

SOCS3 IS ESSENTIAL IN THE REGULATION OF FETAL LIVER ERYTHROPOIESIS

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ABSTRACT

SOCS3 (CIS3/JAB2) is an SH2-containing protein that binds to the activation loop of Janus kinases, inhibiting kinase activity, and thereby suppressing cytokine signaling. During embryonic development, SOCS3 is highly expressed in erythroid lineage cells and is Epo independent. Transgene-mediated expression blocks fetal erythropoiesis, resulting in embryonic lethality. SOCS3 deletion results in an embryonic lethality at 12–16 days associated with marked erythrocytosis. Moreover, the in vitro proliferative capacity of progenitors is greatly increased. SOCS3-deficient fetal liver stem cells can reconstitute hematopoiesis in lethally irradiated adults, indicating that its absence does not disturb bone marrow erythropoiesis. Reconstitution of lymphoid lineages in JAK3-deficient mice also occurs normally. The results demonstrate that SOCS3 is critical in negatively regulating fetal liver hematopoiesis.

Introduction

The appropriate regulation of erythropoiesis is essential for both embryonic development and adult red cell production. At least three distinct stages of erythropoiesis characterize embryonic development. Erythropoiesis first occurs within the blood islands of the embryo beginning at approximately day 7. This stage is characterized by the production of nucleated red cells expressing the embryonic pattern of globins and is not dependent upon erythropoietin (Epo), the major cytokine regulating subsequent stages of erythropoiesis (Wu et al. 1995b). Nor does this stage of erythropoiesis require JAK2, a kinase that is required for the function of a number of cytokines, including Epo (23, 26). The second stage of erythropoiesis begins at about day 10 and involves a dramatic expansion of erythroid lineage cells in the fetal liver. This expansion is highly dependent upon Epo, the Epo receptor, and JAK2. At this stage, the definitive adult pattern of globin expression is observed. Lastly, adult erythropoiesis is characterized by the localization of progenitors in the bone marrow and is similarly dependent upon Epo signaling for expansion.

A number of genes affect fetal liver erythropoiesis and can be conveniently grouped as those that cause a complete block in differentiation when deleted (2, 25, 24). This group includes a variety of transcription factors that are responsible for controlling the differentiation sequence. A second group of gene products are involved in regulating the numbers of red cells, and their deletion results in anemias or erythrocytosis. As noted above, this group includes Epo, the receptor for Epo, and the Epo receptor-associated kinase JAK2. Similarly, mutations of stem cell factor (SCF) and its receptor c-Kit are associated with anemia (Galli et al. 1994), and while an interaction between c-Kit and EpoR (Wu et al. 1995a) may be responsible, other possibilities, independent of EpoR, also exist.

Mice deficient in BclX_L die embryonically due to a severe anemia associated with increased apoptosis of erythroid cells (Motoyama et al. 1995). Curiously, embryos deficient in DNA ligase I also die at 12–15 days with a profound anemia (Bentley et al. 1996), suggesting that this enzyme may be important in protecting cells from the apoptotic pathways or that the high replicative rate of the cells cannot be supported without the enzyme. Consistent with a role for apoptosis in regulating red cell production, mice deficient in either FADD (Yeh et al. 1998) or Caspase 8 (Hakem et al. 1998) exhibit an accumulation of erythroid cells. These examples serve to emphasize the possibility that a number of factors contribute both positively and negatively to the rapid expansion of erythroid cells that occurs in the fetal liver at 10–15 days of embryonic development.

Erythropoiesis may also be negatively regulated through the interaction of proteins with the Epo receptor complex. It has been proposed that the tyrosine phosphatase SHP-1 can interact with the Epo receptor and downregulate JAK2 activity (Klingmuller et al. 1995). More recently, the *CIS* gene was identified as an Epo-induced immediate early gene (40, 20). The CIS protein binds the tyrosine phosphorylated Epo receptor through its SH2 domain and when overexpressed in transiently transfected cells can block Epo signaling. However, CIS-deficient mice are phenotypically normal in all regards (unpublished data).

Following the discovery of CIS, a number of related proteins were identified and given multiple names, including JAB (JAK binding), SOCS (suppressor of cytokine signaling), and SSI (Stat induced

Stat inhibitor). The family is characterized by an SH2 domain and a carboxy-terminal, unique conserved motif referred to as the SOCS box. SOCS1 and SOCS3 share the ability to bind directly to the activation loop phosphotyrosine of the JAKs and thereby block their catalytic activity (27, 38). Mice deficient in SOCS1 die perinatally with a complex pathology (22, 31). However, our recent studies (Marine et al., 1999 [this issue of *Cell*]) demonstrate that this lethality is due to altered lymphocyte function and have suggested that SOCS1 may be essential for blocking cytokine signaling at specific stages of lymphocyte development.

A potential role for SOCS3 has been less obvious. SOCS3 expression is transiently induced in a variety of situations, and overexpression studies have frequently been used to propose a functional role for this expression. In particular, roles in growth hormone function (Adams et al. 1998), lymphocyte proliferation (Cohney et al. 1999), leptin (Bjorbaek et al. 1998), CNTF (Bjorbaek et al. 1999), IL-11 (Auernhammer and Melmed 1999), and IL-10 signaling (Ito et al. 1999) have been proposed. In the studies presented here, we use transgenic and gene disruption approaches to provide evidence that SOCS3 is a critical negative regulator of fetal liver erythropoiesis.

