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TRANSCRIPTIONAL ACTIVATION OF THE SUPPRESSOR OF CYTOKINE SIGNALING-3 (SOCS3) GENE VIA STAT3 IS INCREASED IN F9 REX1 (ZFP-42) KNOCKOUT TERATOCARCINOMA STEM CELLS RELATIVE TO WILD TYPE CELLS

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SUMMARY

Rex1 (Zfp42), first identified as a gene that is transcriptionally repressed by retinoic acid (RA), encodes a zinc finger transcription factor expressed at high levels in F9 teratocarcinoma stem cells, embryonic stem (ES) cells, and other stem cells. Loss of both alleles of *Rex1* by homologous recombination alters the RA-induced differentiation of F9 cells, a model of pluripotent ES cells. We identified Suppressor of Cytokine Signaling-3 (SOCS3) as a gene which exhibits greatly increased transcriptional activation in RA, cyclicAMP and theophylline (RACT) treated F9 *Rex1*^{-/-} cells (~25-fold) as compared to wild type (Wt) cells (~2.5-fold). By promoter deletion, mutation and transient transfection analyses, we have shown that this transcriptional increase is mediated by the STAT3 DNA binding elements located between -99 to -60 in the SOCS-3 promoter. Overexpression of STAT3 dominant negative mutants greatly diminishes this SOCS-3 transcriptional increase in F9 *Rex1*^{-/-} cells. This increase in SOCS-3 transcription is associated with a 4–5 fold higher level of tyrosine phosphorylated STAT3 in the RACT treated F9 *Rex1*^{-/-} cells as compared to Wt. Dominant negative Src tyrosine kinase, Jak2, and protein kinase A (PKA) partially reduce the transcriptional activation of the SOCS-3 gene in RACT-treated F9 *Rex1* null cells. In contrast, parathyroid hormone peptide enhances the effect of RA in F9 *Rex1*^{-/-} cells, but not in F9 Wt. Thus, *Rex-1*, which is highly expressed in stem cells, inhibits signaling via the JAK/STAT pathway, thereby modulating the differentiation of F9 cells.

Keywords

Retinoic Acid; dibutyryl cyclic AMP; RAR (retinoic acid receptor); zinc finger transcription factor; stem cells

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INTRODUCTION

Retinoids such as all-trans retinoic acid (RA) have been shown to play important roles during mammalian development and in the processes of cell differentiation in various cell types^{1; 2; 3; 4; 5}. The diverse biological effects of RA are mediated by its ability to act as both an activator and repressor of gene transcription. Rex1 (Zfp42) (for reduced expression) was first discovered as a gene whose expression is greatly reduced upon RA treatment in F9 teratocarcinoma stem cells⁶, cells which resemble the pluripotent, embryonic stem (ES) cells of the inner cell mass (ICM) of the mouse blastocyst⁷. F9 stem cells differentiate into primitive endoderm when treated with RA, and into parietal endoderm when treated with RACT (retinoic acid plus cyclic AMP and theophylline). Rex1 encodes a protein which contains four “cys-his” zinc finger motifs, indicating that Rex1 functions as a transcription factor^{8; 9}. When wild type F9 cells are cultured in the presence of exogenous RA and differentiate along the primitive endoderm (PrE), parietal endoderm (PE), or visceral endoderm (VE) pathways, Rex1 mRNA levels begin to decline at 12 h, and by 96 h the Rex1 mRNA level is reduced several-fold relative to the level in untreated F9 stem cells^{6; 10}. In addition to its high level of expression in F9 stem cells, the Rex1 gene is expressed at high levels in murine ES cells¹⁰ and has been reported to be transcriptionally regulated by nanog¹¹. Rex1 expression has frequently been used as a molecular marker of both ES and adult murine and human stem cells^{12; 13; 14; 15; 16; 17; 18; 19; 20}. In adult male mice, the Rex1 gene is expressed in the testis at a specific stage during the process of spermatogenesis¹⁰. We've also shown that Rex1 expression is reduced in human kidney tumor specimens as compared to normal tissue²¹.

Current models suggest that Rex1 modifies differentiation by its effects on the transcription of target genes, though the relevant target genes haven't been described. Through the use of DNA microarray analyses (Xu and Gudas, unpublished) we have identified genes that are differentially expressed between RA-treated F9 Wt cells and F9 Rex1^{-/-} cells, which were generated in our laboratory *via* homologous recombination²². One of these genes is the suppressor of cytokine signaling-3 (SOCS-3). One function of SOCS-3 is in the regulation of the JAK/STAT pathway, which is activated by several cytokines. Cytokines such as LIF (leukemia inhibitory factor) regulate cellular behavior by interacting with receptors on the plasma membrane of target cells and activating intracellular signal transduction cascades, including the JAK-STAT pathway^{23; 24; 25; 26}. STAT proteins such as STAT3 control cell cycle progression and apoptosis, and thus overexpression or misregulation of STAT gene expression can contribute to oncogenesis^{27; 28; 29; 30; 31; 32; 33}. Conversely, STAT3 blockade decreased tumor growth in a xenograft model of squamous carcinoma of the head and neck³⁴ and in various human cultured cancer cell lines³⁵. The ablation of STAT3 results in an embryonic lethal phenotype. Homozygous STAT3-null embryos degenerate rapidly immediately after blastocyst implantation³⁶.

Among at least three classes of negative regulators of the JAK-STAT pathway is the family of SOCS proteins^{37; 38}. SOCS-3 is a potent inhibitor of the JAK-STAT signaling cascade, negatively regulating signal transduction of a variety of cytokines³⁹. SOCS-3 negatively regulates LIF signaling by directly binding to the JAK/LIFR/gp130 complex⁴⁰, and inhibiting JAK kinase activity⁴¹. A STAT3 binding element is functionally critical for the positive transcriptional regulation of murine SOCS-3 promoter activity⁴², and this autoregulatory circuit between SOCS-3 and STAT3 maintains proper balance between cell renewal and cell differentiation^{43; 44}. Although SOCS3 has primarily been considered to be a negative regulator of the JAK-STAT signaling cascade, in some cell types SOCS-3 transcription greatly increases during the differentiation process and SOCS-3 has been shown to be required for proper myoblast differentiation⁴⁵. SOCS-3 gene expression is high in some tumors, including breast⁴⁶ and cutaneous T-cell lymphoma⁴⁷. The SOCS-3 gene is often methylated in head and neck squamous cell carcinoma⁴⁸ and in hepatocellular carcinoma⁴⁹. SOCS-3 overexpression

in liver causes insulin resistance⁵⁰ and SOCS-3 expression is induced by resistin, a hormone which antagonizes the effects of insulin in adipocytes⁵¹. SOCS-3-deficient mice die as a result of placental defects during embryonic development^{52; 53}.

Understanding the mechanisms by which the lack of Rex1 protein results in aberrantly high SOCS-3 expression will better define the roles of Rex1 in stem cell renewal, differentiation, and early embryogenesis. In this series of experiments, we examine the mechanisms by which the murine SOCS-3 gene is transcriptionally activated in response to RA and dibutyryl cyclic AMP (db cAMP) in F9 Wt cells versus F9 Rex1^{-/-} cells. We demonstrate that the SOCS-3 gene is induced by RA and dibutyryl cAMP via a transcriptional mechanism involving PKA, Src, and Jak2. This transcriptional activation is greatly increased in the absence of Rex1.

RESULTS

Identification of SOCS-3 as a Rex1 Target Gene in F9 Cells by Microarray Analysis

We used DNA microarray assays to identify Rex1 target genes by comparison of F9 Wt and F9 Rex1^{-/-} knockout cells cultured with or without RA. From the microarray experiments (Xu and Gudas, in preparation), the murine SOCS-3 gene scored the highest level of difference, showing a 17.7-fold higher expression in RA-treated R21 Rex1^{-/-} cells as compared to RA-treated F9 Wt cells. This high level of difference in expression and the fact that SOCS-3 is a key regulator of the STAT3 signaling pathway resulted in the selection of the SOCS-3 gene for further analysis.

Induction of the SOCS-3 gene expression by RA, CT, and RACT

F9 WT cells and two independently isolated F9 Rex1^{-/-} knockout cell lines (R21 and R5) were cultured for 24 h, 48 h, or 72 h in the presence of 1 μ M RA, 250 μ M dibutyryl cAMP, and 250 μ M theophylline (RACT) as previously described^{54; 55; 56; 57; 58}. SOCS-3 mRNA was present in untreated F9 Wt cells, and RA or CT alone had only a small effect on the levels of SOCS-3 transcripts at 72 hr. (Figure 1, lower panel). The combination of RA and CT (RACT) induced the expression of SOCS-3 transcripts in F9 Wt (for 48 h: 2.43 ± 0.58 fold, $n=3$, $p=0.0038$; for 72 h: 5.74 ± 0.53 fold, $n=3$, $p=0.0004$) (Fig. 1).

The expression of SOCS-3 transcripts was just detectable in untreated F9 Rex1^{-/-} (R21) cells (Fig. 1). After 48 h of RA, SOCS-3 mRNA expression was induced (for 48 h: 5.78 ± 0.83 fold, $n=3$, $p=0.035$; for 72 h: 4.3 ± 0.53 fold, $n=3$, $p=0.005$) (Figure 1). CT treatment alone also induced SOCS-3 expression (for 24 h: 2.32 ± 0.06 fold, $n=3$, $p=0.023$; for 72 h: 3.32 ± 0.22 fold, $n=3$, $p=0.003$), although the effect of CT was smaller than the effect of RA on SOCS-3. RACT treatment was able to increase SOCS-3 mRNA levels in Rex1^{-/-} cells to a much greater degree than in F9 Wt cells (for 24 h: 6.54 ± 1.83 fold, $n=3$, $p=0.021$; for 48 h: 25.95 ± 3.53 fold, $n=3$, $p=0.001$; for 72 h: 16.56 ± 0.78 fold, $n=3$, $p=0.0005$). The same results were observed in two independently isolated F9 Rex1^{-/-} null cell lines R21 and R5) (Figure 1). Thus, RACT treatment of F9 Rex1^{-/-} cells resulted in a ~25-fold increase in SOCS-3 transcripts at 48 hr. vs. a ~2.5-fold increase in F9 Wt.

To confirm that the increase in SOCS-3 transcripts resulted from the absence of Rex1^{-/-}, the full-length Rex1 cDNA was stably transfected back into the F9 Rex1^{-/-} (R21) cells (Fig. 2). In part because the exogenous Rex1 gene was driven by a SV40 promoter and SV40-driven transcription is not efficient in F9 cells⁵⁹, the expression of Rex1 in the Rex1 “put-back” lines was only ~10% of its expression in F9 Wt cells. However, this low level of Rex1 mRNA was sufficient to partially reverse the expression pattern of SOCS-3 mRNA to its expression pattern in F9 Wt cells (Figure 2), suggesting that only a low level of Rex1 expression is required to reduce the level of SOCS-3 gene induction.

