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The p53 tumour suppressor inhibits glucocorticoid-induced proliferation of erythroid progenitors

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Hypoxia encountered at high altitude, blood loss and erythroleukemia instigate stress erythropoiesis, which involves glucocorticoid-induced proliferation of erythroid progenitors (ebls). The tumour suppressor p53 stimulates hematopoietic cell maturation and antagonizes glucocorticoid receptor (GR) activity in hypoxia, suggesting that it may inhibit stress erythropoiesis. We report that mouse fetal liver ebls that lack p53 proliferate better than wild-type cells in the presence of the GR agonist dexamethasone. An important mediator of GR-induced ebl self-renewal, the c-*myb* gene, is induced to higher levels in p53$^{-/-}$ ebls by dexamethasone. The stress response to anemia is faster in the spleens of p53$^{-/-}$ mice, as shown by the higher levels of colony forming units erythroids and the increase in the CD34/c-kit double positive population. Our results show that p53 antagonizes GR-mediated ebl expansion and demonstrate for the first time that p53–GR cross-talk is important in a physiological process *in vivo*: stress erythropoiesis.

INTRODUCTION

Physiological stresses to the erythroid system, such as blood loss, hemolysis, erythroleukemia and oxygen deprivation at high altitudes, induce rapid proliferation of erythroid progenitors that is driven by erythropoietin (Epo) and glucocorticoids. Glucocorticoids control the activity of the glucocorticoid receptor (GR), a member of the steroid receptor superfamily. They play an essential role in maintaining basal- and stress-related homeostasis and, in particular, stimulate erythropoiesis. They enhance the formation of murine erythroid colonies (Golde *et al.*, 1976), increase the proliferation of erythroid cells in the presence of limiting amounts of Epo (Udupa *et al.*, 1986) and restore normal erythropoiesis in humans afflicted by certain anemias (Zito and Lynch, 1977; Liang *et al.*, 1994). Pathological changes of glucocorticoid levels affect erythropoiesis. Insufficient corticosteroid production, in Morbus Addison disease, is associated with anemia, whereas elevated glucocorticoid levels, in Cushing's syndrome patients, is connected with increased red blood cell (RBC) count, hemoglobin and hematocrit values. Glucocorticoids cooperate with the activated receptors for Epo (EpoR) and stem cell factor (c-kit) to induce long-term proliferation and differentiation arrest of normal erythroblasts *in vitro* (Wessely *et al.*, 1997a, 1999; Reichardt *et al.*, 1998; von Lindern *et al.*, 1999). This requires the DNA-binding and transactivation capacities of the GR (Wessely *et al.*, 1997b; Reichardt *et al.*, 1998).

The tumour suppressor p53 is frequently inactivated in human cancers, including leukemia and lymphoma (Hainaut and Hollstein, 2000). Amongst its different functions, p53 may be involved in hematopoietic re-population. Numerous *in vitro* studies indicate that p53 is involved in proliferation, differentiation and apoptosis of hematopoietic cells (Kastan *et al.*, 1991; Lotem and Sachs, 1993; Prokocimer and Rotter, 1994). p53 and glucocorticoids are key mediators of stress responses with opposing activities that repress each other's functions in normal cells, notably in responses to hypoxia (Sengupta and Wasylyk, 2001). These observations raised the possibility that p53 may antagonize the activity of glucocorticoids in stress erythropoiesis. Our results show that antagonism between p53 and GR helps to maintain the balance between proliferation and differentiation of erythroid cells *in vivo*. Specific inhibitors of p53–GR interactions may be useful to replenish the RBC pool, for example in anemic patients.

