

# Thyroid hormone T3 acting through the thyroid hormone $\alpha$ receptor is necessary for implementation of erythropoiesis in the neonatal spleen environment in the mouse

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## Summary

Thyroid hormones (THs) mediate many physiological and developmental functions in vertebrates. All these functions are mediated by binding of the active form of the TH T3 to the specific nuclear receptors TR $\alpha$  and TR $\beta$ , which are transcription factors. Using mutant mice lacking TRs or deficient for TH production, we show that T3 influences neonatal erythropoiesis through TR $\alpha$ . The effect of T3 and TR $\alpha$  is restricted to this developmental window and is specific for the spleen but not for other erythropoietic organs. We show that T3 via TR $\alpha$  affects late steps of erythrocytic development, promoting the proliferation of late basophilic erythroblasts. *In vitro*, this effect is exerted directly on erythrocytic cells. *In vivo*, the action of T3 is

also intrinsic to spleen erythrocytic progenitors, as shown by grafting experiments of splenocytes derived from wild-type and TR $\alpha$  knockout (TR $\alpha^{0/0}$ ) mice into wild-type and TR $\alpha^{0/0}$  irradiated recipients. Our results indicate that defective spleen erythropoiesis in hypothyroid and TR $\alpha^{0/0}$  mice results from impaired recognition of the spleen environment by the mutant erythrocytic progenitors. The data presented support a model in which T3 signaling through TR $\alpha$  is essential for the implementation of the transient spleen erythropoiesis at birth.

Key words: Thyroid hormone, Nuclear receptor, Erythropoiesis, Mouse

## Introduction

Thyroid hormones (TH) are produced from the thyroid gland and coordinate a multitude of physiological responses and developmental processes in all vertebrates (Yen, 2001). The biologically active form of TH, T3, binds to two specific members of the nuclear hormone receptors family, the thyroid hormone receptors TR $\alpha$  and TR $\beta$  (Thra and Thrb – Mouse Genome Informatics). These are transcription factors that constitutively bind to target gene promoters and either repress or activate gene expression, depending on the presence of T3: unliganded TRs (apo-receptors) behave as transcription repressors, but when bound to T3 the receptors (holo-receptors) switch into transcription activators on these same target genes (Wu and Koenig, 2000). Thus, TH is able to finely tune the controlled gene network of the TRs both positively and negatively. The investigation of the phenotype of TR knockout mice and of congenital hypothyroid *Pax8*<sup>-/-</sup> mice has revealed multiple defects in intestine, bone, brain and hematopoietic tissues during early postnatal development (Flamant and Samarut, 2003). Yet, evidence for a role of TH/TRs in erythropoiesis has been so far unclear and mostly indirect. The *erbA* oncogene, a dominant negative natural

mutant of TR $\alpha$  that retains DNA binding activity but cannot bind its ligand induces erythroleukemia in chickens by subverting the normal balance between proliferation and differentiation of chicken erythroblasts (Gandrillon et al., 1989; Bauer et al., 1999; Bartunek and Zenke, 1998), and in humans, hypothyroidism is frequently associated with certain forms of anemia (Green and Ng, 1986). Nevertheless, in adult mice lacking TR $\alpha$  and/or TR $\beta$ , steady state erythropoiesis does not seem to be affected (Forrest et al., 1996; Wikstrom et al., 1998; Gauthier et al., 1999; Gothe et al., 1999). In mouse, red blood cells are generated through two successive waves, first in fetal liver at the embryonic stage, and then in bone marrow and spleen from embryonic day (E) 16.5 to adulthood (Delassus and Cumano, 1996; Robb, 1997; Dzierzak et al., 1998; Keller et al., 1999; Wolber et al., 2002). The physiological mediators of these changes are not yet known.

Here, we have studied the role of TH and TRs on mouse erythropoiesis *in vivo* under the hypothesis that they play a transient role during early postnatal development.

Soon after birth the TH blood level, extremely low in the fetus, increases sharply by about 2000-fold, peaks at 2 weeks after birth and then decreases to the low levels observed under

normal conditions in adults (Morreale de Escobar et al., 1994, Campos-Barros et al., 2000, Hadj-Sahraoui et al., 2000). During the transition from fetal to newborn life, which resembles TH-mediated amphibian metamorphosis, TR $\alpha$ s switch from apo-receptors, due to the hypothyroid fetal environment, into holo-receptors after the sudden burst of TH production at birth, and turn on target gene expression and physiologic functions, as recently demonstrated in the heart (Mai et al., 2004).

Using mice lacking TRs, or the congenital hypothyroid *Pax8*<sup>-/-</sup> mice lacking TH (Mansouri et al., 1998), we now provide evidence that TH and TR $\alpha$ , but not TR $\beta$ , are specifically required for normal spleen erythropoiesis during early postnatal development. Moreover, we show that the effect of TH is exerted on spleen erythrocytic precursor cells in a cell-autonomous fashion. The precursors lacking TR $\alpha$  do not recognize the spleen environment to complete their differentiation. These data demonstrate that T3, signaling through its receptor TR $\alpha$ , is necessary for the development of erythropoiesis within the postnatal spleen environment.

