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Fluorescent SOCS3 Reporter of STAT5 Activation

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FLUORESCENT SOCS3 REPORTER OF STAT5 ACTIVATION

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ABSTRACT

The activation of signaling by erythropoietin (Epo) is a critical part of the differentiation and maturation of blood cell progenitors. Stat5, a transcription factor that is phosphorylated indirectly by the binding of Epo to the erythropoietin receptor (EpoR), has been shown to be constitutively active in leukemia and other myelo-proliferative disorders. Using PCR amplification and vector cloning, a fusion protein of emerald GFP and SOCS3 (a negative regulator of Stat5) was created to study the time course of activation of Stat5 activation in erythroid progenitors in both normal and abnormal erythropoiesis. The results of further studies using this fusion protein may eventually lead to improved or novel treatment options for patients with leukemia.

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Finally, I would like to thank Professor Dave Adams for his help initiating this project, providing suggestions when things got difficult, and editing this report.

BACKGROUND

Erythropoiesis

The maturation of erythrocytes from hematopoietic stem cells is known as erythropoiesis. In the mouse, erythroid progenitors are found in bone marrow, spleen, and fetal liver tissues (Richmond et al., 2005). Under hypoxemic conditions, a stress response causes an increase in erythropoietin (Epo) which accelerates red cell production rate. This causes a many-fold increase in the numbers of proerythroblasts and early erythroblasts (Liu et al., 2006). The progression of erythropoiesis is shown in Figure 1.

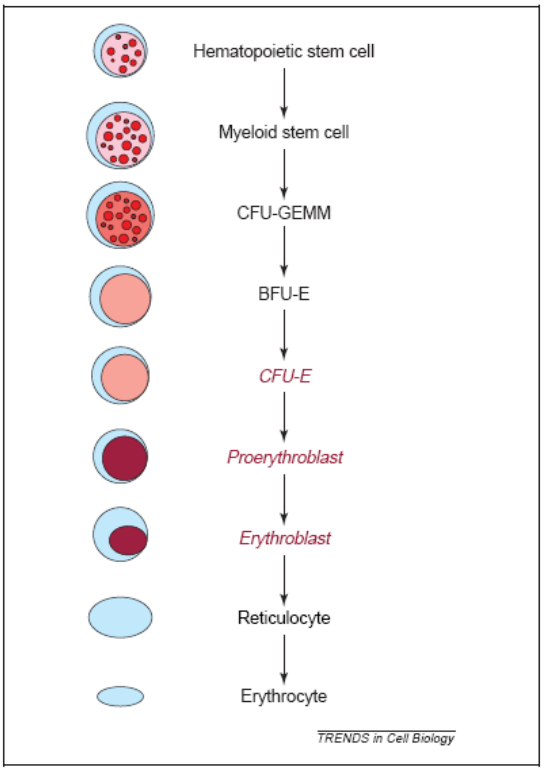

Figure 1. Maturation of Hematopoietic Stem Cells into Erythrocytes. Stages represented in red italics are those dependent on erythropoietin. CFU-GEMM: colony forming unit-granulocyte, erythrocyte, monocyte, megakaryocyte; BFU-E: burst forming unit-erythroid; CFU-E: colony forming unit-erythroid (Richmond et al., 2005).

Epo is one of many cytokines, glycoproteins secreted to regulate various processes, such as hematopoiesis, wound healing, central nervous system development,

and immune responses (Krebs and Hilton, 2001). Epo regulates erythroid development and the erythropoietic response to stress. Knock-out and truncation studies show that Epo is essential for these processes (Liu et al., 2006).

