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Jak2 Deficiency Defines an Essential Developmental Checkpoint in Definitive Hematopoiesis

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Summary

Janus kinases (Jaks) play an important role in signal transduction via cytokine and growth factor receptors. A targeted inactivation of Jak2 was performed. *Jak2*$^{-/-}$ embryos are anemic and die around day 12.5 postcoitum. Primitive erythrocytes are found, but definitive erythropoiesis is absent. Compared to erythropoietin receptor–deficient mice, the phenotype of Jak2 deficiency is more severe. Fetal liver BFU-E and CFU-E colonies are completely absent. However, multilineage hematopoietic stem cells (CD34^{low}, c-kit^{pos}) can be found, and B lymphopoiesis appears intact. In contrast to IFNα stimulation, *Jak2*$^{-/-}$ cells do not respond to IFNγ. *Jak2*$^{-/-}$ embryonic stem cells are competent for LIF signaling. The data provided demonstrate that Jak2 has pivotal functions for signal transduction of a set of cytokine receptors required in definitive erythropoiesis.

Introduction

Cytokines are critical regulators for differentiation and proliferation of a variety of cells (Paul and Seder, 1994). It is now recognized that tyrosine kinases of the Janus kinase (Jak) family play a pivotal role in signal transduction via cytokine receptors (Ihle et al., 1995). At present, this family consists of the four mammalian members Jak1, Jak2, Jak3, and tyk2 (Ihle, 1995). Jaks appear constitutively associated with membrane proximal intracellular domains of cytokine receptors and become phosphorylated after ligand binding and dimerization or oligomerization of the respective receptor chains. Activated Jaks consecutively phosphorylate tyrosine residues at the distal part of the receptor chains, thereby generating docking sites for native signal transducers and activators of transcription (STATs) and other intracellular signaling molecules (Ihle and Kerr, 1995; Briscoe et al., 1996). STATs, which are recruited to specific phosphotyrosine-containing motifs located in the cytoplasmic part of the receptors, are in turn phosphorylated by activated Jaks at distinct tyrosine residues. After this modification, STATs either homodimerize or heterodimerize, leave the receptor complex, enter the nucleus, and bind to response elements of dependent genes, thus influencing their transcriptional activation (Ihle, 1996; Darnell, 1997).

Jaks have a molecular mass of about 130 kDa and are composed of seven Jak homology domains. A characteristic feature is the presence of a kinase and an adjacent pseudokinase domain. Jak1, Jak2, and tyk2 are ubiquitously expressed, whereas the expression of Jak3 appears restricted to hematopoietic cells (Witthuhn et al., 1994; Ihle, 1995). Mice lacking Jak3 exhibit a SCID-type immunodeficiency due to profound defects in lymphoid cell development (Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995).

The function of Jaks was initially discovered by generating cell lines resistant to the effects of interferons (IFNs). These defects could be reconstituted by expressing different cDNAs encoding for Jaks (Darnell et al., 1994). It was shown that cellular responses to IFNα require Jak1 and tyk2, whereas a response to IFNγ requires Jak1 and Jak2 (Velazquez et al., 1992; Müller et al., 1993; Silvennoinen et al., 1993a, Watling et al., 1993). More recent findings demonstrated that Jaks and STATs are utilized by a variety of cytokine and growth factor receptors (Watowich et al., 1996). To date, it is known that Jak2 is associated with receptors binding to the following factors: erythropoietin (Epo) (Witthuhn et al., 1993), growth hormone (GH) (Argetsinger et al., 1993), thrombopoietin (Tpo) (Drachman et al., 1995, 1997), and, furthermore, common β chain–containing receptors binding granulocyte/macrophage colony-stimulating factor (GM-CSF) (Quelle et al., 1994), interleukin-3 (IL-3) (Silvennoinen et al., 1993b), and IL-5 (Pazdrak et al., 1995). Other receptors associated with Jak2 are cardiotrophin-receptor (CT-R) (Pennica et al., 1995), prolactin-receptor (PRL-R) (Campbell et al., 1994), granulocyte CSF-R (G-CSF-R) (Shimoda et al., 1994), and cytokine receptors containing gp130 in their receptor chain complex (IL-6-R, CNTF-R, LIF-R, and OSM-R) (for review, O'Shea, 1997).

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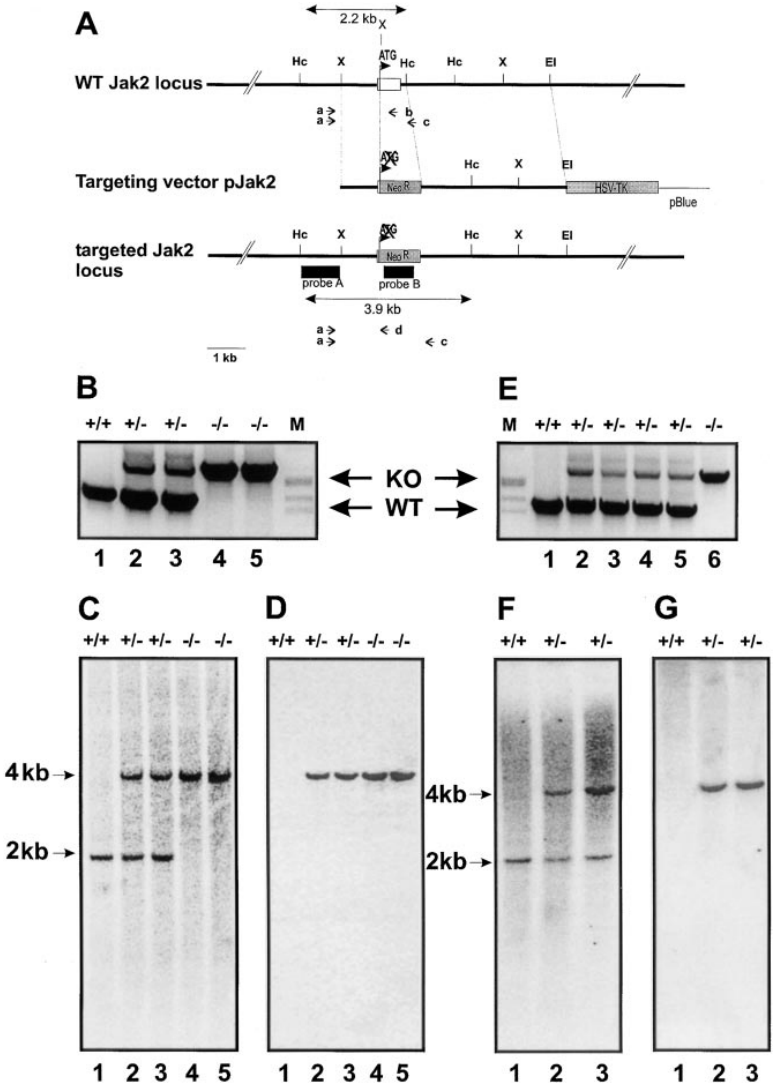

Figure 1. Targeted Disruption of the Murine *Jak2* Gene

(A) The 5′ part of the murine *Jak2* gene and relevant restriction sites are depicted at the top. The targeting vector was designed to replace part of the first coding exon and part of the following intron by a pMC1neopA cassette. The putative translation start site was deleted, but the 5′ splice accepter site was kept intact. Location of probes for hybridization and primers for screening PCR of ES cells are indicated. For Southern blot analysis, DNA was digested with HincII. The expected fragment size after hybridization with the flanking probe (probe A) is approximately 2 kb for the wild-type and approximately 4 kb for the targeted allele. After hybridization of the same blot with a *neo*-probe (probe B), an approximately 4 kb signal is expected. Hc, HincII; X, XbaI; and EI, EcoRI.

(B) Screening PCR of targeted ES cell clones. Genomic DNA of ES cells was used in a PCR with primers a and c (see Figure 1A and Experimental Procedures). Lane 1, $Jak2^{+/+}$ control; lane 2, targeted $Jak2^{+/-}$ ES cell clone 24; lane 3, targeted $Jak2^{+/-}$ ES cell clone 72; lane 4, $Jak2^{-/-}$ ES cell clone 8; and lane 5, $Jak2^{-/-}$ ES cell clone 22. M, marker (λ-phage DNA cut with HindIII and EcoRI).

