TECHNOLOGY REPORT

Generation of a Conditional Knockout Allele for the *Janus Kinase 2* (*Jak2*) Gene in Mice

Andrea Krempler,[1] Yongyue Qi,[1] Aleata A. Triplett,[1] Jianqiong Zhu,[2] Hallgeir Rui,[2] and Kay-Uwe Wagner[1]*

[1]Eppley Institute for Research in Cancer and Allied Diseases and the Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska
[2]Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC

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Summary: To study biologically relevant functions of the Janus kinase 2 (Jak2) in multiple cytokine and hormone receptor signal transduction pathways, we generated a conditional knockout (floxed) allele of this gene by placing *lox*P sites around the first coding exon of *Jak2*. Homozygous floxed animals developed normally and exhibited no phenotypic abnormalities. The conversion of the floxed allele into a null mutation was achieved by transmitting the targeted allele through the female germline of *MMTV-Cre* (line A) mice. Embryos that carry two *Jak2* null alleles died around midgestation and exhibited impaired definitive erythropoiesis, which is a hallmark of Jak2 deficiency reported previously in conventional knockouts. This observation suggested that the Cre-mediated deletion of the first coding exon results in a true null mutation that is incapable of mediating signals through the erythropoietin receptor. Using mouse embryonic fibroblasts derived from Jak2 null embryos and their wildtype littermate controls, we demonstrated that Jak2-deficiency decouples growth hormone-receptor signaling from its downstream mediators, the signal transducer and activator of transcription (Stat) 5a and 5b. genesis 40:52–57, 2004.

Key words: Janus kinase 2; Stat5; embryonic development; Cre recombinase; gene targeting

Jak-Stat pathways mediate extracellular polypeptide signals, such as hormones and cytokines, from transmembrane receptors to target gene promoters in the nucleus. Consequently, cells respond to these signals by altering their gene expression and, in turn, change their growth properties and their physiological activities (Aaronson *et al.*, 2002). Four members of the Janus kinase (Jak) family have been identified in mammals: Jak1, Jak2, Tyk2, and Jak3. Genetic studies in mice revealed that the biological functions of individual Janus kinases are pleiotropic, which is consistent with their suggested coupling to multiple cytokine receptors in specific target cells creviewed by Kisseleva *et al.*, 2002). In particular, Jak2 is suggested to mediate signals through single-chain receptors for ligands such as prolactin (PRL), growth hormone (GH), erythropoietin (EPO), and thrombopoietin (TPO) as well as to the multichain IL-3 receptor family (e.g., IL-3R and GM-CSF-R), and members of the gp130 receptor family. Although more than one Jak family member was suggested to interact with certain cytokine receptors in vitro, knockout studies in mice revealed a remarkable physiological specificity of individual Janus kinases and their associated Stat proteins (Ihle, 2001; Kisseleva *et al.*, 2002; Levy *et al.*, 2002).

Among gene deletion models of Janus kinase family members, Jak2-deficient mice exhibit the most severe phenotype. Embryos with a complete null mutation of *Jak2* die around day 12.5 of gestation due to impaired definitive erythropoiesis (Neubauer *et al.*, 1998; Parganas *et al.*, 1998). Conventional knockout models for Jak2 exhibit similarities to EPO and EPO-R-deficient mice (Wu *et al.*, 1995), suggesting that Jak2 is essential for transducing signals through the EPO receptor. In vitro differ-

A. Krempler and Y. Qi contributed equally to this work.

Present address for A. Krempler: Department of Medical Biochemistry and Molecular Biology, University of the Saarland, Building 44, Homburg, 66424, Germany.