Induction of SOCS-3 protein expression by RA, CT, and RACT

We also examined SOCS-3 protein levels in F9 Wt, F9 Rex1^{-/-} (R21 and R5), and the stably transfected R21JX12 cells. Cells from each cell line were cultured in the presence of 1 μ M RA, 250 μ M dibutyryl cAMP, and 250 μ M theophylline (RACT). After 72 h, total proteins were harvested for Western analysis of SOCS-3. In F9 Wt cells the SOCS-3 protein was not expressed at a detectable level in control, RA-treated, and CT-treated cells (Figure 3), while RACT treatment resulted in induction of SOCS-3 protein expression to a limited extent (<2 fold). In the two independently-isolated F9 Rex1^{-/-} null cell lines, SOCS-3 protein was not expressed in control cells (Figure 3). RA or CT alone did not have any effect on SOCS-3 protein expression, while RACT greatly increased the SOCS-3 protein level (Fig. 3). In the cells in which Rex1 was stably transfected back into the F9 Rex1^{-/-} (R21) cells, the expression of the SOCS-3 protein was partially reversed to its expression pattern in F9 Wt cells (Fig. 3). Thus, the expression of SOCS-3 protein correlates with the expression of SOCS-3 transcripts.

RA, CT, and RACT activate the SOCS-3 promoter/reporter constructs, and this activation is greatly augmented in the absence of Rex1

Constructs 2, 6 and 6D2C (referred as 6D2 in the original paper)⁴² which use 5' flanking DNA of the SOCS-3 gene to drive luciferase reporter expression, were provided to us by Dr. S. Melmed at Cedars-Sinai Medical Center. For measurement of SOCS-3 promoter activity, the F9 Wt cells, the two F9 Rex1^{-/-} null lines (R21, R5), and the Rex1 stably transfected line (R21JX12) were transiently transfected with pGL3Basic alone, construct 2 (nucleotides -2757 to -714), construct 6 (nucleotides -2757 to +929), or construct 6D2C (nucleotides -2757 to +929, but with the complete tandem STAT binding region from nucleotides -99 to -60 deleted) (Figure 4A). The vehicle, pGL3Basic, was used as a control (V). Construct 2 did not contain an essential region of the minimal promoter of SOCS-3 (-714 to +929), and hence it served as another control. The transfected cells were either untreated, or treated with 1 μ M RA, 250 μ M dibutyryl cAMP, and 250 μ M theophylline (RACT) for 48 h. Cells were then harvested for luciferase assays. The durations of the treatments were determined based on the Northern results (Figure 1) in which the expression of SOCS-3 transcript was not induced until after 48 h of RA treatment of F9 Rex1^{-/-} cells.

In **F9 Wt cells**, the effects of RA, CT, and RACT on construct 6 were not significant (for all values: fold change <2, n=4, p>0.05). The deletion of the STAT3 binding repeats (construct 6D2C) did not alter the response of SOCS-3 promoter to RA, CT, and RACT treatments significantly (for all values: fold change <2, n=4, p>0.05) (Fig. 4B, inset).

In **F9 Rex1^{-/-} (R21) cells**, RA addition activated the SOCS-3 promoter (construct 6) by at most two-fold (2 ± 0.19 fold, n=4, p=0.05). The addition of CT increased the SOCS-3 promoter activity greatly (18.2 ± 2.5 fold, n=4, p=0.00015). The induction of SOCS-3 promoter activity by the RACT drug combination was even greater (34.4 ± 7.8 fold, n=4, p= 1.5×10^{-6}) (Fig. 4B). When the STAT3 binding repeats were deleted, the effect of RA on the promoter 6D2C/reporter activity was no longer significant (1.2 ± 0.8 fold, n=4, p>0.05); the effect of CT on this SOCS-3 promoter activity was no longer significant (3.2 ± 1.1 fold, n=4, p>0.05); and the effect of RACT on construct 6D2C SOCS-3 promoter activity was reduced to an activation of only 8 ± 2.7 fold (n=4, p=0.02), which was much lower than the induction of construct 6 seen with RACT treatment (34.4 ± 7.8 fold) (Fig. 4B). Similar results were observed in the F9 Rex1^{-/-} (R5) cells (Fig. 4B). The activities of the SOCS-3 promoter constructs in the exogenous Rex1 stably transfected ("Put-back") cells were like those in F9 Wt cells (Fig. 4B). Similarly, SOCS-3 promoter activity decreased when increasing levels of a Rex1 expression vector were cotransfected into F9 Rex1^{-/-} cells (Fig. 4C). Thus, the SOCS-3 promoter is substantially more active in the absence of Rex1, and re-addition of Rex1 to the Rex1^{-/-} cells reduced SOCS-3 promoter activity.

STAT3-dependent induction of SOCS-3 by RA, CT, and RACT in WT and Rex1^{-/-} teratocarcinoma cells

When the tandem STAT binding sites were deleted from the 5' flanking region of murine SOCS-3 gene, the RA-, CT-, and RACT-induced activation of the SOCS-3 promoter activity decreased by more than six-fold (Fig. 4B). We hypothesized that this decrease resulted from the inability of positively acting STAT3 proteins to bind to this regulatory region of the SOCS-3 promoter. To test this hypothesis, F9 Wt cells and R21 Rex1^{-/-} cells were co-transfected with construct 6 and a number of STAT3 expression plasmids (Fig. 5). These plasmids include the expression vector construct 6 alone (V), the WT STAT3 (STAT3), two dominant negative (DN) forms of STAT3 (Y705F, D715)⁶⁰, and the constitutively active form of STAT3 (STAT3C)⁶¹. For Y705, the tyrosine residue that is phosphorylated by JAK kinases in the WT STAT3 is mutated to a phenylalanine. Therefore, Y705 can no longer be phosphorylated. D715 lacks the C-terminal transcriptional activation domain and hence, D715 can no longer transcriptionally activate target genes. Y705 is more potent than D715 as a DN mutant⁶⁰. For STAT3C, an alanine residue and an asparagine residue within the C-loop of the SH2 domain were both mutated to cysteine residues. As a result of the mutations, two STAT3C molecules dimerize, bind to DNA with a greater affinity, and activate transcription persistently^{61, 62}. The transfected cells were either untreated or treated with 1 μ M RA, 250 μ M db cAMP, and 250 μ M theophylline (RACT) for 48 h. Then cells were harvested for luciferase assays.

In F9 Wt cells, the co-transfection of WT STAT3 and the two DN mutants of STAT3 (Y705F and D715) did not alter SOCS-3 promoter (construct 6) activity significantly (for all values: fold change < 2, n=3, p>0.05) (Figure 5). The co-transfection of the constitutively active mutant, STAT3C, induced SOCS-3 promoter activity in the control, RA-, CT-, and RACT-treated F9 Wt cells by around two-fold each (Fig. 5, inset).

In F9 Rex1^{-/-} (R21) cells, the co-transfection of WT STAT3 (STAT3 in Fig. 5B) reduced SOCS-3 promoter activity in CT- (-2.7 ± 0.8 fold, n=3, p=0.035) and RACT-treated (-2.54 ± 0.56 fold, n=3, p=0.017) cells relative to transfection of construct 6 alone (V). There was no significant effect in control (-1.67 ± 0.2 fold, n=3, p>0.05) or RA-treated (-1.88 ± 0.76 fold, n=3, p>0.05) cells (Fig. 5). A possible explanation for this result is that the transfected WT STAT3 behaved as a mild dominant negative in the cells. It has been reported that a naturally truncated STAT3 could bind to phosphorylated STAT3 monomers and act as a dominant negative^{63; 64}.

The co-transfection of the Y705F expression construct reduced the SOCS-3 promoter activity in CT- (-13.3 ± 3.76 fold, n=3, p=0.001) and RACT-treated cells (-24.8 ± 3.76 fold, n=3, p=0.002) relative to the transfection of construct 6 alone (V) or construct 6 plus Wt STAT3 (STAT3) (Fig. 5B). There was no significant effect in control (-2.16 ± 0.26 fold, n=3, p>0.05) or RA-treated (-2.4 ± 0.75 fold, n=3, p>0.05) cells (Fig. 5B). Similarly, the co-transfection of a construct harboring another DN mutant of STAT3, Y715, also reduced SOCS-3 promoter activities in CT- and RACT- treated cells relative to the transfection of construct 6 alone (V); this reduction was not as great as that seen with the Y705F mutant STAT3 (Fig. 5B). Thus, the large increase in SOCS-3 promoter activity in F9 Rex1^{-/-} cells is dependent on activated STAT3.

In RACT treated F9 Wt cells co-transfection of the plasmid encoding the constitutively active mutant STAT3C resulted in a statistically significant increase in activity over that of construct 6 transfected alone (V) (Fig. 5B). In contrast, compared with the same treatments in the Rex1^{-/-} cells transfected with only the SOCS-3 promoter/luciferase construct 6 (V in Fig. 5B), the co-transfection of the constitutively active mutant, STAT3C, greatly induced SOCS-3 promoter activity in control (5.3 ± 0.47 fold, n=3, p=0.02), RA- (13.03 ± 3.2 fold, n=3, p=0.001), and CT- treated (2.4 ± 0.13 fold, n=3, p=0.023) F9 Rex1^{-/-} cells. Strikingly,

transfection of this constitutively active STAT3C construct induced SOCS-3 promoter/reporter activity in the presence of RA alone *only* in the F9 Rex1^{-/-} cells. In RACT-treated STAT3C transfected Rex1^{-/-} cells the induction over the activity of the construct 6 transfected alone (V) was not significant (1.08 ± 0.08 fold, $n=3$, $p>0.05$) (Fig. 5B). Since RACT does not cause a further increase in the presence of constitutively activated STAT, we hypothesized that the effects of RACT are mediated by STAT3 in F9 Rex1^{-/-} cells. To test this hypothesis, we examined how the loss of Rex1 influenced STAT3 activity.

The expression of STAT3 transcripts and protein in WT and Rex1^{-/-} teratocarcinoma cells

F9 Wt and two independently-isolated F9 Rex1^{-/-} null lines (R21 and R31) were cultured for 24 h or 72 h in the presence of 1 μ M RA, 250 μ M dibutyryl cAMP, and 250 μ M theophylline (RACT). Total RNA was extracted for the Northern analysis of STAT3 (Figure 6A). RACT treatment of F9 Wt and the two F9 Rex1^{-/-} cell lines resulted in the induction of STAT3 transcripts to a much greater level at 72 h (~7.5-fold). However, no differences in STAT3 mRNA expression were detected between F9 Wt cells and the two F9 Rex1^{-/-} cell lines (Fig. 6A, lower panel for quantitation).

F9 Wt, the F9 Rex1^{-/-} null lines R21 and R5, and the R21JX12 cell line were untreated, or treated with RA, CT, and RACT, for 48 hours. Total proteins were harvested for Western analyses of total STAT3 and phosphorylated STAT3 protein (Figure 6B). There was no difference in *basal* STAT3 protein expression among the four cell lines (Fig. 6B). The treatments with RA, CT, or RACT did not result in a statistically significant increase in the *total* STAT3 protein level in any of the four cell lines (Fig. 6B, second gel panel). Thus, total STAT3 protein levels are regulated differently, and are not correlated with STAT3 transcript levels in RACT treated F9 Wt and Rex1^{-/-} cell lines.