(C) Southern blot analysis of Jak2-targeted ES cells. Hybridization with flanking probe (probe A). Lane 1, $Jak2^{+/+}$ control; lane 2, targeted $Jak2^{+/-}$ ES cell clone 24; lane 3, targeted $Jak2^{+/-}$ ES cell clone 72; lane 4, $Jak2^{-/-}$ ES cell clone 8; and lane 5, $Jak2^{-/-}$ ES cell clone 22. The sizes for the wild-type allele (2 kb) and for the targeted allele (4 kb) are indicated.

(D) Southern blot analysis of Jak2-targeted ES cells. Stripped blot from (C) was rehybridized with *neo*-probe (probe B). One specific integration is detected.

(E) Screening PCR after germline transmission of the Jak2 mutation. Genomic DNA from tail clips was used in a PCR with primers a and c. Lane 1, $Jak2^{+/+}$ control; lanes 2–5, tail DNA from mice 55, 58, 59, and 62; lane 6, $Jak2^{-/-}$ ES cell control. M, marker.

(F) Southern blot analysis after germline transmission of the Jak2 mutation. Hybridization with flanking probe (probe A). Lane 1, $Jak2^{+/+}$ control; lanes 2 and 3, tail DNA from mouse 55 and 58.

(G) Rehybridization of blot (F) with *neo*-probe.

Epo-R, Tpo-R, GM-CSF-R, and IL-3R play an important role in controlling the differentiation of hematopoietic stem cells (HSCs) (Emerson et al., 1985; Morrison et al., 1995). Starting at day 7.5 postcoitum (p.c.) of murine ontogeny, hematopoiesis takes place in the yolk sac (YS) (Moore and Metcalf, 1970), followed by hematopoiesis in the paraaortic splanchnopleura/aorta-gonads-mesonephros region (P-Sp/AGM) (Godin et al., 1993; Medvinsky et al., 1993; Müller et al., 1994).

Only hematopoietic cells derived from the P-Sp/AGM region give rise to definitive hematopoiesis (Cumano et al., 1996). After establishment of systemic circulation in the embryo at day 9 p.c., HSCs contributing to definitive hematopoiesis are simultaneously present in the YS and P-Sp/AGM during preliver stage of hematopoiesis (Yoder et al., 1997). Starting on day 10 p.c., cells from both origins colonize the fetal liver (FL) via the blood stream, with YS-derived cells undergoing primitive erythropoiesis, whereas intraembryonic precursors give rise to definitive hematopoiesis after day 12 p.c. (Cumano et al., 1996).

The erythroid lineage has at least two separate classes of progenitor cells: the erythroid burst-forming unit (BFU-E), which is a more primitive progenitor cell, and the more differentiated erythroid colony-forming unit (CFU-E) (Gregory and Eaves, 1978). The earliest morphologically definable precursor cell of the erythroid lineage is the proerythroblast, which develops from CFU-E (Metcalf, 1989). In Epo and $Epo\text{-}R^{-/-}$ mice, a block in definitive erythropoiesis was revealed, leading to embryonic lethality at day 13 p.c., caused by a severe anemia (Wu et al., 1995).

To elucidate the in vivo function of Jak2, a mouse strain deficient in this kinase was generated by gene

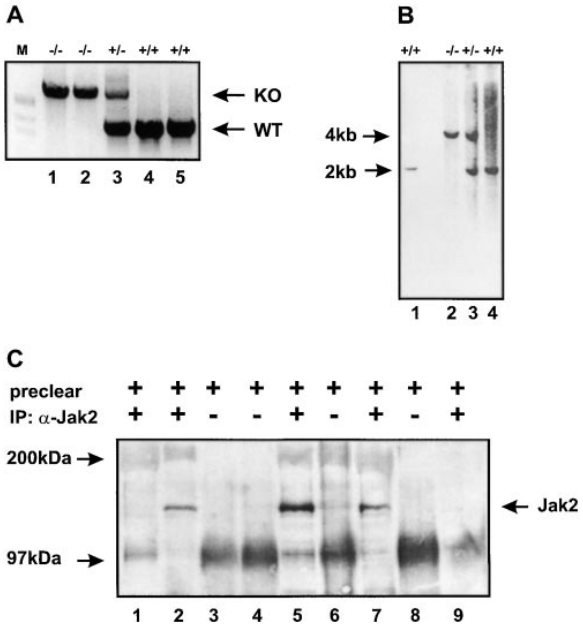

Figure 2. Embryonic Lethality of the Jak2 Inactivation

(A) Screening PCR of embryos at day 11.5 p.c. Genomic DNA of explanted embryos was used in a PCR with primers a and c (see Figure 1 and Experimental Procedures). Lane 1, *Jak2*$^{-/-}$ ES cell control; lane 2, *Jak2*$^{-/-}$ embryo; lane 3, *Jak2*$^{+/-}$ embryo; lane 4, *Jak2*$^{+/+}$ embryo; and lane 5, *Jak2*$^{+/+}$ control. M, marker.

(B) Southern blot analysis of EF cells. Hybridization with flanking probe (probe A). Lane 1, *Jak2*$^{+/+}$ control; lane 2, *Jak2*$^{-/-}$ EF cells; lane 3, *Jak2*$^{+/-}$ EF cells; and lane 4, *Jak2*$^{+/+}$ EF cells. For explanation of hybridization signal, please refer to Figure 1.

(C) Immunoprecipitation of Jak2 protein from EF cells. Jak2 was immunoprecipitated from cell homogenates and detected on Western blots employing anti-Jak2 antibodies. In our hands, the background of the Western blots (especially the band of ~97 kDa) was greatly reduced by preclearing the cell homogenates (see Experimental Procedures). Preclear: mixture of rabbit preimmune serum, anti-Jak1, and anti-Jak3 antibodies. Lane 1: γ2A, negative control; lanes 2 and 3: 2C4, positive control; lanes 4 and 5: *Jak2*$^{+/+}$ EF cells; lanes 6 and 7: *Jak2*$^{+/-}$ EF cells; lanes 8 and 9: *Jak2*$^{-/-}$ EF cells.

targeting. Timed pregnancy studies revealed that *Jak2*$^{-/-}$ animals die between day 12 and 13 p.c. The lack of enucleated erythrocytes in mutant animals defines a nonredundant function of Jak2 in definitive erythropoiesis. Embryonic fibroblasts (EF) and embryonic stem (ES) cells devoid of functional Jak2 reveal that Jak2 is indispensable for IFNγ but not for IFNα and LIF signaling. Flow cytometry demonstrates the loss of c-kit^{neg}Ter-119^{pos}CD44^{low} progenitor cell populations in the FL of *Jak2*$^{-/-}$ animals.

Results

Disruption of the Murine *Jak2* Gene

A replacement-type targeting vector pJak2 that contains a 1 kb short arm and a 3.8 kb long arm of homolog sequences was constructed. For positive selection, a pMC1neopA-cassette was inserted in antisense, and a HSV-tk-cassette was added for negative selection (see Experimental Procedures). The splice accepter site of the first coding exon was retained, whereas the start codon and the remaining part of the exon were deleted in the targeting vector to ensure that no Jak2 protein was produced from the mutant allele (Figure 1A). After electroporation and drug selection, double-resistant colonies of ES cells were picked. Screening PCR was performed using a primer outside of the construct and a primer hybridizing to the neo′-cassette. PCR products were separated on an agarose gel, blotted, and hybridized with the short arm of the construct. Southern blot analysis was performed after HincII digestion and hybridization with an external probe from the 5′-flanking region (Figure 1C, probe A). The expected 4 kb hybridization signal, corresponding to the targeted allele, was detected in about 13% of ES cell clones. Afterward, the filters were stripped and reprobed with part of the neomycin-resistance gene cDNA; only a single integration was detectable (Figure 1D, probe B). Two independently isolated and correctly targeted ES cell clones (24 and 72) were used for generating mutant mice.

Jak2$^{-/-}$ Mice Die during Midgestation

The correctly targeted ES cell clones were aggregated with CD1 morulae or injected into C57BL/6 blastocysts. The resulting chimeric mice were backcrossed, and germline transmission of the mutant allele was verified by PCR and Southern blot analysis of tail DNA from F1 offspring (Figures 1E, 1F, and 1G). Both ES cell clones transmitted the mutant *Jak2* allele into the germline. Heterozygous mice carrying the deletion of the *Jak2* gene appeared normal, viable, and fertile.