Results

SOCS3 IS EXPRESSED IN ERYTHROID PROGENITORS IN THE FETAL LIVER

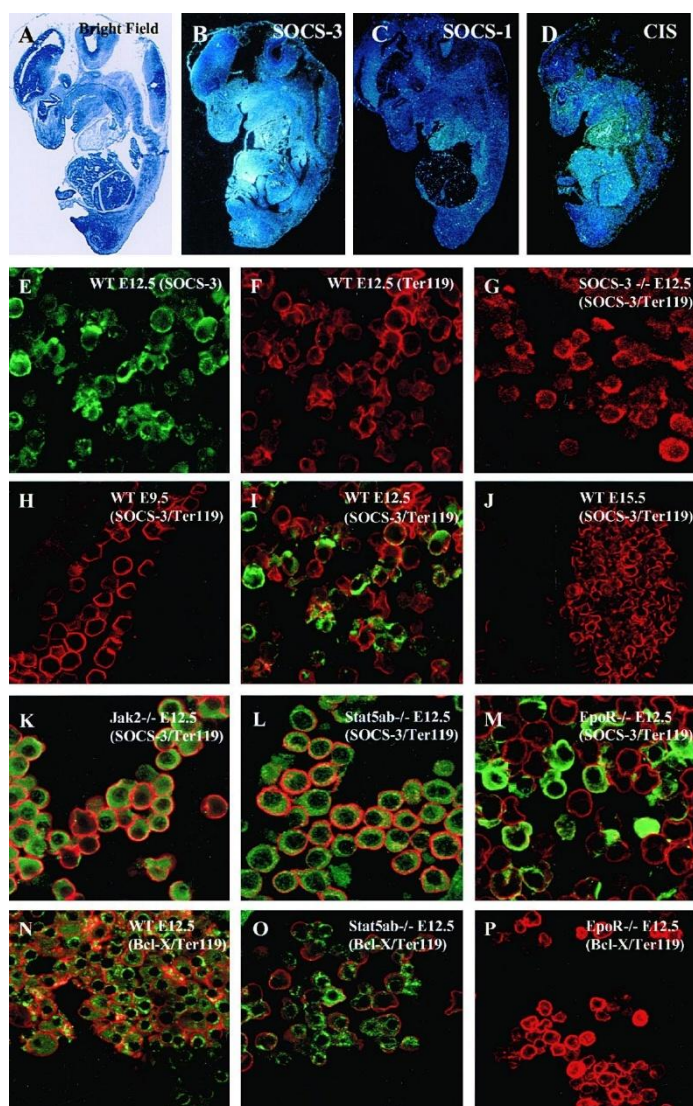
SOCS3 is expressed at low levels in adult tissues (Starr et al. 1997) and induced by a number of cytokines (1, 5, 8, 29, 9). However, by in situ hybridization of E12.5 embryos, SOCS3 was highly expressed in the fetal liver as well as other ill-defined sites (Figure 1B). In contrast, *SOCS1* was expressed at low levels (C), while *CIS* was expressed at detectable levels in the fetal liver and other sites (D). The relevance of *CIS* expression is unknown, since *CIS*-deficient mice have no detectable phenotype (unpublished data).

To further assess SOCS3 protein expression, sections of fetal livers from 12.5-day-old embryos were examined by immunofluorescence. To identify the erythroid progenitors and lineage committed cells, the sections were also exposed to an antibody against Ter119 directly conjugated with phycoerythrin. Ter119 is expressed on all erythroid lineage committed cells (Ikuta et al. 1990) that require Epo for survival and differentiation (Wu et al. 1995b). As illustrated (Figures 1E, 1F, 1I), SOCS3 was highly expressed in Ter119-positive fetal liver cells at day 12.5. SOCS3 was not expressed in the Ter119-negative hepatocytes within the fetal liver. The specificity of detection is indicated by the absence of SOCS3 expression in Ter119-positive fetal liver cells from a SOCS3-deficient embryo (G). However, there was a great variation in the levels of SOCS3 expression in individual Ter119-positive fetal liver cells. The variable levels of expression suggested that expression is regulated. To assess the role of Epo signaling, SOCS3 expression was examined in Ter119-positive fetal liver cells from Epo receptor- (M), Stat5a/b- (L), or JAK2-deficient mice (K). As illustrated, SOCS3 expression was observed in all cases. As a control we also examined BclX_L expression. In contrast to SOCS3, BclX_L expression is not seen in Ter119-positive cells in Epo receptor-deficient embryos, demonstrating the

critical role of Epo signaling in its expression (P). However $BclX_L$ is observed in $Stat5a/b$ -deficient embryos (O) at levels comparable to the levels seen in wild-type embryos (N). Therefore, unlike $BclX_L$, which requires Epo receptor signaling but not $Stat5a/b$ function, SOCS3 expression is independent of Epo regulation.

The developmental specificity of expression was assessed by examining erythroid progenitors in the blood islands of 9.5-day-old embryos. As illustrated (Figure 1H), Ter119-positive cells in the blood islands did not express SOCS3. Similarly, no SOCS3 expression was observed in the Ter119-positive cells, predominantly enucleated mature red cells from the liver sinuses of 15.5-day-old embryos (J). Therefore, SOCS3 is primarily expressed during the stage of fetal liver erythropoiesis that is characterized by the dramatic expansion of the early erythroid lineage cells, and, although variable in individual cells, this expression is independent of Epo receptor signaling.

Figure 1. *In Situ Hybridization and Immunofluorescence to Detect SOCS3 Expression in Embryonic Erythroid Progenitors*



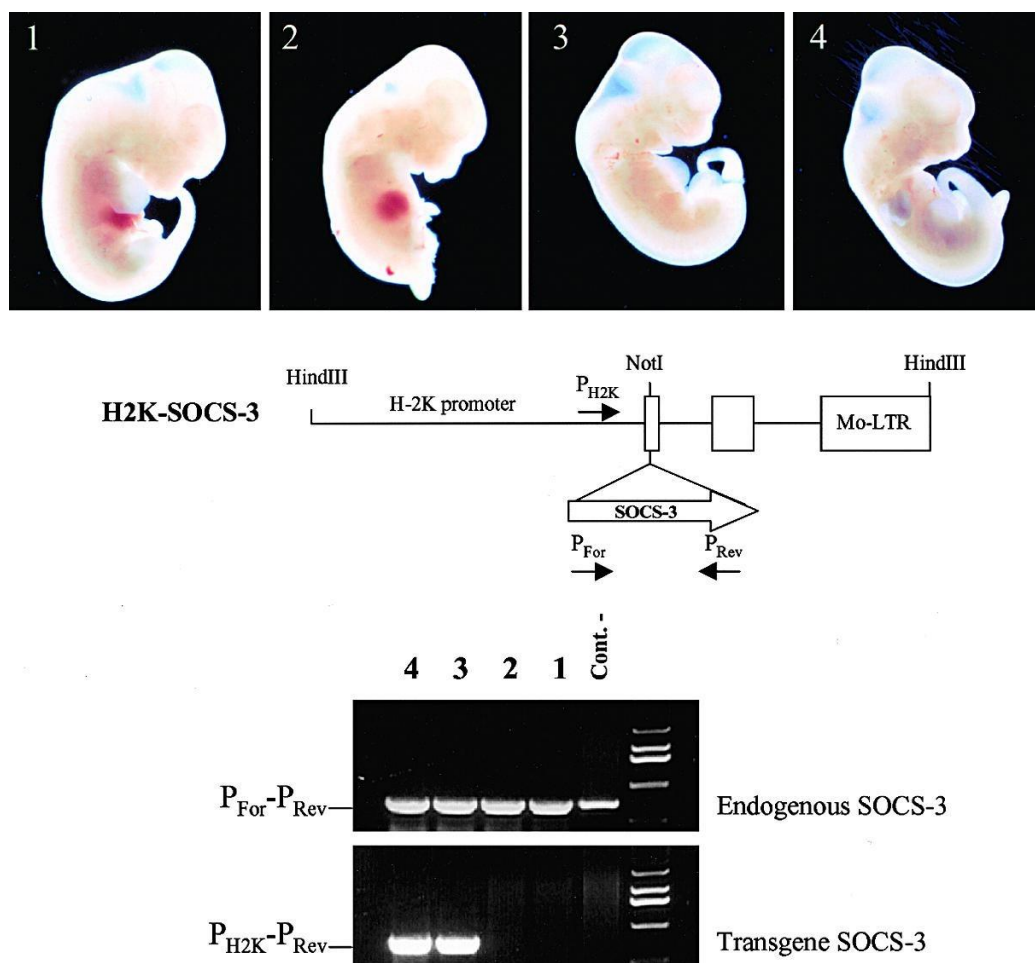
(A) Bright field image of wild-type, day 12.5 embryo sagittal section. Wild-type E12.5 sections hybridized with cRNA probes for SOCS3 (B), SOCS1 (C), and CIS (D). SOCS3 protein expression ([E]; green) in Ter119⁺ erythroid progenitors ([F]; red) in wild-type E12.5 fetal liver sinusoid. (I) SOCS3/Ter119 overlay in wild-type E12.5 fetal liver sinusoid. Ter119⁺ cells at stages E9.5 (H) and E15.5 (J) do not express SOCS3 protein. Ter119⁺ cells from SOCS3^{-/-} E12.5 (G) are negative for SOCS3 protein, whereas similar cells from $Stat5ab^{-/-}$ (L), $JAK2^{-/-}$ (K) and $EpoR^{-/-}$ (M) E12.5 fetal livers retain expression of SOCS3 protein. $BclX_L$ expression, used here as a control, is very high in E12.5 wild-type embryos (N) and $Stat5a/b$ - (O) deficient fetal liver Ter119-positive cells, whereas similar cells from an Epo receptor-deficient embryo lacked expression (P).