* Correspondence to: Dr. Kay-Uwe Wagner, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Rm. 8009, Omaha, NE 68198-6805.
E-mail: kuwagner@unmc.edu

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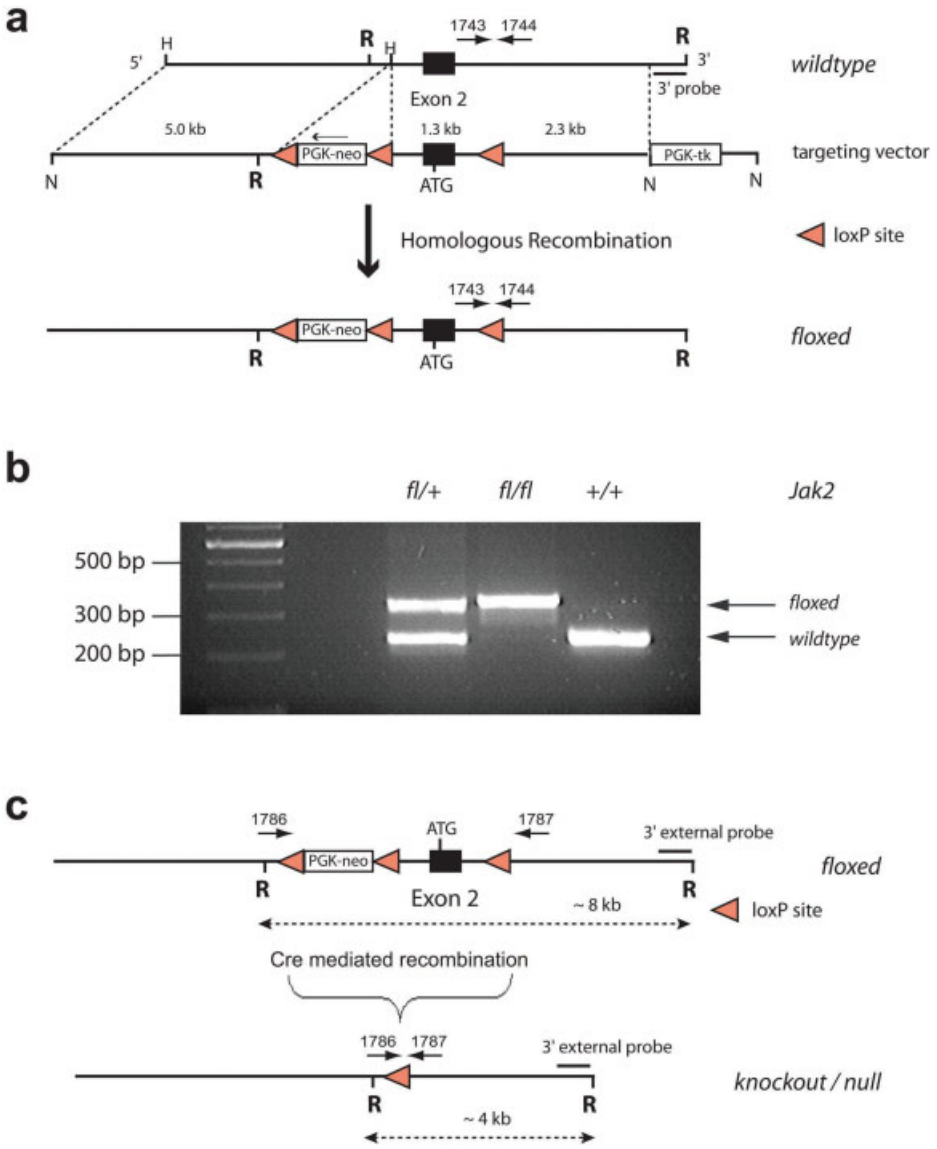