Heterozygotes were then intercrossed. No living offspring homozygous for the mutated *Jak2* allele was found at 3 weeks of age. The genotype of viable offspring showed a Mendelian ratio typical for an embryonically lethal phenotype (1:2:0) (Table 1). Thus, it was assumed that a *Jak2*$^{-/-}$ mutation leads to embryonic lethality. Examination of embryos at different stages of development revealed that *Jak2*$^{-/-}$ embryos died between day 12 and

Table 1. Embryonic Lethal Phenotype of *Jak2*$^{-/-}$ Animals

Genotypes of Living Offspring from *Jak2*$^{+/-}$ Breeding Pairs				Genotypes of Embryos from Timed Pregnancies			
ES cell clone	*Jak2*$^{+/+}$	*Jak2*$^{+/-}$	*Jak2*$^{-/-}$	Stage	*Jak2*$^{+/+}$	*Jak2*$^{+/-}$	*Jak2*$^{-/-}$
#24	11	16	0	E11.5	4	14	7
#72	3	10	0	E12.5	3	12	4
#72	7	10	0	E13.5	7	14	4
#24	5	11	0	E15.5	10	14	6[a]
Total	26	47	0	Total	24	54	21

[a]Embryos were almost completely resorbed in utero.

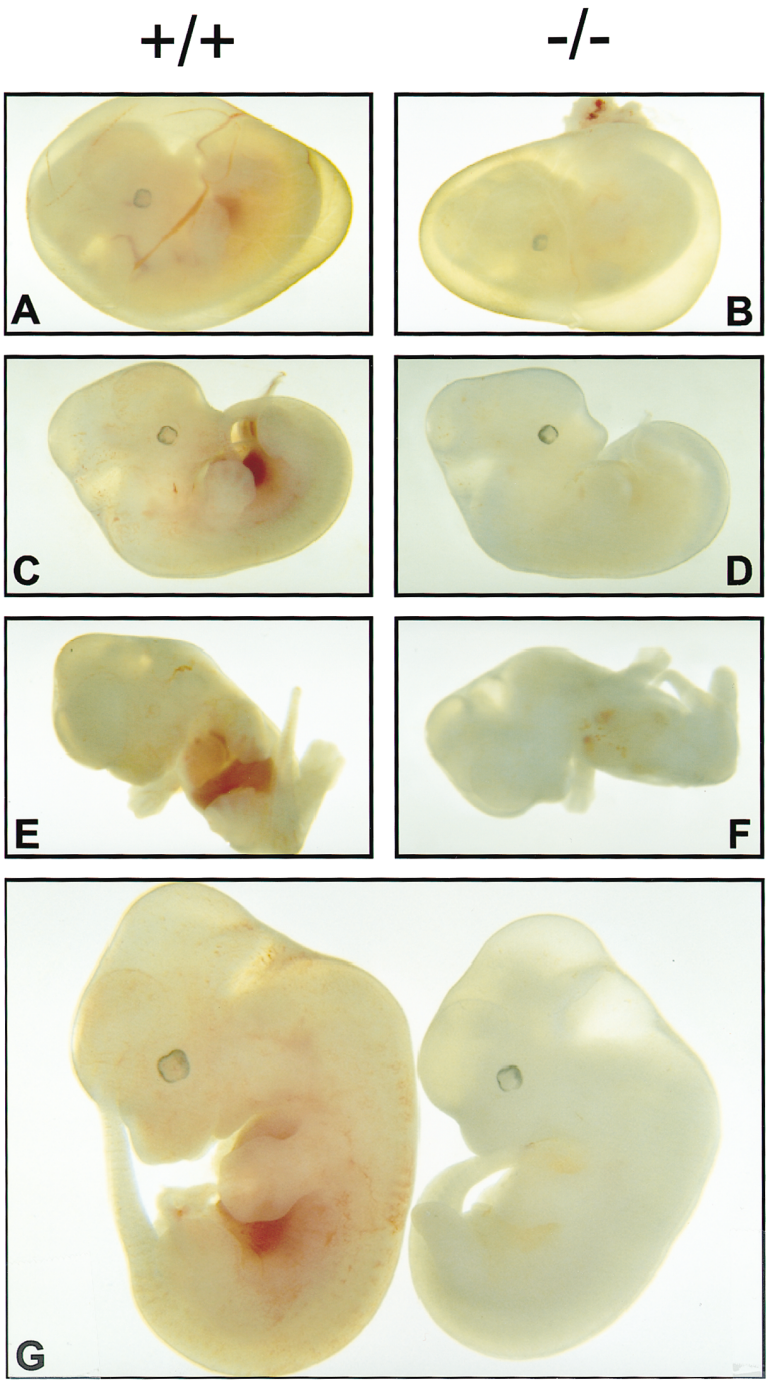

Figure 3. Severe Anemia in *Jak2*$^{-/-}$ Mice

(A) *Jak2*$^{+/+}$ embryo (day 12.5 p.c.) inside the YS. (B) *Jak2*$^{-/-}$ embryo (day 12.5 p.c.) inside the YS.

(C) *Jak2*$^{+/+}$ embryo (day 12.5 p.c.). (D) *Jak2*$^{-/-}$ embryo (day 12.5 p.c.).

(E) *Jak2*$^{+/+}$ embryo (day 13.5 p.c.). (F) *Jak2*$^{-/-}$ embryo (day 13.5 p.c.).

(G) Body size comparison of *Jak2*$^{+/+}$ (left side) and *Jak2*$^{-/-}$ embryos (right side) at day 12.5 p.c.

Original magnification for the *Jak2*$^{+/+}$ embryos ([A], [C], and [E]) was 10×, and original magnification for *Jak2*$^{-/-}$ embryos ([B], [D], and [F]) was 11×.

13 p.c. At day 15 p.c., all *Jak2*$^{-/-}$ embryos were almost completely resorbed in the uterus. Genotyping showed a normal Mendelian ratio (1:2:1) up to day 15 p.c. (Figure 2A and Table 1). The phenotype observed was identical in mouse lines derived from the two different ES cell clones and the different genetic backgrounds, making it unlikely that embryonic lethality resulted from a spontaneous mutation in an ES cell line. Thus, it was concluded that Jak2 defines a critical checkpoint during ontogeny at day 12.5 p.c.

Jak2 Gene–Targeted Mice Do Not Produce Jak2 Protein

EF cells were obtained from E11.5, E12.5, and E13.5 animals. In accordance with results mentioned above, no EF cell lines could be grown from *Jak2*$^{-/-}$ embryos at day 15.5 p.c. The genotype of EF cell lines used for further investigation was determined by Southern blotting (Figure 2B). EFs were analyzed for expression of Jak2 protein (Figure 2C). To this end, Jak2 protein was immunoprecipitated from cell homogenates and

Table 2. Number of Nucleated Cells Per FL

E11.5		E12.5	
Jak2$^{+/-}$	*Jak2*$^{-/-}$	*Jak2*$^{+/-}$	*Jak2*$^{-/-}$
$2.6 \times 10^5 \pm 1.3 \times 10^5$ (n = 15)	$0.8 \times 10^5 \pm 0.24 \times 10^5$ (n = 6)	$35 \times 10^5 \pm 9 \times 10^5$ (n = 9)	$1.6 \times 10^5 \pm 0.9 \times 10^5$ (n = 6)

detected on Western blots employing anti-Jak2 antibodies. For negative control, a mutagenized human fibrosarcoma cell (γ2A) was used that lacks Jak2 mRNA and protein (Kohlhuber et al., 1997). The parental cell line 2C4 was used for positive control (Watling et al., 1993). In 2C4, in *Jak2*$^{+/+}$, and in *Jak2*$^{+/-}$ EF cells, a 130 kDa Jak2 protein could readily be precipitated. In contrast, in *Jak2*$^{-/-}$ EFs, no Jak2 protein was detectable. Based on this result, it was concluded that Jak2 was successfully inactivated in the *Jak2*$^{-/-}$ embryos.

Definitive Erythropoiesis in *Jak2*$^{-/-}$ Fetal Liver Is Completely Impaired

The most dramatic morphological consequence of the Jak2 inactivation turned out to be a severe anemia (Figure 3). Due to the low number of circulating hemoglobinized cells, the embryos appeared pale. Additionally, the liver size of mutant embryos was dramatically reduced compared to that of normal littermates. The number of nucleated cells was reduced more than 10-fold in the FL of *Jak2*$^{-/-}$ animals compared to that in the FL of littermates (Table 2). The typical red color of the FL was not visible in *Jak2*$^{-/-}$ embryos (Figure 3F). No significant difference was observed between wild-type and heterozygous littermates (data not shown). YS enclosing homozygous embryos were of normal size, but YS vessels contained only very few circulating erythrocytes (Figure 3B).