ENFORCED EXPRESSION OF SOCS3 IN VIVO SPECIFICALLY SUPPRESSES FETAL LIVER ERYTHROPOIESIS

Since SOCS3 was predominantly expressed in erythroid populations, we determined the consequences of constitutive expression resulting from an H2K, MHC class I promoter-containing transgene construct (Figure 2). This promoter, in combination with a Moloney long terminal repeat promoter, has been shown to drive expression of a transgene in virtually all stages of hematopoiesis including stem cells (Kondo et al. 1997). Embryos of 4–12 cells were injected with the *H2K-SOCS3* construct, and the progeny were initially examined for founders. Since no founders were obtained, recipient females were sacrificed at 12.5 days and the embryos examined. As illustrated in Figure 2, embryos were observed that had no evidence of fetal liver erythropoiesis (3 and 4) in addition to the normal appearing embryos (1 and 2). This phenotype is strikingly similar to that seen in embryos deficient in either JAK2 (Parganas et al. 1998) or the Epo receptor (Wu et al. 1995b), both of which are associated with embryonic lethality.

To establish that the lack of fetal liver erythropoiesis was associated with the transgene, the embryos were genotyped by PCR. As illustrated in Figure 2 (lower panel), the two normally appearing embryos did not contain the transgene, while both embryos that lacked detectable fetal liver erythropoiesis contained the transgene. Among a total of 46 embryos, 38 embryos did not contain the transgene and were phenotypically normal. Among the 8 embryos containing transgenic sequences, 2 were phenotypically normal, while the remainder lacked detectable fetal liver erythropoiesis. Since it would be anticipated that not all the hematopoietic stem cells would be transduced, the results suggest that the high-level, constitutive expression of SOCS3 can suppress erythropoiesis.

Figure 2. Ectopic Expression of SOCS3 In Vivo Alters Fetal Liver Erythropoiesis



The full-length SOCS3 cDNA was cloned in the pSP65-H2K-I-LTR vector to generate the transgenic construct illustrated here. Recipient foster females were sacrificed at day 12.5 of gestation, and the embryos were examined. Integration of the DNA transgenic construct was assessed by PCR using primers P_{H2K} and P_{Rev} . The quality of the genomic DNA template isolated from each embryo was confirmed using primers P_{For} and P_{Rev} (lower panel). The transgene-positive embryos (3 and 4) are characterized by the visually obvious absence of red cells in the fetal liver and surrounding tissues when compared to wild-type littermates (embryos 1 and 2).

DELETION OF SOCS3 CAUSES EMBRYONIC LETHALITY ASSOCIATED WITH ERYTHROCYTOSIS

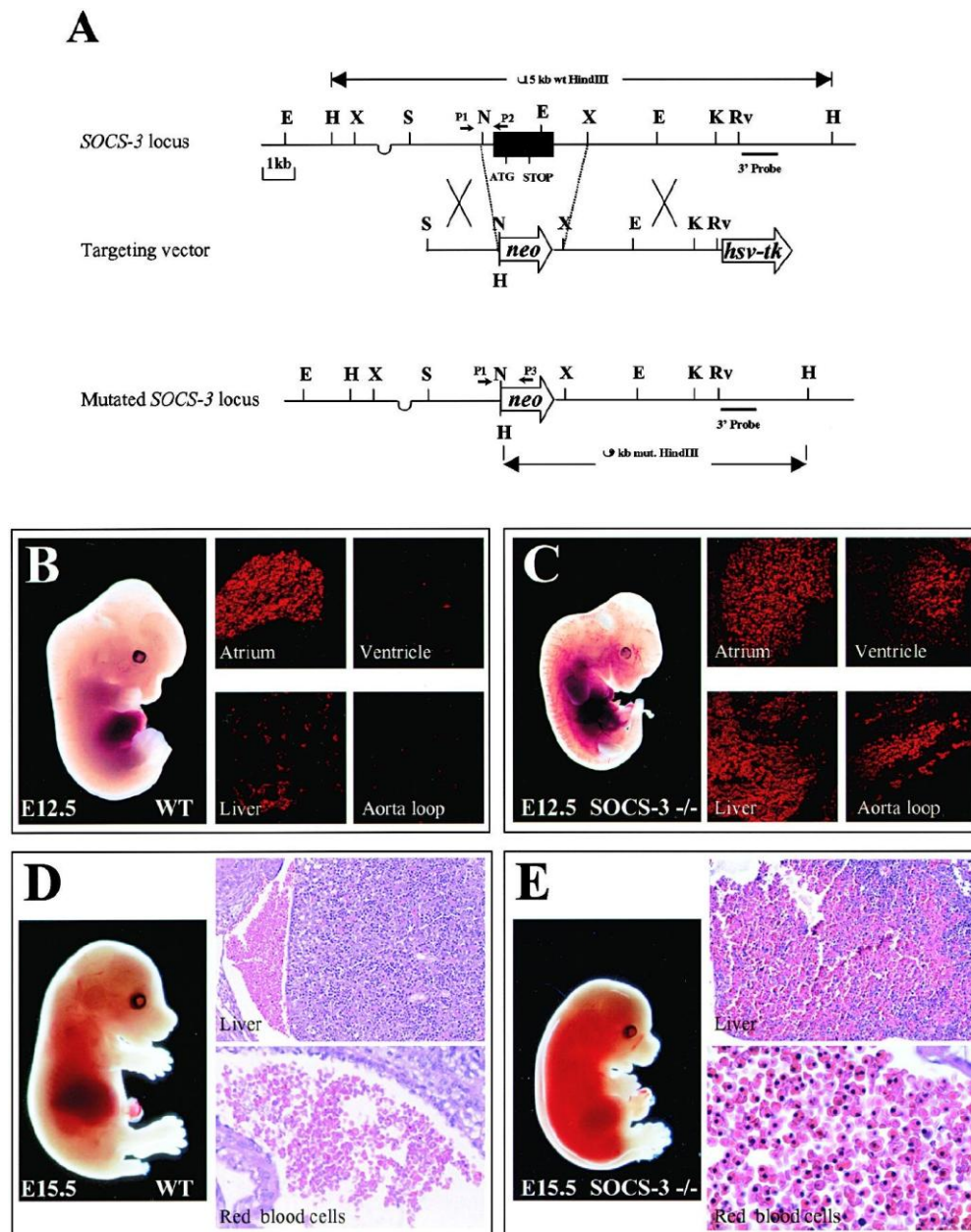
To disrupt the SOCS3 gene, a targeting vector was constructed (Figure 3A) that would delete the exon containing the entire coding region of the gene and thus create a completely protein null mutation. Three independently derived targeted ES clones gave rise to mutant strains, all of which had identical phenotypes. Heterozygous mice were phenotypically normal and were bred to obtain mice homozygous for the disrupted allele. As illustrated in Figure 1G, SOCS3 protein is not detectable in the homozygously deficient embryos. Typing of the progeny failed to detect homozygous individuals, with one exception, indicating that deletion of the gene resulted in an embryonic lethality. In the one exception, the pup was born runted and died within 24 hr. To establish the period