FIG. 1. Gene targeting and generation of a conditional knockout (floxed) allele of the *Janus kinase 2* (*Jak2*) allele. **a:** Targeting strategy to flank the promoter and first coding exon of *Jak2* with *loxP* sites. The targeting vector was constructed by placing a PGK-neo selectable marker flanked by *lox*P sites ~570 bp upstream of the coding exon. A third *lox*P site was introduced 500 bp downstream of the coding sequence within the following intron. **b:** PCR analysis using the primer set 1743/1744 illustrated in **a** in order to determine the presence of the wildtype and/or floxed alleles in heterozygous (fl/+) and homozygous mutants (fl/fl) as well as wildtype controls (+/+). **c:** Cre-mediated deletion of the *Jak2* gene. A Southern blot in combination with the external 3′ probe used to monitor the homologous recombination (**a**) can be employed to verify the Cre-mediated conversion of the floxed allele into a *Jak2* null allele. Arrows indicate the location of PCR primers (1743/1744 and 1786/1787) in **a** and **b**. R, *Eco*R1; H, *Hin*D3; N, *Not*1; tk, thymidine kinase.

entiation studies and reconstitution experiments with Jak2$^{-/-}$ hematopoietic precursors demonstrate that Jak2 also possesses nonredundant functions for IL-3, GM-CSF, IL-5, IFN-γ, and TPO signaling (Parganas *et al.*, 1998). The analysis of other biologically relevant functions of Jak2 as a mediator for peptide hormone receptor signaling in adult tissues, such as GH and PRL, remains difficult to investigate due to the early embryonic lethality of Jak2 conventional knockouts.

To address essential functions of Jak2 during organogenesis in adult mice, we generated a conditional knockout (floxed) allele of the *Jak2* gene (Fig. 1a). A BAC clone encompassing the *Jak2* locus was isolated from a mouse 129SvJ genomic library (Incyte Genomics, St. Louis, MO). Two overlapping DNA fragments with a contiguous sequence of about 10 kb harboring the first coding exon of *Jak2* were subcloned and sequenced (GenBank AY157991). A targeting vector was constructed by placing a PGK-neomycin (PGK-neo) selectable marker flanked by *lox*P sites (floxed) ~570 bp upstream of the first coding exon. A third *lox*P site was introduced 500 bp downstream of the coding sequence within the consecutive intron. The PGK-tk cassette was used for negative selection against random integration events. The floxed allele of the *Jak2* gene was generated through homologous recombination in isogenic mouse embryonic stem (ES) cells (i.e., RW-4 cells from Incyte Genomics). Twelve correctly targeted clones were identified by Southern blot using *Eco*R1 or *Eco*RV restriction digest of genomic DNA and a 3′ external probe that was not part of the targeting vector. The correct insertion of the outermost *lox*P site into the endogenous *Jak2* locus was confirmed by PCR using the 1743/1744 primer set (see Materials and Methods). Two ES cell clones (#2 and