Erythropoietin Signal Transduction

Much is known about Epo signaling within progenitor cells (Figure 2). The erythropoietin receptor (EpoR) is closely associated with Janus kinase (Jak)-2 (Liu et al., 2006), which becomes phosphorylated upon binding of Epo to EpoR. Jak2 phosphorylates several tyrosine residues in the cytoplasmic domain of EpoR. These phosphotyrosines act as binding sites for several proteins, including a family of proteins termed signal transducers and activators of transcription (Stats). One member of this family, Stat5, binds Y343 and Y401 (Richmond et al., 2005) via its Src homology (SH2) domain. Stat5 is then phosphorylated by Jak2, which causes it to dissociate from EpoR and form a homodimer through its SH2 domain. The Stat5 dimer then enters the nucleus where it acts to induce transcription of several genes (Krebs and Hilton, 2001), including suppressor of cytokine signaling (SOCS)-3. SOCS3 also contains an SH2 domain, which allows it to bind to Y401 of EpoR, thus acting as a competitive inhibitor of Stat5 in a negative feedback loop (Krebs and Hilton, 2001; Wormald and Hilton, 2004). SOCS3 also inhibits Stat5 activation by directly inhibiting Jak2 kinase activity (Krebs and Hilton, 2001) and possibly also by promoting degradation of the activated EpoR (see below).

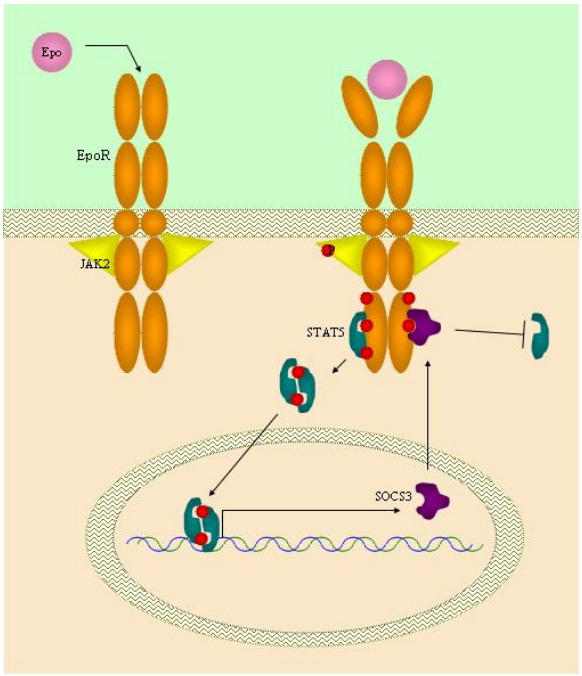

Figure 2. Diagram of Stat5 Signaling Pathway Showing Activation and Inhibition of Stat5. Erythropoietin (purple) binds to the erythropoietin receptor (orange) causing JAK2 (yellow) to become phosphorylated. The phosphorylated JAK2 phosphorylates tyrosine residues on the cytoplasmic domain of the EpoR (diagram right side). Stat5 binds to Y410, becomes phosphorylated, dissociates (diagram lower center), dimerizes, moves to the nucleus, and binds promoter sequences for genes such as SOCS3.

SOCS Proteins

SOCS proteins, negative regulators of cytokine signaling, contain an SH2 domain and have no discernable N-terminal motifs. Their expression is controlled by Stat proteins at the level of transcription, and varies among tissue types and cell lines. For example, SOCS3 is present in low levels in adult tissues, but is found mainly in fetal liver Erythroid progenitors. SOCS3 transcription is induced by over a dozen cytokines, including multiple interleukins (IL), growth hormones, ciliary neurotropic factor, leukemia inhibitory factor (LIF), and Epo (Krebs and Hilton, 2001). SOCS3 acts to

lessen or inhibit signaling by these molecules (Krebs and Hilton, 2001; Wormald and Hilton, 2004).

To help uncover the function of SOCS3, several transgenic and knockout studies have been done with the SOCS3 gene. These showed that both SOCS3 transgenic over-expressers and SOCS3-null mice die prior to birth (Krebs and Hilton, 2001). The $SOCS3^{-/-}$ mice show normal (Krebs and Hilton, 2001) or increased erythropoiesis. Like the related protein SOCS1, SOCS3 has been shown to bind to and inhibit the activity of Jak2 (Krebs and Hilton, 2001), further supporting the idea that SOCS3 acts as an inhibitor of cytokine, and more specifically Epo, signaling.