Histological examination of tissue sections obtained from wild-type, *Jak2*$^{-/-}$, and *Epo-R*$^{-/-}$ embryos at day 12.5 p.c. allowed a comparison of circulating hemoglobinized cells and erythropoietic foci in FL (Figure 4). Embryo sections revealed a tremendous reduction of circulating nucleated erythrocytes in blood vessels of *Jak2*$^{-/-}$ embryos. Peripheral blood vessels of *Epo-R*$^{-/-}$ embryos still contained substantial numbers of nucleated erythrocytes. *Jak2*$^{-/-}$ FLs contained only very few erythropoietic foci within the parenchym (Figure 4H). In comparison, erythropoietic islands were numerous in the liver of wild-type animals (Figure 4G). In wild-type FL, erythroid cells of all stages of differentiation could be seen. In contrast, in *Jak2*$^{-/-}$ and *Epo-R*$^{-/-}$ FLs, almost no erythroid cells were visible (Figures 4H and 4I). All tissue sections from wild-type embryos contained many YS-derived primitive erythrocytes, whereas very few ones were visible in the FL of both mutant embryos. Furthermore, wild-type animals contained FL-derived enucleated erythrocytes originating from definitive erythropoiesis. In contrast, in Jak2 and Epo-R mutant embryos, enucleated erythrocytes could never be discovered. Thus, it appears that in the absence of Jak2, definitive erythropoiesis is blocked.

To confirm these results, cytospin preparations of peripheral blood and YS of normal and mutant embryos were analyzed after Giemsa staining (Figure 5). As expected, cell preparations of *Jak2*$^{+/-}$ animals showed all stages of erythropoietic differentiation, including numerous FL-derived enucleated erythrocytes and a few YS-derived nucleated erythrocytes (Figures 5A and 5C). Only proerythroblasts and very few nucleated erythrocytes were identifiable in preparations from *Jak2*$^{-/-}$ YS and peripheral blood. Enucleated FL-derived erythrocytes could never be seen (Figures 5B and 5D). These results suggest that Jak2 is essential for Epo-R signaling and definitive erythropoiesis in FL.

Analysis of Hematopoietic Cell Populations in Fetal Liver

Hematopoietic cell populations and differentiation stages can be distinguished by expression of characteristic markers such as CD34, CD44, c-kit, and Ter-119 (Ikuta et al., 1990; Krause et al., 1994; Sanchez et al., 1996; Aguila et al., 1997). Flow cytometry was performed to verify the expression of these cell surface markers. Single-cell suspensions of FL cells isolated from wild-type, *Jak2*$^{-/-}$, or *Epo-R*$^{-/-}$ embryos at day 12.5 p.c. were used. At this stage of embryonic development, CD34^{low} and CD34^{neg} cells can be detected in FLs (Yoder et al., 1997). Three different cell populations with regard to CD44 expression can be distinguished in wild-type FL: CD44^{neg}, CD44^{low}, and CD44^{high}. Interestingly, the proportion of CD34^{low}CD44^{high} cells was slightly increased in *Epo-R*$^{-/-}$ mice, whereas CD34^{low}CD44^{high} cells were markedly increased in *Jak2*$^{-/-}$ FL (Figure 6). While in control FL, CD44^{low} cells constitute the majority of cells, in *Epo-R*$^{-/-}$ and in *Jak2*$^{-/-}$ FL, CD44^{high}-expressing cells are predominant (Figure 6). A similar staining pattern could be obtained with c-kit. Here again, c-kit^{neg} cells constituted the majority of cells in control FL, whereas in *Epo-R*$^{-/-}$ and *Jak2*$^{-/-}$ FLs, c-kit^{pos} cells appeared to be predominant. Labeling with Ter-119, expressed on precursors of erythropoiesis after the CFU-E stage, indicated that the reduction of CD44^{low} cells in *Jak2*$^{-/-}$ FLs can be attributed to a lack of Ter-119^{pos} cells. Interestingly, a small population of Ter-119^{pos}CD44^{neg} primitive erythroid cells can be detected in control and *Jak2*$^{-/-}$ FLs. These data provide evidence that definitive erythropoiesis is defective in *Jak2*$^{-/-}$ animals. However, the population harboring the long-term reconstituting HSCs, coexpressing CD34 and c-kit, is present in *Jak2*$^{-/-}$ mice.

Blocked Hemoglobinization of Erythroid Cells in *Jak2*$^{-/-}$ Embryos

To determine at which stage during erythropoiesis Jak2 plays a crucial role, in vitro colony assays of erythroid progenitors were performed (Cumano et al., 1996). FL cells from wild-type, *Jak2*$^{-/-}$, and *Epo-R*$^{-/-}$ animals were incubated in methylcellulose supplemented with IL-3,

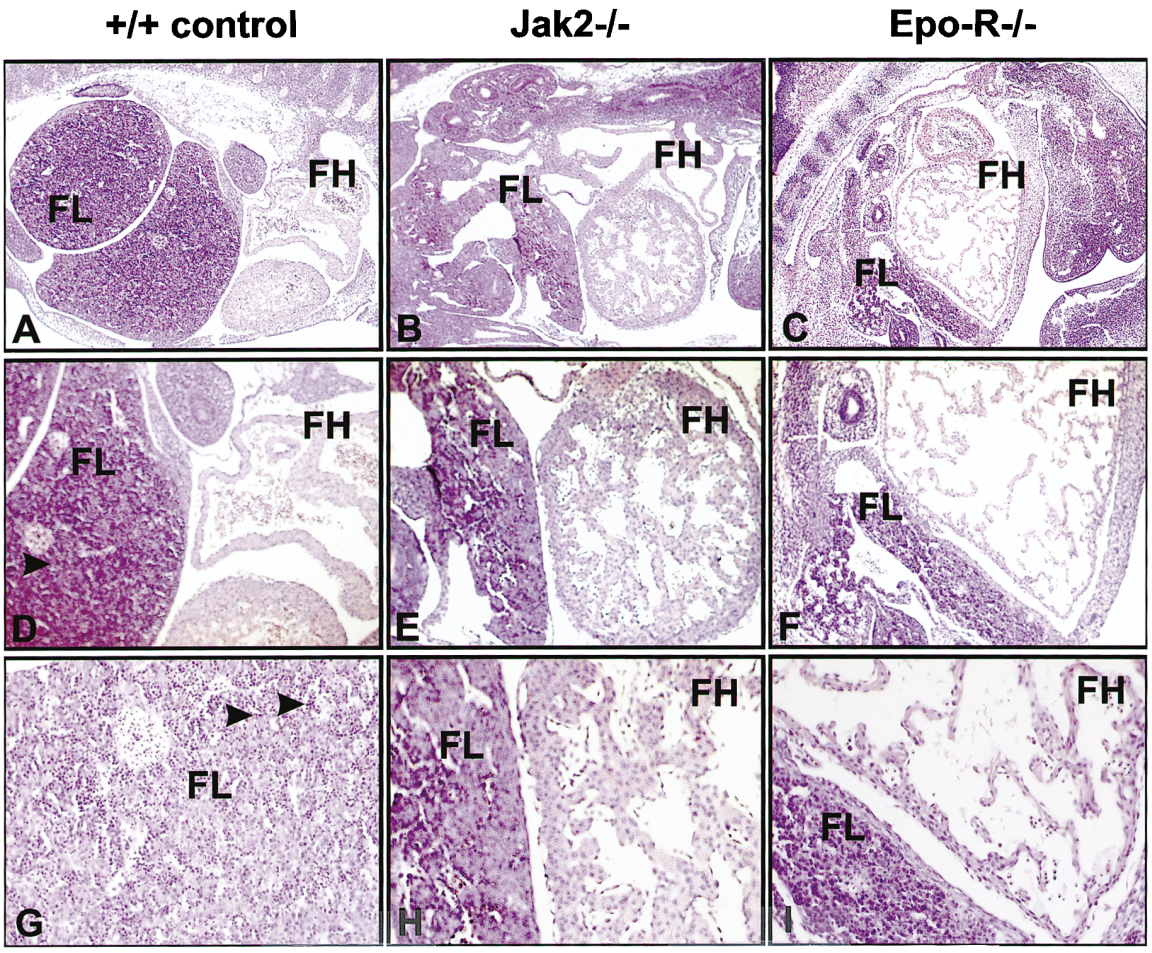

Figure 4. Histology of Wild-Type, *Jak2*$^{-/-}$, and *Epo-R*$^{-/-}$ Embryos

Hematoxylin- and eosin-stained longitudinal tissue paraffine sections of E12.5 embryos. (A) Low (50×), (D) middle (100×), and (G) high (200×) power magnification of sections of wild-type embryos. FL is of normal size, high hematopoietic activity; many nucleated and enucleated erythrocytes are visible.