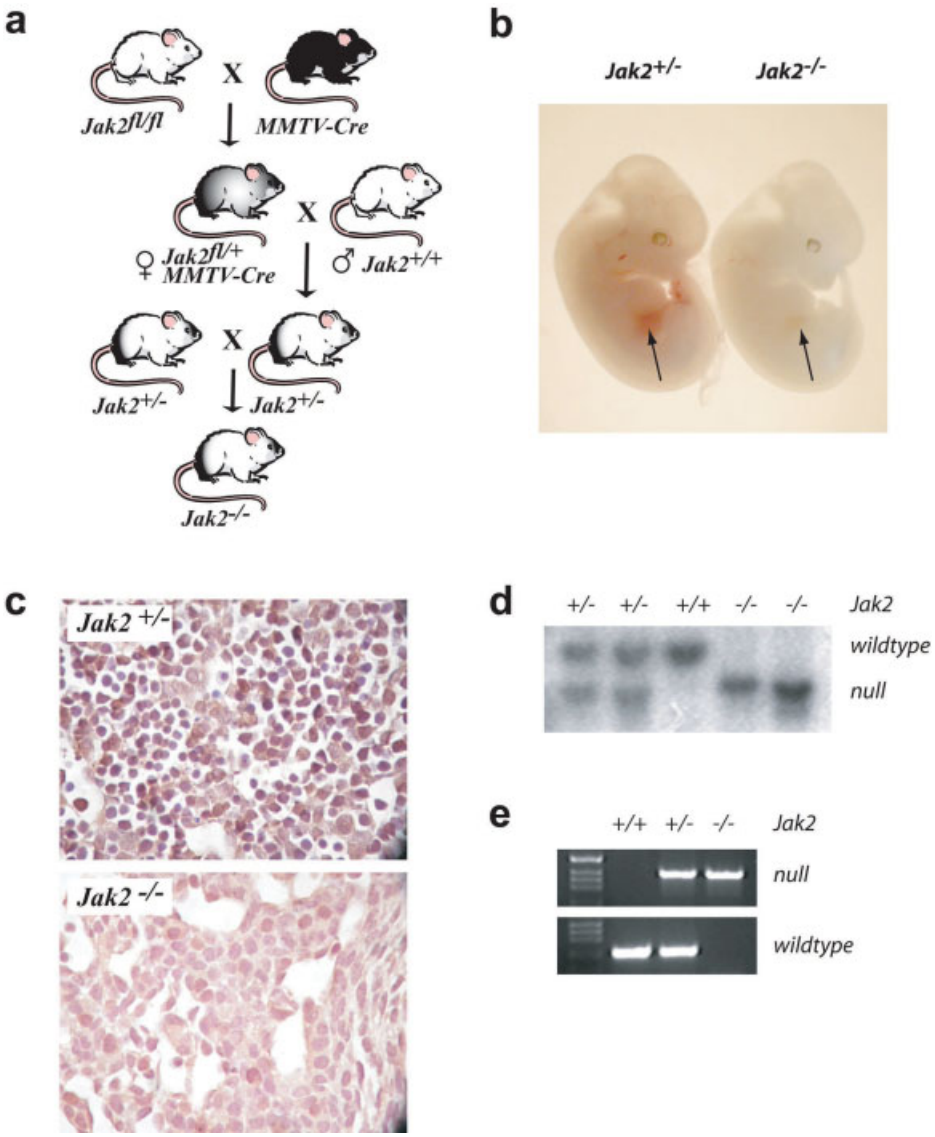

FIG. 2. Deletion of *Jak2* in the female germline and phenotypic consequences of Jak2 deficiency during embryogenesis. **a:** Breeding strategy to convert the floxed allele ($Jak2^{fl}$) into a null mutation ($Jak2^{-}$). *MMTV-Cre* (line A) mice express Cre recombinase in developing oocytes. Thus, heterozygous Jak2fl/+ *MMTV-Cre* females transmit a null allele to their offspring whether the Cre transgene is segregated out or not. **b:** Phenotypic consequences of Jak2 deficiency in developing embryos. $Jak2^{-/-}$ mutants exhibit impaired definitive erythropoiesis at day E12.5. Arrows indicate the location of the fetal liver, which is the site of erythropoiesis at this stage of ontogenesis. **c:** Immunohistochemistry of Stat5, which is an important downstream target of Jak2, in the fetal liver of Jak2-deficient mice (lower panel) and their heterozygous littermate controls (upper panel). Stat5 is highly expressed in nucleated cells of the erythroid lineage in the controls, whereas these cells are absent in the Jak2 mutants. **d,e:** Results of a Southern blot and PCR assay to verify the presence of two knockout alleles ($Jak2^{-/-}$) in anemic embryos. The strategy of these assays is illustrated in Figure 1c.

#88) were expanded and used for the production of chimeras. After germline transmission of the targeted mutation, we bred the floxed gene into homozygosity. Homozygous mutant mice that carry two floxed *Jak2* alleles (Fig. 1b) developed normally until adulthood. Both males and females were fertile, and they exhibited no phenotypic abnormalities, suggesting that the insertion of the selectable marker upstream of the first coding exon had no obvious effect on the transcriptional regulation of the *Jak2* locus.