Tyrosine phosphorylated STAT3 was then measured in the same experiment, and a slight increase in expression of phosphorylated STAT3 was seen in RACT-treated, but not in control, RA-, or CT-treated F9 Wt cells (Fig. 6B, top gel panel, and bar graph below for quantitation). In F9 Rex1^{-/-} cells, RA induced the phosphorylation of STAT3, and RACT greatly increased the level of phosphorylated STAT3 (Fig. 6B, top gel panel, bar graph below for quantitation). The RACT-induced STAT3 phosphorylation in F9 Rex1^{-/-} cells was ~4–5 fold higher than in F9 Wt cells (Fig. 6B, bar graph). In the Rex1 “put-back” stably transfected line, the expression pattern of phosphorylated STAT3 was reversed to a pattern similar to that in F9 Wt cells (Fig. 6B, top gel panel, and bar graph below for quantitation). SOCS-3 protein levels in the same cell extracts are also shown (Fig. 6B, third gel panel). Thus, the level of tyrosine phosphorylated STAT3, and, as expected, the level of SOCS-3 protein were much higher in RACT treated F9 Rex1^{-/-} cells than in RACT treated F9 Wt cells.

PKA activates SOCS-3 transcription in F9 cells

Many actions of cyclic AMP are mediated by protein kinase A (PKA) (for rev.65). This serine/threonine kinase exists as an inactive tetramer composed of two regulatory and two catalytic subunits. Binding of cyclic AMP to the regulatory subunits results in the release of the active catalytic subunits and subsequent phosphorylation of target substrates⁶⁶. Regulatory subunits with mutations in the cAMP-binding domains confer a dominant inhibition of the PKA system in cells⁶⁷. Recent evidence suggests that cyclic AMP signaling also occurs through pathways other than the activation of PKA⁶⁸.

To determine whether the action of cyclic AMP in this system was mediated by PKA, a construct encoding a PKA dominant negative (DN) mutant MT Rev(AB) (Figure 7A) was co-transfected with the SOCS-3 promoter/reporter (construct 6 from Fig. 4). After transient transfection, cells were either untreated, or treated with RA, CT, or RACT, for 48 hours. The

PKA DN mutant did not significantly alter SOCS-3 promoter/reporter activity in either control or RA treated F9 Wt or Rex1^{-/-} cells (Figure 7B). However, in CT-treated F9 Wt cells, the PKA DN mutant reduced SOCS-3 promoter/reporter activity by 3.52 ± 0.29 fold (n=3, p=0.003). In CT-treated R21 Rex1^{-/-} cells, the PKA DN mutant reduced SOCS-3 promoter/reporter activity by 2.92 ± 0.086 fold (n=3, p=0.02). In RACT-treated F9 Wt cells, the PKA DN mutant reduced SOCS-3 promoter/reporter activity by 3.5 ± 0.46 fold (n=3, p=0.003). In RACT-treated R21 Rex1^{-/-} cells, the PKA DN mutant reduced SOCS-3 promoter/reporter activity by 2.68 ± 0.41 fold (n=3, p=0.05). The results from these experiments indicate that treatment with CT or RACT activates SOCS-3 transcription through a PKA-dependent pathway.

Jak2 and Src activate SOCS-3 transcription in F9 cells

STAT3 was originally identified as the acute phase response factor⁶⁹ and STAT3 is tyrosine phosphorylated by JAKs following the binding of members of the IL-6 family of cytokines to their receptors. Four members of the JAK family have been identified so far: Jak1, Jak2, Jak3, and Tyk-2⁷⁰. Jak1, Jak2 and Tyk-2 are ubiquitous, and Jak3 is expressed primarily in T lymphocytes⁷⁰. Recent studies have shown that STAT proteins can also be activated by a variety of receptor and non-receptor protein tyrosine kinases^{71; 72; 73; 74}. These protein tyrosine kinases include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR)^{75; 76; 77}, colony stimulating factor-1 receptor⁷⁰, and the Src tyrosine kinase⁷⁸.

To determine the pathway by which STAT3 is activated and consequently, SOCS-3 transcription is activated, Jak1 DN, Jak2 DN, and Src DN mutants (Figure 8A) were co-transfected with the SOCS-3 promoter/reporter (construct 6). After transfection, cells were either untreated or treated with RACT for 48 hours. In F9 Wt cells, the Jak1 DN, Jak2 DN, and Src DN constructs did not alter the SOCS-3 promoter/reporter activity significantly in either control cells or RACT-treated cells (Figure 8B).

Jak1 DN, Jak2 DN, and Src DN mutants did not affect SOCS-3 transcription significantly in Rex1^{-/-} cells under control (untreated) conditions. In contrast, in RACT-treated R21 Rex1^{-/-} cells, the Src DN mutant reduced the SOCS-3 promoter/reporter activity by 2.79 ± 0.22 fold (n=3, p=0.023) and the Jak2 DN mutant reduced SOCS-3 promoter/reporter activity by 3.21 ± 0.27 fold (n=3, p=0.034). The apparent reduction of SOCS-3 promoter/reporter activity by the Jak1 DN mutant in RACT-treated R21 cells was not statistically significant (p>0.05). We conclude that Src and Jak2 are involved in the enhanced activation of SOCS-3 transcription by RACT treatment in Rex1^{-/-} cells.

RAR α Stimulates SOCS-3 Transcription through the STAT3 Binding Sites

To assess whether the RA:RAR complex played a role in the activation of SOCS3 transcription we transiently co-transfected the murine RAR α in an expression vector into F9 Wt vs. Rex1^{-/-} R21 cells, and then cultured the cells $\pm$ RACT for 48 hr. pSG/RAR α transfection increased the activity of the SOCS3 promoter (construct 6) by ~2-fold over the activity with empty pSG5 vector co-transfected in both the F9 Wt and the R21 mutant. This increase did not take place when the SOCS3 promoter construct 6D2C, which lacks the STAT3 binding sites, was co-transfected (Fig. 9). Thus, in the presence of RA RAR α can stimulate SOCS3 promoter activity, but this stimulation requires the STAT3 binding sites.

Parathyroid hormone (PTH) peptide enhances the effect of RA in F9 Rex1^{-/-} cells but not in F9 Wt cells

To examine the effect of PTH on RA-induced SOCS-3 mRNA in F9 cells, PTH was added to the cells at the same time that RA was added. As a control, PTH peptides were added to the

cells alone. In F9 Wt cells, the addition of PTH alone did not have any effect on SOCS-3 mRNA levels. When PTH was used in combination with RA, PTH peptide did not enhance the action of RA on SOCS-3 mRNA expression in F9 Wt cells. In control F9 Rex1^{-/-} (R21) cells, the addition of PTH alone did not have any effect on SOCS-3 transcript level. In contrast, the combination of PTH and RA greatly increased SOCS-3 mRNA levels in R21 Rex1^{-/-} cells (Fig. 10A).

β-mercaptoethanol (BME) partially blocks the effect of RACT in F9 cells

BME is a membrane permeant reducing agent which has been shown to inhibit the actions of reactive oxygen species (ROS), and thereby, inhibit activation of the JAK/Stat pathway^{75; 79; 80}. To examine the effect of BME on RACT-induced SOCS-3 transcript levels, BME was added to the cells at the same time RACT was added. In both F9 Wt and F9 Rex1^{-/-} cells, the addition of BME partially blocked the effect of RACT on SOCS-3 mRNA expression (Fig. 10A). These results suggest that ROS is involved in RACT-induced SOCS-3 mRNA expression though tyrosine phosphorylation.

DISCUSSION

The STAT3 binding sites are required for a high level of SOCS-3 transcriptional activation in RACT-treated F9 Rex1^{-/-} cells

In the absence of Rex1, the transcriptional activation of the *SOCS-3* gene by RA, CT, and RACT was greatly augmented (Figure 4). Using transient transfection of promoter/reporter constructs, we showed that the STAT3 tandem binding sites, located between -99 and -60 in the 5' genomic region, were required for the high activity of the SOCS-3 promoter/reporter in the F9 Rex1^{-/-} cells, but not in the F9 WT cells (statistically not significant) (Figure 4B). Deletion of the STAT3 binding sites in the SOCS-3 promoter (construct 6D2C) lowered the activity of the SOCS-3 promoter/reporter activity by ~6 to ~8-fold in F9 Rex1^{-/-} cells (Figure 4B).

The two STAT3 DN mutants, Y705F and D715, also reduced SOCS-3 promoter (construct 6, Fig. 4A, B) activity in Rex1^{-/-} cells to a level that was similar to the activity of construct 6D2C (Figure 5B). The reduction by the STAT3 DN mutant Y705 was greater than that seen with the STAT3 DN mutant D715. These data also indicate that STAT3 activation is required for the high level of transcriptional activation of the SOCS-3 gene in F9 Rex1^{-/-} cells after CT or RACT treatment.

The co-transfection of the constitutively active mutant of STAT3 (STAT3C) activated the SOCS-3 promoter by two-fold in F9 Wt cells; this most likely results from a greater number of STAT3 dimers bound to the SOCS-3 regulatory region (Fig. 5). A similar twofold increase in the presence of STAT3C was observed in RA, CT, and RACT-treated F9 Wt and F9 Rex1^{-/-} cells (Figure 5B). In F9 Rex1^{-/-} cells RA alone had an effect similar to CT alone or RACT on SOCS-3 promoter/reporter activity (Fig. 5B, see STAT3C, RA treatment).

The Role of cyclic AMP in SOCS-3 Transcription

According to the negative autoregulatory circuit proposed for STAT3/SOCS-3, tyrosine phosphorylated STAT3 activates SOCS-3 transcription. Subsequently, SOCS-3 protein inhibits the activation of STAT3^{37; 81; 82}. We report here that in F9 Rex1^{-/-} cells, the levels of *both* phosphorylated STAT3 and SOCS-3 greatly increase in response to RACT treatment (Figs. 1, 2, 3). This is in contrast to the reduction in phosphorylated STAT3 that would generally be expected with increased SOCS-3 protein.

To test the involvement of PKA in SOCS-3 transcriptional activation, we co-transfected an expression construct containing a PKA DN mutant with the SOCS-3 promoter/reporter. The PKA DN mutant did not have any effect on control or RA-treated cells (both Wt and Rex1^{-/-} cells) (Figure 7). In contrast, the transfection of the PKA DN mutant lowered the SOCS-3 promoter/reporter activity by ~3.5 fold in CT and RACT-treated F9 Wt and F9 Rex1^{-/-} cells (Fig. 7). Similarly, Bousquest *et al.*^{41; 83} reported that in addition to cytokines, cAMP agonists, alone or with LIF, increased SOCS-3 promoter activity in AtT20 cells. A consistent, additive effect was seen between cAMP analogs and LIF, and the cAMP actions were shown to be mediated *via* PKA. A dominant negative PKA reduced the cAMP mediated induction of the SOCS-3 promoter by 50%⁸³. Bousquest *et al.*^{41; 83} reported that cAMP activated SOCS-3 transcription in AtT20 cells *via* an AP-1 site (TGACTAA) at -105/-99. C-fos and Jun B, but not phospho-CREB, bound to this site in gel shift assays⁴¹. The AP-1 site and the STAT3 sites are very close together in the SOCS-3 promoter. However, we have shown that the enormous activation of the SOCS-3 promoter in the Rex1^{-/-} cells is completely dependent on the STAT-3 sites in the promoter (Figs. 4 and 5). In addition, a dominant negative c-jun expression vector, transfected with the SOCS3(6) promoter into RACT treated Rex1^{-/-} cells, did not decrease the promoter activity (data not shown). Thus, our data are more consistent with the effect of cyclic AMP in the Rex1^{-/-} cells being exerted through an increased level of tyrosine phosphorylated STAT-3 protein (Fig. 6B) rather than through c-fos, junB, or CREB binding at a separate site on the SOCS-3 promoter.