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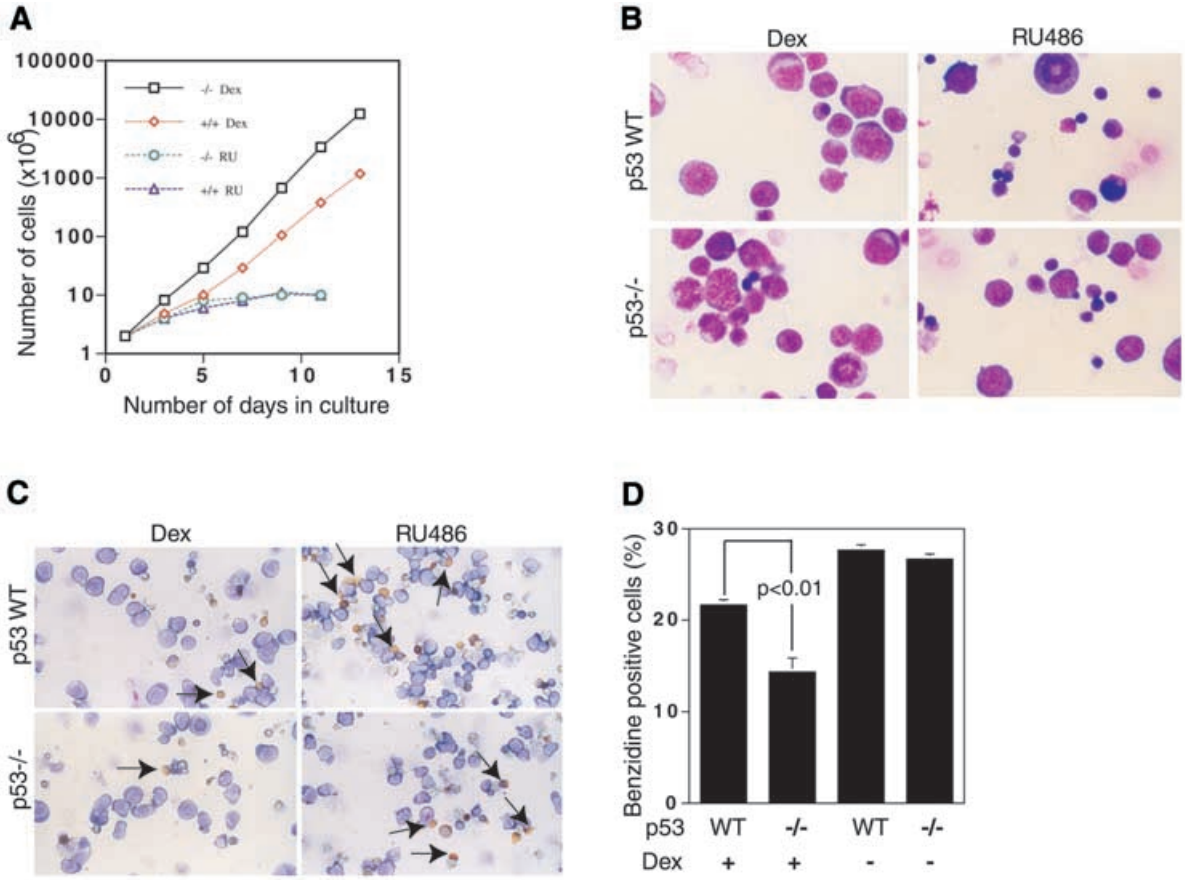

Fig. 1. Proliferation of fetal liver cell erythroblasts in culture. (**A**) Cells were counted daily and cumulative cell numbers determined. (**B** and **C**) Aliquots on day 6 were centrifuged on slides and stained with May Grunwald (B) or neutral benzidine (C) and counter-stained with Giemsa. The number of benzidine positive cells was counted blindly. (**D**) The proportion of benzidine positive cells (yellow indicated with arrows) was significantly lower in the $p53^{-/-}$ than in the wild-type cultures (p <0.01).

RESULTS AND DISCUSSION

Increased proliferation of $p53^{-/-}$ erythroblasts (ebls)

We compared the proliferative capacity of wild-type and $p53^{-/-}$ ebls from mouse fetal livers at E14.5 d.p.c. (after the onset of definitive erythropoiesis). Fetal liver cell suspensions were cultured in StemPro-34 medium supplemented with Epo, SCF and IGF. In the presence of Dex, the cells divided actively for at least 14 days, with a doubling time of about 24 h (Figure 1A). The $p53^{-/-}$ cells proliferated better than their wild-type counterparts from the earliest times in culture, and after 14 days the total number of cells was at least 10-fold higher (similar results were obtained in five independent experiments). In the presence of the GR antagonist RU486, $p53^{-/-}$ and wild-type cells grew much more slowly and at similar rates. These results show that p53 decreases the velocity (doubling time) of GR-dependent proliferation of ebls in fetal liver cultures.

Spontaneous differentiation during renewal was analysed after 12 days in culture by staining with May Grunwald for morphology (Figure 1B) and neutral benzidine for hemoglobin (Figure 1C). In the presence of Dex, $p53^{-/-}$ cultures contained a larger proportion of proliferating immature cells with morphological features of ebls (basophilic cytoplasm and large central nucleus) and significantly fewer (p <0.01) hemoglobin containing cells (Figure 1B–D). In the absence of Dex or in the presence of the antagonist RU486, $p53^{-/-}$ and wild-type cultures were very similar, with fewer ebls and more hemoglobinized reticulocytes/erythrocytes (Figure 1B–D; data not shown).