Excision of the promoter and the first coding exon of *Jak2* can be achieved through Cre-mediated recombination in male or female germ cells, as well as in any somatic tissues (Fig. 1c). The site-specific recombination event can be verified using either the *Eco*R1 Southern blot strategy described above or a PCR assay using the 1786/1787 primer set (see Materials and Methods). We generated mutant mice that lack *Jak2* completely in all organs ($Jak2^{-/-}$) to assess phenotypic abnormalities that were reported previously in Jak2 conventional knockout mice with targeted deletions of exons 2 or 3 (Neubauer *et al.*, 1998; Parganas *et al.*, 1998). Identical phenotypes would indicate that the targeting strategy and Cre-mediated deletion results in a true null mutation of *Jak2*. The conversion of the floxed allele into a null mutation was achieved by transmitting the targeted allele through the female germline of *MMTV-Cre* (line A) mice that have been shown to express Cre recombinase in 100% of developing oocytes (Wagner *et al.*, 1997, 2001). The specific breeding strategy that was employed to generate heterozygous and homozygous *Jak2* null mutants is illustrated in Figure 2a. Female *MMTV-Cre* $Jak2^{fl/+}$ mice were mated with wildtype males ($Jak2^{+/+}$) to produce heterozygous offspring and to segregate out the *MMTV-Cre* transgene. Heterozygous knockout mice were fertile and did not display any

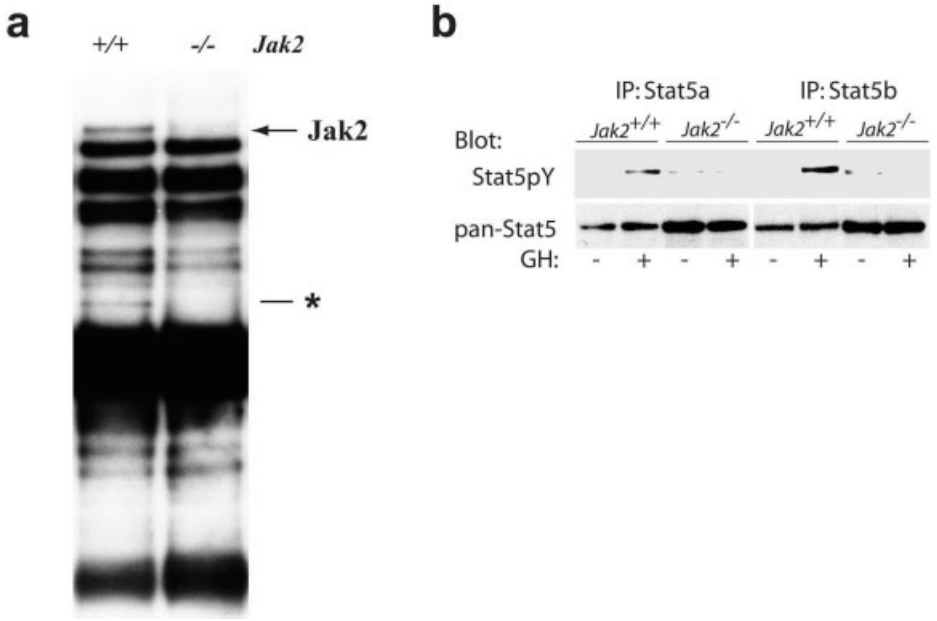

FIG. 3. Verification of the absence of a functional Jak2 protein in mutant embryos. **a:** Immunoprecipitation and western blot analysis of Jak2 derived from embryos carrying two wildtype or two knockout alleles. Note the absence of the full-length Jak2 protein (arrow) and smaller degradation products (asterisk) in *Jak2*$^{-/-}$ animals. **b:** Immunoprecipitation and western blot analysis of both Stat5 isoforms (Stat5a and Stat5b) in growth hormone (GH)-treated mouse embryonic fibroblasts (MEFs) to monitor the loss of Jak2 function. Note that Jak2 deficiency decouples signaling from the GH receptor to its downstream mediator Stat5, suggesting that the targeting strategy leads not only to the absence of the Jak2 protein but also to the complete inhibition of Jak2-mediated signaling events.