## Materials and methods

### Mice

*Pax8*<sup>-/-</sup>, TR $\alpha$ <sup>0/0</sup> and TR $\beta$ <sup>-/-</sup> mice were progeny of 129/C57BL/6 mice. Because *Pax8*<sup>-/-</sup> and TR $\alpha$ <sup>0/0</sup> mutants have a different genetic background (Mansouri et al., 1998; Gauthier et al., 2001), each mutant was compared to its own littermate heterozygote or homozygote wild type as control. Experiments were performed on 2-week-old (D15) animals kept with their mothers. When indicated, TH (1.6 $\times$ 10<sup>-5</sup> mol of T3 and 1.6 $\times$ 10<sup>-4</sup> mol of T4, Sigma, St Louis, in 100  $\mu$ l of PBS) was injected intraperitoneally on days 13 and 14 and animals were sacrificed on day 15.

### Flow cytometry

Single cell suspensions obtained from spleens and bone marrow were analyzed with the following antibodies: FITC-B220, PE-TER119, FITC-CD71 and AnnexinV (Pharmingen, San Diego). Cells were analyzed on a FACScan machine (Becton Dickinson) using Cell Quest software. The four different erythrocytic populations were defined according to Socolovsky et al. (Socolovsky et al., 2001): I: Ter119<sup>med</sup>CD71<sup>high</sup>, II: Ter119<sup>high</sup>CD71<sup>high</sup>, III: Ter119<sup>high</sup>CD71<sup>med</sup>, IV: Ter119<sup>high</sup>CD71<sup>low</sup>. For the calibration and standardization of the procedures, 60 000 cells were analyzed. For the comparative analyses between animals the number of analyzed cells was reduced to 10,000 and was the same for all genotypes and experimental conditions. No significant differences were observed in the frequencies of the different populations between the two numbers of tested cells. The reduction in the number of tested cells was decided to preserve most of the cells for other purposes, mainly molecular biology studies.

KI-67 nuclear staining to identify proliferating cells was performed as described at <http://icg.cpmc.columbia.edu/cattoretto/Protocol/flowcytometry/IntracellularStaining.html>.

### Primary cell isolation, culture and differentiation

Primary splenocytes and bone marrow cells were isolated from 10-day-old (P10) SV129 mice, passed through a 70  $\mu$ m cell strainer and washed. To analyze their proliferation potential, the cells were seeded and expanded in the proliferation medium containing serum-free erythroid medium (StemPro34TM; Life Technologies) supplemented with 2 units/ml human recombinant erythropoietin (EPO; Janssen-Cilag), 100 ng/ml murine recombinant stem cell factor (R&D Systems), 10<sup>-6</sup> mol dexamethasone (Sigma) and 40 ng/ml insulin-like growth factor 1 (Promega), as described for fetal liver and bone

marrow cells (Dolznic et al., 2001; von Lindern et al., 2001; Kolbus et al., 2002). Cultures were kept between 3 and 5 $\times$ 10<sup>6</sup> cells/ml, counted daily and cultured for 6 days to enrich for erythrocytic progenitors.

To induce differentiation, cells were centrifuged (7 minutes at 700 g) to remove dead and differentiated cells and to obtain a 99% homogenous population of proliferating erythroid progenitors. Cells were washed with PBS and reseeded at 3 $\times$ 10<sup>6</sup> cells/ml in differentiation medium containing serum-free erythroid medium (StemPro34TM; Life Technologies) supplemented with 10 units/ml Epo, insulin (4 $\times$ 10<sup>-4</sup> IE=10 ng/ml, Actrapid<sup>®</sup> HM; Novo Nordisk), and 1 mg/ml iron-saturated human transferrin (Sigma). When indicated, 10<sup>-7</sup> mol of T3 (Sigma-Aldrich) was added to the cultures. The percentage of viable cells was determined as a ratio of the number of Trypan-Blue-excluding cells over total cell number.

### CFU-Es and BFU-Es colony assay

For evaluation of burst-forming unit erythrocytic (BFU-Es) and colony-forming unit erythrocytic (CFU-Es), spleen cells were seeded at various concentrations (2 $\times$ 10<sup>5</sup> to 2 $\times$ 10<sup>6</sup> cells/well) into semisolid medium MethoCultM334 (Stem Cell Technologies) in 12 well dishes. For evaluation of CFU-Es, small compact colonies were counted 48 hours later and for BFU-Es burst were counted 6 days later.

### Cell morphology and histological staining

Erythroblasts at various stages of differentiation were cytocentrifuged onto glass slides and stained with neutral benzidine (Sigma), to detect hemoglobin-expressing cells, and with May-Gruenwald Giemsa (MGG) staining. Differentiated and undifferentiated cells were distinguished in the following manner: differentiated hemoglobin-positive cells are either nucleated cells with brown cytoplasm, or enucleated brown stained erythrocytes; undifferentiated hemoglobin-negative cells are larger cells with blue cytoplasm. Following these criteria, differentiated and undifferentiated cells were counted after visual inspection under the microscope, evaluating >300 cells per sample on multiple, randomly selected fields.