(B) Low, (E) middle, and (H) high power magnification of sections of *Jak2*$^{-/-}$ embryos: FL is very small, almost no hematopoietic activity, only few nucleated erythrocytes, and no enucleated erythrocytes are visible.

(C) Low, (F) middle and (I) high power magnification of sections of *Epo-R*$^{-/-}$ embryos: FL is small, almost no hematopoietic activity, and reduced numbers of nucleated erythrocytes are visible. FH, fetal heart; arrowheads depict erythropoietic foci.

SCF, GM-CSF, and Epo. After incubation, BFU-Es and CFU-Es were counted (Tables 3 and 4). In *Jak2*$^{-/-}$ FL cell cultures, the number of BFU-E and CFU-E colonies was below the detection limit of the assay. In contrast to *Jak2*$^{-/-}$ FL cell cultures, colonies were readily detected in *Epo-R*$^{-/-}$ FL cell cultures. However, their number was about 10-fold reduced compared to that of FL cell cultures of wild-type embryos. Part of the FL cells was incubated in a liquid culture with S17 stromal cells, IL-3, SCF, GM-CSF, and Epo for 5 days. Then, benzidine staining was performed, and hemoglobin-containing cells were scored. In *Jak2*$^{-/-}$ FL cell cultures, almost no benzidine-positive cells were detected. In *Epo-R*$^{-/-}$ FL cell cultures, about 23% of the cells were benzidine positive, which was less than half of the amount of wild-type controls. These data suggest that erythropoiesis is more severely blocked in *Jak2*$^{-/-}$ mice than in *Epo-R*$^{-/-}$ mice and indicate that Jak2 is indispensible for definitive erythropoiesis. Interestingly, B lymphopoiesis appears intact in the absence of Jak2 (Table 3).

IFN-R and LIF-R Signaling in the Absence of Jak2

Jak2 is constitutively associated with the IFNγ-R β chain (Kotenko et al., 1995; Bach et al., 1996). Conflicting data about the association of Jak2 with gp130 was reported (Narazaki et al., 1994; Stahl et al., 1994; Guschin et al., 1995). To address the functional role of Jak2, EFs were stimulated with IFNα or IFNγ, and, subsequently, IFNα-responsive (IRF-1) as well as IFNγ-responsive genes (IRF-1, GBP-2) were monitored. For LIF signaling via gp130, homozygously targeted *Jak2*$^{-/-}$ ES cells were used (see Experimental Procedures). As indicated by Northern blot analysis, up-regulation of IRF-1 could be readily verified in *Jak2*$^{+/-}$ as well as in *Jak2*$^{-/-}$ EFs after IFNα stimulation (Figure 7B). In marked contrast, IFNγ stimulation did not result in induction of IRF-1 or GBP-2

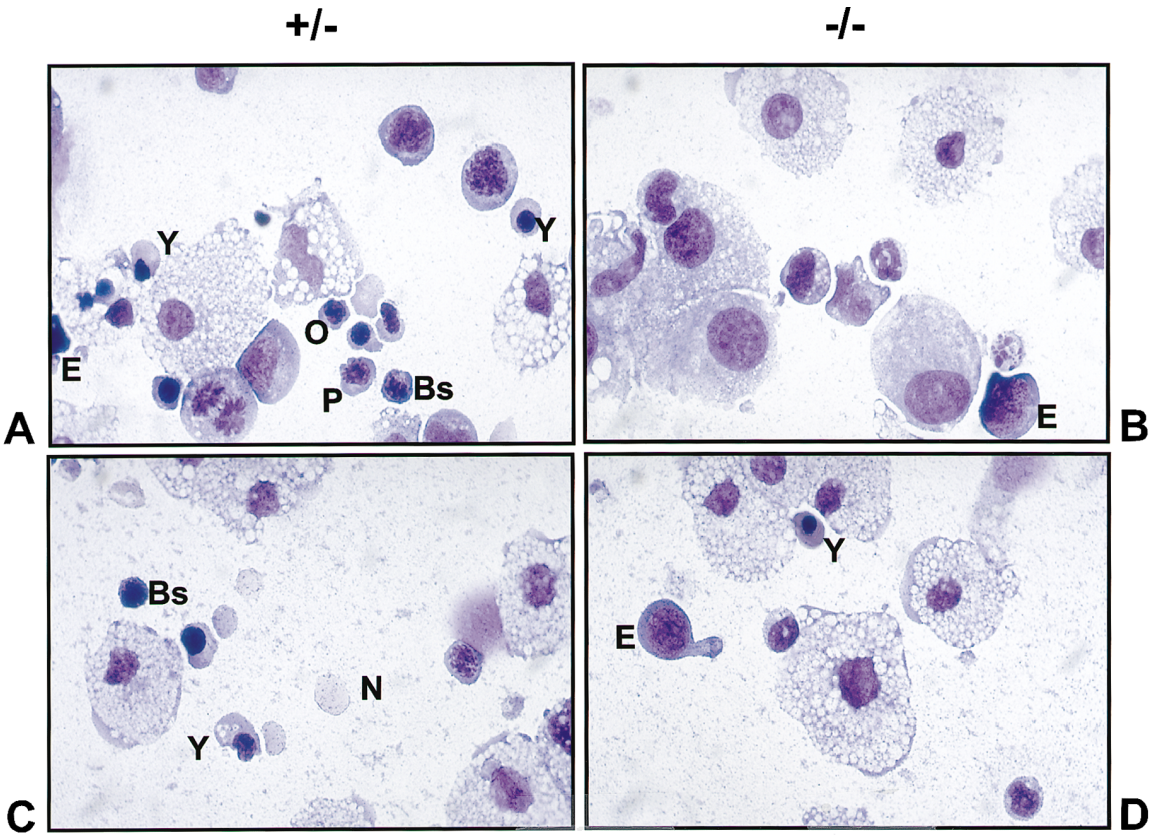

Figure 5. Cytospin Preparations of YS and Fetal Blood

(A) YS and (C) peripheral blood cytospin preparations from a representative *Jak2*$^{+/-}$ E12.5 embryo after Giemsa staining. Numerous erythropoietic cells in all stages of differentiation are present, including proerythroblasts (E), basophilic erythroblasts (Bs), polychromatic erythroblasts (P), orthochromatic erythroblasts (O), and enucleated erythrocytes (N). YS-derived nucleated erythrocytes are visible as well (Y).

(B) YS and (D) peripheral blood cytospin preparations from a representative *Jak2*$^{-/-}$ E12.5 embryo after Giemsa staining. Proerythroblasts (E) are found. Very rarely, nucleated erythrocytes from the YS are observed (Y); no enucleated erythrocytes were detectable.

in *Jak2*$^{-/-}$ EFs, whereas in control cell lines, IRF-1 and GBP-2 were readily transcribed (Figure 7A). These results indicate that signaling via type II IFN-R, but not via type I IFN-R, is essentially dependent on Jak2.

To examine the involvement of Jak2 in signaling via LIF-R, *Jak2*$^{+/+}$ and *Jak2*$^{-/-}$ ES cells were grown for 4 days without an EF cell layer and in the absence of LIF. Then, LIF-containing medium was added, and upregulation of IRF-1 was analyzed. Induction of IRF-1 mRNA could readily be detected in *Jak2*$^{+/+}$ and in *Jak2*$^{-/-}$ ES cells (Figure 7C), indicating that Jak2 is not involved in the LIF-R/gp130 signal cascade. Further evidence along this line is provided by the observation that *Jak2*$^{-/-}$ ES cells do not differentiate in LIF-containing medium after long-term culture (data not shown).