Another mechanism by which cyclic AMP could enhance SOCS-3 transcription in the presence of RA is via phosphorylation of the activation functions of the RARs by pka^{84; 85}. Additional mechanisms of crosstalk between the RA and the STAT3 signaling pathways have been reported in leukemia cells^{31; 86; 87}.

STAT3 activation by intracellular signaling pathways

STAT proteins can be activated by a variety of receptor and non-receptor protein tyrosine kinases^{71; 72; 73; 75; 76; 86; 88; 89}. To delineate the pathway that activates STAT3 in RACT-treated F9 cells, we further explored the signaling pathway upstream of STAT3. First, we examined the effects of Src DN, Jak1 DN, and Jak2 DN mutant expression constructs on SOCS-3 promoter/reporter activity (Fig. 8). The transfection of each of these mutants did not significantly alter the SOCS-3 promoter/reporter activity in F9 WT cells (Figure 8B). In RACT-treated F9 Rex1^{-/-} cells, the transfection of either the Jak2 DN or Src DN lowered the SOCS-3 promoter/reporter activity by ~3-fold. However, neither the Src DN expression construct nor the Jak2 DN construct had as potent an effect as the STAT3 DN mutants (Fig. 5) on the SOCS-3 promoter/reporter activities. A dominant negative MEK expression vector, transfected into RACT treated F9 Rex1^{-/-} cells with the SOCS3(6) promoter vector, did not cause any decrease in SOCS3 promoter activity (data not shown). This indicates that the MEK pathway is not required for the enhanced SOCS3 promoter activity seen in the Rex1 null cells.

The classical pathway of STAT3 activation is through LIF/LIFR/gp130/Jak2^{90; 91}. However, there are several pieces of evidence that LIF is not involved in the enhanced STAT3 activation in F9 Rex1^{-/-} cells. First, LIF is not required for F9 cells to remain undifferentiated⁹², and LIF is not present in the culture medium. Second, the LIF pathway is rapid, e.g. only thirty minutes are required for STAT3 phosphorylation following LIF addition⁹³. Third, LIFR levels are similar in F9 WT cells and F9 Rex1^{-/-} cells (Tighe and Gudas, unpublished data).

It has been reported that the stimulation of the G-protein coupled receptor (GPCR) family members leads to tyrosine phosphorylation of JAKs and STATs, and subsequently induces STAT binding activity in target cells⁹⁴. Pelletier *et al.*⁹⁴ reported that the GPCR agonists thrombin and angiotensin II could stimulate tyrosine phosphorylation of STAT3 in a Rac-dependent manner in smooth muscle cells. They provided solid data suggesting that the late,

second phase of STAT3 activation by GPCRs depends on the autocrine production of a ligand, possibly IL-6, by a Rac-dependent mechanism. Pettelier *et al.* suggested that GPCR activation leads to Rho activation, and subsequently, reactive oxygen species (ROS) are activated, resulting in the activation of the Jak2/STAT3 pathway⁹⁴. PTHR (parathyroid hormone receptor) is a member of the GPCR family⁹⁵ and the expression of PTHR mRNA is higher in F9 Rex1^{-/-} cells than in F9 WT cells after RA treatment (Fig. 10A). It was reported that PTH (parathyroid hormone) activates Src kinase activity⁹⁶ and Src kinase activates Rho family members by phosphorylation⁹⁷. Thus, PTHR may be involved in the activation of Jak2 and STAT3 in RACT treated F9 Rex1^{-/-} cells (Fig. 10B).

The Role of Rex1 in the STAT3 Pathway

In RACT treated F9 Rex1^{-/-} cells phosphorylated STAT3 levels are very high (Fig. 6), indicating that the Rex1 protein plays an inhibitory role in this pathway in F9 cells. In the absence of Rex1 and with RACT treatment, then, higher levels of expression of receptors such as PTHR (Fig. 10A, B) would lead to increased tyrosine phosphorylation and activation of STAT3. This increase in activated STAT-3 would then result in the increased transcription of the SOCS-3 gene observed in the RACT treated Rex1^{-/-} cells. We found no functional evidence of Rex1 directly binding to the SOCS-3 promoter, i.e. a promoter element, which when deleted, would elevate the level of SOCS-3 promoter driven luciferase activity (Xu and Gudas, unpublished). Thus, our data suggest an indirect role for Rex1 protein in this pathway, possibly, for example, via stimulating the transcription of an inhibitor of STAT3 such as GRIM1⁹⁸; 99; 100; 101 (Fig. 10B). This model is consistent with our experimental data which indicate that the enhanced levels of SOCS-3 mRNA in Rex1 null cells occur at later times (>48 hr.) after RA addition (Figs. 1, 2).

MATERIALS AND METHODS

Cell Culture

F9 WT cells, two homozygous knockout lines [Rex1^{-/-}-2-1 (R21), Rex1^{-/-}-5 (R5)]²², and an exogenous Rex1 expression “put back” line (R21JXR12) were maintained in DMEM (Dulbecco's Modified Eagles Medium) containing 10% heat-activated calf serum and 2 mM glutamine. To culture cells in monolayer, the flasks and dishes were treated with 0.3% gelatin before plating the cells. To generate primitive endoderm (PrE), F9 cells were grown in monolayer culture in the above medium containing 1 μ M all-trans RA (Cat# R-2625, Sigma, St. Louis, MO). Medium on the cells was replaced every 48 hours with fresh medium containing 1 μ M RA. To generate parietal endoderm (PE), F9 cells were grown in the above medium containing 1 μ M RA, 250 μ M dibutyryl cyclic AMP (db cAMP) (N⁶, 2'-O-dibutyryl adenosine 3':5'-cyclic monophosphate, Sigma Cat# D-0627), and 250 μ M theophylline (Sigma Cat# T-1633). Medium on the cells was replaced every 48 hours with fresh medium containing RA, db cAMP, and theophylline (RACT). PTH 1-34 was from Sigma (#P4056).

DNA Microarray Analysis

F9 WT cells and F9 Rex1^{-/-} cells were either untreated or treated with 1 μ M RA for 48 hours, and total RNA was then extracted. Microarray analysis was carried out according to the Affymetrix Genechip expression analysis technical manual. Briefly, total RNA from untreated and RA-treated (1 μ M RA for 48 h) F9 WT and F9 Rex1^{-/-} cells was reverse transcribed into cDNA. After second-strand synthesis, cDNAs were then *in vitro* transcribed into cRNAs with biotinylated ribonucleotides (Enzo Diagnostics, Farmingdale, NY). cRNA (20 μ g) was fragmented by heating at 94 °C for 35 minutes. A cocktail containing fragmented cRNA, control oligonucleotide B2, control cRNA (Biotin B, Biotin C, Biotin D, and Cre), and herring sperm DNA was hybridized to microarray chips (MG-U74Av2, 11,938 mouse genes) for 16 hours at 45 °C. After washing and staining, the distribution of fluorescent material on the array

was measured using a laser scanner. The resultant image was processed with MAS (Microarray Suite) software (Affymetrix, Santa Clara, CA). The microarray experiments were repeated two times with different RNA preparations. Only genes which exhibited reproducible differences in expression patterns among the four groups of cells were further analyzed.

Microarray Data Analysis

The Microarray Core Facility of Weill Medical College of Cornell University performed the data analysis. The expression level of each gene and the fold change in expression in untreated versus RA-treated F9 Wt cells and F9 Rex1^{-/-} (R21) cells were calculated using a computer program (GeneSpring, Redwood, CA). This computer program was also used for functional gene grouping analyses (not shown).

Stable Transfection and Generation of the Rex1 “Put Back” or “Exogenous Rex1 Expression” Lines

F9 Rex1^{-/-}-2-1 cells (4×10^6 cells/150 μ m plate were set up the night before) were co-transfected with 40 μ g of pREX-SG plasmid and 4 μ g pSV2Neo plasmid by the calcium phosphate precipitation method¹⁰². This pREX-SG plasmid was constructed by Dr. Betsy Hosler¹⁰³. pREX-SG contains the full length mouse *Rex1* cDNA driven by the SV40 promoter; the *Rex1* full length cDNA is in the correct orientation for expression of the murine Rex1 protein. Both plasmids were prepared using the QIAGEN EndoFree™ Maxi Kit (QIAGEN Inc., Valencia, CA), and supercoiled DNAs were used for the transfection. Three hours prior to transfection, the old medium was replaced with 20 ml of fresh medium. For the transfection, DNA was suspended in 840 μ l 1mM Tris (pH 7.4) first; then, 120 μ l of 2M CaCl₂ was added dropwise with agitation to the DNA; finally, the DNA-CaCl₂ was added to 960 μ l 2 $\times$ HBS (pH=7.05) with bubbling, the reaction (Tris + DNA + CaCl₂ + 2 $\times$ HBS) was incubated for 20 minutes at room temperature, and then added dropwise to the cells. The precipitates were removed after 16 hours, and then the cells were washed with 1 $\times$ TBS. Approximately 48 hours after transfection, G418 (Gibco, Carlsbad, CA, Cat#11811-0311) was added to a final concentration of 390 μ g/ml active drug. The cells were cultured in medium with 300 μ g/ml active G418 for three weeks, and 70 G418 resistant cell colonies were picked for analysis. Each resistant colony was individually expanded, a duplicate well was frozen, and total RNA was extracted for Northern analyses to assay for the Rex1 positive clones.