### Reconstitution of lethally irradiated mice

SV129 wild-type or TR $\alpha$ <sup>0/0</sup> mice (8-12 weeks old) were lethally irradiated with 10 Gy and received a retro-orbital injection of spleen suspension in PBS from 10-day-old mice. Eleven days after grafting, mice were sacrificed and reconstituted spleen analyzed by histology (hematoxylin and eosin staining of paraffin sections), counting total cell number, determining CFU-E numbers and cell morphology analysis on cytospin smears after MGG staining.

### Statistical analyses

Values reported are means  $\pm$  standard deviation (s.d.). *P*-values were calculated by two-way ANOVA when mice of different genotypes and treatments were compared and by Student's *t*-test when comparisons were made within the same genotype.

## Results

### Two-week-old hypothyroid mice present a severely decreased spleen cellularity

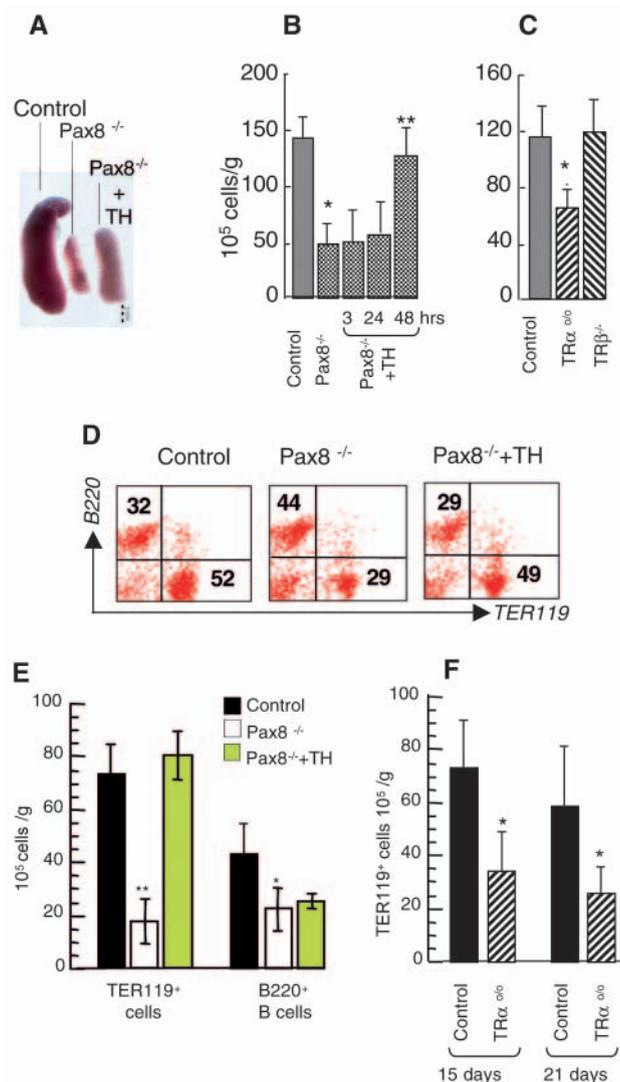
To address the role of TH and TRs during erythropoiesis in vivo, genetically modified mice TR $\alpha$ <sup>0/0</sup> and TR $\beta$ <sup>-/-</sup>, lacking the TR $\alpha$  and TR $\beta$  receptors, respectively (Gauthier et al., 1999), and mice lacking TH production (*Pax8*<sup>-/-</sup>) (Mansouri et al., 1998), were examined. The *Pax8* gene controls specifically the development of the follicles in the developing thyroid. Its knockout induces a lack of thyroid follicle development without any significant developmental defects in any other organs. The *Pax8*<sup>-/-</sup> mutant mice show a deep congenital

hypothyroidism throughout their short life and die by the third week after birth (Mansouri et al., 1998). By contrast, the  $TR\alpha^{0/0}$  and  $TR\beta^{-/-}$  mice are viable, although they suffer some developmental defects (reviewed by Flamant and Samarut, 2003). As previously described, fetal and adult TR knockout mice display normal erythropoiesis, suggesting that TH plays no role in fetal liver and bone marrow erythropoiesis (Forrest et al., 1996; Wikstrom et al., 1998) (data not shown). On the contrary, when 2-week-old mutant mice were examined closely, a drastic reduction in spleen cellularity was observed in both  $Pax8^{-/-}$  and  $TR\alpha^{0/0}$  mice (40 and 60%, respectively, compared with littermate control mice) but not in  $TR\beta^{-/-}$  (Fig. 1A,C). In  $Pax8^{-/-}$  mice this defect could be rescued by TH injection within 48 hours (Fig. 1A,B). To identify the resident cell population affected by the lack of either TH or  $TR\alpha$ , splenocytes obtained from 2-week-old  $Pax8^{-/-}$  mice treated or not treated with TH were compared with littermate controls by flow cytometry. For this, B220 and TER119 cell surface markers were used to identify B and erythrocytic cells, respectively, the two major cell types resident in the spleen. In addition to a lower number of B220<sup>+</sup> B cells, as described previously (Arpin et al., 2000), a more drastic decrease (5-fold) of total TER119<sup>+</sup> cells was observed in the spleen of  $Pax8^{-/-}$  (Fig. 1E) and of  $TR\alpha^{0/0}$  mice, but not  $TR\beta$  mice (data not shown). Indeed, the relative ratio of B220<sup>+</sup> to TER119<sup>+</sup> cells was inverted in  $Pax8^{-/-}$  mice compared with control littermates (Fig. 1D). This defect was fully restored upon TH injection into  $Pax8^{-/-}$  mice, suggesting that the lack of erythrocytic cells was mainly responsible for the size reduction of spleens in these mice. Overall, these results suggest that spleen erythropoiesis is influenced by TH via  $TR\alpha$  during early postnatal development.

