCη- and PKCε-dependent extracellular signal-regulated kinase (ERK) activation. The minimal, ERK-responsive element of the *SOCS-3* promoter was localized to a region spanning nucleotides −107 to the transcription start site and contains conserved binding sites for AP-1 and SP1/SP3 transcription factors, as well as proximal and distal signal transducer and activator of transcription (pSTAT and dSTAT) binding elements. All three classes of transcription factor were activated in response to ERK activation. Moreover, representative protein components of each of these transcription factor binding sites, namely c-Jun, STAT3, and SP3, were found to undergo ERK-dependent phosphorylation within their respective transactivation domains. Mutational analysis demonstrated an absolute requirement for the SP1/SP3 binding element in controlling basal transcriptional activity of the minimal *SOCS-3* promoter. In addition AP-1, pSTAT, and SP1/SP3 binding sites were required for ERK-dependent, PMA-stimulated *SOCS-3* gene activation. The dSTAT site seems to be important for supporting activity of the AP-1 site, because combined deletion of both sites completely blocks transcriptional activation of *SOCS-3* by PMA. Together these results describe novel, ERK-dependent regulation of transcriptional activity that requires codependent activation of multiple transcription factors within the same region of the *SOCS-3* gene promoter.

Introduction

The suppressors of cytokine signaling (SOCS) constitute a family of eight related Src homology 2-(SH2) containing proteins, namely CIS and SOCS-1 to SOCS-7 (Krebs and Hilton, 2001). Only SOCS-1 and SOCS-3 proteins have been intensely studied and have been shown to function as end points in a classic negative feedback loop whereby activation of STAT transcription factors triggers the induction of SOCS proteins, which then bind and terminate signaling from activated cytokine receptors (Kubo et al., 2003). The SOCS-3 protein is known to inhibit signal transduction from various receptors, including IL-6Rα, interferon-γR, IL-12 receptor β2, granulocyte-colony stimulating factor, erythropoietin, and leptin receptors (Dalpke et al., 2008; Dimitriou et al., 2008). SOCS-3 exerts its negative feedback through at least two mechanisms: 1) by binding to JAK-phosphorylated receptors via an SH2 domain, SOCS-3 inhibits JAK activity and, consequently, activation of STATs 1 and 3 (Sasaki et al.,

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ABBREVIATIONS: C/EBP, CCAAT/enhancer-binding protein; cPKC, conventional PKC; CRE, cAMP response element; CREB, cAMP response element-binding protein; DAG, diacyl glycerol; EPAC, exchange protein activated by cAMP; ERK, extracellular signal regulated kinase; F/R, forskolin/rolipram; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gö6983, 3-[1-(3-dimethylamino-propyl)-5-methoxy-1*H*-indol-3-yl]4-(1*H*-indol-3-yl)pyrrolidine-2,5-dione; HUVEC, human umbilical vascular endothelial cell; IL, interleukin; JAK, Janus tyrosine kinase; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein kinase; MEK, mitogen activated protein kinase kinase; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; MSH, melanocyte stimulating hormone; nPKC, novel PKC; PCR, polymerase chain reaction; PGE_2, prostaglandin E_2; AP-1, activator protein 1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Ro 31-7549, bisindolylmaleimide VIII acetate; RT, reverse transcriptase; SH2, Src homology 2; siRNA, small interfering RNA; SOCS, suppressors of cytokine signaling; SP, specificity protein; SP600125, 1,9-pyrazoloanthrone; STAT, signal transducers and activators of transcription; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene.

1999); and 2) by targeting SH2-bound proteins for ubiquitination and proteosomal degradation (Kamura et al., 1998; Zhang et al., 1999).

The importance of SOCS-3 negative feedback is highlighted by the fact that dysregulation of SOCS-3 and increased levels of STAT3 activation contributes to the development of cancer in multiple neoplasias, including cholangiocarcinoma, hepatocellular carcinomas, and breast and lung cancer (Silver and Hunter, 2010). Indeed, SOCS-3 has been reported to be a tumor suppressor in breast cancer cells (Barclay et al., 2009), and methylation of CpG islands within the SOCS-3 promoter regions occurs frequently in a variety of cancers, including melanoma (Tokita et al., 2007), glioblastoma (Martini et al., 2008), head and neck squamous cell carcinoma (Weber et al., 2005), and cancers of the lung (He et al., 2003b) and gut (Tischoff et al., 2007), thereby preventing SOCS-3 induction and limiting its damping actions on cell growth. Despite this, inhibition of SOCS-3 induction in macrophages may actually be therapeutic for the suppression of tumor metastasis, because hyperactivation of STAT3 in these cells simultaneously exerts anti-inflammatory as well as antitumor effects through the concomitant suppression of IL-6 and TNFα production and increased production of monocyte chemotactic protein 2 (Hiwatashi et al., 2011).

SOCS-3 expression is increased, however, at sites of acute and chronic inflammation (White et al., 2011), and IL-6 has been reported to promote acute and chronic inflammatory disease in the absence of SOCS-3 (Croker et al., 2012). Moreover, conditional deletion of the *SOCS-3* gene in hematopoietic and endothelial cells of transgenic mice results in death caused by severe inflammatory lesions in the peritoneal and pleural cavities (Croker et al., 2008). Consequently, cell-permeant forms of recombinant SOCS-3 have been used as therapy to effectively suppress pathogen-induced, acute inflammation, by reducing the production of inflammatory cytokines, attenuating liver apoptosis, and limiting hemorrhagic necrosis (Jo et al., 2005). It is clear, therefore, that by understanding and manipulating the molecular control of *SOCS-3* gene induction, we may find novel therapies for diverse diseases ranging from chronic inflammation to cancer.

In this respect, we have found efficient induction of the *SOCS-3* gene by cAMP in human umbilical vascular endothelial cells (HUVECs) and COS1 cells requires coincident activation of the ERK MAP kinase cascade and ERK-dependent phosphorylation of C/EBPβ on Thr235 seems to be a prerequisite for efficient *SOCS-3* induction (Sands et al., 2006; Borland et al., 2009; Woolson et al., 2009). In this case, the pathway leading from cAMP to ERK in these cells is not known; however, it does seem to be independent of activation of both cAMP-dependent protein kinase and the Rap1 guanine nucleotide exchange factor EPAC1 (Woolson et al., 2009). In the current study, we use HUVECs and COS1 cells, both of which have been shown to exhibit SOCS-3 induction in response to elevations in intracellular cAMP, to investigate how the ERK MAP kinase cascade serves to integrate these diverse cAMP-regulated pathways during the regulation of *SOCS-3* promoter activity in an effort to determine how we could manipulate SOCS-3 protein production for therapeutic benefit. In this respect, we have investigated the individual roles of STAT, AP-1, and SP1/SP3 transcription factors in mediating *SOCS-3* induction in response to ERK activation. Given the central roles that both the ERK MAP kinase cascade and SOCS-3 play in regulating inflammatory and cell proliferative responses, our findings cast new light on a potentially important new gene-regulatory signaling pathway.

Materials and Methods

Materials. Primary antibodies to PKCα, PKCδ, PKCη, PKCε, ERK, phospho-ERK (Thr202/ Tyr204), c-Jun, phospho-c-Jun (Ser63), STAT3, and phospho-STAT3 (Ser727) were obtained from New England Biolabs UK Ltd (Hertfordshire, UK). The anti-SP3 and anti-SOCS-3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the anti-GAPDH antibody was from Ambion (Austin, TX). The primary antibody that recognizes ERK-phosphorylated SP3 (Ser73) was a generous gift from Dr Giles Pagès (University of Nice Sophia Antipolis, Nice, France). Protein A-Sepharose beads, enhanced chemiluminescence reagents, secondary antibodies anti-rabbit-IgG horseradish -peroxidase conjugate and anti-mouse-IgG horseradish peroxidase conjugate were bought from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). HUVECs and endothelial cell growth medium 2 were obtained from PromoCell (Heidelberg, Germany). HiPerFect transfection reagent was purchased from QIAGEN (West Sussex, UK), and PGE_2, αMSH, Dulbecco's phosphate-buffered saline were from Sigma-Aldrich (Dorset, UK). Forskolin, rolipram, phorbol 12-myristate 13-acetate (PMA), *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132), and 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126) were obtained from MSD (Hertfordshire, UK).