Spontaneous differentiation during renewal was also studied by FACS analysis of immature (c-kit, CD117) and late erythroid (Ter119) cell surface markers (Table I). In the presence of Dex at day 10, there was a larger proportion of immature erythroid cells in the $p53^{-/-}$ cultures (58 versus 43%) and fewer late erythroid cells (23 versus 29%). Similarly, at day 15, there were more immature erythroid cells in the $p53^{-/-}$ cultures (61 versus 34%) and fewer late erythroid cells (30 versus 40%). These cultures did not express the macrophage and granulocyte markers Mac-1 and Gr-1 (data not shown). In the absence of Dex, there were fewer immature erythroid and more Ter119 positive late erythroid cells (data not shown), as expected from the lack of Dex stimulation of ebl proliferation. These results show that the loss of p53 favours the expansion of immature erythroblasts at the expense of the more differentiated cells. Hence, p53 is important for the maturation of hematopoietic cells.

Table I. FACS analysis of fetal liver cell cultures (%) in the presence of Dex

	10 days		15 days	
Cell surface markers	WT	KO	WT	KO
Early hematopoietic c-kit (CD117)	43 (±2.0)	58 (±3.5)	34 (±3.5)	61 (±3.6)
Late erythroid Ter119	29 (±2.0)	23 (±3.5)	40 (±2.4)	30 (±2.5)

Fetal liver cells from day 10 and 15 cultures were immunostained with fluorochrome-labelled antibodies against c-kit and Ter119. Three independent cultures were analysed by FACS. The results are expressed as the mean (±SD) of the three separate analyses.

Effect of p53 on expression of genes involved in erythroid cell proliferation and differentiation

A number of genes have been identified that have roles in erythroid cell differentiation and proliferation, including c-myb, c-kit, RBTN2 and GATA-1. c-myb codes for a transcription factor that regulates the proliferation and differentiation of various hematopoietic cell lineages and is required for proliferation of erythroid progenitors (Introna *et al.*, 1994; Wessely *et al.*, 1997a; Weston, 1998; von Lindern *et al.*, 1999). The c-kit receptor ligand SCF stimulates the proliferation of erythroid progenitors (Muta *et al.*, 1995; Wessely *et al.*, 1997b). c-myb and c-kit are downregulated upon differentiation of erythroid progenitors. The RBTN2 transcription factor is selectively expressed in immature erythroid cells and is required for progression past the proerythroblast stage (Warren *et al.*, 1994). The GATA-1 transcription factor is required for terminal differentiation (Pevny *et al.*, 1995; Fujiwara *et al.*, 1996). Dex activation of GR in cultured erythroid progenitors enhances the expression of c-myb, c-kit and RBTN2 and inhibits the expression of GATA-1 (von Lindern *et al.*, 1999). We tested whether p53 affects Dex induction of the expression of these genes, using real-time RT–PCR. Day 10 cultures of fetal liver cells were incubated for 48 h in medium containing growth factors but lacking steroid hormone to withdraw them from the inducer (Wessely *et al.*, 1997a; von Lindern *et al.*, 1999). Dex was then added and relative mRNA levels were measured after 24 h. As expected, in wild-type cultures ($p53^{+/+}$), Dex induced the expression of c-myb, c-kit and, to a lesser extent, RBTN2 and decreased GATA-1 (Figure 2). In $p53^{-/-}$ cultures, Dex induced c-myb to significantly higher levels (p <0.02), whereas c-kit, RBTN2 and GATA-1 were not significantly different from the $p53^{+/+}$ cultures. The control, 28S RNA, did not vary. Interestingly, c-myb expression has been shown previously to be repressed by p53, in one of three cell lines studied (Zhao *et al.*, 2000). Our results show that c-myb, a key regulator of ebl proliferation, could be an important intermediary in a p53–GR-mediated decision between proliferation and differentiation.