Western Blotting

Cells were either untreated, treated with RA, CT, or RACT for 6 hr, 24 hr, 48 hr, or 72 hr. After 48 hours, medium was changed and fresh drugs were added. Cell monolayers were washed twice with ice-cold PBS and harvested in 200 μ l 2X SDS-PAGE buffer (240 mM Tris-HCl, pH6.8, 8% SDS (sodium dodecylsulfate), 40% glycerol, without bromphenol blue). Cell lysates were then passed through 26G5/8 needles (VWR, West Chester, PA, #309597). Then, cell lysates were boiled for 5 minutes, and cell debris were removed by centrifuging the cell lysates for 30 seconds in a microfuge at maximum speed in the cold. Some lysates were used for protein concentration measurements using the DC Protein Assay (Bio-Rad, Hercules, CA, #500-0114). Dithiothreitol was added to the remainder of the cell lysates to a final concentration of 100 μ M, and lysates were stored at -20 °C or at -70°C. Then, 45 μ g (for STAT3 analysis) or 100 μ g (for SOCS-3 analysis) of total cell extract was mixed 1:1 with 2X SDS-PAGE buffer with 0.1% bromphenol blue, boiled for 5 minutes, and separated on a 7.5% SDS-PAGE gel for STAT3 and a 12% gel for SOCS-3. The gel was electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, #162-0090). The membrane was blocked in 5% BLOTTO (Santa Cruz Biotechnology, Santa Cruz, CA, #sc-2325) made in PBS-0.5% Tween 20 for 3 hours at room temperature. Then, the membranes were incubated with primary antibodies to STAT3 (Upstate Biotechnology, Inc., Charlottesville, VA, #06-596) 1:2,000; Phospho-STAT3

(Cell Signaling Technology, Beverly, MA, #9131) 1:2,000; SOCS-3 (Santa Cruz Biotechnology, #sc-7009) 1:200; and actin (Santa Cruz Biotechnology, #sc-1616) 1:2,000, overnight at 4°C, and with secondary antibodies: anti-rabbit IgG-horseradish peroxidase (HRP) (Santa Cruz Biotechnology, #sc-2030) 1:5,000; or anti-goat IgG-HRP (Santa Cruz Biotechnology, #sc-2056) 1:7000 for 2 hours at room temperature. Results were visualized by enhanced chemiluminescence reaction using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc., Rockford, IL, #34080), according to the manufacturer. To detect the luminescent signal, blots were exposed to film and developed.

Northern Analysis

Northern analysis was performed as previously described¹⁰⁴. Total RNA was prepared from cells using the RNA-BEE™ (TEL-TEST "B", Inc., Friendswood, TX, #CS-104B). Each RNA sample (10 µg) was run on a 6.2% formaldehyde agarose gel. RNA was transferred to a Hybond-N nylon membrane (Amersham Biosciences, Piscataway, NJ, #RPN303) and hybridized to individual probes in standard hybridization solution containing 5X SSC, 5 mM EDTA, 10X Denhardt's, 0.1% SDS, 10% dextran sulfate, 100 µg/ml Salmon Sperm DNA, and 50% formamide. Probes were radiolabeled using the Random Primed DNA Labeling Kit (Roche Diagnostics, Indianapolis, IN, #1004760). Hybridized filters were washed two times under low stringency conditions (0.1% SDS and 2X SSC) at 42 °C for 20 minutes each and then were washed once under high stringency (0.1% SDS and 0.2X SSC) at 60 °C for another 20 minutes. After washing, the filters were exposed to Kodak BioMax film, and a Phosphorimager. Each filter was also hybridized to GAPDH as a loading control. We obtained a cDNA for murine SOCS-3 from Dr. J. Ihle at St. Jude Children's Research Hospital, and sequenced this to confirm that it was SOCS-3.

Construction of Reporter Plasmids

PCR was performed in a total volume of 20 µl using 10 ng of the template (clone 6), 10 pmol each of either the forward or reverse primer, 200 µmol dNTP, 1X Cloned Pfu DNA polymerase reaction buffer (Stratagene, La Jolla, CA, Cat# 600250), and 2.5 U PfuTurbo DNA Polymerase (Stratagene, Cat# 600250). PCR reactions were carried out in a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA) for 30 cycles with the following parameters: 94 °C for 45 seconds, 55 °C for 45 seconds, 72 °C for various lengths of time (1 minute per kb). Before the 30 cycles, PCR reactions were heated at 94 °C for 5 minutes to separate double-stranded DNA. After 30 cycles, PCR reactions were extended for another 10 minutes at 72 °C for final nucleotide extensions.

Murine SOCS-3 promoter clones 2, 6, and 6D2C were kindly provided by Dr. S. Melmed from Cedars-Sinai Research Institute. All three of these constructs were cloned into the pGL3-Basic vector by Auernhammer *et al*^{42, 83}. Clone 6 contains the -2757 to +929 5' genomic region of murine SOCS-3. Clone 2 is a truncation of clone 6 (nucleotides -2757 to -714) and lacks the minimal promoter region. In clone 6D2C (referred to as 6D2 by Auernhammer *et al*⁴²), the complete tandem STAT binding region from nucleotides -99 to -60 was deleted⁴².

All of the inserts for the SOCS-3 promoter deletions and mutations were obtained by PCR. PCR products were run on a 1% agarose gel and the PCR products were gel-purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, Cat# 28706). Purified PCR products and either the pGL3-Basic vector (Promega, Madison, WI, lacks eukaryotic promoter and enhancer sequences) or pGL3-Promoter vector (Promega, contains SV40 promoter located upstream of the luciferase gene) were cut by designated restriction enzymes overnight. Then, restriction digested PCR products and vectors were run on a 1% agarose gel and gel-purified. The gel-purified PCR products and vectors were ligated using the Rapid DNA Ligation Kit (Roche

Diagnostics, Cat# 1635379). The ligated products were transformed into the host strain, DH5 α . The products were confirmed by DNA sequencing.

Transient Transfections and Dual Luciferase Assays

1.5×10^6 F9Wt cells/100mm plate and 1.3×10^6 F9 Rex1^{-/-} cells/100 mm plate were plated the night before transfection. Three hours before transfection, the medium was changed. Then, cells were transiently transfected with 4 μ g of reporter plasmid and 1.5 μ g of Renilla luciferase plasmid (Promega) by the calcium phosphate precipitation method. Plasmids were prepared using the QIAGEN EndoFreeTM Maxi Kit (QIAGEN Inc., Valencia, CA), and supercoiled DNAs were used for the transfection. For the transfections: first, DNA was brought up to 420 μ l in 1 mM Tris (pH 7.4); then, 60 μ l of 2M CaCl₂ were added dropwise with agitation to DNA; finally, DNA-CaCl₂ was added to 480 μ l 2XHBS with bubbling, the reaction was incubated for 20 minutes at room temperature, and then was added dropwise to the cells. The precipitates were removed after 16 to 18 hours and then the cells were washed with 1X TBS. Then, RA, CT, and RACT were added with fresh medium immediately after washing. Forty-eight hours after drug treatments, cells were harvested for dual-luciferase assays.

To study the effects of the retinoic acid receptors (RARs), expression vectors containing the full-length murine RAR α or RAR γ downstream of the SV40 promoter were transiently co-transfected. In some experiments an expression plasmid with the full-length murine Rex1 cDNA downstream of the SV40 promoter was co-transfected. To study the effects of these various constructs: WTSTAT3, Y705F (STAT3 dominant negative (DN) mutant), D715 (STAT3 DN mutant), STAT3-C (constitutively active mutant of STAT3), a Jak1 DN mutant, a Jak2 DN mutant, and a Src DN mutant, 4 μ g of each of the above constructs was cotransfected with 1.5 μ g of the Renilla luciferase construct and 4 μ g of clone 6 of the SOCS-3 promoter. For a negative control, 4 μ g of the pUC9 vector was co-transfected with 1.5 μ g of the Renilla luciferase construct and 4 μ g of clone 6 of the SOCS-3 promoter. To study the effect of MT-REV(AB) (protein kinase A DN) on SOCS-3 promoter activity, 3 μ g of the MT-REV(AB) construct was co-transfected with 1.5 μ g of the Renilla luciferase construct and 4 μ g of clone 6 of the SOCS-3 promoter. For the negative control, MT64AA [the MT-REV(AB) empty expression vector] was co-transfected with 1.5 μ g of the Renilla luciferase construct and 4 μ g of clone 6 of the SOCS-3 promoter. To induce the expression of MT-REV (AB), ZnCl₂ was added to the cells to a final concentration of 50 μ M at the same time that RA, CT, and RACT were added.

Forty-eight hours after drug treatments, transiently transfected cells were harvested in 1X Passive Lysis Buffer (Promega, Cat# E194A). Cell lysates were first stored at -20 °C overnight. Before luciferase assays, cell lysates were thawed and the cell debris were removed by centrifuging at maximum speed in a microfuge for 30 seconds at room temperature. For F9 Wt and Rex1^{-/-} samples 20 μ l (~5 μ g) of cell lysates were used to perform luciferase assays according to the manufacturer's protocol for Dual-Luciferase[®] Assay System (Promega, Cat# E1960). The amounts of cell lysates used for the luciferase assays were determined by running concentration curves and the amounts used were within the linear ranges for luciferase activity/ μ g protein. For each assay, the luminometer was programmed to provide a 2-second pre-read delay, followed by a 10-second measurement period. The luciferase values were recorded by a TD-20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA).

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REFERENCES

1. Gudas LJ. Molecular mechanisms of retinoid action. *Am J Respir Cell Mol Biol* 1990;2:319–20. [PubMed: 2157472]
2. Gudas LJ. Retinoids and vertebrate development. *J Biol Chem* 1994;269:15399–402. [PubMed: 7910825]
3. Means AL, Gudas LJ. The roles of retinoids in vertebrate development. *Ann. Rev. Biochem* 1995;64:210–233.
4. Mongan NP, Gudas LJ. Diverse actions of retinoid receptors in cancer prevention and treatment. *Differentiation*. 2007 In press.
5. Mark M, Ghyselinck NB, Chambon P. Function of retinoid nuclear receptors: lessons from genetic and pharmacological dissections of the retinoic acid signaling pathway during mouse embryogenesis. *Annu Rev Pharmacol Toxicol* 2006;46:451–80. [PubMed: 16402912]
6. Hosler BA, LaRosa GJ, Grippo JF, Gudas LJ. Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Mol Cell Biol* 1989;9:5623–9. [PubMed: 2511439]
7. Stevens, LC. The origin and development of testicular, ovarian and embryo-derived teratocarcinomas. *Teratocarcinoma Stem Cells*, 10, Cold Spring Harbor Lab. Press; New York: 1983.
8. Kaczynski J, Cook T, Urrutia R. Sp1- and Kruppel-like transcription factors. *Genome Biol* 2003;4:206. [PubMed: 12620113]
9. Mongan NP, Martin KM, Gudas LJ. The putative human stem cell marker, Rex-1 (Zfp42): structural classification and expression in normal human epithelial and carcinoma cell cultures. *Mol Carcinog* 2006;45:887–900. [PubMed: 16865673]
10. Rogers MB, Hosler BA, Gudas LJ. Specific expression of a retinoic acid-regulated, zinc-finger gene, Rex-1, in preimplantation embryos, trophoblast and spermatocytes. *Development* 1991;113:815–24. [PubMed: 1821852]
11. Shi W, Wang H, Pan G, Geng Y, Guo Y, Pei D. Regulation of the pluripotency marker Rex-1 by Nanog and Sox2. *J Biol Chem* 2006;281:23319–25. [PubMed: 16714766]
12. Scherer CA, Chen J, Nachabe A, Hopkins N, Ruley HE. Transcriptional specificity of the pluripotent embryonic stem cell. *Cell Growth Differ* 1996;7:1393–401. [PubMed: 8891343]
13. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. “Stemness”: transcriptional profiling of embryonic and adult stem cells. *Science* 2002;298:597–600. [PubMed: 12228720]
14. Goolsby J, Marty MC, Heletz D, Chiappelli J, Tashko G, Yarnell D, Fishman PS, Dhib-Jalbut S, Bever CT Jr, Pessac B, Trisler D. Hematopoietic progenitors express neural genes. *Proc Natl Acad Sci U S A* 2003;100:14926–31. [PubMed: 14634211]
15. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* 2004;10:55–63. [PubMed: 14702635]
16. Verfaillie CM. Adult stem cells: assessing the case for pluripotency. *Trends Cell Biol* 2002;12:502–8. [PubMed: 12446111]
17. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol* 2002;30:896–904. [PubMed: 12160841]
18. Jiang Y, Henderson D, Blackstad M, Chen A, Miller RF, Verfaillie CM. Neuroectodermal differentiation from mouse multipotent adult progenitor cells. *Proc Natl Acad Sci U S A* 2003;100 (Suppl 1):11854–60. [PubMed: 12925733]
19. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;418:41–9. [PubMed: 12077603]