Plasmids. Mouse SOCS-3 promoter constructs were a generous gift from Professor J. G. Bode (Heinrich-Heine University, Dusseldorf, Germany) with permission from Professor Shlomo Melmed (Ceders-Sinai Medical Center, Los Angeles, CA). These included pGL3-SOCS3-2757Luc, which contains the promoter region −2757 to +929 of the murine *SOCS3* gene fused to the coding region of firefly luciferase as described previously (Auernhammer et al., 1999), as well as promoter truncates pGL3-SOCS3-511Luc, -107Luc, -79Luc, -68Luc, and -49Luc and pGL3-SOCS3-107Luc constructs mutated to disrupt the putative SP1/SP3, dSTAT, and pSTAT, as described previously (Ehlting et al., 2005). The QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to introduce mutations (underlined) into vectors pGL3-SOCS3-107Luc, pGL3-SOCS3-107-pSTAT, pGL3-SOCS3-107-SP1/SP3, and pGL3-SOCS3-107-pSTAT-SP1/SP3, using primers 5′-GCCTTTCAGTGCAGAGTAAAGCTTAAACATTACAAGAAGACCGGCCGGGC-3′ (forward) and 5′-GCCCGGCCGGTCTTCTTGTAATGTTTAAGCTTTACTCTGCACTGAAAGGC-3′ (reverse) to disrupt the putative AP1 site ($^{-105}$GTGACTAA^{-99}– $^{-105}$AAGCTTAA^{-99}). Mutations (underlined) were also introduced into vectors pGL3-SOCS3-107Luc, pGL3-SOCS3-107-pSTAT, pGL3-SOCS3-107-SP1/SP3, and pGL3-SOCS3-107-pSTAT-SP1/SP3 using primers 5′-GCCTTTCAGTGCAGAGTAAAGCTTAAACATCCCAGGAAGACCGGCCGGGC-3′ (forward) and 5′-GCCCGGCCGGTCTTCCTGGGATGTTTAAGCTTTACTCTGCACTGAAAGGC-3′ (reverse) to disrupt both the putative AP1 binding site ($^{-105}$GTGACTAA^{-99}–$^{-105}$AAGCTTAA^{-99}) together with the putative dSTAT site ($^{-95}$TTACAAGAA^{-88}–$^{-95}$TCCCAGGAA^{-88}). The AP1-Luc (fn34), STAT-Luc, SP1/SP3-Luc [pAldGCB^{4}luc (Ehlting et al., 2005)], and CRE-Luc reporter constructs were generous gifts from Professor Walter Kolch (University College Dublin, Dublin, Republic of Ireland), Dr. Timothy Palmer (University of Glasgow, Glasgow, Scotland, UK), Professor Gerald Thiel (University of Saarland, Hamburg, Germany), and Professor Ferenc Antoni (University of Edinburgh, Edinburgh, Scotland, UK).

Cell Culture. HUVECS were grown in endothelial cell growth medium 2 (Promocell) at 37°C and 5% (v/v) CO_2. Cells were passaged weekly to a maximun of six. COS1 cells were cultured at 37°C in

5% (v/v) CO_2 in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich), 2 mM-glutamine, and 2% (v/v) penicillin/streptomycin (Sigma-Aldrich).

Transfection of Cells with siRNA. The day before transfection, HUVECs were seeded into six-well plates at a density of approximately 2×10^5 cells/cm^2 and grown to approximately 90% confluence. Cells were then transfected with 200 nM FlexiTube siRNA (QIAGEN) to either PKCα (Hs_PRKCA_6 or Hs_PRKCA_7 FlexiTube siRNA), PKCδ (Hs_PRKCD_11 or Hs_PRKCD_7 FlexiTube siRNA), PKCη (Hs_PRKCH_5 FlexiTube siRNA), PKCε (Hs_PRKCE_5 FlexiTube siRNA), or nontargeting control oligonucleotides (AllStars Negative Control siRNA) using HiPerFect (QIAGEN) transfection reagent, according to the manufacturer's instructions. The following day, cells were treated with pharmacological agents, harvested in SDS-polyacrylamide gel electrophoresis sample buffer, and analyzed by Western blotting.

Western Blotting. For Western blotting, cells were harvested by scraping directly into 200 μl of SDS-polyacrylamide gel electrophoresis sample buffer [20 mM Tris-HCl, pH 8.0, 2% (w/v) SDS, 2 mM EDTA, 20% (v/v) glycerol, 2.5% (v/v) β-mercaptoethanol, and 0.01% (w/v) bromphenol blue], separated on 10% (w/v) resolving gels, and electroblotted onto nitrocellulose membranes. Membranes were blocked in 5% (w/v) milk powder [or 5% (w/v) bovine serum albumin for phosphospecific antibodies] in Tris-buffered saline containing 0.1% (v/v) Tween 20. Blots were incubated in primary antibodies overnight at 4°C followed by appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were then developed using enhanced chemiluminescence reagent (GE Healthcare) according to the manufacturer's instructions.

Reverse Transcriptase PCR. HUVECs were grown to 15×10^4 cells/well in six-well plates and transfected with siRNA oligonucleotides as described previously (Borland et al., 2009). Cells were then stimulated for 5 h with diluent, a combination of 10 μM forskolin plus 10 μM rolipram or 10 μM PMA for 5 h. Cells were then washed twice with ice-cold phosphate-buffered saline, and then total RNA was extracted using the RNeasy Mini Kit (QIAGEN), according to the manufacturer's instructions. Extracted RNA (2–10 ng) was then converted to cDNA and amplified using the OneStep RT-PCR Kit (QIAGEN), with a total reaction volume of 25 μl, containing 0.4 μM dNTPs and 0.6 μM primers (SOCS3: forward, 5′-CACATGGCACAAGCACAAGA-3′; reverse, 5′-AAGTGTCCCCTGTTTGGAGG-3′; Actin: forward, 5′-CTGGCACCCAGCACAATG-3′; reverse, 5′-GCCGATCCACACGGAGTACT-3′). The RT-PCR reaction was initiated by 1 cycle at 50°C for 30 min, followed by 15 min at 95°C to activate the hot start DNA polymerase. The amplification reaction involved a denaturation step (94°C, 30 s), an annealing step (50°C, 30 s), and 30 cycles of amplification (72°C, 1 min) followed by a single amplification step (72°C, 10 min). DNA fragments were visualized using 1.5 to 2% (w/v) agarose gels.

Dual Luciferase Reporter Assays. COS1 cells were grown on 12-well plates until around 80 to 90% confluence and then transfected with 0.125 μg of *Renilla reniformis* luciferase (pGL4.74) together with 1.125 μg of either AP1-Luc, STAT-Luc, CRE-Luc, or SP1/SP3 reporter constructs or murine SOCS3-Luc promoter deletion/mutation constructs using the *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (Roche Diagnostics, Basel, Switzerland) transfection agent. Cells were incubated with luciferase reporter constructs for 24 h, and then the medium was changed for Dulbecco's modified Eagle's medium, after which the cell treatments were applied and incubated for a further 24 h. Cells were then harvested according to the protocols in the Dual Luciferase Reporter Assay kit (Promega, Southampton, UK) and analyzed using a BMG Labtech GmbH (Offenburg, Germany) luminometer.

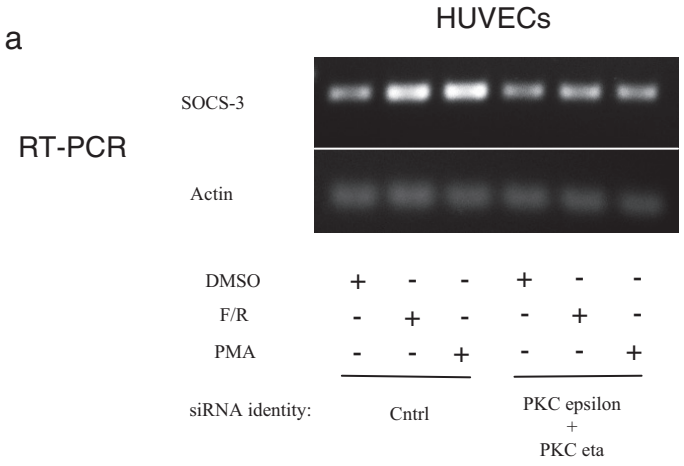
Densitometry and Statistical Analysis. Nonsaturating immunoblots from multiple experiments were quantified densitometrically using ImageJ software (http://rsbweb.nih.gov/ij/). Statistical significance was determined by one-way ANOVA using InStat Software (GraphPad Software, San Diego, CA).