of lethality, embryos were taken at various stages of development and typed. As illustrated in Table 1, approximately the expected numbers of homozygous embryos were present at 10–12 days, but the percentage of homozygous individuals decreased with age.

The only obvious pathology associated with the homozygously deleted embryos was a marked erythrocytosis (Figure 3C and Figure 3E). This was most striking in the abdominal region and surrounding the fetal liver. In addition, the structure of the fetal liver was completely distorted by the abundance of erythroid cells, particularly in older embryos (data not shown). Erythemia was also evident throughout the embryo, often including the head and even the lens of the eye. Histological examination revealed extensive erythrocytosis in the fetal liver and in other thoracic organs. For example, at 12.5 days the ventricles and aortic loop normally contain relatively few erythroid cells; however, both are filled with red cells in SOCS3-deficient embryos (compare Figure 3B and Figure 3C). In normal embryos at day 15.5, the majority of the erythrocytes in the fetal liver are enucleated, major red cells (Figure 4D), whereas in SOCS3-deficient embryos a significant fraction of the cells are still nucleated (Figure 4E). As indicated in Table 1, the frequency of marked erythrocytosis in the SOCS3-deficient embryos increased with age such that after day 14, all the SOCS3-deficient embryos that had survived display erythrocytosis. Lastly, all the SOCS3-deficient embryos are smaller than heterozygous or wild-type embryos at the same age. The delayed development may be secondary to the erythrocytosis.

One consequence of the extensive erythrocytosis in the embryos might be a shift from the more immature populations to the more mature, nonproliferative populations of cells. As illustrated in Figure 4A, such an effect was observed in colony assays. The numbers of the most differentiated erythroid colony-forming cells (CFU-E) and the lesser differentiated burst-forming cells (BFU-E) were reduced approximately 10-fold as were the number of multilineage progenitors that formed colonies in the presence of IL-3. However, there was a marked difference in the proliferative capacity of the early, IL-3 responsive cells. As illustrated in Figure 4B, the sizes of the IL-3/Epo-induced BFU-E and CFU-Mix colonies from fetal liver cells of SOCS3-deficient embryos were typically 5–10 times larger than the colonies derived from fetal liver cells from wild-type embryos.

Figure 3. Targeted Disruption of the SOCS3 Gene Results in a Phenotype of Erythrocytosis



(A) Structure of the SOCS3 locus, SOCS3-neo targeting vector, and predicted SOCS3 mutated locus is shown.

(B) The morphology of an E12.5 wild-type embryo; the presence of cells of erythroid origin in various organs of the embryo was visualized by immunofluorescence using a Ter119 antibody.

(C) An E12.5 SOCS3-deficient embryo. The mutant embryo shows sign of severe erythrocytosis; Ter119-positive cells were ectopically detected in large numbers and in many different organs including the ventricle of the heart (as shown here).

(D) The morphology of an E15.5 wild-type embryo; the histological appearance of its liver is also illustrated. At that stage of development, the majority of the erythroid cells are fully mature enucleated red blood cells.

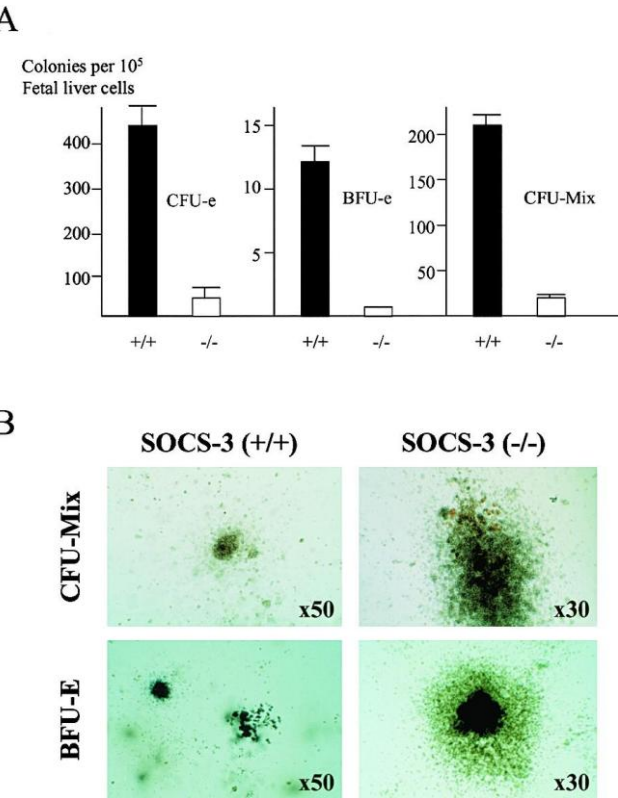
(E) An E15.5 SOCS3-deficient embryo. The structure of the liver is completely altered; this organ is full of red blood cells. As opposed to the littermate controls, the vast majority of those cells are still nucleated.

Table 1. Genotypic Distribution of Newborn Mice and Embryos from SOCS3 Heterozygous Intercrosses

Stage	No. of Litters	Total No.	+/+	+/-	-/-	Fetal Liver Erythrocytosis (%)	-/- (%)
10.5	1	10	3	4	3	0	30
11.5	5	42	13	20	9	33	21
12–12.5	30	211	76	102	33	50	16
13–13.5	14	83	24	49	10	66	12
14.5	5	30	11	16	3	100	10
15.5	6	30	10	18	2	100	7
16.5	5	31	11	19	1	100	3
F2	36	193	70	122	1		<1

Postnatal mice and embryos were genotyped by PCR, as shown in Figure 3B. The embryos were collected between day 10.5 and 16.5 of pregnancy from SOCS3^{+/-} females crossed to SOCS3^{+/-} males. Histological examination of some mutant embryos revealed extensive erythrocytosis in the fetal liver (as shown in Figure 4); the frequency at which this phenotype was observed is indicated in the table in percent of the total number of embryos analyzed.