It has been proposed that SOCS proteins also act as adaptor molecules for the binding of ubiquitin to mark associated proteins for degradation (Krebs and Hilton, 2001) by associating with elongins B and C (Zhang et al., 1999). This interaction takes place between the elongins and the SOCS box (Wormald and Hilton, 2004), a 40-amino acid sequence found in SOCS proteins (Kile et al., 2002). Together with a cullin-family protein and a RING protein, the SOCS box and elongins form an E3 ubiquitin ligase complex (Wormald and Hilton, 2004). The E3 complex acts to catalyze the transfer of ubiquitin from E2 to the target protein: the protein bound by SOCS (Kile et al., 2002) (Figure 3).

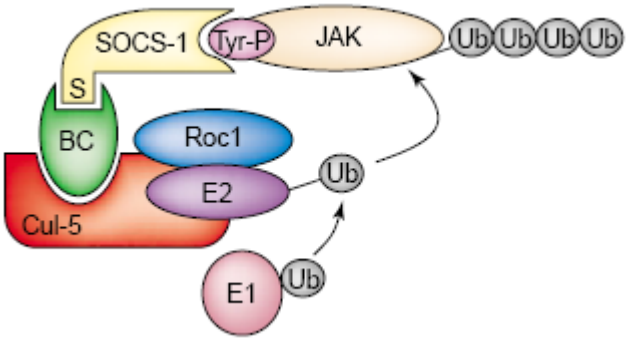

Figure 3. The Role of SOCS Proteins in Protein Degradation. SOCS acts as an adapter for the E3 ubiquitin ligase complex. In this specific example, the SOCS box (S) of SOCS1 (yellow) is bound to the elongin B/C complex (BC) (green), which interacts with Roc1 (a RING protein) (blue) and E2 (purple). E1 (violet) transfers ubiquitin to E2. The SOCS1-BC-Cul-5-Roc1 complex acts to catalyze the transfer of ubiquitin to the target protein, JAK (Kile et al., 2002).

As mentioned previously, SOCS3 transcription is induced by Stat5, which acts to regulate survival (anti-apoptosis) (Benekli et al., 2003; Debierre-Grokiego, 2004) in mature cells, and proliferation and differentiation in progenitors at the transcriptional level (Buitenhuis et al., 2004). Constitutively active Stat5 has been shown to be responsible for certain types of leukemia (see below), and an increase in the production of antiapoptotic factors (Coffer et al., 2000), such as Bcl-X_L (Liu et al., 2006).

Stat5 deficiency in mice has been implicated in some forms of anemia (Liu et al., 2006), perinatal death, and aberrant growth, lactation, and T-cell development (Richmond et al., 2005; Liu et al., 2006). A decrease in the number of mature erythroid progenitors has been noted in Stat5$^{-/-}$ mice, caused by an increase in apoptosis, rather than a decrease in proliferation (Debierre-Grokiego, 2004), further supporting Stat5 as a regulator of antiapoptosis genes. While decreases in Stat5 activity can be harmful, large increases can be just as detrimental. Constitutive activation of Stat5 has been implicated in the formation and proliferation of several forms of cancer, such as cutaneous lymphomas

(Benekli et al., 2003), head and neck squamous cell carcinoma (HNSCC) (Buitenhuis et al., 2004), breast cancer, prostate cancer (Debierre-Grokiego, 2004), and various types of leukemias (Gouilleux-Gruart et al., 1996; Weber-Nordt et al., 1996; Lin et al., 2000; Benekli et al., 2003; Buitenhuis et al., 2004). The inhibition of Stat5 has been shown to induce apoptosis in tumor cells, which may be a key to future therapies (Buitenhuis et al., 2004; Debierre-Grokiego, 2004).