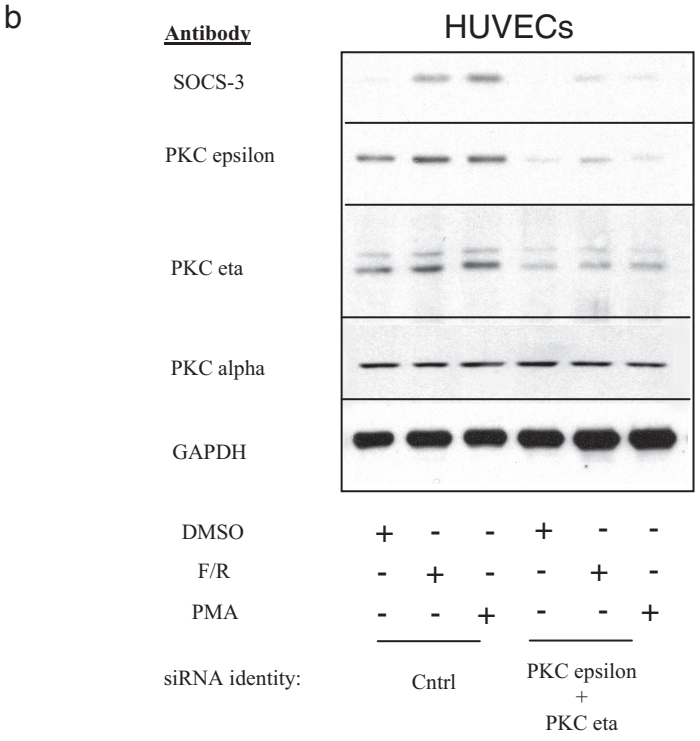
Results

PKC Isoforms Regulate SOCS-3 Induction through the ERK MAP Kinase Pathway in HUVECs. In an ongoing effort to delineate the signaling pathways that regulate the induction of the *SOCS-3* gene, we previously demonstrated that in COS1 cells PKC isoforms α and δ act in a cAMP-activated gene regulatory pathway upstream of ERK MAP kinase (Borland et al., 2009). ERK then induces transcriptional activation of the *SOCS-3* gene through the phosphorylation of the transcription factor C/EBPβ on Thr235, both in COS1 cells and in HUVECs (Borland et al., 2009; Woolson et al., 2009). It is not known whether PKC isoforms are required for ERK activation in HUVECs and hence contribute to anti-inflammatory signaling in this model of endothelial dysfunction (Sands and Palmer, 2005). HUVECs normally express the conventional PKC (cPKC; DAG and Ca^{2+}-dependent), PKCα; the novel PKCs (nPKCs; DAG but not Ca^{2+}-dependent) PKCδ, PKCη, and PKCε; and the atypical PKC (DAG and Ca^{2+}-independent) PKCζ (Mellor and Parker, 1998). Of these, the nPKCs PKCη and PKCε are not expressed in COS1 cells (Borland et al., 2009), which prompted us to investigate the importance of PKCη and PKCε for *SOCS-3* gene regulation in HUVECs. Preincubation of cells with individual siRNAs toward PKCη or PKCε had little effect on the ability of cAMP elevation with a combination of the adenylyl cyclase activator forskolin and the cAMP-specific phosphodiesterase inhibitor rolipram (F/R) to increase SOCS-3 protein levels (results not shown). However, coincubation with PKCη and PKCε siRNAs significantly impaired the ability of F/R to induce SOCS-3 mRNA, as determined by RT-PCR (Fig. 1a), and SOCS-3 protein expression, as determined by Western blotting (Fig. 1b), but not actin mRNA (Fig. 1a), GAPDH protein (Fig. 1b), or PKCα protein (Fig. 1b). Moreover, PKCη and PKCε siRNA blocked the ability of PGE_2, a physiological stimulus capable of elevating cAMP in HUVECs (Sands et al., 2006), to induce SOCS-3 protein, indicating that these PKC isoforms can modulate to response of the *SOCS-3* gene to a physiological agonist (Fig. 1c).

We next tested the involvement of PKCη and PKCε, or a combination of PKCα and PKCδ, in controlling ERK activation in HUVECs (Fig. 2a). We monitored ERK using phosphospecific antibodies and found that, as with SOCS-3 induction, a combination of PKCη and PKCε siRNAs significantly inhibited ERK activation in response to F/R or the cell-permeant diacylglycerol analog PMA (10 μM; Fig. 2a). Moreover, F/R- or PMA-stimulated ERK activation seemed to be also sensitive to inhibition by a combination of PKCα and PKCδ siRNAs (Fig. 2a). Individually, these two siRNAs had no significant effect on ERK activity or SOCS-3 induction (results not shown); however, a combination of the two siRNAs effectively inhibited SOCS-3 induction in response to F/R and PMA (Fig. 2b) as well as PGE_2 (Fig. 2b). Together these observations suggest that, as in COS1 cells (Borland et al., 2009), both PKCα and PKCδ are required for SOCS-3 protein induction by cAMP in HUVECs.

To support the idea that PKC isoforms are required for SOCS-3 induction in HUVECs, two chemical inhibitors of cPKC





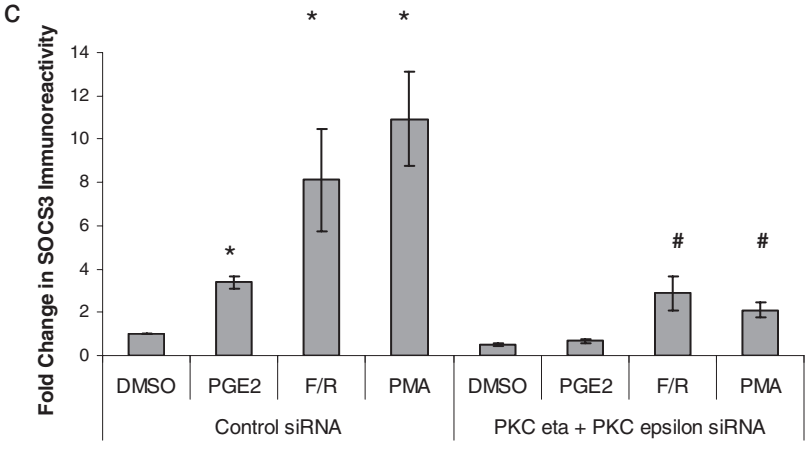


Fig. 1. Involvement of the PKC isoforms η and ε in cAMP-dependent SOCS-3 induction in HUVECs. a, HUVECs were treated with a combination of PKCη- and PKCε-specific siRNAs (Sequence 1) and then stimulated with either F/R or PMA for 5 h. Total RNA was then extracted from cells and subjected to one-step RT-PCR, with specific primers toward SOCS-3 or actin, as described under *Materials and Methods*. Amplified DNA fragments were visualized by agarose gel electrophoresis. b, HUVECs were treated with control (cntrl) siRNA or a combination of PKCη- and PKCε-specific siRNAs. Cells were then stimulated with F/R or PMA for 5 h in the presence of MG132 (10 μM). Cell lysates were then prepared and immunoblotted with antibodies to SOCS-3, PKCη, PKCε, PKCα, and GAPDH, as indicated. C, densitometric units were obtained from SOCS-3 immunoblots from three separate experiments and presented as a histogram in the lower panel. Significant differences in SOCS-3 expression in PKC siRNA-treated cells compared with cntrl siRNA cells are indicated (#, $p < 0.05$), as are significant increases in SOCS-3 expression relative to DMSO-treated cells (*, $p < 0.05$).

and nPKC, 3-[1-(3-dimethylamino-propyl)-5-methoxy-1*H*-indol-3-yl]4-(1*H*-indol-3-yl)pyrrolidine-2,5-dione (Gö 6983; 10 μM) and bisindolylmaleimide VIII acetate (Ro-31-7549; 10 μM), were found to robustly inhibit ERK activation and SOCS-3 protein induction in response to either F/R or PMA (Fig. 3a). It is noteworthy that coincubation with the inhibitor of ERK ac-