Effect of p53 on ebl upregulation in spleen in response to anemia

GR is required for the rapid expansion of ebls in the spleen in response to hemolytic anemia (Bauer *et al.*, 1999). We studied whether p53 affects this response. Anemia was induced by injection of phenylhydrazine into wild-type and $p53^{-/-}$ mice, and

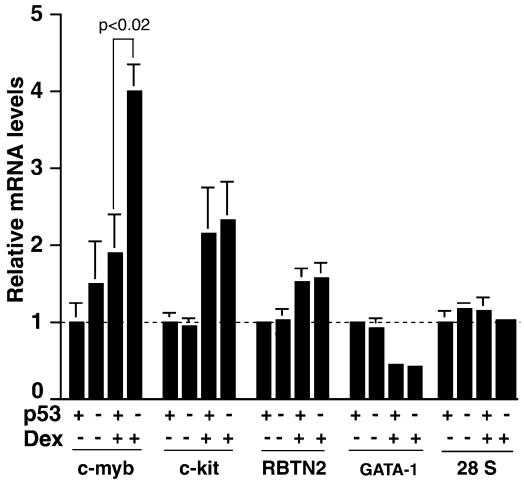

Fig. 2. Relative mRNA levels. Day 10 cultures were withdrawn from Dex for 48 h, Dex was added and 24 h later total RNA was extracted. The levels of c-myb, c-kit, RBTN2, GATA-1 and 28S RNAs were measured by real-time RT–PCR and plotted relative to the wild-type samples (p53 +) in the absence of Dex (Dex –). Each column represents the average of three amplifications (error bars show the standard deviation). The relative levels of c-myb mRNA are significantly higher in the $p53^{-/-}$ compared to the wild-type cultures (p <0.02). Comparable results were obtained in a replicate experiment using a different set of mice.

spleen cell suspensions were seeded in methylcellulose medium. Three days after the injection, the number of early erythroid progenitors (colony forming units erythroids, CFU-Es) induced by hemolysis was higher in the $p53^{-/-}$ mice (Figure 3A and B; $p < 0.01$). We also studied a blood parameter, the hematocrit. Five days after the first phenyhydrazine injection, the hematocrit values of $p53^{-/-}$ mice were consistently but transiently higher than in the wild-type mice (Figure 3C; p <0.03). These results show that $p53^{-/-}$ mice recover faster from the anemic stress, with increased numbers of progenitors (CFU-Es) by day 3 and mature erythroid cells by day 5. Apparently, the p53 restraint on GR-mediated proliferation is lost, allowing faster recovery.

We examined the differentiation status of the spleen cells by FACS analysis of the early hemopoietic markers c-kit (CD117) and CD34 and the late erythroid marker Ter119 (Figure 4). The proportion of c-kit positive cells was found to be elevated to a significantly greater extent in the $p53^{-/-}$ anemic mice (Figure 4B; p <0.03). A distinct cell population, double stained by CD34 and c-kit, was upregulated to a greater extent in the $p53^{-/-}$ anemic mice (Figure 4A). As expected (Bauer *et al.*, 1999), a fraction of these double positive cells also coexpressed Ter119 (Figure 4C), showing that they belong to the erythroid lineage. Thus, loss of p53 enhances the expansion of the particular type of spleen ebls (c-kit/CD34/Ter119 triple positive) that are responsible for the glucocorticoid- and GR-mediated response to erythrolytic stress (Axelrod and Reisine, 1984; Tronche *et al.*, 1998; Bauer *et al.*, 1999).

In conclusion, our results show that stress erythropoiesis is a physiological process in which p53 antagonizes the GR. We showed previously that p53 antagonizes the activity of GR in normal cells in culture (Sengupta and Wasylyk, 2001), but the

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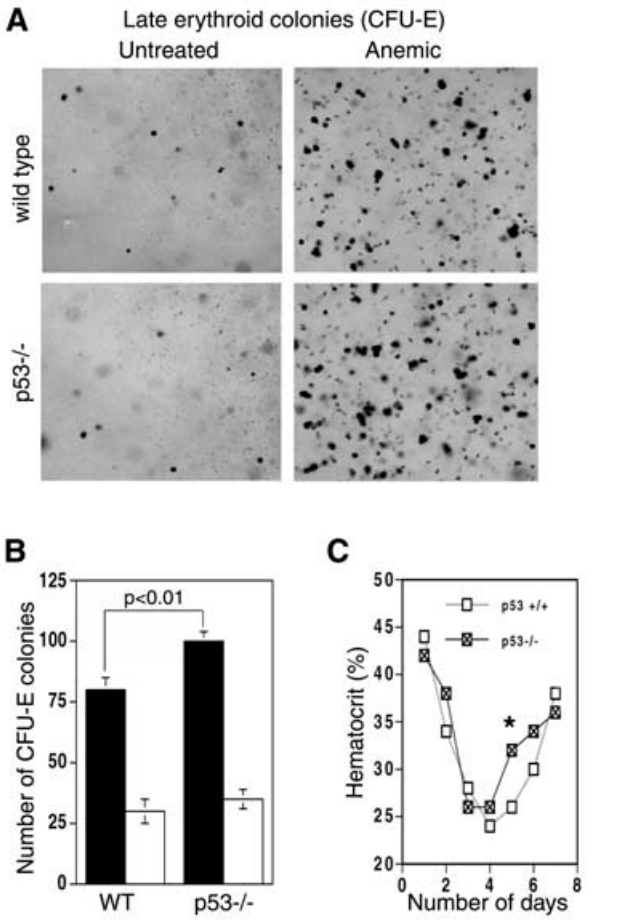