noticeable phenotypic abnormalities, suggesting that the Cre-mediated deletion of the floxed *Jak2* does not create a hypermorphic or hypomorphic allele. The breeding of heterozygous mutants did not produce progeny with two *Jak2* null alleles (*Jak2*$^{-/-}$). Based on previous reports (Neubauer *et al.*, 1998; Parganas *et al.*, 1998), we expected that homozygous mutants that lack *Jak2* in all tissues die during mid-pregnancy. Indeed, Jak2-deficient embryos exhibited impairment of definitive erythropoiesis at day 12.5 of gestation (Fig. 2b). Nucleated red blood cells normally seen in the fetal liver of wildtype mice or heterozygous mutants (Fig. 2b, arrow, and Fig. 2c) were absent in Jak2-deficient embryos. Hence, these mice resemble phenotypic features of the EPO and EPO receptor knockout mice (Wu *et al.*, 1995). The presence of two knockout alleles in anemic embryos was verified by Southern blot and PCR analysis (Fig. 2d,e). Since lack of definitive erythropoiesis is a hallmark of Jak2 deficiency (Neubauer *et al.*, 1998; Parganas *et al.*, 1998), we concluded that the Cre-mediated, conditional deletion of the entire first coding exon results in a true null mutation of *Jak2*.

Tissue lysates of entire Jak2-deficient embryos and their littermate controls were analyzed by Western blot to confirm the absence of the Jak2 protein in mutant animals (Fig. 3a). Since it is technically challenging to visualize Jak2, we concentrated this low abundant protein by performing an immunoprecipitation (IP) prior to the Western blot analysis. Using this technique, we were able to confirm that the full-length protein (Fig. 3b, arrow) and smaller degradation products of Jak2 (Fig. 3a, asterisk) were absent in *Jak2*$^{-/-}$ embryos. More important, we also did not detect any smaller protein variants of Jak2 in the knockouts, suggesting that other downstream ATG codons were insufficient to initiate translation. The evidence that the Jak2 null allele does not produce a smaller protein with limited functionality is in agreement with the phenotypic resemblance of our mutant mice with other conventional *Jak2* knockout models (Neubauer *et al.*, 1998; Parganas *et al.*, 1998).

Stat5 is one of the main targets for phosphorylation by Jak2 in response to growth factor and cytokine receptor activation (reviewed by Kisseleva *et al.*, 2002). In particular, nucleated erythroid cells in the fetal liver express high levels of active Stat5 (Fig. 2c, upper panel). These cells are absent in Jak2-deficient embryos. Consequently, the fetal liver of Jak2 knockouts contains significantly less active Stat5 in hepatocytes and other cell types (Fig. 2c, lower panel). Based solely on these cellular imbalances, it would be less informative to use fetal liver tissue or the whole embryo to compare activation levels of Stat5 in the mutants and their wildtype controls. Therefore, we chose mouse embryonic fibroblasts (MEFs) as an in vitro model system to examine whether growth factor-mediated activation of Stat5 is impaired in the Jak2 conditional knockouts under controlled conditions. Unlike erythroid cells, MEFs do not exclusively depend on functional Jak2 for proliferation, differentiation, and cell survival. However, MEFs express the growth hormone receptor, which is capable of activating Stat5a and Stat5b in a Jak2-dependent manner. *Jak2*$^{-/-}$ MEFs and heterozygous control cells were grown in the presence or absence of growth hormone (GH). Stat5a and Stat5b were immunoprecipitated using isoform-specific antibodies and A-Sepharose beads as described previously (Liu *et al.*, 1996). Immunoprecipitates were resolved by SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes. A monoclonal antibody, which is specific to tyrosine (Y694/699) phosphorylated Stat5, was used to deter-