20. Kucia M, Reza R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, Ratajczak MZ. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. *Leukemia* 2006;20:857–69. [PubMed: 16498386]
21. Raman JD, Mongan NP, Liu L, Tickoo SK, Nanus DM, Scherr DS, Gudas LJ. Decreased expression of the human stem cell marker, Rex-1 (zfp-42), in renal cell carcinoma. *Carcinogenesis* 2006;27:499–507. [PubMed: 16344273]
22. Thompson JR, Gudas LJ. Retinoic acid induces parietal endoderm but not primitive endoderm and visceral endoderm differentiation in F9 teratocarcinoma stem cells with a targeted deletion of the Rex-1 (Zfp-42) gene. *Mol Cell Endocrinol* 2002;195:119–33. [PubMed: 12354678]
23. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science* 1994;264:95–8. [PubMed: 8140422]
24. Horvath CM. STAT proteins and transcriptional responses to extracellular signals. *Trends Biochem Sci* 2000;25:496–502. [PubMed: 11050435]
25. Levy DE, Lee CK. What does Stat3 do? *J Clin Invest* 2002;109:1143–8. [PubMed: 11994402]
26. Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002;3:651–62. [PubMed: 12209125]
27. Dauer DJ, Ferraro B, Song L, Yu B, Mora L, Buettner R, Enkemann S, Jove R, Haura EB. Stat3 regulates genes common to both wound healing and cancer. *Oncogene* 2005;24:3397–408. [PubMed: 15735721]
28. Calo V, Migliavacca M, Bazan V, Macaluso M, Buscemi M, Gebbia N, Russo A. STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol* 2003;197:157–68. [PubMed: 14502555]
29. Bromberg J. Stat proteins and oncogenesis. *J Clin Invest* 2002;109:1139–42. [PubMed: 11994401]
30. Chan KS, Sano S, Kiguchi K, Anders J, Komazawa N, Takeda J, DiGiovanni J. Disruption of Stat3 reveals a critical role in both the initiation and the promotion stages of epithelial carcinogenesis. *J Clin Invest* 2004;114:720–8. [PubMed: 15343391]
31. Dong S, Chen SJ, Tweardy DJ. Cross-talk between retinoic acid and STAT3 signaling pathways in acute promyelocytic leukemia. *Leuk Lymphoma* 2003;44:2023–9. [PubMed: 14959844]
32. Quadros MR, Peruzzi F, Kari C, Rodeck U. Complex regulation of signal transducers and activators of transcription 3 activation in normal and malignant keratinocytes. *Cancer Res* 2004;64:3934–9. [PubMed: 15173005]
33. Leeman RJ, Lui VW, Grandis JR. STAT3 as a therapeutic target in head and neck cancer. *Expert Opin Biol Ther* 2006;6:231–41. [PubMed: 16503733]
34. Xi S, Gooding WE, Grandis JR. In vivo antitumor efficacy of STAT3 blockade using a transcription factor decoy approach: implications for cancer therapy. *Oncogene* 2005;24:970–9. [PubMed: 15592503]
35. Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL, Cheng JQ, Jove R, Yu H. Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. *Oncogene* 2005;24:5552–60. [PubMed: 16007214]
36. Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S. Targeted disruption of the mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci U S A* 1997;94:3801–4. [PubMed: 9108058]
37. Hilton DJ. Negative regulators of cytokine signal transduction. *Cell Mol Life Sci* 1999;55:1568–77. [PubMed: 10526574]
38. Krebs DL, Hilton DJ. SOCS: physiological suppressors of cytokine signaling. *J Cell Sci* 2000;113 (Pt 16):2813–9. [PubMed: 10910765]
39. Auernhammer CJ, Melmed S. The central role of SOCS-3 in integrating the neuroimmunoendocrine interface. *J Clin Invest* 2001;108:1735–40. [PubMed: 11748254]
40. Nicholson SE, De Souza D, Fabri LJ, Corbin J, Willson TA, Zhang JG, Silva A, Asimakis M, Farley A, Nash AD, Metcalf D, Hilton DJ, Nicola NA, Baca M. Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proc Natl Acad Sci U S A* 2000;97:6493–8. [PubMed: 10829066]

41. Bousquet C, Susini C, Melmed S. Inhibitory roles for SHP-1 and SOCS-3 following pituitary proopiomelanocortin induction by leukemia inhibitory factor. *J Clin Invest* 1999;104:1277–85. [PubMed: 10545526]
42. Auernhammer CJ, Bousquet C, Melmed S. Autoregulation of pituitary corticotroph SOCS-3 expression: characterization of the murine SOCS-3 promoter. *Proc Natl Acad Sci U S A* 1999;96:6964–9. [PubMed: 10359822]
43. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 2002;109(Suppl):S121–31. [PubMed: 11983158]
44. Forrai A, Boyle K, Hart AH, Hartley L, Rakar S, Willson TA, Simpson KM, Roberts AW, Alexander WS, Voss AK, Robb L. Absence of suppressor of cytokine signalling 3 reduces self-renewal and promotes differentiation in murine embryonic stem cells. *Stem Cells* 2006;24:604–14. [PubMed: 16123385]
45. Spangenburg EE. SOCS-3 induces myoblast differentiation. *J Biol Chem* 2005;280:10749–58. [PubMed: 15653674]
46. Raccurt M, Tam SP, Lau P, Mertani HC, Lambert A, Garcia-Caballero T, Li H, Brown RJ, McGuckin MA, Morel G, Waters MJ. Suppressor of cytokine signalling gene expression is elevated in breast carcinoma. *Br J Cancer* 2003;89:524–32. [PubMed: 12888825]
47. Brender C, Nielsen M, Kaltoft K, Mikkelsen G, Zhang Q, Wasik M, Billestrup N, Odum N. STAT3-mediated constitutive expression of SOCS-3 in cutaneous T-cell lymphoma. *Blood* 2001;97:1056–62. [PubMed: 11159537]
48. Weber A, Hengge UR, Bardenheuer W, Tischhoff I, Sommerer F, Markwarth A, Dietz A, Wittekind C, Tannapfel A. SOCS-3 is frequently methylated in head and neck squamous cell carcinoma and its precursor lesions and causes growth inhibition. *Oncogene*. Jun 27;2005 Epub ahead of print.
49. Niwa Y, Kanda H, Shikauchi Y, Saiura A, Matsubara K, Kitagawa T, Yamamoto J, Kubo T, Yoshikawa H. Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. *Oncogene*. Jun 27;2005 Epub ahead of print.
50. Ueki K, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol Cell Biol* 2004;24:5434–46. [PubMed: 15169905]
51. Steppan CM, Wang J, Whiteman EL, Birnbaum MJ, Lazar MA. Activation of SOCS-3 by resistin. *Mol Cell Biol* 2005;25:1569–75. [PubMed: 15684405]
52. Takahashi Y, Carpino N, Cross JC, Torres M, Parganas E, Ihle JN. SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. *Embo J* 2003;22:372–84. [PubMed: 12554639]
53. Roberts AW, Robb L, Rakar S, Hartley L, Cluse L, Nicola NA, Metcalf D, Hilton DJ, Alexander WS. Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. *Proc Natl Acad Sci U S A* 2001;98:9324–9. [PubMed: 11481489]
54. Wang SY, LaRosa GJ, Gudas LJ. Molecular cloning of gene sequences transcriptionally regulated by retinoic acid and dibutyl cyclic AMP in cultured mouse teratocarcinoma cells. *Dev Biol* 1985;107:75–86. [PubMed: 2981185]
55. Wang SY, Gudas LJ. Isolation of cDNA clones specific for collagen IV and laminin from mouse teratocarcinoma cells. *Proc Natl Acad Sci U S A* 1983;80:5880–4. [PubMed: 6310600]
56. Strickland S, Smith KK, Marotti KR. Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyl cAMP. *Cell* 1980;21:347–55. [PubMed: 6250719]
57. LaRosa GJ, Gudas LJ. Early retinoic acid-induced F9 teratocarcinoma stem cell gene ERA-1: alternate splicing creates transcripts for a homeobox-containing protein and one lacking the homeobox. *Mol Cell Biol* 1988;8:3906–17. [PubMed: 2906112]
58. Hogan BLM, Taylor A, Adamson E. Cell interactions modulate embryonal carcinoma cell differentiation into parietal or visceral endoderm. *Nature* 1981;291:235–237. [PubMed: 6164928]
59. Nomiya H, Fromental C, Xiao JH, Chambon P. Cell-specific activity of the constituent elements of the simian virus 40 enhancer. *Proc Natl Acad Sci U S A* 1987;84:7881–5. [PubMed: 2825168]