The defect of spleen erythropoiesis observed in the  $TR\alpha^{0/0}$  mutants seems to be constitutive and not the result of a delay in onset of splenic erythropoiesis. Indeed in 3-week-old  $TR\alpha^{0/0}$  animals, the total number of spleen TER119-positive cells did not increase compared with 2-week-old animals and was still deeply below that of wild type of the same age (Fig. 1F).

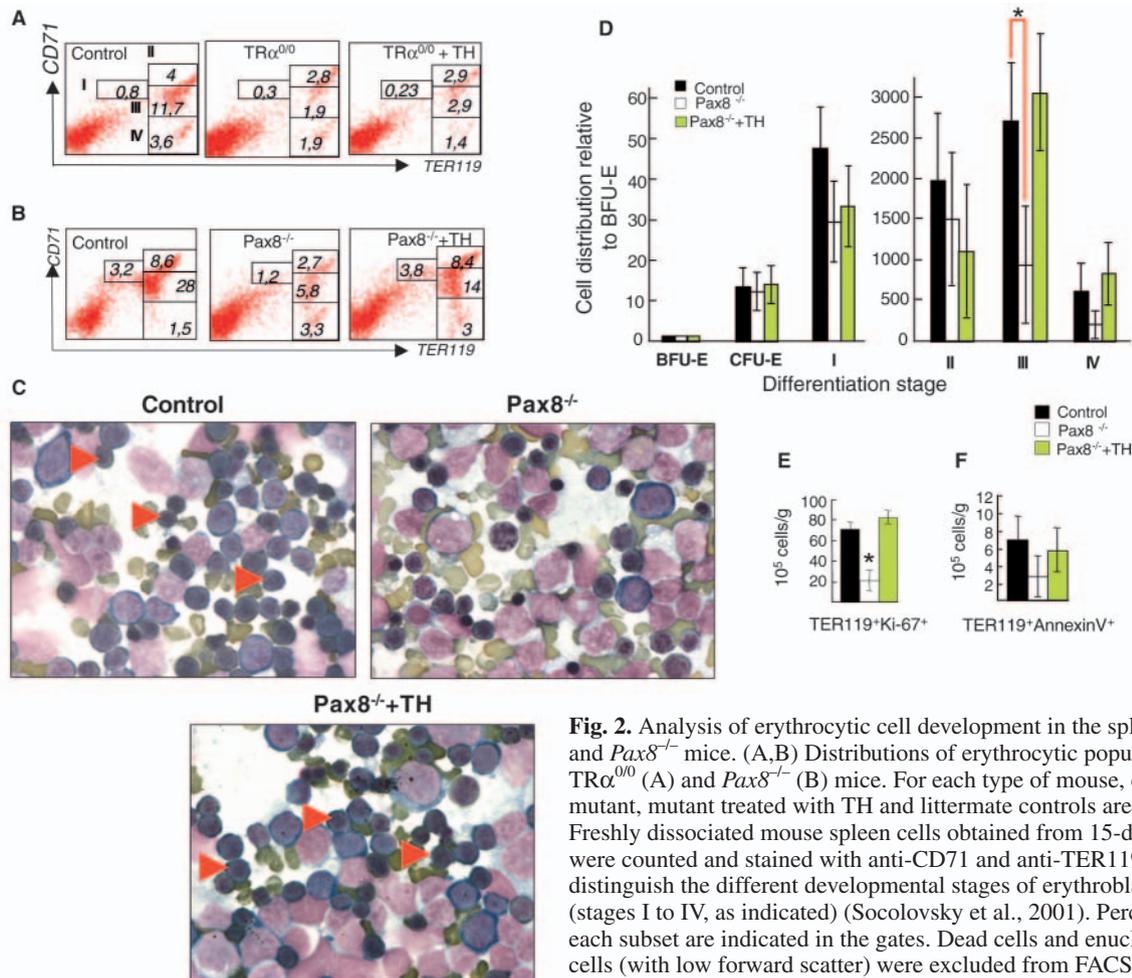
### Late basophilic erythroblasts are the spleen cells mainly affected by TH/ $TR\alpha$ deficiency