Discussion

Since the original description of Jak2 involvement in Epo-R and IFNγ-R signaling (Watling et al., 1993; Witthuhn et al., 1993), it has become clear that Jak2 participates in signal transduction of a variety of cytokine and growth factor receptors (Darnell et al., 1994; Ihle et al., 1995; Briscoe et al., 1996). To detect the biological role of Jak2, a gene-targeted mouse strain was established. *Jak2*$^{-/-}$ mice reveal that Jak2 plays an important role in definitive erythropoiesis. Definitive erythropoiesis is disrupted at the differentiation to the acidophilic erythroblast. Embryos die between day 12 and day 13 p.c. probably due to severe anemia. This phenotype of *Jak2*$^{-/-}$ mice appears to be comparable to *Epo* and *Epo-R*$^{-/-}$ animals. However, clear differences were found: *Jak2*$^{-/-}$ embryos contained fewer circulating primitive erythrocytes, and almost no benzidine-positive erythroid colonies were detectable in BFU-E and CFU-E assays. This indicates that the defect in *Jak2*$^{-/-}$ embryos is more severe than that in *Epo-R*$^{-/-}$ embryos, leading to the conclusion that Epo-R signaling is not the only deficiency in *Jak2*$^{-/-}$ mice. Besides the Epo-R, Jak2 is associated with the IL-3-R, GM-CSF-R, and Tpo-R. Mice deficient in $\beta_{IL\text{-}3}$ (Nishinakamura et al., 1995), common β chain (β_c) (Nishinakamura et al., 1995), GM-CSF (Dranoff et al., 1994), and IL-5 (Kopf et al., 1996) demonstrated that IL-3, GM-CSF, and IL-5 are not crucial for hematopoiesis. *Tpo*$^{-/-}$ mice contain fewer thrombocytes due to a reduced differentiation of megacaryocytes but show normal levels of other hematopoietic

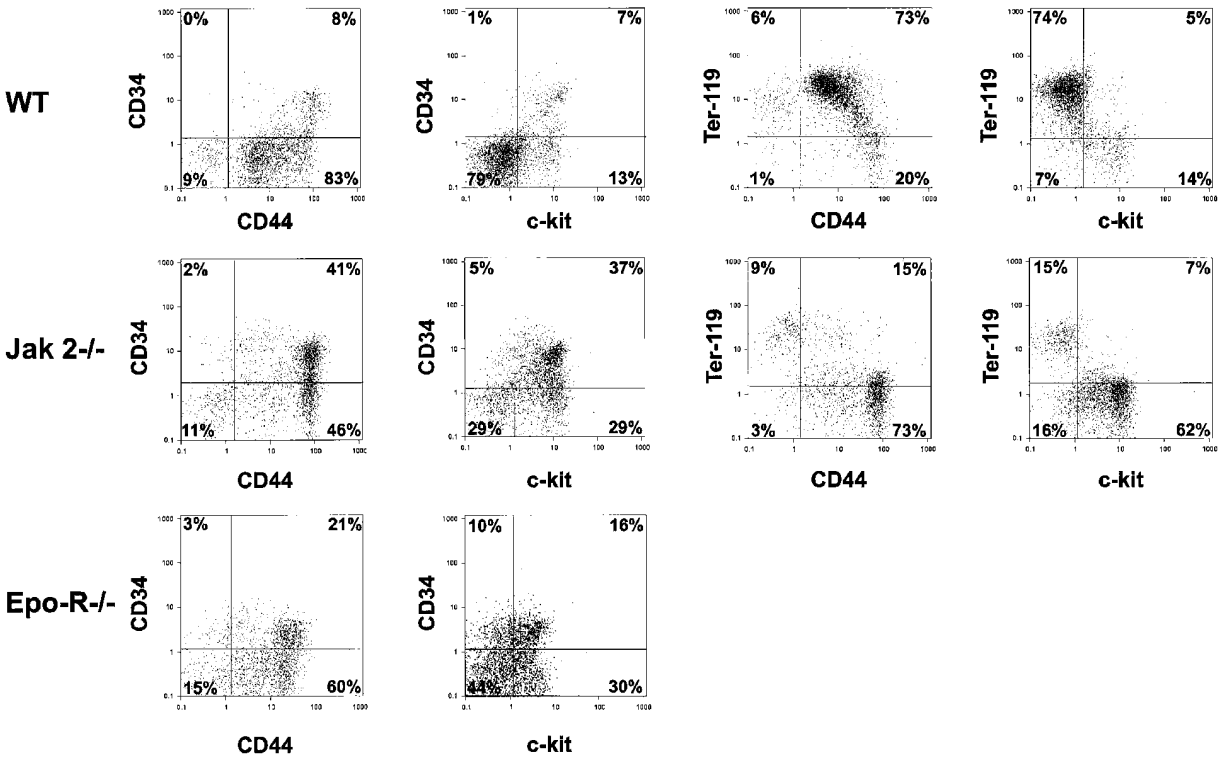

Figure 6. Flow Cytometry of Hematopoietic Cells in the FL of Day 12.5 p.c. Mouse Embryos

The cell surface expression of different markers is depicted. Cell suspensions from day 12.5 p.c. FL were labeled with anti-c-kit, anti-CD44, anti-Ter-119, or anti-CD34 antibodies. Representative results from 1 out of 5 embryos are shown. Eight thousand live events are gated.

cell types (Gurney et al., 1994). In stem cell assays, Tpo, especially in combination with SCF and/or IL-3, acts synergistically on the propagation of multiple types of hematopoietic colonies, including multilineage colonies from a highly purified population of hematopoietic progenitors (Kobayashi et al., 1996; Ku et al., 1996; Yoshida et al., 1997). The lack of BFU-E and CFU-E colonies in *Jak2*$^{-/-}$ FL cell cultures and the presence of BFU-E and CFU-E colonies in *Epo-R*$^{-/-}$ FL cell cultures indicate that the lack of Epo-R signaling at this stage of erythropoiesis might, at least partially, be overcome by IL-3-R and GM-CSF-R signaling via Jak2.

Mice lacking SCF (Sl mutant) or its receptor c-kit (W mutant) exhibit a significant reduction of CFU-E in the FL (Nocka et al., 1989). Conflicting evidence is provided on Jak2 involvement in c-kit signaling (Tang et al., 1994; Weiler et al., 1996; Deberry et al., 1997). Thus, at the current stage of investigation, it might be suggested that the severe defect in *Jak2*$^{-/-}$ erythropoiesis is additionally caused by a lack of SCF signaling. Taken together, at this early stage of hematopoiesis it appears that Epo, IL-3, GM-CSF, and SCF are at least partially redundant due to their capacity of Jak2 activation.

The defects of *Jak2*$^{-/-}$ embryos are also distinct from other mouse mutations affecting hematopoiesis. *GATA-2*$^{-/-}$ embryos die at E10-E11 with severe anemia due to an impairment of both primitive and definitive erythropoiesis (Tsai et al., 1994). At this point of embryonic development, *Jak2*$^{-/-}$ embryos are vital but anemic. Mice deficient in c-myb and the retinoblastoma gene (*Rb*$^{-/-}$) die at E15-16 exhibiting anemia (Mucenski et al., 1991; Clarke et al., 1992). Mice with a c-myb deficiency appear normal at day 13 of gestation, suggesting that c-myb is not essential for early development. The erythropoietic defect seen in *Rb*$^{-/-}$ mice seems to be partly due to abnormalities in the hemopoietic microenvironment of FLs where hepatocytes undergo apoptosis. *Gp130*$^{-/-}$ mice die between day 12.5 p.c. and day 20 p.c., and some of the mutant embryos show a severe anemia (Yoshida et al., 1996). *Gp130*$^{-/-}$ embryos show a hypoplastic ventricular myocardium. In *Jak2*$^{-/-}$ animals, the heart is enlarged. This heart alteration might be a

Table 3. Number of BFU-E Progenitors and B-Lymphocyte Precursors in *Jak2*$^{-/-}$ and *Epo-R*$^{-/-}$ Embryos

BFU-E Assay (Day 7)			B-Lymphocyte Precursors		
Wild-Type	*Jak2*$^{-/-}$	*Epo-R*$^{-/-}$	Wild-Type	*Jak2*$^{-/-}$	*Epo-R*$^{-/-}$
5250 ± 250 (n = 3)	<10 (n = 2)	550 ± 50 (n = 2)	771 ± 130 (n = 4)	355 ± 168 (n = 5)	540 (n = 1)

Number of colonies per FL.