Fig. 3. Effect of p53 on the induction by anemia of spleen CFU-Es and circulating blood cells. (**A**) On day 4 (after the first phenylhydrazine injection), spleen cells were isolated and equal numbers of cells were seeded in duplicate into semi-solid media containing factors required for CFU-E formation. After 2–3 days, pictures were taken (A) and the numbers of CFU-Es were counted (**B**; solid bars, treated; empty bars, untreated). (**C**) Hematocrit values after phenylhydrazine injection. There was a reproducible and significant (*p <0.03) increase in the hematocrit values at day 5 in the $p53^{-/-}$ mice in four separate experiments.

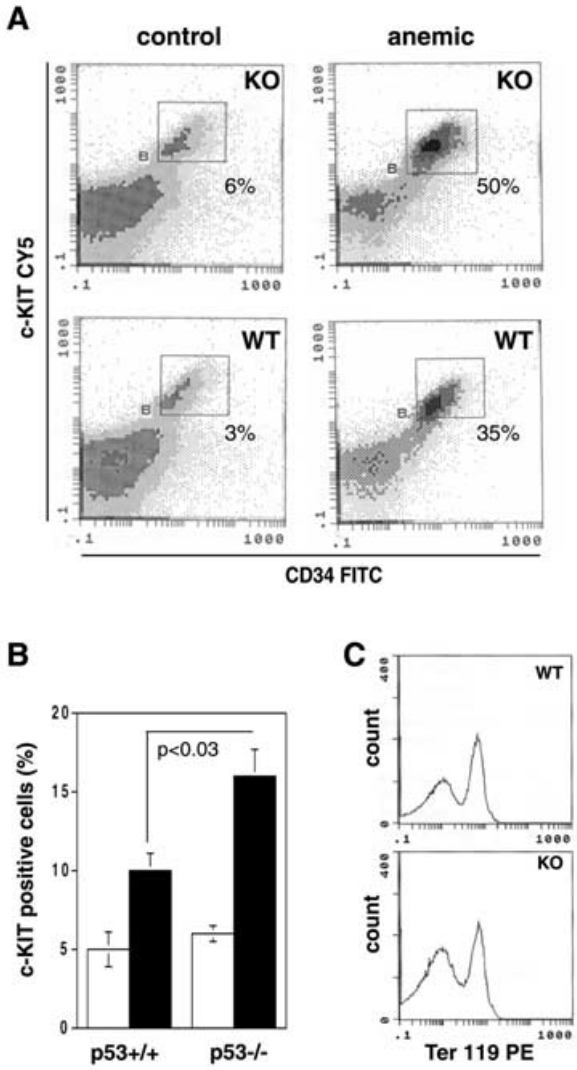

Fig. 4. Effect of p53 on the induction by anemia of erythroid progenitors in the spleen. Aliquots of spleen cell suspensions, prepared from mice treated with phenylhydrazine, were immunostained with fluorochrome-labelled antibodies against CD34, c-Kit (CD117) and Ter119. (**A**) Viable cells were gated and evaluated by FACS analysis. The CD34/c-kit double positive cells are outlined (squares), and the percentages of double positive cells are indicated. One representative result, from three independent experiments, is shown. (**B**) The fraction of c-kit positive cells from the untreated (empty bars) and the anemic (solid bars) animals are plotted. The percentages of c-kit positive cells are calculated from several independent experiments, and a p-value is indicated. (**C**) The CD34/CD117 double positive cells [the rectangle outlined in (A)] were gated and analysed for Ter119 expression. WT, wild type; KO, $p53^{-/-}$.