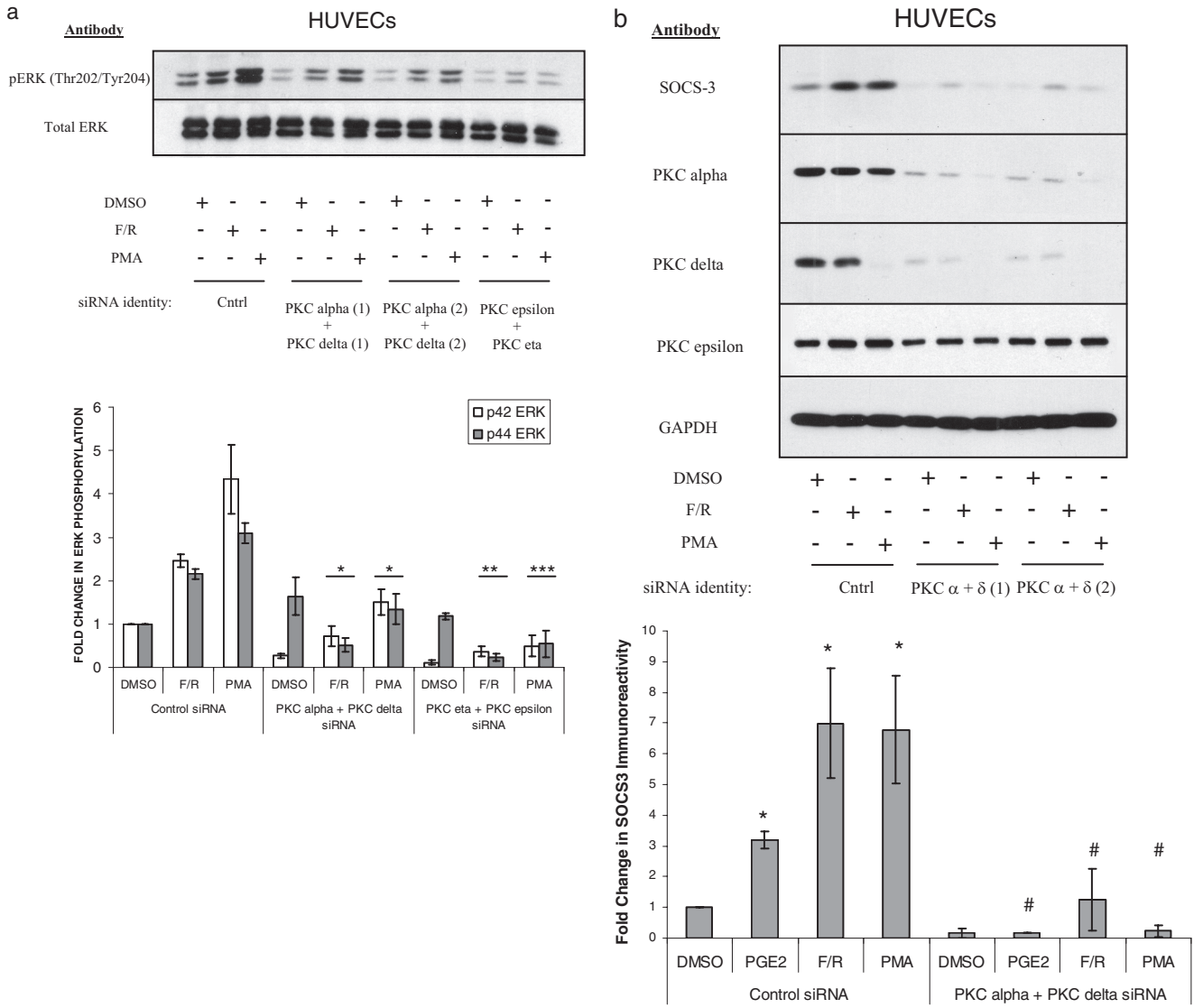


Fig. 2. Involvement of PKC isoforms in cAMP-dependent ERK activation in HUVECs. a, HUVECs were treated with combinations of PKCα- and PKCδ-specific siRNAs (sequence 1, Hs_PRKCA_6 + Hs_PRKCD_11; sequence 2, Hs_PRKCA_7 + Hs_PRKCD_7), or a combination of PKCη- and PKCε-specific siRNAs, and then stimulated with F/R or PMA as described above. Cell extracts were immunoblotted with anti-phospho-ERK (Thr202/Tyr204) or total ERK antibodies and immunoblots from three separate experiments were quantified and densitometric units ($n = 3$) presented in the histogram in the lower panel. Significant differences in ERK phosphorylation in siRNA-treated cells compared with control siRNA cells with equivalent treatment are indicated $*$, $p < 0.05$; $**$, $p < 0.01$; or $***$, $p < 0.001$. b, HUVECs were treated with control (cntrl) siRNA, PKCα-specific siRNA (sequences 1 and 2), PKCδ-specific siRNA (sequences 1 and 2) or a combination of PKCα- and PKCδ-specific siRNAs. Cells were then stimulated with F/R or PMA for 5 h in the presence of MG132 (10 μM). Cell lysates were then prepared and immunoblotted with antibodies to SOCS-3, PKCα, PKCδ, PKCε, and GAPDH, as indicated. Bottom, SOCS-3 immunoblots from three separate experiments were quantified and densitometric units ($n = 3$) are presented as a histogram. Significant differences in SOCS-3 expression in siRNA-treated cells compared with control siRNA cells with equivalent treatment are indicated (#, $p < 0.05$). Significant increases in SOCS-3 expression relative to DMSO-treated cells are also indicated ($*$, $p < 0.05$).

tivation, U0126 (10 μM), dramatically inhibited SOCS-3 induction in response to PGE_2, αMSH, F/R, and PMA (Fig. 3, a and b), demonstrating that ERK is required for SOCS-3 induction by both cAMP and PMA in HUVECs. Moreover, the fact that treatment with U0126 completely ablated SOCS-3 induction by PMA (Fig. 1a) indicates that the ability of cPKCs and nPKCs to induce *SOCS-3* gene activity occurs through regulation of the ERK MAP kinase pathway. This indicates that in HUVECs, as in COS1 cells (Borland et al., 2009), the response of SOCS-3 to cAMP stimulation is also dependent on PKC-regulated ERK activation. Moreover, the ERK MAP kinase cascade seems to be vital for coordinating signals from both cAMP- and PKC-activated pathways to induce SOCS-3 expression and the nPKCs (PKCη, PKCε, and PKCδ) and the cPKC (PKCα) play a vital role in controlling this gene regulatory cross-talk.

Identification of the Minimal, PKC-, and ERK-Regulated SOCS-3 Promoter. Having determined a central role for ERK in regulating SOCS-3 protein expression in HUVECs, and previously in COS1 cells (Borland et al., 2009), we next sought to determine which region of the *SOCS-3* gene promoter is specifically targeted by PKC-regulated ERK. A deletion series of the murine *SOCS-3* promoter (Auernhammer et al., 1999), cloned into a promoterless firefly luciferase expression vector (pGL3-Basic), was transfected into COS1 cells and then stimulated in the presence or absence of 10 μM PMA for 16 h. COS1 cells were used in place

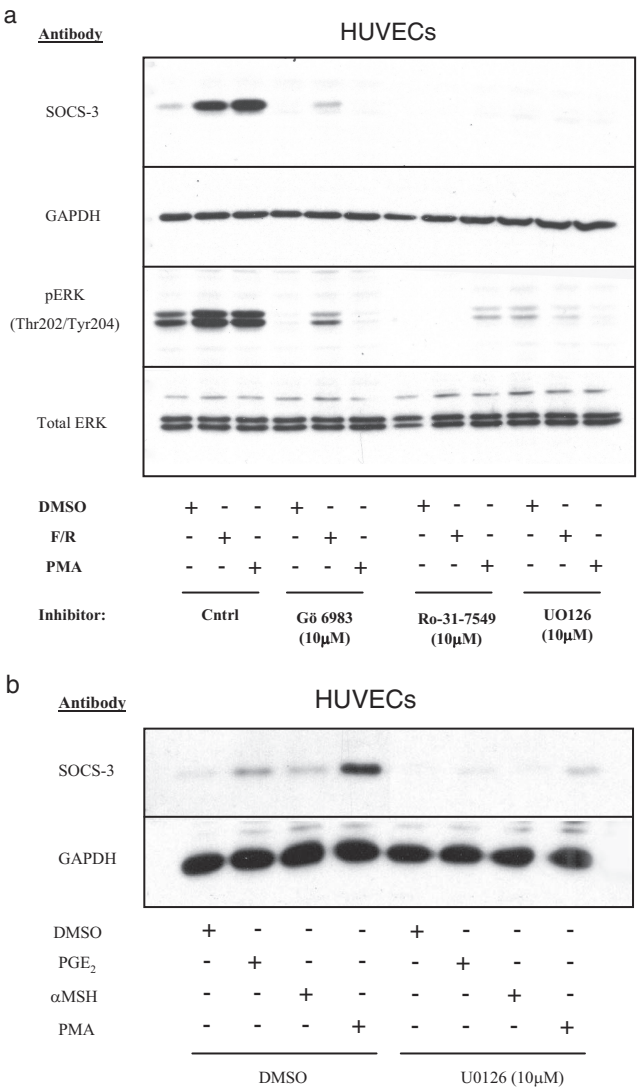


Fig. 3. Involvement of ERK in SOCS-3 induction in HUVECs. a, HUVECs were stimulated for 5 h with MG132 (10 μM) plus either a combination of 10 μM forskolin plus 10 μM rolipram (F/R) or 10 μM PMA or in the presence or absence of the PKC inhibitors 10 μM Ro-31-7549 (RO) or 10 μM Gö 6983 or the MEK inhibitor 10 μM U0126. Cell extracts were then prepared and immunoblotted with the indicated antibodies. b, HUVECs were stimulated for 5 h with MG132 and either 10 μM PGE_2, 10 μM αMSH, or 10 μM PMA in the presence or absence of the ERK inhibitor U0126 (10 μM) and then immunoblotted with anti-SOCS-3 and GAPDH antibodies as indicated.