Previous Results

Our lab previously developed a novel method for the measuring of Stat5 activation in erythroid progenitors using flow cytometry (FACS). This method allows for the measure of Stat5 activation in individual cells; however, the cells must be fixed and permeablized in order to do this. Therefore, the levels of Stat5 activation cannot be measured in the same cells over time. FACS analysis of Stat5 activation in erythroid progenitors has revealed unexpected oscillations in Stat5 activity (unpublished) (Figure 4). Other results suggest these oscillations may be caused, at least in part, by the induction of the negative regulator SOCS3 (unpublished) (Figure 5).

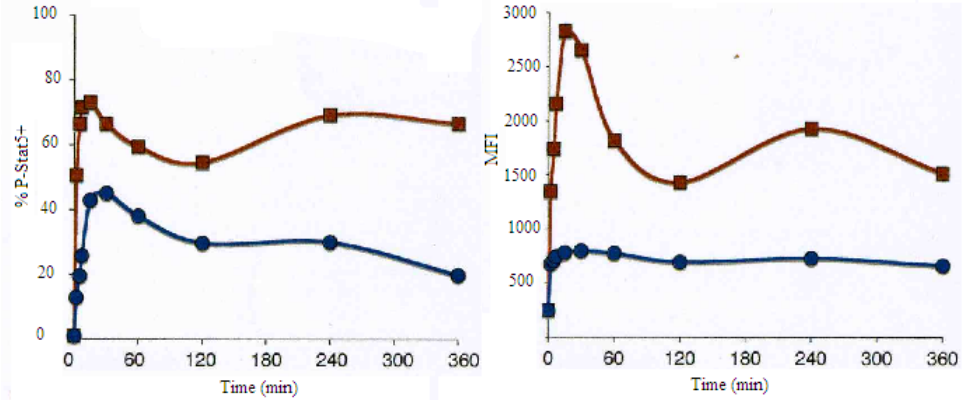

Figure 4. Stat5 Activity in E14.5 Murine Fetal Liver Cells. FACS analysis of early (red) and late (blue) erythroblasts shows oscillations in Stat5 activity (Y-axis) over time. (X-axis) in the percent of activated cells in the population (left panel) and the average signal per cell (right panel) in the early stage of erythroblast maturation, which are diminished in later cells.