mine the activation of the Stat5 proteins (Fig. 3b). In addition, membranes were incubated with an anti-pan-Stat5 antibody to verify the presence of total Stat5 protein independent of the activation status. The results of this study indicate that GH was able to induce the phosphorylation of Stat5a and Stat5b in the wildtype controls, whereas Jak2-deficiency decouples GH-receptor signaling from its downstream mediator Stat5. Using the Jak2 conditional knockout mice, we recently demonstrated that a Jak2-dependent mechanism of signal transduction also applies to the PRL-receptor-mediated activation of Stat5 in mammary epithelial cells (Wagner *et al.*, submitted). The *MMTV-Cre*-mediated deletion of *Jak2* from the developing mammary gland resulted in phenotypic abnormalities that correspond to PRL-receptor mutants or Stat5a/b double knockouts. More importantly, Jak2-deficient mammary epithelial cells were incapable of activating Stat5 in response to endogenous and supraphysiological levels of circulating PRL. In summary, our combined studies suggested that, in addition to the effects of cytokines on hematopoietic cells mentioned earlier, Jak2 also possesses nonredundant functions for GH and PRL-mediated activation of Stat5 in cells of mesodermal and ectodermal origin (i.e., fibroblasts and mammary epithelial cells).

MATERIALS AND METHODS

Construction of the *Jak2* Targeting Vector

A BAC clone encompassing the *Jak2* locus was isolated from a mouse 129SvJ genomic library. A 5.1 kb *Eco*RI fragment harboring the first coding exon and a 5′-overlapping 5.2-kb *Hin*DIII fragment were isolated and cloned into pBluescript and pZErO, respectively. Both clones were sequenced and used as a template to generate the floxed targeting vector. The entire *Hin*DIII fragment was released by *Asp*I and *Bam*HI digestion and cloned into pLoxpNeo upstream of the PGK-neomycin cassette. A 1.3-kb fragment with the first coding exon was amplified by Pfx polymerase, introducing an *Xho*I site on the 5′-end of the amplification product. The PCR fragment was cloned blunt into the *Hin*DIII(blunt) site of a pBS64 vector, placing it 5′ of the single *loxP* site. A second 3.1-kb fragment was amplified by Pfx polymerase to introduce a *Not*I site at the 3′ end. The PCR product was cloned blunt into the *Eco*RV site of pZErO and released by *Eco*RI digest. The fragment was then cloned into the *Eco*RI site of the pBS64 vector, 3′ from the *loxP* site. The entire 4.4-kb fragment was released from the pBS64 vector by *Xho*I/*Not*I and subcloned into the pLoxpNeo vector containing the 5.2-kb 5′ homology region. The final targeting construct contained a floxed PGK-neomycin selection marker ~570 bp upstream and a third *loxP* site 450 bp downstream of the first coding exon. The final plasmid was linearized using *Not*I, phenol-chloroform extracted, and electroporated into RW-4 cells (20 μg DNA per 10^7 ES cells). The selection and expansion of ES cell clones were performed by Incyte Genomics.

Southern Blot Analysis

Genomic DNA from 192 ES cell clones was prepared using standard phenol/chloroform extraction. Fifteen μg of DNA was digested with *Eco*RI or *Eco*RV at 37°C overnight and separated on a 0.8% agarose gel. The DNA was denatured and blotted onto a nylon membrane (Genescreen plus, NEN, Boston, MA), and hybridized with a ^{32}P-labeled probe. The 3′ external probe, ~643 bp in size, was generated by PCR using the 1724/1725 primer pair (5′-CCA GGT TCA TAC ATC TCA AAA CC-3′ and 5′-GTC ACA GTA GTC CTT TGT CAG G-3′). Membranes were washed in 0.1× SSC buffer containing SDS and exposed for 16 h to Kodak XOMAT-AR film. The *Eco*RV Southern analysis yielded two distinct bands of 18 kb (wildtype allele) and 13 kb in size (floxed allele). The *Eco*R1 Southern blot exhibited bands of 5.1 kb (wildtype allele) and ~8 kb in size (floxed allele). The correct insertion of the outermost *loxP* site into the endogenous *Jak2* locus was confirmed by PCR using the primer set 1743/1744 illustrated in Figure 1a (5′-ATT CTG AGA TTC AGG TCT GAG C-3′ and 5′-CTC ACA ACC ATC TGT ATC TCA C-3′). This assay yielded a 230-bp PCR fragment without the *loxP* site (wildtype allele) and a band of ~310 bp in size with the *loxP* site (floxed allele). Both PCR bands are easily distinguishable on a 2.5% agarose gel.