The expression of the surface markers CD71 and TER119, which define specific steps of differentiation of erythroblast precursors toward terminally differentiated enucleated red blood cells, was used to identify the population affected by TH/ $TR\alpha$  deficiency. CD71<sup>high</sup>TER119<sup>med</sup> (fraction I) defines pro-erythroblasts; CD71<sup>high</sup>TER119<sup>high</sup> (fraction II) defines basophilic erythroblasts; CD71<sup>med</sup>TER119<sup>high</sup> (fraction III) defines late basophilic erythroblasts; and CD71<sup>low</sup>TER119<sup>high</sup> (fraction IV) defines the orthochromatophilic erythroblasts (Socolovsky et al., 2001). A strong defect in the distribution of TER119<sup>+</sup> cells was observed in splenocytes of 2-week-old hypothyroid  $Pax8^{-/-}$  and  $TR\alpha^{0/0}$  mice (Fig. 2A,B): fraction III (CD71<sup>med</sup>TER119<sup>high</sup>) representing late basophilic erythroblasts was reduced by at least 5-fold in these mutant mice. TH injection into  $Pax8^{-/-}$  mice, but not into  $TR\alpha^{0/0}$ , resulted in an increase of this fraction after only 48 hours, suggesting that this is the erythrocytic population mostly affected by TH. Cytological analysis confirmed the decreased number of late basophilic erythroblasts in  $Pax8^{-/-}$  mice and their reappearance upon TH treatment (Fig. 2C, arrows). These



**Fig. 1.** Spleen cellularity and composition of spleens in control and mutant mice. (A) Spleens of control,  $Pax8^{-/-}$  and  $Pax8^{-/-}$  TH-treated mice. (B,C) Spleen cellularity in controls and  $Pax8^{-/-}$  mice, treated or not with TH, and in controls,  $TR\alpha^{0/0}$  and  $TR\beta^{-/-}$  mice. Nucleated splenocytes from 15-day-old mice were counted and cell numbers plotted as a cellularity index expressed as  $10^5$  cells/g of body weight. [\* $P < 0.005$  compared with control; \*\* $P < 0.005$  compared with  $Pax8^{-/-}$ ,  $n = 20$  for control,  $n = 20$  for  $Pax8^{-/-}$ ,  $n = 4$  for  $Pax8^{-/-}$  after 3 and 24 hours,  $n = 10$  for  $Pax8^{-/-}$  after 48 hours,  $n = 13$  for  $TR\alpha^{0/0}$  and  $n = 5$  for  $TR\beta^{-/-}$ ]. (D) Analysis of spleen populations in wild-type,  $Pax8^{-/-}$  and TH-treated  $Pax8^{-/-}$  mice. Splenocytes were analyzed by flow cytometry using anti-B220 and anti-TER119 antibodies to identify cells belonging to the B and erythrocytic compartments, respectively. Numbers in the FACS profiles indicate percentages of the respective populations. (E) Total number of TER119<sup>+</sup> and B220<sup>+</sup> positive spleen subpopulations in wild-type,  $Pax8^{-/-}$  and TH-treated  $Pax8^{-/-}$  mice, plotted as a cellularity index (\*\* $P < 0.002$ ; \* $P = 0.05$  compared with control;  $n = 7$  for control,  $n = 9$  for  $Pax8^{-/-}$  and  $n = 5$  for  $Pax8^{-/-}$  + TH). (F) Comparison of numbers of TER119<sup>+</sup> spleen subpopulations between 15-day-old and 21-day-old wild-type and  $TR\alpha^{0/0}$  mice (\* $P = 0.05$  compared with control;  $n = 3$ ).

results suggest that TH through  $TR\alpha$  influences the late and not the early phases of erythrocytic differentiation. To have an



**Fig. 2.** Analysis of erythrocytic cell development in the spleen of  $TR\alpha^{0/0}$  and  $Pax8^{-/-}$  mice. (A,B) Distributions of erythrocytic populations in  $TR\alpha^{0/0}$  (A) and  $Pax8^{-/-}$  (B) mice. For each type of mouse, data from mutant, mutant treated with TH and littermate controls are presented. Freshly dissociated mouse spleen cells obtained from 15-day-old mice were counted and stained with anti-CD71 and anti-TER119 antibodies to distinguish the different developmental stages of erythroblast maturation (stages I to IV, as indicated) (Socolovsky et al., 2001). Percentages of each subset are indicated in the gates. Dead cells and enucleated red blood cells (with low forward scatter) were excluded from FACS analysis. (C) Benzidine-MGG-stained cytopsin preparations of freshly isolated

spleen cells from wild-type,  $Pax8^{-/-}$  and TH-treated  $Pax8^{-/-}$  mice, as indicated. The red arrowheads point to representative late basophilic erythroblasts. (D) Graphical representation of the distribution of the respective erythrocytic populations relative to the BFU-E population. For each tested mouse, the frequency of each cell population was divided by that of the BFU-Es. Numbers of animals analyzed were 5 for the control and  $Pax8^{-/-}$  and 4 for the  $Pax8^{-/-}$ +TH. (\*,  $P=0.003$ ). (E,F) Total numbers of nucleated cells  $TER119^{+}KI67^{+}$  and  $TER119^{+}AnnexinV^{+}$ , respectively, plotted as a cellularity index in control,  $Pax8^{-/-}$  and TH-treated  $Pax8^{-/-}$  mice. Spleen cells from 15-day-old mice were counted and analyzed by flow cytometry using anti-TER119 together with either anti-KI67 (E) or anti-Annexin V (F) antibody (\*  $P<0.01$ ;  $n=3$  for each genotype and treatment).