60. Minami M, Inoue M, Wei S, Takeda K, Matsumoto M, Kishimoto T, Akira S. STAT3 activation is a critical step in gp130-mediated terminal differentiation and growth arrest of a myeloid cell line. *Proc Natl Acad Sci U S A* 1996;93:3963–6. [PubMed: 8632998]
61. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. Stat3 as an oncogene. *Cell* 1999;98:295–303. [PubMed: 10458605]
62. Li L, Shaw PE. Elevated activity of STAT3C due to higher DNA binding affinity of phosphotyrosine dimer rather than covalent dimer formation. *J Biol Chem* 2006;281:33172–81. [PubMed: 16956893]
63. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* 1998;67:227–64. [PubMed: 9759489]
64. O'Shea JJ. Jaks, STATs, cytokine signal transduction, and immunoregulation: are we there yet? *Immunity* 1997;7:1–11. [PubMed: 9252115]
65. Cooper DM. Regulation and organization of adenylyl cyclases and cAMP. *Biochem J* 2003;375:517–29. [PubMed: 12940771]
66. Steinberg RA, O'Farrell PH, Friedrich U, Coffino P. Mutations causing charge alterations in regulatory subunits of the cAMP-dependent protein kinase of cultured S49 lymphoma cells. *Cell* 1977;10:381–91. [PubMed: 191196]
67. Correll LA, Woodford TA, Corbin JD, Mellon PL, McKnight GS. Functional characterization of cAMP-binding mutations in type I protein kinase. *J Biol Chem* 1989;264:16672–8. [PubMed: 2550452]
68. Kopperud R, Krakstad C, Selheim F, Doskeland SO. cAMP effector mechanisms. Novel twists for an 'old' signaling system. *FEBS Lett* 2003;546:121–6. [PubMed: 12829247]
69. Akira S, Nishio Y, Inoue M, Wang XJ, Wei S, Matsusaka T, Yoshida K, Sudo T, Naruto M, Kishimoto T. Molecular cloning of APRF, a novel IFN-stimulated gene factor 3 p91-related transcription factor involved in the gp130-mediated signaling pathway. *Cell* 1994;77:63–71. [PubMed: 7512451]
70. Aringer M, Cheng A, Nelson JW, Chen M, Sudarshan C, Zhou YJ, O'Shea JJ. Janus kinases and their role in growth and disease. *Life Sci* 1999;64:2173–86. [PubMed: 10374907]
71. Ihle JN. STATs: signal transducers and activators of transcription. *Cell* 1996;84:331–4. [PubMed: 8608586]
72. Darnell JE Jr. STATs and gene regulation. *Science* 1997;277:1630–5. [PubMed: 9287210]
73. Zong CS, Chan J, Levy DE, Horvath C, Sadowski HB, Wang LH. Mechanism of STAT3 activation by insulin-like growth factor I receptor. *J Biol Chem* 2000;275:15099–105. [PubMed: 10747872]
74. Liu AM, Lo RK, Wong CS, Morris C, Wise H, Wong YH. Activation of STAT3 by G alpha(s) distinctively requires protein kinase A, JNK, and phosphatidylinositol 3-kinase. *J Biol Chem* 2006;281:35812–25. [PubMed: 17008315]
75. Simon AR, Rai U, Fanburg BL, Cochran BH. Activation of the JAK-STAT pathway by reactive oxygen species. *Am J Physiol* 1998;275:C1640–52. [PubMed: 9843726]
76. Wang YZ, Wharton W, Garcia R, Kraker A, Jove R, Pledger WJ. Activation of Stat3 preassembled with platelet-derived growth factor beta receptors requires Src kinase activity. *Oncogene* 2000;19:2075–85. [PubMed: 10815799]
77. Bowman T, Broome MA, Sinibaldi D, Wharton W, Pledger WJ, Sedivy JM, Irby R, Yeatman T, Courtneidge SA, Jove R. Stat3-mediated Myc expression is required for Src transformation and PDGF-induced mitogenesis. *Proc Natl Acad Sci U S A* 2001;98:7319–24. [PubMed: 11404481]
78. Garcia R, Jove R. Activation of STAT transcription factors in oncogenic tyrosine kinase signaling. *J Biomed Sci* 1998;5:79–85. [PubMed: 9662066]
79. Aitken RJ, Buckingham DW, Irvine DS. The extragenomic action of progesterone on human spermatozoa: evidence for a ubiquitous response that is rapidly down-regulated. *Endocrinology* 1996;137:3999–4009. [PubMed: 8756577]
80. Chiarugi P. Reactive oxygen species as mediators of cell adhesion. *Ital J Biochem* 2003;52:28–32. [PubMed: 12833635]
81. Starr R, Hilton DJ. SOCS: suppressors of cytokine signalling. *Int J Biochem Cell Biol* 1998;30:1081–5. [PubMed: 9785473]
82. Nicholson SE, Willson TA, Farley A, Starr R, Zhang JG, Baca M, Alexander WS, Metcalf D, Hilton DJ, Nicola NA. Mutational analyses of the SOCS proteins suggest a dual domain requirement but

- distinct mechanisms for inhibition of LIF and IL-6 signal transduction. *Embo J* 1999;18:375–85. [PubMed: 9889194]
83. Bousquet C, Chesnokova V, Kariagina A, Ferrand A, Melmed S. cAMP neuropeptide agonists induce pituitary suppressor of cytokine signaling-3: novel negative feedback mechanism for corticotroph cytokine action. *Mol Endocrinol* 2001;15:1880–90. [PubMed: 11682619]
84. Taneja R, Rochette-Egly C, Plassat JL, Penna L, Gaub MP, Chambon P. Phosphorylation of activation functions AF-1 and AF-2 of RAR alpha and RAR gamma is indispensable for differentiation of F9 cells upon retinoic acid and cAMP treatment. *Embo J* 1997;16:6452–65. [PubMed: 9351827]
85. Huggenvik JI, Collard MW, Kim YW, Sharma RP. Modification of the retinoic acid signaling pathway by the catalytic subunit of protein kinase-A. *Mol Endocrinol* 1993;7:543–50. [PubMed: 8388997]
86. Ruff-Jamison S, Chen K, Cohen S. Epidermal growth factor induces the tyrosine phosphorylation and nuclear translocation of Stat 5 in mouse liver. *Proc Natl Acad Sci U S A* 1995;92:4215–8. [PubMed: 7538664]
87. Dong S, Tweardy DJ. Interactions of STAT5b-RARalpha, a novel acute promyelocytic leukemia fusion protein, with retinoic acid receptor and STAT3 signaling pathways. *Blood* 2002;99:2637–46. [PubMed: 11929748]
88. Grandis JR, Drenning SD, Chakraborty A, Zhou MY, Zeng Q, Pitt AS, Tweardy DJ. Requirement of Stat3 but not Stat1 activation for epidermal growth factor receptor-mediated cell growth *In vitro*. *J Clin Invest* 1998;102:1385–92. [PubMed: 9769331]
89. Olayioye MA, Beuvink I, Horsch K, Daly JM, Hynes NE. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *J Biol Chem* 1999;274:17209–18. [PubMed: 10358079]
90. Niwa H, Burdon T, Chambers I, Smith A. Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. *Genes Dev* 1998;12:2048–60. [PubMed: 9649508]
91. Matsuda T, Nakamura T, Nakao K, Arai T, Katsuki M, Heike T, Yokota T. STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *Embo J* 1999;18:4261–9. [PubMed: 10428964]
92. Lehtonen E, Laasonen A, Tienari J. Teratocarcinoma stem cells as a model for differentiation in the mouse embryo. *Int J Dev Biol* 1989;33:105–15. [PubMed: 2485690]
93. Lang R, Pauleau AL, Parganas E, Takahashi Y, Mages J, Ihle JN, Rutschman R, Murray PJ. SOCS3 regulates the plasticity of gp130 signaling. *Nat Immunol* 2003;4:546–50. [PubMed: 12754506]
94. Pelletier S, Duhamel F, Coulombe P, Popoff MR, Meloche S. Rho family GTPases are required for activation of Jak/STAT signaling by G protein-coupled receptors. *Mol Cell Biol* 2003;23:1316–33. [PubMed: 12556491]
95. Gudas, LJ.; Sporn, MB.; Roberts, AB. Cellular Biology and Biochemistry of the Retinoids. In: Sporn, MB.; Roberts, AB.; Goodman, DS., editors. *The Retinoids: Biology, Chemistry, and Medicine*. 2nd edit.. Raven Press; New York: 1994.
96. Yoneda T, Niewolna M, Lowe C, Izbicka E, Mundy GR. Hormonal regulation of pp60c-src expression during osteoclast formation *in vitro*. *Mol Endocrinol* 1993;7:1313–8. [PubMed: 7505394]
97. Goldshmit Y, Walters CE, Scott HJ, Greenhalgh CJ, Turnley AM. SOCS2 induces neurite outgrowth by regulation of epidermal growth factor receptor activation. *J Biol Chem* 2004;279:16349–55. [PubMed: 14764607]
98. Alchanati I, Nallar SC, Sun P, Gao L, Hu J, Stein A, Yakirevich E, Konforty D, Alroy I, Zhao X, Reddy SP, Resnick MB, Kalvakolanu DV. A proteomic analysis reveals the loss of expression of the cell death regulatory gene GRIM-19 in human renal cell carcinomas. *Oncogene* 2006;25:7138–47. [PubMed: 16732315]
99. Kalakonda S, Nallar SC, Lindner DJ, Hu J, Reddy SP, Kalvakolanu DV. Tumor-suppressive activity of the cell death activator GRIM-19 on a constitutively active signal transducer and activator of transcription 3. *Cancer Res* 2007;67:6212–20. [PubMed: 17616678]
100. Lufei C, Ma J, Huang G, Zhang T, Novotny-Diermayr V, Ong CT, Cao X. GRIM-19, a death-regulatory gene product, suppresses Stat3 activity via functional interaction. *Embo J* 2003;22:1325–35. [PubMed: 12628925]

101. Zhang J, Yang J, Roy SK, Tininini S, Hu J, Bromberg JF, Poli V, Stark GR, Kalvakolanu DV. The cell death regulator GRIM-19 is an inhibitor of signal transducer and activator of transcription 3. *Proc Natl Acad Sci U S A* 2003;100:9342–7. [PubMed: 12867595]
102. Vasios GW, Gold JD, Petkovich M, Chambon P, Gudas LJ. A retinoic acid-responsive element is present in the 5' flanking region of the laminin B1 gene. *Proc Natl Acad Sci U S A* 1989;86:9099–103. [PubMed: 2556699]
103. Ben-Shushan E, Thompson JR, Gudas LJ, Bergman Y. Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol Cell Biol* 1998;18:1866–78. [PubMed: 9528758]
104. Boylan JF, Lohnes D, Taneja R, Chambon P, Gudas LJ. Loss of retinoic acid receptor gamma function in F9 cells by gene disruption results in aberrant Hoxa-1 expression and differentiation upon retinoic acid treatment. *Proceedings of the National Academy of Sciences of the United States of America* 1993;90:9601–5. [PubMed: 8105479]

Figure 1. The expression of the SOCS-3 gene was induced in response to RA and dibutyryl cAMP in F9 Wt cells and F9 Rex1^{-/-} cells: Northern analysis of SOCS-3

F9 Wt cells and two independent Rex1^{-/-} lines (R21 and R5) were cultured for 24 h, 48 h, or 72 h in the presence of 1 μ M RA, 250 μ M dibutyryl cAMP, and 250 μ M theophylline (RACT). Total RNA was extracted for Northern analysis. The same filter was used for Northern analysis of Rex1. The higher molecular mass Rex1 signals in the two knockout lines occur because these transcripts contain a neomycin^R gene which disrupts the Rex1 protein coding sequence²². GAPDH was used as a loading control. Below the Northern analyses is the quantitative analysis of the effect of RA, CT, and RACT on SOCS-3 mRNA levels in F9 WT and F9 Rex1^{-/-} cells based on three repeats of the same experiment with different RNA preparations. The data are shown as the mean $\pm$ SD.

Quantitation of SOCS-3 Northern Analyses

Figure 2. Stable Transfection of Exogenous Rex1 into the Rex1^{-/-} Cells Partially Restored the Pattern of SOCS-3 Expression

F9 Wt and F9 Rex1^{-/-} (R21), and the Rex1 stable transfectant (R21JX12, which contains the Rex1 cDNA driven by the SV40 promoter stably transfected into the Rex^{-/-} R21 line) cells were cultured for 24 h, 48 h, or 72 h in the presence of 1 μ M RA, 250 μ M db cAMP, and 250 μ M theophylline (RACT). Total RNA was extracted for Northern analysis. The same filter was used for the Northern analysis of Rex1. The Rex1 transcript is 1.74kb. (The larger transcript in the Rex1^{-/-} cell lines contains the neomycin resistance gene in a position which interrupts the Rex1 open reading frame.) GAPDH was used as a loading control. Below the Northern analyses is the quantitative analysis of the effect of RA, CT, and RACT on SOCS-3 mRNA levels in F9 WT, R21, and R21JX12 cells, based on three repeats of the same experiment starting with different RNA preparations. The data are shown as the mean $\pm$ SD.