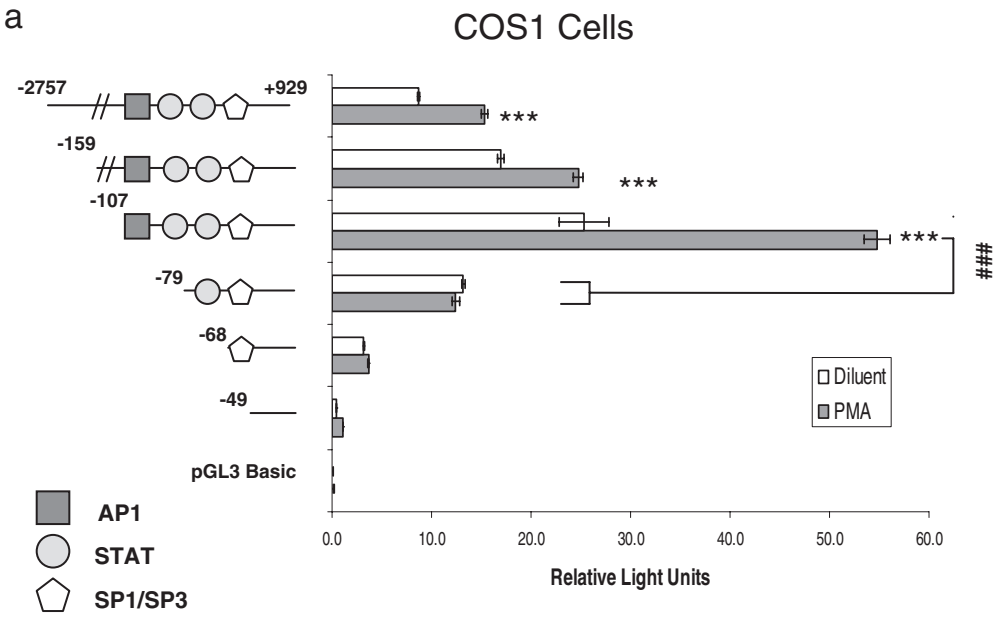
of HUVECs for these experiments because they are comparably easier to transfect and SOCS-3 is regulated in an ERK- and PKC-dependent manner in these cells (Borland et al., 2009). PMA treatment was used because it induces a robust activation of ERK in both HUVECs (Fig. 2a) and COS1 cells (Borland et al., 2009).

Luciferase activities were measured in PMA-stimulated cells extracts and results demonstrated that the full-length promoter (−2757/+929) and two truncated promoters (−159/+929 and −107/+929) showed significant increases in promoter activity after PMA stimulation (Fig. 4a). The −107/+929 region showed slightly higher activity than −159/+929 and −2757/+929 (Fig. 3a), which probably represents the deletion of a repressor element between nucleotides −107 and −159. Further deletion beyond position −107 to position −79 and beyond resulted in a loss of PMA responsiveness, indicating that the PMA-responsive element lies between nucleotides −107 and −79, a region that contains a putative AP-1 and dSTAT transcription factor binding site (Fig. 4, a and c).

Having determined that the mSOCS3/−107 luciferase construct contains the minimal PMA-responsive promoter, we then tested whether this region was in fact regulated through activation of the ERK MAP kinase pathway. Cells were transfected with the mSOCS3/−107 minimal promoter construct and then stimulated with PMA in the presence or absence of the MEK inhibitor 10 μM U0126 (Fig. 4b). Results demonstrated that inhibition of ERK significantly reduced the ability of PMA to induce the activity of the minimal SOCS-3 promoter and also significantly reduced basal activity (Fig. 4b). These results demonstrate that the ERK MAP kinase pathway is vital for the regulation of SOCS-3 transcriptional activity through interactions with a minimal segment of the *SOCS-3* promoter contained within −107 nucleotides relative to the transcription start site (+1). It is noteworthy, however, that U0126 did not completely abolish transcriptional activation of the minimal SOCS-3 promoter (Fig. 4b) but is still highly effective at inhibiting SOCS-3 protein induction in response to PMA treatment and increased cAMP in HUVECs (Fig. 3a). This suggests that PMA may act through pathways in addition to the ERK cascade to induce SOCS-3 expression in COS-1 cells. A potential candidate pathway for this is the c-Jun N-terminal kinase (JNK) MAP kinase pathway, which may also be important for SOCS-3 induction in these cells (J. Dunlop and S. J. Yarwood, unpublished observations).

ERK Regulates the Activity of AP-1 Complex, STAT, and SP1/SP3 Transcription Factors. Analysis of the minimal mSOCS3 −107/+929 promoter fragment reveals the presence of putative consensus sites for AP-1 complex, STAT (dSTAT and pSTAT), and SP1/SP3 transcription factors, which are conserved within the promoter sequences from both human and mouse (Fig. 4c). It has been demonstrated previously that the AP-1 site is required for promoter responsiveness to cAMP analogs (Bousquet et al., 2001), the pSTAT site for responsiveness to IL-6 and leukemia inhibitory factor (Auernhammer et al., 1999; Ehlting et al., 2005), and the SP1/SP3 site is also required for the action of IL-6 (Ehlting et al., 2005) as well as PGE_2 (Barclay et al., 2007).

To confirm that these transcription factor binding sites can be activated in our cell system, we transfected COS1 cells with specific luciferase reporter constructs and found that transcriptional activity could be elicited from each of the AP-1, STAT, and SP1/SP3 reporters after either F/R or PMA treatment (Fig. 5a). In contrast, using a CRE reporter to monitor the activation of the cAMP-responsive transcription factor CREB showed that this was activated by F/R alone and not PMA (Fig. 5b). This demonstrates that it is unlikely that CREB activation is responsible for the activation of the mSOCS3 −107/+929 promoter construct in response to PMA-stimulated ERK activation and that transcription factor interaction with the AP-1, STAT, and SP1/SP3 sites may be involved. This idea is supported by the observation that the activation of each of the AP-1, STAT, and SP1/SP3 reporter constructs by PMA was significantly reduced by coincubation with the inhibitor of ERK activation, 10 μM U0126 (Fig. 5c).



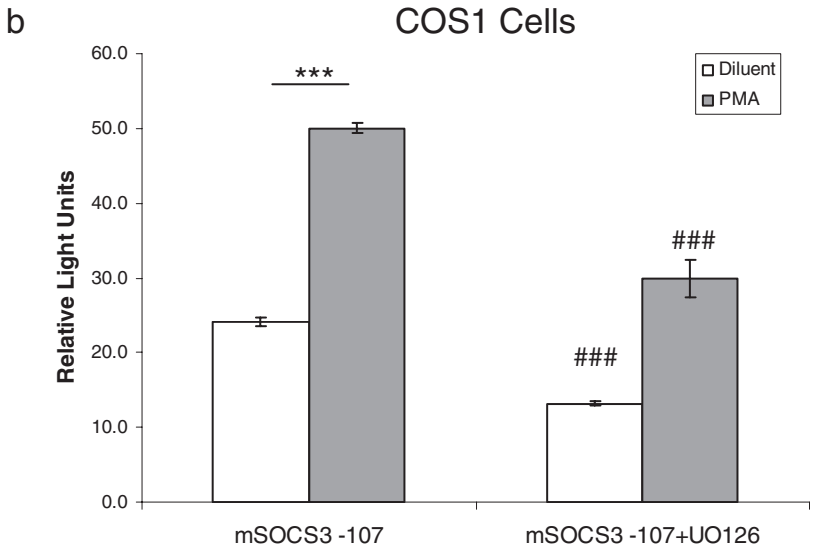


Fig. 4. Identification of the minimal ERK-responsive *SOCS-3* promoter region. a, COS1 cells were transfected with firefly luciferase reporter constructs containing truncates of the murine SOCS3 promoter. In addition, cells were cotransfected with *R. reniformis* luciferase vector to normalize luciferase activity and to correct for transfection efficiency. Cells were then stimulated for 16 h with 10 μM PMA, after which cells were harvested and luciferase activities determined. The relative positions of putative transcription factor binding sites identified in Fig. 3 are shown in the schematic on the left side of the histogram. Results are expressed means± S.E.M. of absolute relative light units from three separate experiments and significant differences relative to cells stimulated with diluent alone are indicated (***, $p < 0.001$). The significant difference in luciferase activity between reporter construct mSOCS-3 −107 and mSOCS-3 −79 is also indicated (###, $p < 0.0001$). b, COS1 cells were transfected with the luciferase reporter vector, mSOCS-3 −107, which represents the minimal PMA-responsive region of the murine *SOCS-3* promoter. Cells were then stimulated with either diluent (DMSO) or 10 μM PMA in the presence or absence of the ERK inhibitor U0126 (10 μM). Significant differences in luciferase activity between diluent- and PMA-treated cells and between diluent- and U0126-treated cells are shown [***, $p < 0.001$; ###, $p < 0.001$ ($n = 3$), respectively].