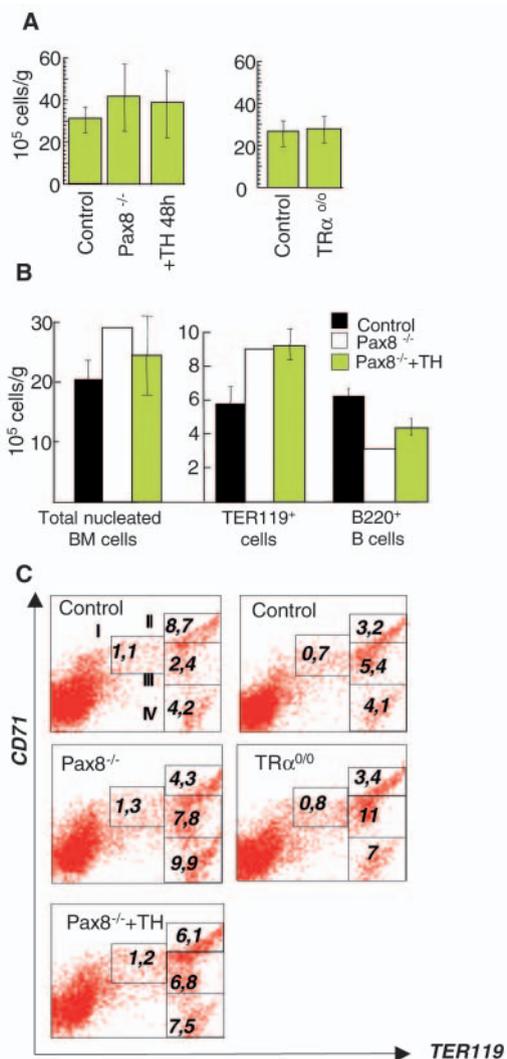
overview of the whole sequence of differentiation, we determined the relative frequencies of each differentiation step, including CFU-Es and BFU-Es (these two last progenitors being determined by in-vitro colony assays). The respective frequencies of all erythrocytic progenitors were normalized to that of the BFU-Es. As shown in Fig. 2D, the  $Pax8^{-/-}$  mice showed a specific defect in the relative number of late basophilic erythroblasts (fraction III) compared with control mice. By contrast,  $Pax8^{-/-}$  mice treated with TH for 48 hours showed a strong enhancement in the representation of these same cells. This observation confirms that the defect in erythropoiesis in the mutant  $Pax8^{-/-}$  mice resides at the level of production of late basophilic erythroblasts. To determine if TH controls proliferation or survival of late basophilic erythroblasts,  $TER119^{+}$  splenocytes obtained from control,  $Pax8^{-/-}$ , and  $Pax8^{-/-}$  mice treated with TH, were stained with antibodies against the Ki-67 antigen or Annexin V, markers of proliferation and apoptosis respectively. Although not

statistically different, the number of apoptotic cells was slightly decreased in  $Pax8^{-/-}$  mutant mice compared with controls (Fig. 2F). Clearly the major effect of the mutation was a decrease in the number of  $TER119^{+}KI67^{+}$  proliferating cells (Fig. 2E).  $TER119^{+}KI67^{+}$  double positive cells strongly increased upon TH treatment of  $Pax8^{-/-}$  mice. Thus, TH affects mostly the proliferation of late basophilic erythroblasts.

### Bone marrow erythropoiesis compensates the spleen erythropoiesis defect in young hypothyroid mutant mice

Despite a defect in spleen erythropoiesis, hypothyroid  $Pax8^{-/-}$  mutant mice are, with rare exception, not anemic, as measured by hematocrit values (not shown). This suggests that the defect in red blood cell production in the spleen may be compensated in another erythropoietic organ. Given that fetal liver ceases its activity at birth, the bone marrow was analyzed in young mutant mice. Overall, bone marrow cellularity of both  $Pax8^{-/-}$

and  $TR\alpha^{0/0}$  mice was normal (Fig. 3A). A small decrease in the number of  $B220^+$  cells was observed (Fig. 3B), as described previously for another  $TR\alpha$ -defective mutant mouse strain (Arpin et al., 2000), together with a concomitant increase in the number of  $TER119^+$  cells (Fig. 3B). By contrast to the spleen, TH treatment of  $Pax8^{-/-}$  mice had no effect on bone marrow erythropoiesis. FACS analysis, using the erythrocytic markers  $CD71$  and  $TER119$ , showed no significant skewing in



**Fig. 3.** Bone marrow erythropoiesis in  $Pax8^{-/-}$  and  $TR\alpha^{0/0}$  mutants. (A) Bone marrow cellularity in  $Pax8^{-/-}$ , TH-treated  $Pax8^{-/-}$  mice,  $TR\alpha^{0/0}$  mice and respective controls. Bone marrow nucleated cells obtained from 15-day-old mice were counted and cell numbers plotted as a cellularity index ( $n=3$  for each genotype). (B) Analysis of resident BM populations in  $Pax8^{-/-}$  mice ( $n=3$  except for  $Pax8^{-/-}$ , where  $n=2$ ). (C) Distribution of the various erythrocytic cell subpopulations in the bone marrow of  $Pax8^{-/-}$  and  $TR\alpha^{0/0}$  mice. Due to the different genetic backgrounds, each mutant was compared with its appropriate control littermate in the same column. Bone marrow cells obtained from 15-day-old mice were analyzed by FACS using the anti- $CD71$  and anti- $TER119$  antibodies to distinguish the different developmental stages of erythroblast maturation (stages I to IV). Percentages of each subset are indicated in the gates. Dead cells and enucleated red blood cells (with low forward scatter) were excluded from FACS analysis. One representative example, out of four, is shown here.