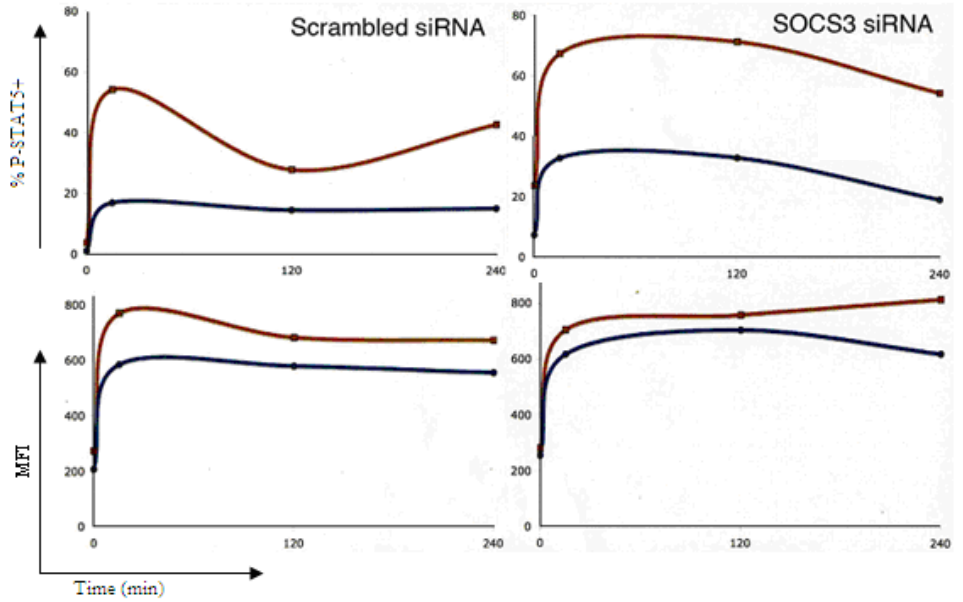

Figure 5. Stat5 Activation in the Presence and Absence of SOCS3. FACS analysis shows the same oscillations seen previously in early erythroblasts (red), but not in (blue) in both the population (top) and individual cells (bottom) of the control group (left panels). Interference using SOCS3 siRNA (right panels) eliminates the oscillations in both fractions of activated cells (top) or in the median signal per cell (bottom).

PROJECT PURPOSE

As discussed in the Background, Epo plays a key role in erythropoiesis, and Stat5 transcription factor plays a key role in Epo cell signaling. Thus the monitoring of Stat5 activity in cell populations or individual progenitor cells over time is important. Previous results suggest that Stat5 activity increases rapidly following Epo stimulation, and subsequently decreases steadily over time. However, unpublished experiments in our lab measuring Stat5 activity using FACS have shown unexpected oscillations in Stat5 activation over time in populations of hematopoietic progenitor cells. This could be due to oscillations in individual cells, or different cells activating at different times. Due to the nature of the FACS assays used that required cell fixation, it has not been possible to monitor Stat5 activation in the same cell over time to determine the mechanism causing the observed oscillations in the total cell population.

The purpose of this MQP project was to create an intracellular fluorescent reporter of Stat5 activity that would allow single cell measurement of that activity over time. We planned to engineer this reporter downstream of an enhancer containing 4 sequential Stat5 binding sites to allow its rapid induction following Stat5 activation. SOCS3 has a short half-life, its rapid decay after its initial Stat5-mediated induction allowing the second peak in Stat5 activity (Figure 4). Therefore, it can faithfully report the activity of Stat5 over time. This inducible SOCS3-GFP fusion should decay at the same rate as endogenous SOCS3, allowing Stat5 oscillations to persist. Nevertheless, it is possible that the SOCS3-GFP fusion may alter some of the Stat5 oscillation characteristics by inhibiting Stat5 activity along with the endogenous protein. For this

reason, we also planned to make a second reporter, in which only the SOCS box region is used. This second reporter would have no direct inhibitory effect on Stat5, be degraded rapidly, and therefore should not interfere with the Stat5 signal.

METHODS

Synthesis of cDNA

Murine fetal liver cells (mFLCs) embryonic day 13.5 (ED 13.5) were obtained from live BAlb/C mice. The mFLCs were starved for 30 minutes, then two groups of cells were stimulated with 2.5 U/ml of erythropoietin for 30 minutes and one hour, respectively, while the third group remained unstimulated. The mRNA was isolated and purified using the RNeasy® MinElute kit (Qiagen), and then the RNA was treated with deoxyribonuclease I (Invitrogen). The RNA samples were split, and a 50 ng aliquot from each sample was incubated with SuperScript™ II reverse transcriptase (Invitrogen) to produce cDNA as a source of the SOCS3 gene.

PCR Amplification of SOCS3

Forward (F-SOCS3) and reverse primers (R-SOCS3) were designed for SOCS3 with the sequences ATGGTCACCCACAGCAAGTTTC and TTAAAGTGGAGCATCATACTGATC, respectively (Invitrogen). Forty cycles of PCR were performed with an annealing temperature of 55°C for a variety of samples including –RT, noEpo+RT, 30'Epo+RT, and 1hEpo+RT samples. The process was repeated under the same conditions for the SOCS box, using the same reverse primer but a new forward primer (F-SOCSBox) with the sequence TCCAACGTGGCCACCCTCCAG. The SOCS3 amplicon products were analyzed using agarose gel electrophoresis.