To further test the idea that ERK activates transcription factors, which then interact with the minimal SOCS-3 promoter, we used Western blotting of HUVEC cell extracts with phosphospecific antibodies to examine whether representative AP-1 complex, STAT, or SP1/SP3 interacting proteins (i.e., c-Jun, STAT3, and SP3) are phosphorylated in their transactivation domains in an ERK-dependent manner (Fig. 5d). It has previously been shown that ERK-dependent phosphorylation of c-Jun on Ser63 (Pulverer et al., 1991), STAT3 on Ser727 (Wen et al., 1995; Kuroki and O'Flaherty, 1999), and SP3 on Ser73 (Pagès, 2007) is required for full activity of each these transcription factors. We found that the inhibitor of ERK activation U0126 effectively blocked phosphorylation of each of these transactivating sites as induced by PMA treatment (Fig. 5d).

We were surprised to note that although PMA promoted phosphorylation of Ser727 of STAT3, we did not detect much increase above basal of Tyr705 (results not shown), which is usually required for full activation of STAT3 (Darnell et al., 1994). This suggests that PMA stimulation leads either to the serine phosphorylation of a pool of STAT3 transcription factors that are already basally phosphorylated on tyrosine or that phosphorylation of Ser727 is sufficient for the activation of STAT3 in HUVECs. Regardless of the mechanism involved, it is clear that stimulation of HUVECs, with either F/R or PMA, leads to recruitment of STAT3 to the *SOCS-3* promoter, as detected by chromatin immunoprecipitation (results not shown). Activation of STAT reporter constructs by F/R and PMA is clearly measurable in COS1 cells (Fig. 5, a and c). Together, these results demonstrate that ERK-regulated transcription factors capable of interacting with AP-1, STAT, or SP1/SP3 consensus sites are strong candidates for transcriptional regulation of the minimal *SOCS-3* promoter. We were also intrigued to note, however, that phosphorylation of c-Jun in response to PMA was also blocked by a chemical inhibitor of JNK MAP kinase, 1,9-pyrazoloanthrone (SP600125; 10 μM), whereas STAT3 and SP3 were insensitive. This suggests for the first time that JNK, in addition to ERK, may play an important role in regulating *SOCS-3* gene activity in HUVECs. Indeed, we found that SP600125 (10

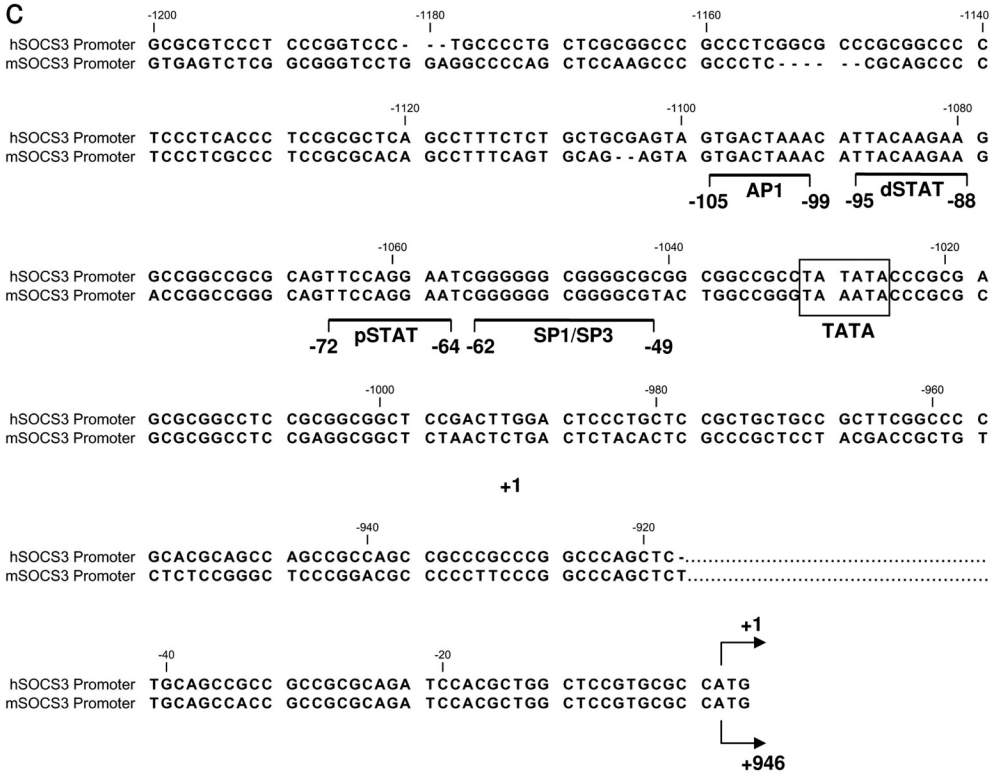


Fig. 4. *Continued.* c, sequence alignment of the comparative coding regions of the murine (mSOCS3; Entrez Gene ID 12702; Genbank accession no. AF117732) and human (hSOCS; Entrez Gene ID 9021) SOCS-3 genes was carried out using CLC Workbench Software and an abbreviated comparison of the first 1.2 kilobase pairs of the 5′-regulatory regions of each sequence is shown here. The transcription initiation site within the murine *SOCS-3* promoter is indicated as +1 (Auernhammer et al., 1999). The corresponding initiation site within the human *SOCS-3* promoter has yet to be formally identified therefore numbering of this sequence was begun at the translation initiation codon. pSTAT- and dSTAT-responsive elements, previously identified (Auernhammer et al., 1999) within the murine promoter, are indicated, as is the GC-rich, specificity protein 1/3 (SP1/SP3) binding site (Ehlting et al., 2005; Barclay et al., 2007). The putative AP-1 site identified by Barclay et al. (2007) and the TATA box are also shown.

μM) is an effective inhibitor of cAMP-induced SOCS-3 protein induction in these cells (Fig. 5e).

Interactions between AP-1, STAT, and SP1/SP3 Transcription Factor Binding Sites Are Required for ERK-Dependent Regulation of the SOCS-3 Promoter. To further test the requirement for AP-1, STAT, and SP1/SP3 transcription factor binding sites for the regulation of the minimal, ERK-responsive SOCS3 promoter (−107/+929), we introduced disruptive point mutations, individually or in combination, into each of these consensus binding motifs to test their requirement for PMA-induced transcriptional activation in COS-1 cells.

In the first set of experiments, we examined the impact of disrupting the putative SP1/SP3 binding site, either alone or in combination with the other consensus motifs (Fig. 6a). In agreement with others (Ehlting et al., 2005; Barclay et al., 2007), we found that ablation of the SP1/SP3 site alone was sufficient to significantly reduce basal transcriptional activity and severely blunt induction of the minimal *SOCS-3* promoter (Fig. 6a). Mutation of any of the AP-1, dSTAT, or pSTAT sites in combination with the SP1/SP3 site caused a further significant reduction in basal transcriptional activity of the promoter region and effectively ablated the ability of PMA to induce transcriptional activation (Fig. 6a). These results demonstrate that the SP1/SP3 transcription factor binding site is vital for basal and ERK-stimulated regulation of the *SOCS-3* promoter, possibly through supporting the activity and promoter recruitment of STAT and AP-1 complex transcription factors.

In the next set of experiments, we sought to determine the individual roles of AP-1 and STAT consensus sites in regulating promoter activity (Fig. 6b). Disruption of the pSTAT site alone caused a significant reduction in PMA-stimulated transcription activity, with little noticeable effect on basal activity of the promoter (Fig. 6b). Ablation of the dSTAT site, alone or in combination with the pSTAT site, seemed to have little effect on PMA-induced promoter activation (Fig. 6b). There was, however, a definite requirement for AP-1 complex transcription factors, because mutation of this site caused a marked decrease in transcriptional activation by PMA (Fig. 6b). We were surprised that codeletion of the AP-1 site with the dSTAT, but not the pSTAT, site abolished the ability of PMA to induce activation of the *SOCS-3* promoter (Fig. 6b). These results demonstrate the importance of the AP-1 and pSTAT sites for the ERK-dependent regulation of the *SOCS-3* promoter and that the activity of the AP-1 site seems to depend on transcription factor interaction with the dSTAT site (Fig. 6b).