the number of bone marrow erythrocytic cell populations in  $TR\alpha^{0/0}$  and  $Pax8^{-/-}$  mutant mice, yet erythropoiesis was enhanced in these mice compared with their littermate controls (Fig. 3C). These results indicate that in young hypothyroid and  $TR\alpha^{0/0}$  mice, the bone marrow compensates the erythropoiesis defect of spleen by a mechanism that is not dependent on TH and  $TR\alpha$ .

### TH exerts a direct effect during erythroblast differentiation in ex-vivo primary cultures

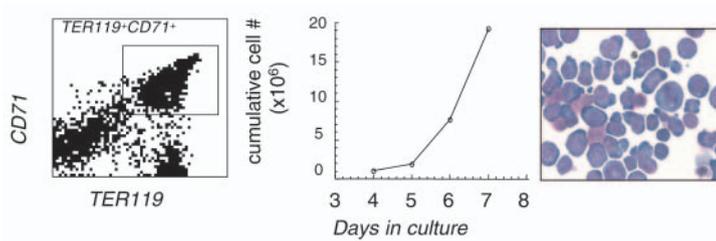
To dissect the role of TH during erythropoiesis, primary erythroblast cultures were developed from spleen and bone marrow. For spleen culture, the technique was adapted from the previously described technique established for fetal liver and bone marrow (von Lindern et al., 2001; Kolbus et al., 2002). Under these conditions, erythroblast proliferation is dependent on the presence of erythropoietin (EPO), stem cell factor (SCF), and dexamethasone (DEX), and differentiation into enucleated erythrocytes is induced upon withdrawal of SCF and DEX and addition of insulin (von Lindern et al., 2001; Kolbus et al., 2002). Ten-day-old SV129 mice were used to obtain splenocytes enriched in  $CD71^+$  and  $TER119^+$  double-positive erythroblasts. At this stage of development, the spleen is highly enriched in erythroblasts double positive for the erythrocytic markers  $CD71^+$  and  $TER119^+$  (Fig. 4A). To further enrich for erythroblasts, total splenocytes were maintained in erythroid medium to favor their proliferation (Fig. 4B), but not their differentiation, as described (von Lindern et al., 2001). Under these conditions, 99% of large, undifferentiated, hemoglobin-negative erythrocytic progenitors were obtained, as shown by cytological analysis (Fig. 4C). Bone marrow cultures were performed exactly as previously described by Kolbus et al. (Kolbus et al., 2002).

Erythroblasts grown in culture were induced to terminally differentiate in the presence or absence of TH and monitored daily for cell proliferation and morphology (Fig. 5). In the spleen cultures, T3 induced an increase in the number of differentiated cells visible 40–43 hours after induction of differentiation compared with non-treated cultures (Fig. 5A). This effect was paralleled by a more rapid accumulation of hemoglobin-positive and enucleated differentiated erythrocytes over hemoglobin-negative cells in T3-treated cultures (Fig. 5B). By contrast, in bone marrow culture, T3 had no effect on the number of differentiated cells (Fig. 5C) and did not increase the proportion of Hb-positive cells (Fig. 5D). No effect of T3 was observed in erythroblast cultures derived from spleen or bone marrow from  $TR\alpha^{0/0}$  mice (Fig. 5E,F).

Taken together, these results indicate that T3 via  $TR\alpha$  directly enhances or accelerates the differentiation of spleen erythroblasts, in their progression into mature hemoglobin-positive cells.