Mouse Models and Genotyping Protocols

Two correctly targeted ES cell clones (#2 and #88) were expanded and used for the production of chimeras. The injection of ES cells into C57/Bl6 blastocytes were carried out by Cell & Molecular Technologies (Phillipsburg, NJ). The germline transmission of the floxed *Jak2* allele (*Jak2^{fl}* or Jak2^{tm1Kuw}) was verified using the Southern blot assays described above. The PCR primer set 1743/1744 was utilized to genotype mice that carry one or two floxed *Jak2* alleles. The PCR protocol for genotyping *MMTV-Cre* (TgN(MMTV-cre)1Mam) mice has been described previously (Wagner *et al.*, 1997, 2003). The *Eco*R1 Southern assay in combination with the 3′ external probe described earlier was used to monitor the Cre-mediated conversion of the floxed allele into a *Jak2* null allele (~8 kb floxed versus ~4 kb knockout). In addition, the recombined *Jak2* null allele (*Jak2^{-}* or Jak2$^{tm1.1Kuw}$) is easily identifiable using PCR with the primer pair 1786/1787 indicated in Figure 1c (5′-GTC TAT ACA CCA CCA CTC CTG-3′ and 5′-GAG CTG GAA AGA TAG GTC AGC-3′). The amplicon of the null allele is ~320 bp in size. All animals used in the studies were treated humanely and in accordance with federal guidelines and institutional policies.

Immunohistochemistry

Jak2 mutant embryos and their wildtype littermate controls were fixed overnight at 4°C in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA). The specimens were embedded in paraffin and sectioned by standard methods. The immunohistological detection of the

Stat5 protein in fetal tissues was performed as described previously (Nevalainen *et al.*, 2002).

Cell Culture

Mouse embryonic fibroblasts (MEFs) were derived from 12.5-day-old embryos and expanded in culture as described previously (Krempler *et al.*, 2002). Cells were starved in serum-free DMEM for 16 h and then incubated with or without ovine 20 nM GH for 15 min at 37°C. Cells were harvested, frozen on dry ice, and stored at –80°C before using them in protein immunoblotting experiments.

Protein Analysis

Whole 12.5-day-old embryos, either $Jak2^{+/+}$ or $Jak2^{-/-}$, were pooled in groups of three and homogenized on ice in a Triton X-100-based lysis buffer (1 g/5 ml) with protease inhibitors and processed as described (Nevalainen *et al.*, 2002). Clarified cell lysates were immunoprecipitated with 4 μg/ml of either the Jak2 antibody or isoform-specific antibodies against Stat5a and Stat5b (Liu *et al.*, 1996). Immunoprecipitates or whole cell extracts were resolved by SDS-PAGE and immunoblotted with either phosphoStat5a/b (Y694/9) antibody (AX1; 0.5 μg/ml; Advantex Bioreagents, Conroe, TX) or a panStat5a/b antibody (AX55; 2 μg/ml; Upstate Biotechnology, Lake Placid, NY), or Jak2 antibody (1:3,000). Horseradish peroxidase-coupled secondary antibodies and enhanced chemiluminescence (Amersham, Piscataway, NJ) were used for detection. Rabbit polyclonal antibodies against both isoforms of Stat5 were a kind gift from Lothar Hennighausen.

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