in vivo significance of these observations was unclear. Various studies have implicated p53 in differentiation (Almog and Rotter, 1997), in addition to cell-cycle arrest and apoptosis (Balint and Vousden, 2001). The levels of p53 protein increase with maturation in human hematopoietic cells, suggesting that p53 may play a role in hematopoietic cell maturation by contributing to the inhibition of proliferation that occurs during terminal differentiation (Kastan *et al.*, 1991). GR is required *in vivo* for the rapid expansion of ebls under stress conditions (Bauer *et al.*, 1999). Our observations fit with these studies and provide a molecular mechanism for the balance between precursor expansion and differentiation of erythroid cells. p53 and GR apparently function as opposing forces that help to maintain homeostasis and to make the important decision between proliferation and differentiation. GR regulates the expression of genes that are required for ebl proliferation, such as c-*myb* (von Lindern *et al.*, 1999). p53 antagonizes the proliferative effects of GR and inhibits the expression of c-myb, thereby favouring differentiation.

METHODS

Isolation and cultures of mouse fetal liver cells. p53 heterozygote mice from mixed background (BL6/sv129) mice were crossed, and fetal livers were isolated from 14.5 d.p.c. embryos (Bauer *et al.*, 1999). The livers were resuspended in 1 ml of

serum free stem cell expansion medium (StemPro-34; Life Technologies Gibco-BRL), supplemented with 100 ng/ml mouse SCF, 2 U/ml Epo (R&D Systems), 40 ng/ml IGF-1 (Sigma) and 1 µM Dex (Sigma). Erythroid cultures were maintained at 2–4 × 10^6 cells/ml by daily medium changes plus re-addition of fresh factors with appropriate dilution. Cell numbers were determined manually on a daily basis.

Cell staining. Cytospins (1–20 × 10^4 cells in 200 µl of growth medium centrifuged on glass slides) were stained with May Grunwald and counter-stained with Giemsa, or fixed for 5 min in methanol, incubated in 1% 3′,3′-dimethoxybenzidine (Sigma) in methanol for 1.5 min, washed with bi-distilled H_2O for 30 s and counter-stained with Giemsa (Beug *et al.*, 1982). Dark yellow cytoplasms are benzidine (hemoglobin) positive, light blue negative.

FACS analysis. The cells were incubated with Fc block (Pharmingen) for 10 min, antibodies for 15 min at 4°C, followed by secondary antibodies coupled to CY5 (for c-kit) or FITC (for CD34) or with streptavidin linked to PE (for Ter119). Flow cytometry was performed with the EPICS profile analyser.

RNA extraction and RT–PCR. Ten-day fetal liver cell cultures were incubated in medium with growth factors but lacking Dex for 48 h, stimulated with Dex for 1 day and total RNA extracted with Trizol Reagent (Life Technologies Gibco-BRL). Real-time RT–PCR was performed with the LightCycler system (Roche Diagnostics) and the SYBR Green protocol. Primers for c-myb, c-kit, RBTN2, GATA-1 and 28S RNA were designed using the Oligo 4.0 programme. The reactions, containing 5 µl of RNA (100–500 ng) and 15 µl of reagents (including the primers, $MgCl_2$, enzymes and SYBR green), were reverse transcribed for 60 s at 55°C, denatured for 30 s at 95°C and cycled 40 times for 2 s at 95°C, 10 s at 55°C and 10 s at 72°C (~1 s per 25 bp depending on the size of the product). Amplification specificity was verified by melting curve analysis, and the data quantified with LightCycler software.

***In vivo* erythropoiesis.** Anemia was induced with phenylhydrazine (60 mg/kg body weight; Sigma) injected intraperitoneally on two consecutive days (Broudy *et al.*, 1996; Bauer *et al.*, 1999). Cell suspensions were prepared by squeezing spleens collected on day 3 through 70 µm cell strainers and washing with cold PBS. For CFU-Es, the cells were resuspended in StemPro-34 medium and seeded (2 × 10^5 to 2 × 10^6 cells per well, four well dishes) into methocult 3236 supplemented with 5 U of human recombinant Epo, 100 ng/ml mouse SCF, 40 ng/ml IGF-1 and 40 mg/ml LDL (Sigma). Small compact colonies were counted on days 2–3. For the hematocrit experiments, blood samples were collected daily from four wild-type and four $p53^{-/-}$ mice, starting from the first phenylhydrazine injection. The blood samples were centrifuged in capillary tubes in a minicentrifuge (Bayers diagnostics).

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