Figure 4. In Vitro Proliferative Capacity of Early Hematopoietic Progenitors in Liver Cells from SOCS3 Wild-Type and Mutant Embryos



(A) In vitro colony-forming ability of fetal liver hematopoietic progenitors from SOCS3-deficient and wild-type embryos. The mean and standard deviation of the number of colonies/10⁵ cells are shown from the assays with four 12.5-day-old embryos of each genotype. The four SOCS3 embryos chosen showed signs of erythrocytosis.

(B) Typical appearance of CFU-Mix (top panel) and BFU-E (lower panel) colonies derived from wild-type (left) and SOCS3-deficient embryos (right) after 18 days of incubation in methylcellulose media containing optimal concentrations of appropriate recombinant growth factors.

SOCS3 DEFICIENCY DOES NOT SIGNIFICANTLY AFFECT ADULT HEMATOPOIESIS

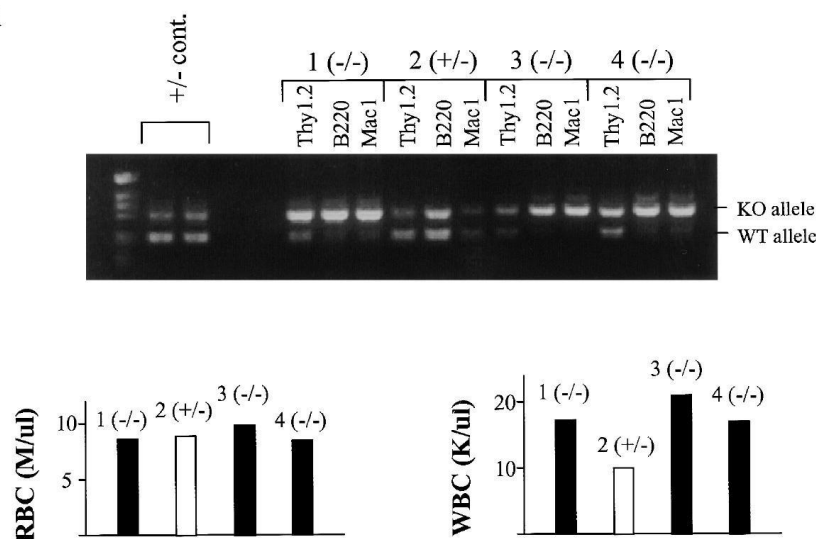
To explore the potential role of SOCS3 in adult hematopoiesis, fetal liver cells from SOCS3-deficient embryos were used to reconstitute lethally irradiated wild-type adult mice (Figure 5). Mice were analyzed at 2 months following reconstitution with fetal liver cells. As illustrated in Figure 5A, PCR analysis of FACS-sorted Thy1.2-, B220-, or Mac1-positive blood cells demonstrated the predominance of cells derived from the SOCS3-deficient fetal liver cells (–/–) and from heterozygous fetal liver cells (+/–). In each of the mice reconstituted with fetal liver cells from homozygously deleted embryos, the wild-type allele was only evident in the Thy1.2-positive population and was estimated to constitute at most 20% of the T cells. As illustrated in (A), there were no significant differences in the number of peripheral red cells or white blood cells in any of the reconstituted animals.

The ability of bone marrow from reconstituted mice to form hematopoietic colonies is illustrated in Figure 5B. PCR analysis of the bone marrow cells from mice reconstituted with fetal liver cells from SOCS3 homozygously deficient embryos (–/–) detected the presence of only the mutant allele. As indicated, there were no detectable differences in the number of IL-7-dependent colonies between animals reconstituted with fetal liver cells from heterozygous or homozygous SOCS3-deficient embryos. However, there was a consistent and significant increase of approximately 2-fold in the number of CFU-E, BFU-E, and CFU-Mix. As observed with the colonies from fetal liver (above), the bone marrow-derived colonies were significantly larger in size (data not shown). The results demonstrate that SOCS3-deficient hematopoietic progenitors can functionally reconstitute lethally irradiated animals without excessive proliferation that results in pathology. However, the cells have an enhanced responsiveness to cytokines.

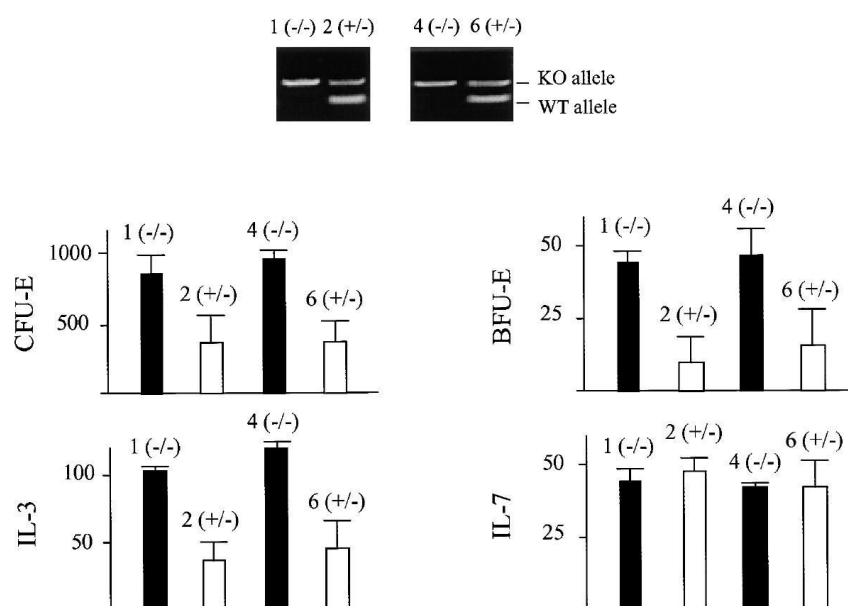
To evaluate the potential role of SOCS3 in the lymphoid lineages, fetal liver cells from SOCS3-deficient embryos were used to reconstitute sublethally irradiated JAK3-deficient mice. Approximately 2 months following reconstitution, thymus and spleen were examined for evidence of reconstitution of the lymphoid populations by FACS analysis. As illustrated in Figure 6A, fetal liver cells from SOCS3-deficient embryos reconstituted the T cell population in the thymus comparable to fetal liver cells from heterozygous embryos. Similarly, the B cell population in the spleen was reconstituted comparably to that seen in mice reconstituted with bone marrow from control mice. Moreover, splenic lymphocytes from mice reconstituted with SOCS3-deficient fetal liver cells were capable of responding to increasing concentrations of IL-2 in the presence of limiting concentrations of anti-CD3 as well as responding to anti-IgM in the presence of increasing concentrations of IL-4 (Figure 6C). The data demonstrate that SOCS3 deficiency does not result in any overt alterations in T or B cell differentiation or function, although it will be important to study these cells in more detail for subtle effects in subset differentiation or function.