Cloning

The 30'Epo+RT PCR product was cloned into the expression plasmid pcDNA6.2/N-EmGFP-GW/TOPO® (Invitrogen) as per the manufacturer's provided instructions. The ligated plasmids were transformed into competent *E. coli* DH5α cells, plated on ampicillin-containing agar plates, and grown overnight. Colonies were isolated and grown in 3 mL of 2X LB + ampicillin. Plasmids were purified using the QIAprep® Spin MiniPrep Kit (Qiagen). Positive clones were identified via restriction enzyme digestion, using BstXI and BamHI. Positive clones were sequenced and compared to the known sequences of both SOCS3 and the SOCS box using the BLAST feature from the National Center for Biotechnology Information (NCBI). One positive SOCS3 plasmid and one positive SOCS box plasmid were transformed again into competent DH5α *E. coli* cells, plated, and isolated as before to make a high titer stock. Large scale plasmid isolations were performed using the EndoFree® Plasmid MaxiPrep Kit (Qiagen). Agarose gel electrophoresis was performed to confirm positives. Positive clones were once again sequenced to confirm identity and rule out mutations. The sequences were entered into BLAST for comparison.

Transfection

Samples of $3x10^5$ Phoenix cells (G. Nolan, Stanford University) were transfected with the following plasmids: EmGFP expression control (Invitrogen), FLAG-tagged SOCS3 (a polypeptide tag that can be detected with a specific antibody), EmGFP-SOCS3, and EmGFP-SOCS box. Fugene6® Transfection Reagent (Roche) was used following the manufacturer's provided protocol. GFP expression was measured by flow cytometry at 24 and 48 hours (GFP antibodies from BD Biosciences).

RESULTS

The aim of this project was to produce a GFP fluorescent SOCS3 marker protein for Stat5 activation to allow its activity to be monitored in cells without fixation. Cells were stimulated with Epo prior to mRNA collection to increase the copy number of SOCS3 transcripts. Previous attempts at amplifying the SOCS3 gene from unstimulated cDNA were unsuccessful. We also produced a shorter fluorescent SOCS box to use in place of the GFP-SOCS3 fusion if a functional fluorescent SOCS3 protein caused a severe decrease in Stat5 activity in the presence of endogenous SOCS3.

Agarose gel analysis of the PCR products using the synthesized cDNA 30'Epo+RT confirmed the expected amplicon size fragments of 678 bp and 129 bp for SOCS3 and the SOCS box, respectively (Figure 6). The 30'Epo+RT and 1hEpo+RT samples showed greater signals than the unstimulated sample (data not shown). From these, 30'Epo+RT was chosen arbitrarily for subsequent procedures.

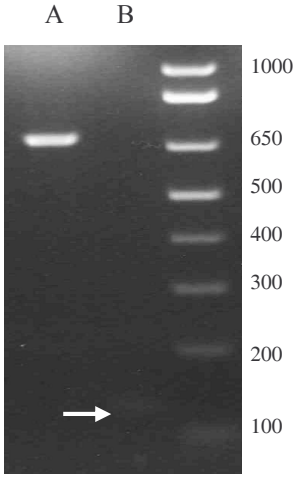

Figure 6. PCR Amplification of the SOCS3 and SOCS Box Genes from Murine Fetal Liver Cells. Agarose gel (2%) analysis of PCR products. Lane A contains the SOCS3 product (678 bp expected size), while lane B contains the SOCS box product (129 bp expected size). White arrow indicates location of faint band for the SOCS box.

Following the successful amplification of the SOCS3 and SOCS box DNAs, the amplicons were ligated directly into the Em-GFP plasmid expression vector already containing the GFP gene. Agarose gel electrophoresis of plasmid DNA isolated from ampicillin-resistent colonies showed the presence of a properly-oriented insert in four of eight SOCS3 samples, and three of eight SOCS box samples (Figure 7).