Table 4. Number of CFU-E in *Jak2*$^{-/-}$ and *Epo-R*$^{-/-}$ Embryos

	CFU-E Assay[a] (Day 3)				CFU-E Assay[b] (Day 5)			
	Jak2$^{+/-}$	*Jak2*$^{-/-}$	*Epo-R*$^{+/-}$	*Epo-R*$^{-/-}$	*Jak2*$^{+/-}$	*Jak2*$^{-/-}$	*Epo-R*$^{+/-}$	*Epo-R*$^{-/-}$
CFU-E (absolute count)	230 ± 10 (n = 2)	<10 (n = 4)	310 ± 10 (n = 2)	105 ± 15 (n = 3)				
Benzidine-positive cells (%)					46.5 ± 1.5 (n = 2)	1 ± 0 (n = 4)	47 ± 1 (n = 2)	23 ± 6 (n = 3)
CFU-E (per FL)	2.3×10^4	<200	3.1×10^4	2.1×10^3				

[a] 1:10 (ko animals) or 1:50 (wild-type animals) of the FL was used for 2 ml of methylcellulose mixture with (2 ng/ml) rIL-3, rSCF, rGM-CSF, and human rEpo (4 U/ml). The numbers reflect independent counts done on the 2 ml plated in 35 mm dishes with 1 ml each of the original mixture. The choice of amount of cells was based on the fact that ko mice had about 10× fewer cells than controls. SCF and IL-3 were obtained from the supernatant of cells transfected with the cDNA encoding the respective genes. They were titrated on the capacity to grow FL cells and generate mast cells efficiently.

[b] FL cells were plated in liquid culture with S17 stromal cells, rIL-3, rEpo, rSCF, and rGM-CSF at the same concentrations. On day 5 of culture, benzidine staining was performed, and hemoglobin-containing cells were counted. Numbers indicate percentages of benzidine-positive cells per total number of cells.

secondary effect due to hypoxemia. This finding, together with the induction of IRF-1 by LIF in homozygously targeted ES cells, further suggests that gp130/LIFRβ signaling is not mediated via Jak2.

PU.1$^{-/-}$ embryos die at a late gestational stage (E16-18), and anemia starts at E14.5 with variable severity (Scott et al., 1994). *AML1*$^{-/-}$ embryos reveal intact hematopoiesis in the primitive YS but are completely deficient in FL hematopoiesis. The *AML1*$^{-/-}$ embryos die around day 12.5 p.c. from multiple bleeding probably due to an absence of circulating platelets (Okuda et al., 1996).

According to recently established models (Cumano et al., 1996; Sanchez et al., 1996; Yoder et al., 1997), multipotent HSC, which are able for long-term reconstitution (LTR) of conditioned newborn recipients, arise in the YS and P-Sp/AGM region. Reconstitution potential of multilineage progenitors in conditioned adult recipients is first found in the AGM at day 10 p.c., then followed on day 11 p.c. by FL and YS (Müller et al., 1994). Reconstitution experiments showed that LTR-HSCs in the AGM and FL are exclusively in the c-kit^{pos}CD34^{low} fraction, which may arise from the c-kit^{pos}CD34^{neg} population (Krause et al., 1994; Sanchez et al., 1996; Aguila et al., 1997). Interestingly, c-kit^{pos}CD34^{low} LTR-HSC cells are still present in *Jak2*$^{-/-}$ mice, and precursors for B lymphocytes could be differentiated under suitable culture conditions, indicating that the lymphocytic lineage, in contrast to the differentiation of erythroid/myeloid lineage, is not dependent on Jak2. At day 12.5 p.c., almost all c-kit^{neg} cells express the erythroid Ter-119 marker (Ikuta et al., 1990). In *Jak2*$^{-/-}$ FLs, c-kit^{neg}Ter-119^{pos} cells were dramatically reduced at day 12 p.c., indicating that erythroid differentiation is severely blocked. Interestingly, some c-kit^{neg}Ter-119^{pos} cells were still present in *Jak2*$^{-/-}$ FLs. These cells might represent cells derived from fetal erythropoiesis having immigrated from the YS. Since erythropoiesis in *Jak2*$^{-/-}$ animals is more severely blocked than in *Epo-R*$^{-/-}$ mice, it seems unlikely that c-kit^{neg}Ter-119^{pos} cells represent early nucleated erythroblasts from definitive erythropoiesis. Thus, it is suggested that erythropoietic stem cells are arrested in *Jak2*$^{-/-}$ embryos at a CD44^{high}c-kit^{pos}Ter-119^{neg} stage.

Experiments addressing the signaling capacity of cytokine receptors in the absence of Jak2 revealed, as expected, that IFNγ-R signals were disrupted, whereas signaling via type I IFN-R, which uses Jak1 and tyk2, remained intact. It might be speculated that RAG/Jak2 chimeras generated from homozygous *Jak2*$^{-/-}$ ES cells might have defective Th1 responses due to the absence of IFNγ-R as well as IL-12-R signaling (Briscoe et al., 1996; Chen, 1996).

The results observed with *Jak2*$^{-/-}$ ES cells clarifies the involvement of Jak2 in LIF-Rβ/gp130 signaling. Long-term culture of *Jak2*$^{-/-}$ ES cells did not lead to differentiation, and LIF stimulation readily triggered upregulation of IRF-1, indicating that the LIF-Rβ/gp130 signaling complex is not dependent on Jak2.

In summary, the defects in *Jak2*$^{-/-}$ mice are more profound than these in *Epo-R*$^{-/-}$ mice (Wu et al., 1995) and only partially overlapping with defects encountered in *Jak1*$^{-/-}$ mice (Rodig et al., 1998 [this issue of *Cell*]). Jak2 functions are crucial in control for erythroid/myeloid lineage differentiation but appear not to be essential for the lymphoid lineage, as in vitro differentiation of B lymphocytes (this study) as well as lymphoid reconstitution of *Jak3*$^{-/-}$ mice with *Jak2*$^{-/-}$ FL cells (Parganas et al., 1998 [this issue of *Cell*]) is possible. The Jak2 absence defines a genetic checkpoint at the transition from primitive to definitve erythropoiesis. Additional studies with conditional inactivation of Jak2 are required to bypass the embryonic lethality and to define the role of this important kinase in an adult organism.

Experimental Procedures

Generation of *Jak2*$^{-/-}$ Mice

A genomic library (129/Sv/λDash2) was screened with a partial Jak2 cDNA clone. A genomic clone containing the first coding exon was isolated, mapped, and partially sequenced (data not shown). The sequenced exon was identical to the corresponding region of the published cDNA (Silvennoinen et al., 1993b; GeneBank accession number L16956). The targeting vector pJak2 was constructed by inserting a 1 kb XbaI-XbaI fragment encoding part of the 5′ intron and the 5′ part of the coding exon into pBlue (Stratagene) yielding pJak2-X4. Then, a 1.1 kb BamHI-XhoI fragment from pMC1neopolyA (Stratagene) containing a neomycin resistance cassette was inserted in antisense into the SmaI site of pJak2-X4, yielding pJak2-X4neopA. Next, a 3.8 kb downstream HincII fragment was cloned into the EcoRV site of pJak2-X4neopA, yielding pJak2-X4neopAE1. Finally, a 2.7 kb herpes simplex virus thymidine kinase (HSV-tk) cassette (XhoI-SalI-SspI digest/pGEM7[TK]) was inserted into the

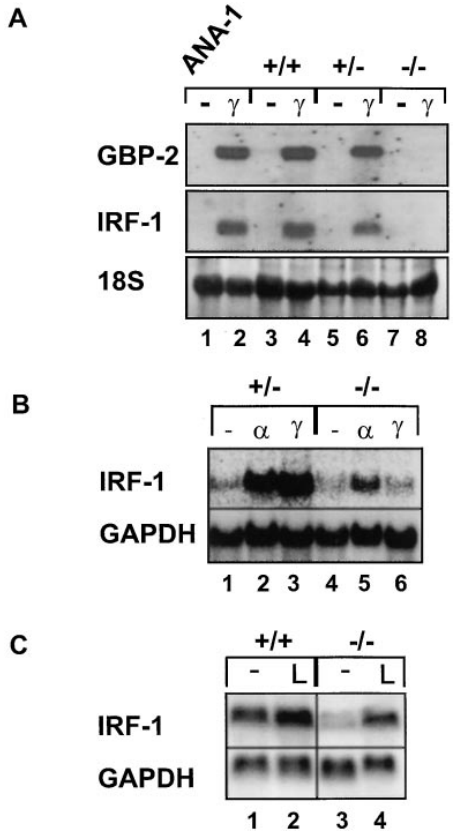

Figure 7. Response of *Jak2*$^{-/-}$ Cells to IFNγ, IFNα, and LIF

(A) Northern blot of EF cells after treatment with medium alone (−) or with 10 ng/ml IFNγ for 19 hr (γ). Lanes 1 and 2: Ana-1 cells, positive control; lanes 3 and 4: *Jak2*$^{+/+}$ EF cells; lanes 5 and 6: *Jak2*$^{+/-}$ EF cells; lanes 7 and 8: *Jak2*$^{-/-}$ EF cells. Hybridization with GBP-2 (upper panel) and IRF-1 (middle panel). The lower panel shows the methylene blue–stained 18S rRNA band as a loading control.