Figure 5. SOCS3 Does Not Play a Major, Nonredundant Role during Adult Hematopoiesis

A. Blood

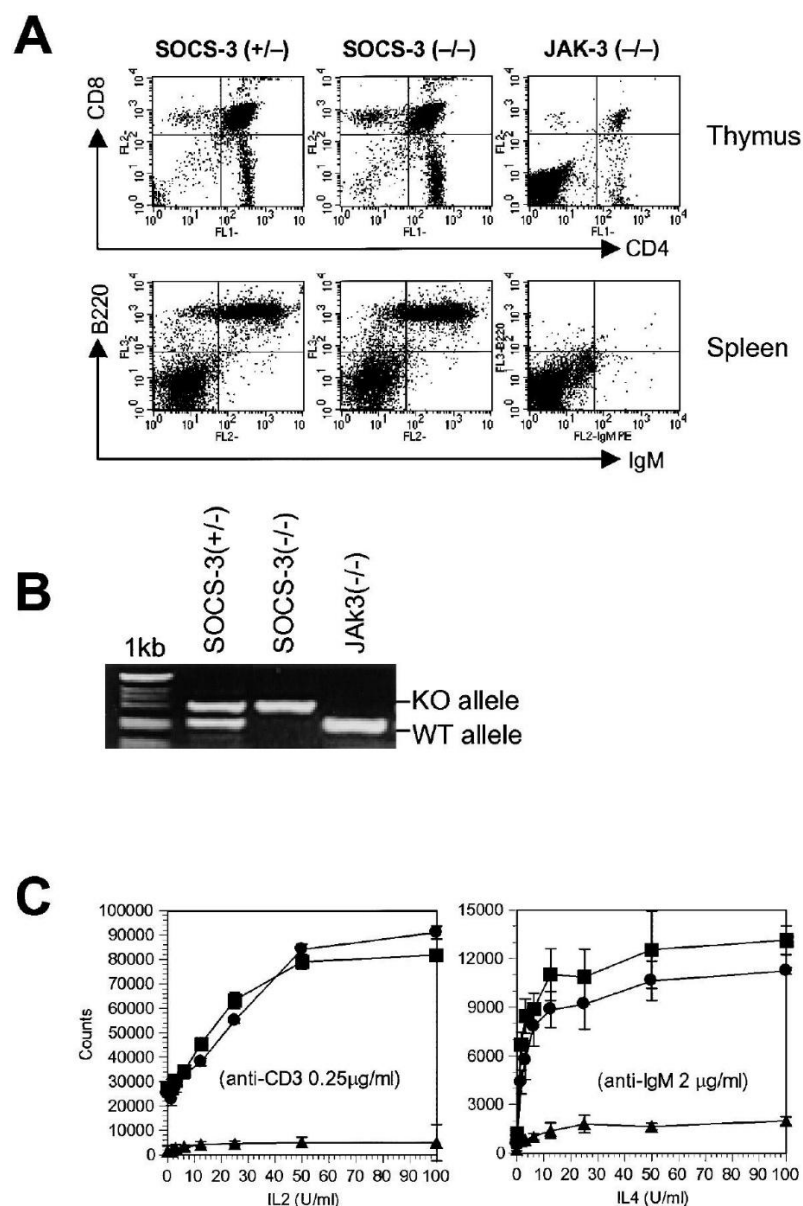


B. Bone Marrow



Fetal liver cells from SOCS3 heterozygous and homozygous mutant embryos were used to reconstitute lethally irradiated wild-type adult mice. (A) summarizes the analysis of peripheral blood from such mice, performed 2 months following reconstitution. To assess the contribution of cells from SOCS3-deficient fetal liver cells, a PCR reaction was used to detect the presence of the altered allele in FACS-sorted Thy1.2-, B220-, or Mac1-positive cells. DNA isolated from two SOCS3 heterozygous mice were used as controls (+/- cont.). The peripheral red blood count (RBC) and white blood count (WBC) of such reconstituted animals are indicated in the lower part of (A). PCR analysis of bone marrow cells from reconstituted mice with SOCS3^{+/-} or SOCS3^{-/-} fetal liver cells is illustrated in (B). A comparison in the numbers of bone marrow-derived CFU-E, BFU-E, CFU-Mix (IL-3), and IL-7-dependent colonies between animals reconstituted with fetal liver cells from heterozygous or homozygous SOCS3-deficient embryos is also shown in (B).

Figure 6. Reconstitution of the Lymphoid Lineages of JAK3-Deficient Mice by Fetal Liver Cells from SOCS3-Deficient Embryos



(A) JAK3-deficient mice were sublethally irradiated and reconstituted by injection of liver cells from wild-type embryos or SOCS3-deficient embryos. Five weeks after reconstitution, the ability to reconstitute the thymocyte and the splenic populations was examined by analysis of the expression of the T lineage markers CD4 and CD8, and the expression of the B lineage markers B220 and IgM, respectively. The thymus and the spleen of a JAK3^{-/-} animal were included in this experiment as a control.

(B) To assess the contribution of cells from SOCS3-deficient fetal liver cells, a PCR reaction was used to detect the presence of the altered allele in FACS-sorted Thy1.2-positive cells.

(C) The functional capabilities of splenic lymphocytes were examined by assessing their ability to proliferate to a limiting concentration of anti-CD3 in the presence of increasing concentrations of IL-2 (left), or to a limiting concentration of anti-IgM in the presence of increasing concentrations of IL-4 (right). The samples shown include cells from JAK3-deficient mice (filled triangles) and JAK3-deficient mice reconstituted with fetal liver cells from heterozygous or homozygous SOCS3-deficient embryos (filled circles and filled rectangles, respectively).

Discussion

The results demonstrate that SOCS3 plays a critical role in fetal liver erythropoiesis at a stage when it is highly expressed in erythroid lineage cells. The pathology seen in the absence of SOCS3 is a dramatic expansion of erythropoiesis within the fetal liver as well as throughout the embryo. Morphologically, the abnormal expansion that occurs within the fetal liver causes, in later stage embryos, a complete loss of the normal architecture of the liver (data not shown). However, the most visually striking phenotype is the greatly expanded numbers of red cells and red cell precursors throughout the embryo. It is possible that the increased numbers of red cell progenitors are a direct consequence of the increased production within the fetal liver. However, it is also possible that in the absence of SOCS3 peripheral sites are able to produce erythrocytes. Another possibility is that erythroid progenitors that migrate out of the fetal liver retain a proliferative capacity that would normally be controlled through the induction of SOCS3 expression.