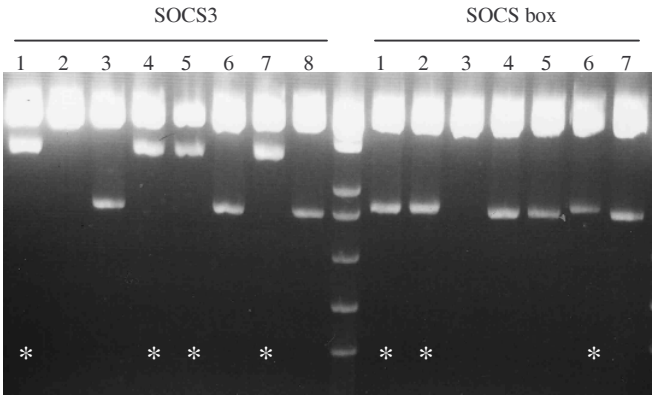

Figure 7. Screening of Expression Plasmids Containing the SOCS3 Gene (Left) or SOCS Box Gene (right). Agarose gel (2%) analysis of BstXI and BamHI digestion of purified plasmids. Lanes marked with an asterisk (*) are samples determined to be positive.

The sequences of these clones showed a point mutation at position 1024 of the SOCS3 gene of the guanine to an adenine, which also corresponds to the mutation found at position 99 of the SOCS box sequence. This is a silent mutation as it does not alter the amino acid sequence of either protein. One clone from the SOCS3 group and one from the SOCS box group were selected for use in subsequent experimentation.

The expression plasmids were transfected into Phoenix cells to test for GFP fluorescence. The plasmids do not have Stat5 binding sites in the GFP promoter so these plasmids simply constitutively express GFP. Transfected cells were analyzed by FACS for GFP fluorescence (Figure-8). FACS analysis of the transfected Phoenix cells revealed an average transfection efficiency of 5.82% after 24 hours post-transfection, and

12.9% after 48 hours post-transfection. The expression of EmGFP more than doubled within the populations in the 24 hours between FACS analyses, but the significance of the differences within individual cells (MFI) has not been determined. Thus the plasmids produced in this MQP project do indeed encode GFP, although it has yet to be determined whether the SOCS3 or SOCS box proteins fused with the GFP are functional.