(B) Northern blot of EF cells after treatment with medium alone (−), with 1000 U/ml IFNα for 6 hr (α), or with 10 ng/ml IFNγ for 19 hr (γ). Lane 1: *Jak2*$^{+/-}$ EF cells, unstimulated; lane 2: *Jak2*$^{+/-}$ EF cells, IFNα stimulated; lane 3: *Jak2*$^{+/-}$ EF cells, IFNγ stimulated; lane 4: *Jak2*$^{-/-}$ EF cells, unstimulated; lane 5: *Jak2*$^{-/-}$ EF cells, IFNα stimulated; lane 6: *Jak2*$^{-/-}$ EF cells, IFNγ stimulated. Note that in (A) and (B) two independent experiments are shown where basal IRF-1 transcription in EF cells slightly varied.

(C) Northern blot of ES cells after treatment with LIF. ES cells were deprived of LIF for 4 days and then incubated with LIF for 1 hr. Lane 1: *Jak2*$^{+/+}$ ES cells deprived of LIF; lane 2: *Jak2*$^{+/+}$ ES cells stimulated with LIF; lane 3: *Jak2*$^{-/-}$ ES cells deprived of LIF; lane 4: *Jak2*$^{-/-}$ ES cells stimulated with LIF.

SalI site of pJak2-X4neopAE1 generating the pJak2 targeting vector. The NotI-linearized pJak2 was electroporated into E14.1 ES cells (129/Sv/J), and transfected cells were cultivated in the presence of G418 (0.2 mg/ml) and gancyclovir (2 μM) as described (Wurst and Joyner, 1993). Double resistant cells were screened for homologous recombination. For PCR screening, the following primers were used: KO1 (5′-GCCATGCCCAAGTTGTAACTC-3′) annealing upstream of the targeting construct and *neo*1500 (5′-TCGCCTTCTATCGCCTTC TTG-3′) annealing in the neomycin resistance cassette. As flanking probe (probe A) a 1 kb EcoRI fragment was used. The *neo*-probe was from pMC1neopolyA. Two ES cell clones containing the correctly targeted *Jak2* allele were both aggregated with CD1 morulae or injected into C57BL/6 blastocysts. Chimeric mice were backcrossed to CD1 or C57BL/6 mice, and germline transmission of the mutant allele was confirmed by PCR and Southern blot analysis.

Homozygous Mutant ES Cell Lines

To generate homozygous mutant ES cell lines, *Jak2*$^{+/-}$ ES cells were incubated in the presence of 1.6 mg/ml G418 (Mortensen et al., 1992). Surviving colonies were assayed for loss of the wild-type allele, and *Jak2*$^{-/-}$ ES cells were subcloned.

Cell Culture

ES cells were grown in DMEM medium (GIBCO-BRL) supplemented with L-glutamin (2 mM, Seromed), leukemia inhibitory factor, Pen/Strep (100 μg/ml, Seromed), 2-ME (0.05 mM, GIBCO-BRL), and FCS (15%, GIBCO-BRL).

Mouse EF cells were prepared from individual 11- to 13-day-old embryos as described (Wurst and Joyner, 1993). EF cells were cultivated in DMEM medium as above, except 5% FCS was used.

RNA Isolation and Northern Blot Analysis

Mouse EF cells were stimulated with murine rIFNγ (10 ng/ml, Genzyme) or murine rIFNα (1000 IRU/ml, BIOSOURCE) for 19 or 6 hr, respectively. Mouse ES cells were deprived of LIF for 4 days and then stimulated with LIF for 1 hr. Cells were lysed in 2 ml lysis buffer consisting of 4 M guanidinium isothiocyanate (Merck), 0,5% (w/v) N-lauryl-sarcosine (Serva), 15 mM Na-citrate (pH 7.0), and 100 mM 2-ME (Sigma). Total RNA was prepared as described (Chomczynski and Sacchi, 1987). Twenty micrograms total RNA was fractionated on formaldehyde agarose gels, blotted onto GeneScreen Plus membrane (Biotechnology Systems), and hybridized with fluorescein- or ^{32}P-labeled cDNA probes (Amersham). The following partial murine cDNA probes were used: interferon regulatory factor-1 (IRF-1, 550 bp) and guanylate binding protein-2 (GBP-2, 800 bp) (Fütterer et al., unpublished data). Methylene blue staining or hybridization with murine GAPDH cDNA (1150 bp) was performed as loading control.

Histological Analysis of Embryos

Embryos were dissected and genotyped. For histology, the embryos were fixed in 10% buffered formalin for 24–48 hr and embedded in paraffine. Sections (5 μm) were stained with hematoxylin and eosin (HE).

Cytospins, BFU, and CFU Assays

YS cells and peripheral blood were prepared by cytospin technique and stained according to May-Grünwald. Colony assays were performed as described (Cumano et al., 1996). For analysis of erythroid differentiation stages, FL cells were grown in methylcellulose containing a mixture of rIL-3, rSCF, rGM-CSF (2 ng/ml), and human rEpo (4 U/ml). Suspension cells were transferred to glass slides by cytospin technique at day 5 of culture and stained with benzidine (Sigma). Briefly, cells were washed in PBS and resuspended in equal volumes of PBS and benzidine/H_2O_2 for 15 min. After several washes with PBS, cells were scored under a microscope.

B-Lymphoid Conditions

Cells were cultured on S17 stromal cells with medium supplemented with rIL-7 and rSCF. Cells were counted by microscopical scoring (Cumano et al., 1993).

Immunoprecipitation and Western Blot Analysis

1×10^7 cells were lysed in a lysis buffer containing 0.5% NP-40 (ICN Biomedicals), 50 mM Tris/Cl (pH 8.0) (Sigma), 10% glycerol (Sigma), 0,1 mM EDTA, 200 mM NaCl (Sigma), 0,1 mM Na_3VO_4 (Sigma), 1 mM DTT (Sigma), 0,4 mM PMSF, 3 μg/ml aprotinin, 2 μg/ml pepstatin, 1 μg/ml leupeptin, and 2 μg/ml bestatin (all Boehringer). The lysates were precleared with protein A-Sepharose (Sigma) and a mixture of rabbit preimmune serum, rabbit anti-mouse Jak1 (Wilks et al., 1991), and rabbit anti-mouse Jak3 polyclonal sera (Upstate Biotechnology). After preclearing, lysates were precipitated with a rabbit anti-mouse Jak2 polyclonal serum (specific for aa 758-776, Upstate Biotechnology) and loaded on an 8% SDS polyacrylamide gel. The gel was semi-dry blotted on nitrocellulose (Protran BA 85, Schleicher and Schuell/BIORAD, Trans-Blot SD). Western detection was performed with a rabbit anti-mouse JH2-Jak2 polyclonal serum (A. Ziemiecki, unpublished data) followed by incubation with mouse anti-rabbit AP-coupled antibody and detection by ECL kit (Amersham).

Flow Cytometry

Embryos were explanted at day 12.5 p.c. FLs were isolated, and a single-cell suspension was obtained. The cell suspensions were incubated on ice with rat anti-mouse CD16/CD32 (Pharmingen) to block Fc receptor binding. Cells were incubated with rat anti-mouse biotin-conjugated CD34 (clone RAM34)/streptavidin-phycoerythrin (PE), fluorescein isothiocyanate (FITC)–conjugated anti CD44, FITC-conjugated anti c-kit, anti Ter-119-PE, or appropriate isotype control antibodies (all Pharmingen) on ice. Subsequently, cells were washed and transferred to PBS 1% PFA. For flow cytometry a Coulter Epics XL cytometer was used.

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