The phenotype raises a general question regarding the mechanisms by which the amount of erythropoiesis is regulated within the fetal liver. The availability of Epo is clearly a critical factor, and ultimately it is the availability of Epo that must define the limiting level of erythropoiesis. However, it is becoming obvious that the disruption of several genes can result in a significant, and pathological, expansion of red cells, suggesting that Epo does not define the amount of erythropoiesis during the period of fetal liver production. In particular, the embryos deficient in either FADD (Yeh et al. 1998) or Caspase 8 (Varfolomeev et al. 1998) exhibit an erythrocytosis that is very similar to that observed in SOCS3-deficient embryos. This observation has suggested the possibility that a TNF-like receptor system may exist that controls the numbers of erythroid lineage cells through induction of apoptosis. Such a mechanism could be envisioned to simply regulate the extent of erythropoiesis, or it could also contribute to defining the sites at which erythropoiesis can occur.

A role for apoptosis is also supported by the critical role that BclX_L plays in fetal liver hematopoiesis (Motoyama et al. 1995). In particular, deletion of BclX_L results in an embryonic lethality due to a dramatic increase in apoptotic cells and hematopoietic failure. In contrast to SOCS3, BclX_L levels are regulated through Epo signaling. Recently, a potential role for Stat5a/b in BclX_L regulation during fetal liver erythropoiesis was proposed based on (1) the presence of Stat-binding sites in the gene, (2) their functionality in expression constructs in cell lines, and (3) the partial inhibition of BclX_L transcripts in a long-term cell line by overexpression of Stat5a dominant-negative protein (Socolovsky et al. 1999). However, the expression of BclX_L in Stat5a/b-deficient or control fetal liver cells was not examined. As assessed by immunofluorescence in our studies (Figure 10), Stat5a/b-deficient cells express BclX_L at levels comparable to wild-type cells. Therefore, we conclude that the Stat5 proteins are not absolutely required for BclX_L expression in fetal erythroid cells.

The mechanism by which SOCS3 negatively influences fetal liver erythropoiesis is hypothesized to be through its ability to inhibit JAK2, which, based on the phenotype of JAK2-deficient embryos (Parganas et al. 1998), is critical for Epo receptor function and erythropoiesis. The SH2 domain of SOCS1 has been shown to bind to the activation loop tyrosine of JAKs and to inhibit their kinase

activity (Yasukawa et al. 1999), and we have recently found that SOCS3 similarly binds the activation loop kinase of JAK2 and inhibits activity (Sasaki et al. 1999). In addition to inhibiting JAK kinase activity, it is also possible that SOCS proteins, through interaction of the SOCS box with the elongin BC complex (16, 41), may target the kinases for degradation. All these experiments, however, have relied on overexpression studies. It will therefore be important to use mice genetically deficient in SOCS3 as well as cytokine signaling to establish the specificity of SOCS3 inhibition.

Perhaps the most important, unanswered question deals with the factors that control SOCS3 expression during erythropoiesis. This is particularly critical, since, as demonstrated with transgenic embryos, overexpression can completely block erythropoiesis. A number of studies have identified cytokines that can induce SOCS3 RNA, including CNTF (Bjorbaek et al. 1999), leptin (Bjorbaek et al. 1998), LIF (5, 6), IL-11 (Auernhammer and Melmed 1999), IL-10 (Ito et al. 1999), and growth hormone (Adams et al. 1998). Thus, the concept has emerged that SOCS proteins are feedback inhibitors of cytokine signaling. However, in none of these situations has the level of protein been examined relative to the levels that are required for inhibition of JAK function. This is particularly important, since, in our experience, the SOCS proteins turn over very rapidly in many of the cell types in which they are induced. Irrespective, the expression of SOCS3 in fetal liver cells does not require Epo receptor signaling. Two possibilities exist: namely that SOCS3 expression is developmentally regulated in a manner which is independent of cellular signaling or, alternatively, that another signaling pathway is involved. One intriguing signaling possibility is that stem cell factor, acting through c-Kit, suppresses a developmentally programmed expression of SOCS3.

The possibility that SOCS3 expression is developmentally regulated is also of considerable interest. In particular, it could be envisioned that it is the activation of SOCS3 expression at later stages of erythropoiesis that is responsible for turning off Epo signaling and promoting the cells into the terminal stages of differentiation. This possibility is consistent with the phenotype of the SOCS3-deficient mice; particularly the predominance of more immature cells in peripheral sites. It is also consistent with the increased colony size as indicated below. In this regard it should also be noted that SOCS1 expression in thymocytes is most likely to be developmentally regulated and, specifically, is not regulated by cytokines (Marine et al. 1999). Moreover, the phenotype of SOCS1-deficient mice strongly supports the concept that the critical role for SOCS1 is in thymocyte differentiation (Marine et al. 1999). Therefore, the developmental regulation of SOCS protein expression may be more biologically relevant than the observation that cytokines can transiently induce their expression.

Associated with the overt erythrocytosis was a decrease in the number of CFU-E and BFU-E in colony assays with fetal liver cells from SOCS3-deficient mice. This was not anticipated based on the observation that there were many more nucleated cells present at various sites in SOCS3-deficient embryos. However, there is also an increased amount of apoptosis, and we would propose that in these embryos Epo becomes limiting and a significant fraction of the cells undergo apoptosis prior to terminal differentiation. Remarkably, a decrease in colony-forming cells was also observed in Caspase 8-deficient embryos that have a virtually identical pathology of erythrocytosis (Varfolomeev et al. 1998). In contrast to the decrease in colony numbers, the numbers of cells

produced in the colonies are greatly increased. This could be due to a decreased cell cycle time or to the ability of the cells to undergo more rounds of proliferation. We favor the later possibility, although additional studies will be needed to substantiate this model. Irrespective, it is possible that SOCS3 expression is turned on with differentiation, and it is this induction that limits the colony size of the wild-type progenitors. However, it should be emphasized that terminal differentiation does occur in colonies from SOCS3-deficient fetal liver cells, demonstrating that SOCS3 expression modifies the program rather than being an essential component for terminal differentiation.

As demonstrated through the reconstitution experiments, a deficiency of SOCS3 has very little effect on erythropoiesis in the adult animal and specifically was not associated with an obvious erythrocytosis or other pathology. However, there was an increase in CFU-E and BFU-E in bone marrow progenitors, and the proliferative capacity of individual progenitors, based on colony size, was increased comparable to that seen in the fetal liver. The absence of erythrocytosis likely reflects a difference in the control of erythropoiesis in the adult animal. In particular, the amount of adult erythropoiesis may be more dependent upon the availability of Epo. This would suggest that mice reconstituted with SOCS3-deficient bone marrow would be much more responsive or sensitive to exogenous administered Epo—a possibility that we are examining.

Reconstitution experiments in JAK3-deficient animals were done to examine the possible role of SOCS3 in lymphoid development. This experiment was prompted by our recent studies (Marine et al. 1999) with SOCS1 as well a recent study that suggested that SOCS3 might be involved in suppressing Stat5 tyrosine phosphorylation and thereby block lymphocyte proliferation (Cohnen et al. 1999). Deficiency of SOCS1 is associated with a perinatal lethality (22, 31). A lymphoid defect for this lethality is indicated by its elimination by introducing a RAG2 deficiency and by the ability to transfer the pathology with lymphoid lineage reconstitution (Marine et al. 1999). These results suggest that the high levels of SOCS1 expression in the thymus play a role in lymphocyte development. This observation emphasizes that while SOCS1 and SOCS3 may function identically in binding JAK kinases and inhibiting their activity, the specificity of biological function is likely to be related to their unique sites of high levels of expression.