Discussion

Our previous work using COS1 cells demonstrated that downstream signaling from cAMP-activated EPAC1 to the *SOCS-3* gene seems to involve a pathway including Rap1 (Sands et al., 2006; Yarwood et al., 2008; Borland et al., 2009), phospholipase C ε and protein kinase C isoforms α and δ (Borland et al., 2009). Our work here expands on these findings and further demonstrates that in HUVECs, cPKCs and nPKCs (namely, PKCs α, δ, η, and ε) play a vital role in governing the induction of *SOCS-3* gene expression through coordinated regulation of the ERK MAP kinase pathway after elevations in intracellular cAMP. We demonstrated the involvement of PKC isoforms and ERK in *SOCS-3* induction by cAMP using relatively high concentrations (10 μM) of two indolylmalemide-based PKC inhibitors, Gö 6983 and Ro-31-7549, in addition to the MEK inhibitor U0126 (Fig. 3a). Whereas the specificity of the PKC inhibitors at this concentration may be questionable, because they have also been

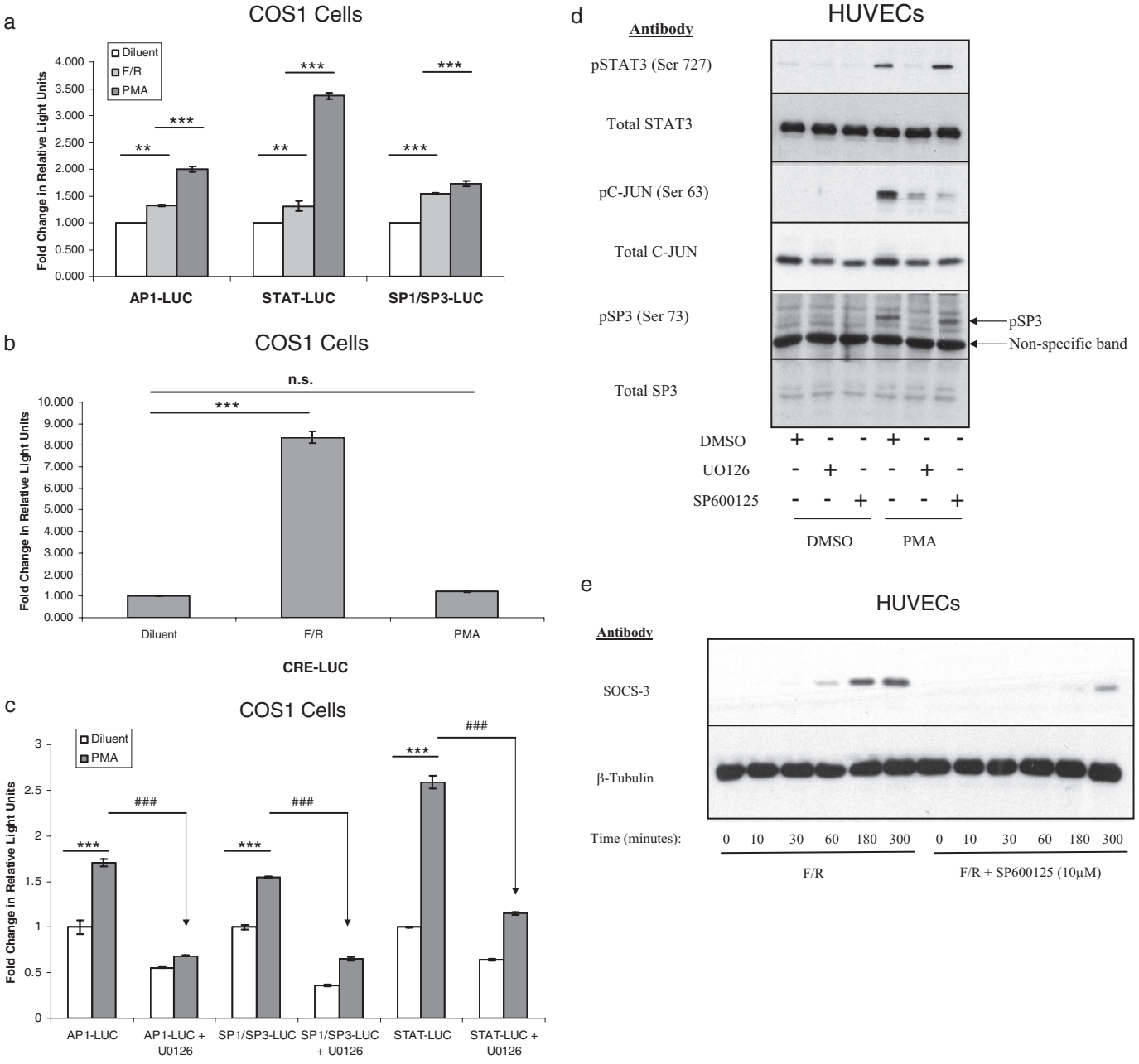


Fig. 5. cAMP or PMA treatment leads to ERK-dependent activation of AP-1, STAT, and SP1/SP3 transcription factors. a, COS1 cells were cotransfected with *R. reniformis* luciferase vector and the indicated firefly luciferase reporter constructs. Cells were then stimulated for 16 h with either a combination of 50 μM forskolin and 10 μM rolipram (F/R) or 10 μM PMA. Cells were then harvested and luciferase activities determined. Significant differences relative to diluent-treated cells are indicated (**, $p < 0.01$; ***, $p < 0.001$). b, COS1 cells that had been transfected with a CRE, firefly luciferase reporter construct were stimulated for 16 h with either F/R or PMA. Luciferase activities were then determined and plotted as a histogram. Significant differences in luciferase activities relative to diluent-stimulated cells are indicated (***, $p < 0.001$). Nonsignificant changes in F/R treated cells are also indicated (n.s.). c, COS1 cells were cotransfected with *R. reniformis* luciferase vector and either an AP-1, STAT, or SP1/SP3 reporter construct. Cells were then stimulated for 16 h with 10 μM PMA, in the presence or absence of 10 μM U0126. Luciferase activities were then determined and plotted in a histogram of means ± S.E.M. for three separate experiments. Significant increases in activity in PMA-treated cells relative to diluent-stimulated are indicated (***, $p < 0.001$). Significant decreases in luciferase activity in U0126-treated cells are also indicated (###, $p < 0.001$). d, HUVECs were stimulated for 30 min with F/R or PMA in the presence or absence of the JNK inhibitor SP600125 (10 μM) or the ERK inhibitor U0126 (10 μM). Cell extracts were then prepared and immunoblotted with the indicated phosphospecific or "total" protein antibodies to c-Jun, STAT3, and SP3 transcription factors. Results are representative of an individual experiment carried out on three separate occasions. e, HUVECs were stimulated for the indicated times with MG132 (10 μM) plus F/R in the presence or absence of the JNK inhibitor, SP600125 (10 μM). Cell extracts were then prepared and immunoblotted for SOCS-3 and β-tubulin as indicated. Results are representative of an individual experiment carried out on three separate occasions.

shown to be able to inhibit other kinases, including S6 kinase β1 and glycogen synthase kinase 3β (Davies et al., 2000), the use of specific siRNAs (Figs. 1 and 2) provides more compelling evidence for the involvement of specific PKC isoforms. U0126, on the other hand, has been shown to display a much more impressive selectivity profile (Davies et al., 2000), and we can be confident that it achieves on-target effects here, particularly because we found that it inhibits known ERK-