Figure 3. SOCS-3 protein is induced in response to RA and dibutyryl cAMP in F9 Wt cells and F9 Rex1^{-/-} cells (monolayer culture): Western analysis of SOCS-3

F9 Wt cells, two Rex1^{-/-} cell lines (R21, R5), and a Rex1 put-back line (R21JX12) were cultured for 72 hours in the presence of various drugs as indicated. 100 µg of total protein was used for SOCS-3 Western analysis and β-actin was used as a loading control. This experiment was repeated three times with similar results.

Figure 4. Induction of the SOCS-3 promoter/reporter activity in F9 Wt and F9 Rex1^{-/-} cells in response to RA, CT, and RACT

(A) Graphic representation of the ≈ 3.7 kb genomic 5' flanking region of the murine SOCS-3 promoter. The tandem STAT3 binding sites (in bold) and the start codon ATG are shown on the top. Diagrams of each pGL3 Basic-derived construct linked to the firefly luciferase reporter gene with different forms of the mouse SOCS-3 promoter are presented. The numbers refer to the 5' and 3' terminal nucleotides included in each construct with respect to the transcription start site of the above sequence. (B) F9 WT, Rex1^{-/-} R21, Rex1^{-/-} R5, and R21JX12 cells were transiently transfected with 4 μ g of pGL3 Basic, clone 2, clone 6, or clone 6D2C. Each cell line was also co-transfected with 1.5 μ g of a Renilla luciferase plasmid as a transfection control. Sixteen hours after the transfection, fresh medium was replaced in the presence of 1 μ M RA, 250 μ M db cAMP, and 250 μ M theophylline (RACT). After 48 h of treatment, cells were harvested for luciferase assay. Firefly luciferase activity was normalized to the

corresponding Renilla luciferase activity. Relative luciferase activity was calculated as a ratio over nonstimulated F9 Wt cells transfected with clone 6. Mean values with error bars as indicated are from four independent experiments. (C) F9 WT and Rex^{-/-} cells were co-transfected with 4 µg of construct 6 and 2, 4, 6 µg, or no psg-Rex1. pSG5 “vector” plasmid was used to equalize the total amount of DNA used for transfection. Each cell line was also co-transfected with 1.5 µg Renilla luciferase plasmid as a transfection control. Sixteen hours after transfection, fresh medium was replaced in the presence of 1 µM RA, 250 µM dibutyl camp and 250 µM theophylline (RACT). After 48 hours, cells were harvested for luciferase assays. This experiment was performed twice; average values are plotted.

Figure 5. Induction of SOCS-3 promoter/reporter activity by RA, CT, and RACT: effect of STAT3 mutants

(A) Schematic representation of various STAT3 constructs. LZ, leucine zipper domain; R, arginine; Y, tyrosine; S, serine; F, phenylalanine; C, cysteine; A, alanine; N, asparagine. These constructs include the WTSTAT3, two DN mutants of STAT3 (Y705F and D715), and the constitutively active mutant of STAT3 (STAT3C). For Y715, the tyrosine residue in the C-terminus is mutated to phenylalanine. D715 lacks the C-terminal transcriptional activation domain. For STAT3C, an alanine residue and an asparagine residue within the C-loop of the SH2 domain are both mutated to cysteine residues. (B) F9 Wt and R21 Rex1^{-/-} cells were co-transfected with 4 μ g of construct 6 and 4 μ g of each of the STAT3 constructs in A (or 4 μ g of pUC19 V=vehicle). As a negative control, F9 WT and R21 cells were co-transfected with 4 μ g of construct 6 and 4 μ g of pSG5. Each cell line was also cotransfected with 1.5 μ g of the Renilla luciferase plasmid as a transfection control. Sixteen hours after the transfection, fresh medium was replaced in the presence 1 μ M RA, 250 μ M dibutyryl cAMP, and 250 μ M theophylline (RACT). After 48 h of treatment, cells were harvested for luciferase assays. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. Relative luciferase activity was calculated as a ratio over unstimulated F9 Wt cells transfected with construct 6 and pSG5. Mean values with error bars ($\pm$ SD) as indicated are from four independent experiments. SD = standard deviation.

Figure 6. The expression of STAT3 mRNA and STAT3 protein in F9 Wt versus F9 Rex1^{-/-} cells
(A) Northern analysis of STAT3. F9 Wt cells and two independent F9 Rex1^{-/-} lines (R21 and R31) were cultured for 24 h, 48 h, or 72 h in the presence of 1 μ M RA, 250 μ M db cAMP, and 250 μ M theophylline (RACT). Total RNA was extracted for Northern analysis. GAPDH was used as a loading control. Below the Northern analyses is the quantitative analysis of the effects of RA, CT, and RACT on STAT3 mRNA levels in F9 Wt and F9 Rex1^{-/-} cells, based on three repeats of the same experiment. **(B)** Western analyses of *total* STAT3 protein and tyrosine phosphorylated STAT3. F9 Wt cells, two independent Rex1^{-/-} lines (R21 and R31), and the Rex1 "put-back" line (R21JX12) were cultured for 48 h, in the presence of 1 μ M RA, 250 μ M dibutyl cAMP, and 250 μ M theophylline (RACT). Total protein was harvested, and 45 μ g protein was used for Western analyses. Western analyses of total STAT3 and p-STAT3 were repeated numerous times with different time points, and very similar results were obtained. Below the Western analyses is the quantitative analysis of the effects of RA, CT, and RACT on p-STAT3 protein level over total STAT3 protein level for the Western analyses shown above. SOCS-3 protein levels are also shown for comparison.

Figure 7. Induction of the SOCS-3 promoter/reporter activity by RA, CT, and RACT: The effect of a PKA Dominant Negative mutant

(A) The schematic representation of the PKA DN mutant in MT-REV(AB) expression vector. This construct contains the cDNA for the RI α of PKA. Point mutations in both cAMP-binding domain sites are present (Gly-200 $\rightarrow$ Glu; Gly-324 $\rightarrow$ Asp; Arg-322 $\rightarrow$ His) which prevent cAMP binding and activation of the kinase⁶⁷. Transcription of the construct is directed by the metallothionein-1 (MT-1) promoter, and a polyadenylation signal is provided by the 3' human growth hormone (hGH) sequence. (B) F9 WT and R21 Rex1^{-/-} cells were co-transfected with 4 μ g of construct 6 (see Fig. 4) and 3 μ g of the DN PKA mutant construct MT-REV(AB). As a negative control, F9 Wt and R21 cells were co-transfected with 4 μ g of construct 6 and 3 μ g of the "empty vector" MT64AA (Veh). Each cell line was also co-transfected with 1.5 μ g of the Renilla luciferase plasmid as a transfection control. Sixteen hours after the transfection, fresh medium was replaced in the presence 1 μ M RA, 250 μ M dibutyryl cAMP, and 250 μ M theophylline (RACT). After 48 h of treatment, cells were harvested for luciferase assays. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. Relative luciferase activity was calculated as a ratio over unstimulated F9 Wt cells transfected with construct 6 and MT64AA. Mean values with error bars as indicated are from three independent experiments.

Figure 8. Induction of the SOCS-3 promoter/reporter activity by RA, CT, and RACT: The effects of the Src DN, Jak1 DN, and Jak2 DN expression vectors

(A) The schematic representation of the DN mutants of Jak1, Jak2, and Src. The DN mutant of Jak1 contains a 3-amino acid change in the conserved region VIII of the kinase domain (FWYAPE → LTYAPV) that impairs its catalytic function⁷³. The DN mutant of Jak2 contains a single amino acid change (Lys → Ala) at the ATP-binding site, resulting in impaired binding of ATP and catalytic activity⁷³. The DN mutant of Src (Upstate Biotechnology, Cat# 21-154) contains two point mutations (Arg-296 → Lys; Tyr-528 → Phe) in the kinase domain that inactivate its catalytic activity. (B) F9 Wt and R21 cells were co-transfected with 4 μg of construct 6 and 4 μg of each of these DN mutant constructs. As a negative control, F9 Wt and R21 cells were co-transfected with 4 μg of construct 6 and 4 μg of pUC9. Each cell line was also co-transfected with 1.5 μg of the Renilla luciferase plasmid as a transfection control. Sixteen hours after the transfection, fresh medium was replaced in the presence 1 μM RA, 250 μM dibutyl cAMP, and 250 μM theophylline (RACT). After 48 h of treatment, cells were harvested for luciferase assays. Firefly luciferase activity was normalized to the corresponding Renilla luciferase activity. Relative luciferase activity was calculated as a ratio over unstimulated F9 WT cells transfected with construct 6 and pUC9. Mean values with error bars as indicated are from three independent experiments.

Figure 9. RARα stimulates SOCS3 promoter activity through the STAT3 binding sites

F9 WT and Rex^{-/-} (R21) cells were transiently transfected with 4 μg of construct 6 or construct 6D2C (see Fig. 4A). Each cell line was also co-transfected with 4 μg pSG-RARα or Psg5 “empty” vector as a negative control. 1.5 μg Renilla luciferase plasmid was co-transfected as a transfection control for each cell line. Sixteen hours after transfection, fresh medium was replaced in the presence 1 μM RA, 250 μM dibutyryl AMP, and 250 μM theophylline (RACT). After 48 hours, cells were harvested for luciferase assays. Relative luciferase activity was calculated. Mean values with error bars are shown.

Figure 10. A. The effect of parathyroid hormone (PTH) and β -mercaptoethanol on RA- and RACT-induced SOCS-3 expression: Northern analysis of SOCS-3

F9 WT cells and R21 cells were cultured for 40 h and 72 h in the presence of 1 μ M RA, 250 μ M db cAMP, and 250 μ M theophylline (RACT). To examine the effects of PTH and BME on SOCS-3 mRNA levels, 5 nM or 50 nM of bPTH, and 0.5 mM BME were added to the cells, together with RA and RACT. Total RNA was extracted for Northern analysis. 28S rRNA was used as a loading control. The lanes represent: 1) control, 2) RA, 3) CT, 4) RACT, 5) 50 nM bPTH, 6) RA + 5 nM bPTH, 7) RA + 50 nM bPTH, 8) RACT + 10 μ M BME. This experiment was repeated twice with similar results; one experiment is shown. B. Model for SOCS3 transcriptional activation in F9 cells In F9 Wt, cyclic AMP acts via pKA, c-Src, and Jak2 to phosphorylate and thereby activate Stat3 protein. RA acts to increase the total amount of Stat3 protein by increasing the transcription of the Stat3 gene. In F9 Rex1 null cells, lower levels of STAT3 inhibitory proteins could result in increased STAT3 activity.