Experimental Procedures

CONSTRUCTION OF SOCS3 TARGETING VECTOR

The *SOCS3* gene was isolated from a 129/SVE mouse genomic library in λ EMBL3 using *SOCS3* full-length cDNA probe. Positive clones were restriction mapped and partially sequenced. For the targeting construct, a 13 kb *Sall*/*HindIII* fragment was subcloned, followed by replacement of a 3 kb *NotI*/*XbaI* fragment containing the entire *SOCS3* coding region with a neomycin resistance cassette previously described (van Deursen et al. 1991). A herpes simplex thymidine kinase (HSV-tk) cassette mediating negative selection was inserted in the 3'-end of the *SOCS3-neo* construct.

TRANSFECTION OF ES CELLS AND GENERATION OF SOCS3-DEFICIENT MICE

E14 (129/Ola mouse strain) ES cells were cultured as described (Parganas et al. 1998). Twenty-five micrograms of SalI linearized SOCS3 plasmid construct was electroporated into the ES cells and grown under double selection as described (Parganas et al. 1998). Conditions for blastocyst injection of correctly targeted and karyotypically normal ES clones and breeding to generate mice homozygous for the mutated SOCS3 gene were performed essentially as described (van Deursen et al. 1993). Seven independent ES clones were injected, of which three gave germline transmission.

GENOTYPING OF SOCS3-DEFICIENT MICE BY SOUTHERN BLOT AND PCR

Genotyping of mice was performed by Southern blot analysis at first, then using a PCR-based assay. For this assay, approximately 200 ng of mouse tail DNA or genomic DNA isolated from mouse embryos was amplified per 25 µl reaction using 2.0 U of Qiagen Taq-polymerase in a final concentration of each dNTP at 0.2 mM, MgCl₂ at 1.5 mM. The PCR primers consisted of P1 primer (5'-AGGGGAAGAGACTGTCTGGGG), P2 (5'-CCGCACAGCGGCCGCTACC), and the neomycin primer P3 (5'-ACCACACTGCTCGACATTGGGT) as shown in Figure 3A. The PCR cycle profile was as follows: 1 cycle at 94°C for 4 min followed by 35 cycles at 94°C for 1 min, 62°C for 30 s, 72°C for 1 min; and finally 1 cycle at 72°C for 5 min. A 200 bp fragment indicates the presence of the wild-type allele, whereas a 300 bp fragment is amplified from the mutated allele.

GENERATION AND ANALYSIS OF SOCS3 TRANSGENIC MICE

We created the *H2K-SOCS3* transgenic construct by introducing *SOCS3* full-length cDNA into the NotI cloning site of the previously described pSP65-H2K-I-LTR vector (Domen et al. 1998). Purified DNA of the construct was microinjected into zygotes and transplanted into oviduct of pseudopregnant foster mothers. Successful integration of the injected DNA was monitored using a PCR-based approach. Since no founders were obtained, recipient females were sacrificed at day 12.5 of gestation and the embryos examined. Primers P_{For} (5'-TCCACGCTGGCTCCGTGCG) and P_{Rev} (5'-GCTCCTTAAAGTGGAGCATCA) were used to detect endogenous *SOCS3*; primer P_{H2K}, chosen in the H2K promoter portion of the transgenic construct (5'-TCACTTCTGCACCTAACCTGG), and primer P_{Rev} were used for the detection of the *SOCS3* transgene (see Figure 2). The PCR cycle profile was as follows: 1 cycle at 94°C for 4 min followed by 35 cycles at 94°C for 1 min, 62°C for 30 s, 72°C for 2 min; and finally 1 cycle at 72°C for 5 min.

COLONY ASSAYS

Fetal liver cells were prepared from livers of E11.5–E12.5 embryos and bone marrow cells from femurs of wild-type lethally irradiated mice reconstituted with either wild-type or *SOCS3* mutant fetal liver cells. The cells were plated and the colonies scored exactly as previously described (26, 32).

HISTOLOGY/IN SITU HYBRIDIZATION AND IMMUNOFLUORESCENCE

Embryos taken at day 12.5 of pregnancy from wild-type, *JAK2^{-/-}*, *Stat5ab^{-/-}*, and *EpoR^{-/-}* mice were fixed in 10% buffered formalin for 24 hr, and 6 µm sections were prepared from frozen tissue samples. Day 9.5, 12.5, and 15.5 embryos were fixed in 10% buffered formalin, paraffin embedded, and sectioned at 4 µm. Slides prepared for histological analysis were stained with hematoxylin and eosin as described previously. RNA in situ hybridization was carried out on frozen cryosections essentially as described (Angerer and Angerer 1991) using [α -³³P]-UTP-labeled antisense cRNA probes transcribed from plasmids containing murine cDNA fragments of *CIS* (216 bp), *SOCS1* (200 bp), and *SOCS3* (300 bp). Immunofluorescence was carried out using a PE-conjugated anti-mouse TER119 antibody to detect numbers of erythroid progenitors in all embryonic sections. SOCS3 protein levels were detected using a rabbit polyclonal antisera anti-SOCS3 N-terminal peptide antibody (aa 5–21 of mouse SOCS3) and Alexa⁴⁸⁸-anti-rabbit polyclonal antibody (Molecular Probes) as a secondary antibody. Briefly, tissue sections were refixed for 20 min in 10% buffered formalin, blocked for 1 hr in a solution of 10% BSA in 1× PBS followed by incubation overnight at 4°C with a 1:100 dilution of primary anti-SOCS3 antibody. Slides were washed with 1× PBS and incubated for a further 2 hr at room temperature with a 1:100 dilution of PE-anti TER119 and Alexa⁴⁸⁸-anti-rabbit antibodies. Slides were washed three times with 1× PBS and mounted under coverslips using Antifade-Fluoromount (Fisher). Ter119-positive cells and SOCS3 expression levels were visualized by confocal microscopy using a Leica DM-IRBE microscope together with Leica TCS-NT software.

RECONSTITUTION

Fetal liver cells from wild-type or SOCS3-deficient embryos were used to reconstitute sublethally irradiated JAK3-deficient mice (900 Rads) as well as wild-type lethally irradiated mice (1200 Rads). Approximately 3×10^6 liver cells in 500 µl of PBS containing 2% of fetal bovine serum were injected into the tail blood vessel of the recipient animals.

FLOW CYTOMETRY AND PROLIFERATION RESPONSES

These assays were performed exactly as previously described (Parganas et al. 1998).

HISTOLOGY

For sectioning, tissues were fixed in 10% phosphate-buffered formalin (Fisher), paraffin embedded, sectioned, and stained in hematoxylin and eosin.

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