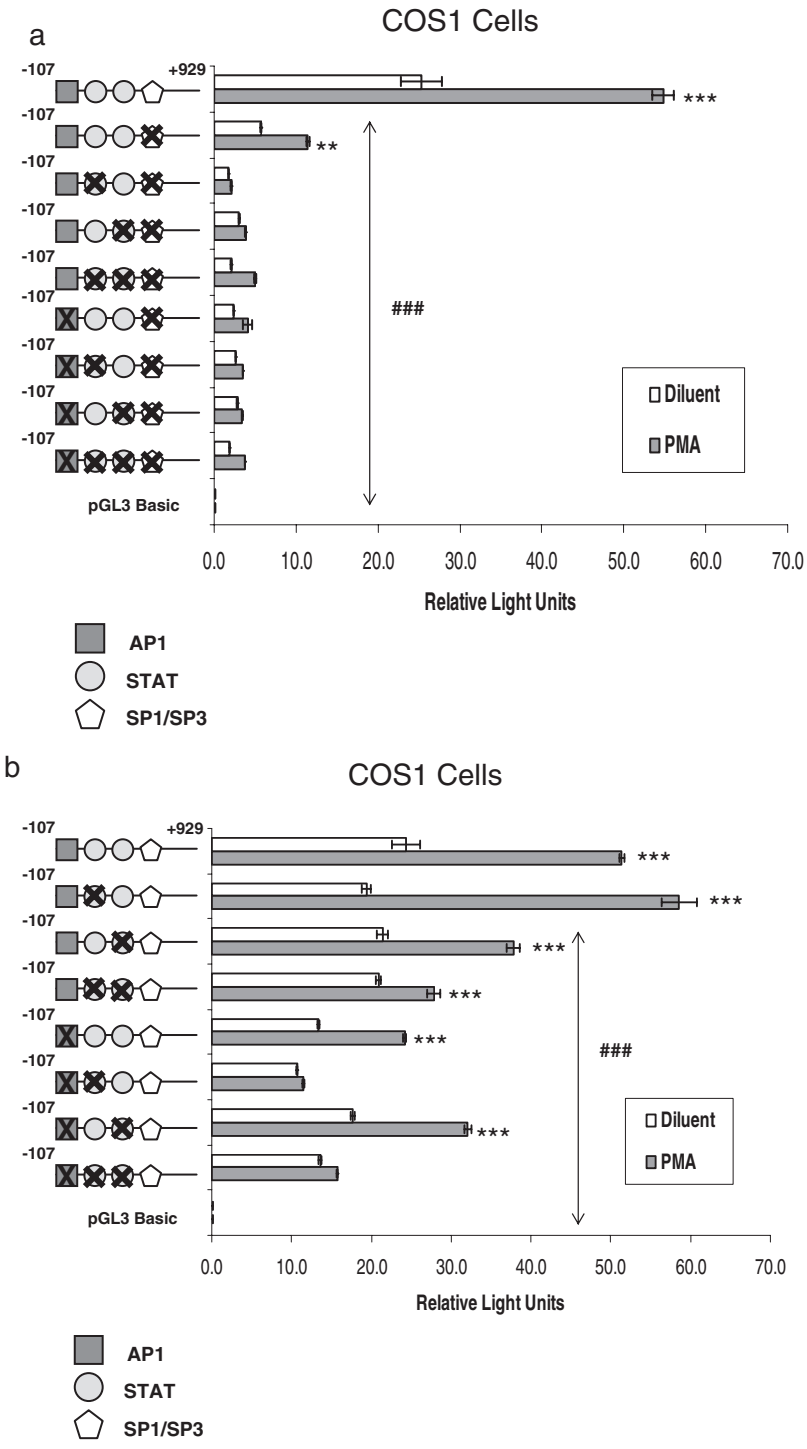


Fig. 6. Role of AP-1, STAT, and SP1/SP3 transcription factor binding sites in the response of the *SOCS-3* promoter to PMA-promoted ERK activation. a, COS1 cells were cotransfected with *R. reniformis* luciferase vector, to normalize luciferase activity, together with firefly luciferase reporter constructs containing the minimal ERK-responsive element of the murine SOCS3 promoter, mSOCS-3 −107, in which various combinations of the putative AP-1, STAT, and SP1/SP3-binding sites had been mutated, as described under *Materials and Methods*. The relative positions of mutated transcription factor binding sites are shown in the schematic on the left side of the histogram. Cells were then stimulated with 10 μM PMA for 16 h, and luciferase activities were determined. Results are expressed as relative light units and represent means ± S.E.M. for three separate experiments. Significant differences relative to cells stimulated with diluent alone are indicated (***, $p < 0.001$), as are differences between mutated promoters and wild-type promoter, mSOCS-3 −107 (###, $p < 0.001$). b, COS1 cells were cotransfected with *R. reniformis* luciferase vector together with wild-type *SOCS-3* promoter construct, mSOCS-3 −107 or mSOCS-3 −107 that had been mutated to ablate various combinations of transcription factor binding sites as indicated on the left side of the histogram. After 16-h treatment with 10 μM PMA, cells were harvested, and luciferase activities were determined. Significant differences relative to diluent-treated cells (***, $p < 0.001$) or wild-type mSOCS-3-107 (###, $p < 0.001$) are indicated.

phosphorylation sites in STAT3, SP3, and c-Jun (Fig. 5d). Likewise, the JNK inhibitor 10 μM SP600125 was shown to block the known Ser63 JNK-phosphorylation site in c-Jun, as well as SOCS-3 induction by cAMP (Fig. 5e), despite having well known off-target effects (Bain et al., 2003). Future work will be necessary, however, to unequivocally determine the involvement of the JNK cascade in the regulation of *SOCS-3* induction.

Deletion analysis of the murine *SOCS-3* gene has previously been used to isolate the minimal functional promoter, which contains an AP-1 site, between nucleotides −105 and −99 from the start of transcription (Bousquet et al., 2001; Barclay et al., 2007), that is reported to bind c-*fos* and JunB (Bousquet et al., 2001), a GC-rich region (−58 to −52) that has the potential to bind either SP1 (Barclay et al., 2007) or SP3 (Ehlting et al., 2005) transcription factors and two binding sites for STAT transcription factors (Auernhammer et al., 1999; Ehlting et al., 2005), one proximal to the transcription start site (pSTAT; −72 to −64) and one distal (dSTAT; −95 to −87). Activation of the *SOCS-3* promoter after cytokine stimulation seems to involve the pSTAT site, which seems to be able to bind STAT 1, 3, or 5, depending on cell context

(Emanuelli et al., 2000; He et al., 2003a; Yang et al., 2010). The function of the dSTAT site remains to be determined. *SOCS-3* responsiveness to IL-6 has also been shown to require SP3 transcription factor binding to the GC-rich region, in addition to STAT3 interaction with the pSTAT site (Ehlting et al., 2005; Yang et al., 2010). In contrast, activation of the *SOCS-3* promoter by cAMP seems to be independent of STAT binding; rather, it relies on the AP-1 site (Bousquet et al., 2001) or SP-1 interaction with the GC-rich region (Barclay et al., 2007), again depending on the cell type studied.

We found that once activated in HUVECs, ERK induces phosphorylation and activation of AP-1, STAT, and SP1/SP3 family transcription factors. Of these, the AP-1 and pSTAT sites seem to be vital for full transcriptional activation by ERK, whereas the SP1/SP3 region seems to be responsible for maintaining basal promoter activity and for supporting the activity of the STAT and AP-1 sites. The molecular basis for this ERK-dependent cooperation between transcription factor binding sites remains to be determined but may involve the recruitment of enzymes, such as acetyltransferases, that are required for full transcription factor activity at the other sites. In this respect, c-Jun, STAT3, and SP3 have all been shown to require acetylation at specific lysine residues to achieve their full activity (Vries et al., 2001; Ammanamanchi et al., 2003; Yuan et al., 2005). It has also been demonstrated that the coactivator acetyltransferase CREB binding protein p300, is recruited to the *SOCS-3* promoter after ERK activation (Qin et al., 2007; Baker et al., 2008). Clearly, further work needs to be done in this area to determine whether CREB binding protein p300, or another acetyltransferase, is required for the regulation of transcription factors targeting the *SOCS-3* promoter. Moreover, the role of transcription factor methylation should also be investigated in light of a recent study demonstrating that STAT3 is reversibly dimethylated after recruitment to the *SOCS-3* promoter, leading to termination of its activity (Yang et al., 2010). In this case, it is thought that phosphorylation of Ser727 on STAT3, which we show here to be ERK-dependent in HUVECs, is thought to provide a binding site for the lysine 4 of histone 3 methyltransferase SET9 (Yang et al., 2010); however, this hypothesis requires to be formally tested.

An additional consideration is what is the role of C/EBPβ and C/EBPδ in this process? We have shown that both transcription factors are required for effective *SOCS-3* induction in HUVECs and that C/EBPβ is also phosphorylated and activated by ERK (Yarwood et al., 2008; Borland et al., 2009; Woolson et al., 2009). Chromatin immunoprecipitation analysis demonstrated that C/EBPβ interacts with the human *SOCS-3* promoter at approximately −2000 (~3000 base pairs from ATG) from the putative transcription start site (Yarwood et al., 2008), which is quite distant from the minimal promoter region identified in this study. We predict, therefore, that there is an additional C/EBP binding site located within the −107/+929 promoter region, perhaps located within the ERK-responsive AP-1 site we identified. This is a credible proposition, because it has been demonstrated that C/EBPβ and c-Jun physically interact to regulate induction of the TNFα gene in myelomonocytic cells (Zagariya et al., 1998); whether the same is true for the *SOCS-3* gene remains to be determined. Overall, this work points toward a central role for ERK-regulated transcription factors in regulating the induction of the *SOCS-3* gene in response to elevations in intracellular cAMP. These findings may have consequences for a broad range of signaling scenarios in which understanding of the molecular basis controlling *SOCS-3* gene induction may have therapeutic benefit; these range from stimulating ERK-dependent *SOCS-3* induction in vascular endothelial cells to combat chronic inflammation (Parnell et al., 2011), to suppression of *SOCS-3* in macrophages to combat the progression of cancer (Hiwatashi et al., 2011).

Authorship Contributions

Participated in research design: Wiejak, Borland, and Yarwood.
Conducted experiments: Wiejak, Dunlop, Gao, and Borland.
Performed data analysis: Yarwood.
Wrote manuscript: Yarwood.

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