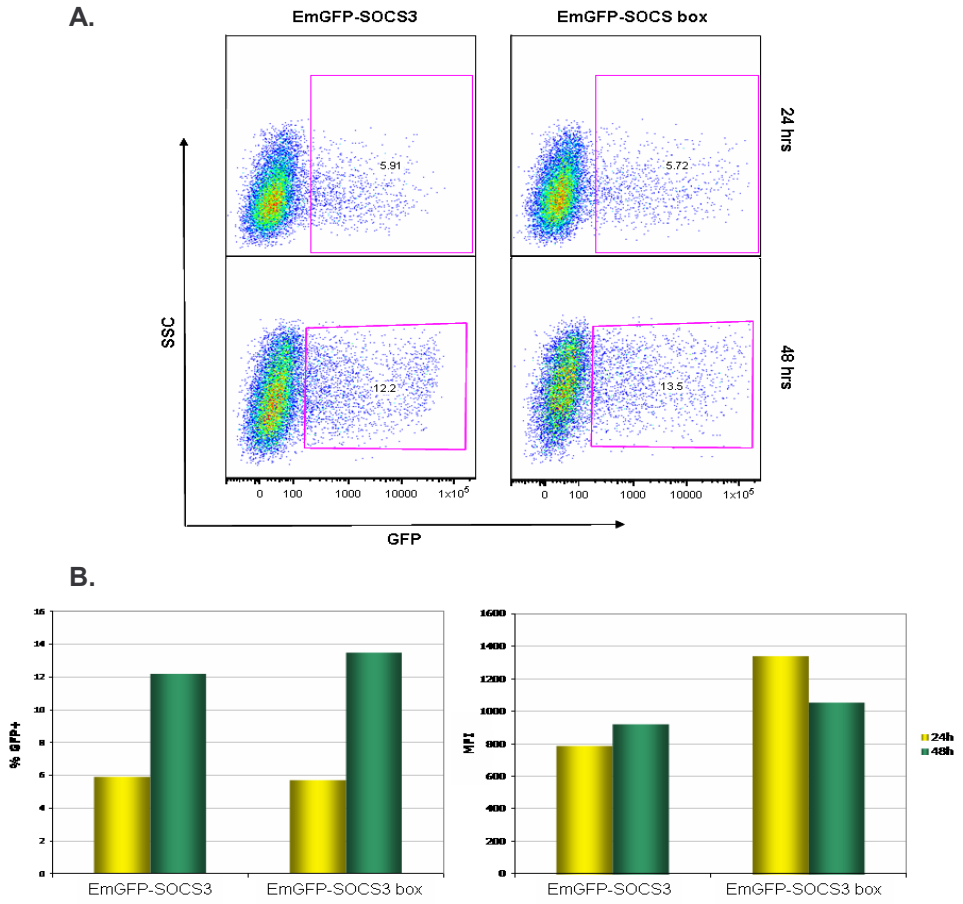

Figure 8. Flow Cytometry Analysis of Phoenix Cells Transfected with the SOCS3 or SOCS Box EmGFP Constructs. (A) Side scatter vs. GFP expression in transfected cells after 24 and 48 hours post-transfection, for both plasmids. Gated populations (enclosed in the pink box) were determined to be EmGFP-positive through comparison with both positive and negative controls (not shown). Clockwise from upper left: 5.91%, 5.72%, 12.2%, and 13.5%. (B) Graphic representation of EmGFP expression and median fluorescence. It is clear that GFP expression increases significantly over time post-transfection. However, we have yet to assay expression in individual cells over time.

DISCUSSION

This project resulted in the successful formation of an EmGFP-SOCS3 fusion protein plasmid, as well as an EmGFP-SOCS box fusion protein plasmid. Both plasmids, when transfected into Phoenix cells, fluoresced green at 24 and 48 hours post-transfection, proving the expression of the GFP gene, but it remains to be determined whether the SOCS3 or SOCS box proteins fused to the GFP are functional. Preliminary data showed a drop in Stat5 activity in transfected cells (not shown), suggesting a functional SOCS3, but further analysis is required to confirm this. A western blot should be performed to confirm the size of the protein products, but blotting so far has been unsuccessful. After further modifications, these plasmids may aid in monitoring Stat5 activity in individual cells over time, without the need for cell fixation.

Eventually the fusion protein will need to be cloned into a vector containing a promoter under the control of Stat5. Thus future experiments may involve subcloning the SOCS genes into an expression vector containing 4X Stat5-binding sites. As of now we have successfully used PCR to amplify the EmGFP fusions out of the original cloning vector and added distinct restriction endonuclease recognitions sites at either end with the intention of inserting the gene into the 4XpT109Luc vector created by Ganguly et al. (1997). This vector contains four Stat5 binding sites and the strong herpes simplex virus thymidine kinase (HSVtk) promoter. Unfortunately this vector is not behaving as we would predict based on the information available regarding the content of the polylinker and the locations of specific restriction endonuclease recognition sites.

It is also unclear how the plasmids created here function in murine fetal liver cells. Our laboratory has transfected the pcDNA6.2/N-EmGFP-SOCS3/SOCS box plasmids into mFLCs with moderate success. No conclusions have been drawn due to a very high background cell self-fluorescence signal during FACS analysis. This experiment will need to be repeated to determine the transfectability of mFLCs and their ability to express EmGFP.

Ultimately the goal of this set of experiments is learn about the activation and function of Stat5 in normal and aberrant erythropoiesis. The more information gathered from laboratory data, the easier it may be to target the over-activated Stat5 in treatments of leukemia and other forms of cancer. There is much more that still needs to be done, but every experiment is one step closer to novel treatments and possibly even a cure for leukemia.

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