### $TR\alpha$ affects the ability of erythrocytic progenitors to develop in spleens of lethally irradiated mice

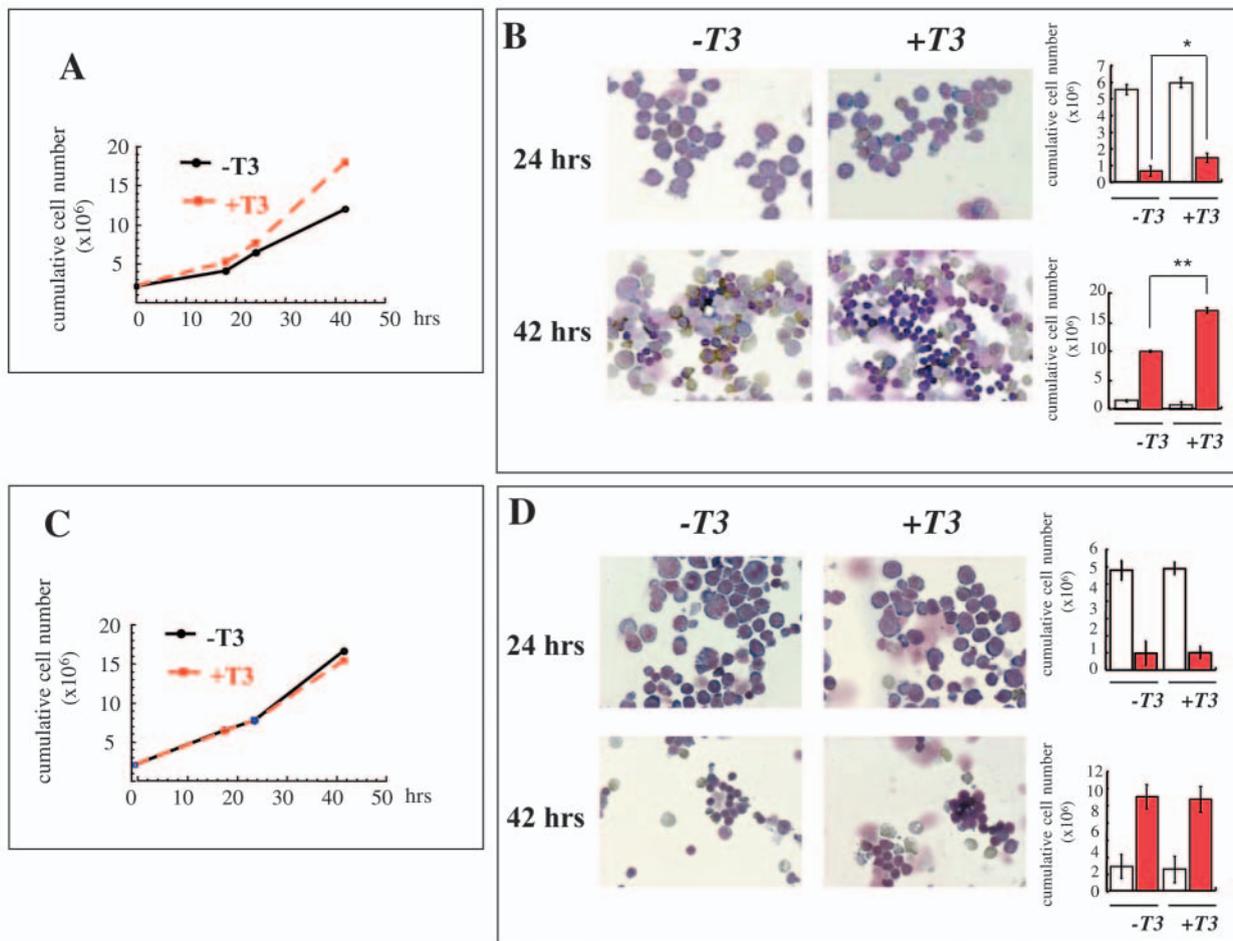
The results described above suggest a direct effect of TH on spleen erythropoiesis in vivo and ex vivo. However, the situation may be more complex in vivo, due to the pleiotropic effects of TH and the possibility that TH influences erythropoiesis indirectly by acting on the spleen environment. To address this question, lethally irradiated adult wild-type and  $TR\alpha^{0/0}$  mice were reconstituted with splenocyte suspensions



**Fig. 4.** Establishment of primary cultures of spleen erythroblasts in proliferation conditions. (A) Freshly dissociated mouse splenocytes obtained from 10-day-old wild-type mice were stained with anti-CD71 and anti-TER119 antibodies to estimate the amount of TER119<sup>+</sup>/CD71<sup>+</sup> erythroblasts before plating in serum-free medium under conditions that favor proliferation of erythroblasts. (B) Cumulative cell number of erythroblasts determined daily by Trypan Blue exclusion. (C) MGG and neutral benzidine staining of cytospin preparations obtained from proliferating erythroblasts analyzed on day 7.

isolated from 10-day-old TR $\alpha^{0/0}$  or wild-type mice. Eleven days after reconstitution, grafted mice were sacrificed and their spleens analyzed. At this time, the hematopoietic reconstitution consisted mainly of cells of the erythrocytic lineage, as observed by benzidine and MGG staining (Fig. 6D-F) and by

FACS analysis (not shown). Macroscopically, spleens obtained from wild-type mice reconstituted with TR $\alpha^{0/0}$  splenocytes were smaller than spleens reconstituted with wild-type cells, while no major differences were observed in TR $\alpha^{0/0}$  mice reconstituted with wild-type cells (not shown). In wild-type



**Fig. 5.** Effect of T3 on the differentiation of spleen and bone marrow erythroblast primary cultures. After 7 days in culture, in proliferation conditions, erythroblasts derived from either spleen or bone marrow were switched to medium that favors their differentiation in the presence or absence of  $10^{-7}$  mol T3. (A,C) Growth curves of respectively spleen- and bone-marrow-derived erythroblasts. Cell numbers during differentiation were determined at the indicated timepoint by Trypan Blue exclusion. (B) Spleen culture and (D) bone marrow culture show the morphology of differentiating cells after staining with MGG and neutral benzidine together with the corresponding cumulative numbers of hemoglobin-positive (red bars) and hemoglobin-negative (white bars) cells in the presence or absence of T3 (\* $P < 0.05$ ; \*\* $P < 0.01$ ;  $n = 3$  for each condition). The numerous small brownish bodies in B at 42 hours in the presence of T3 represent orthochromatophilic erythroblasts with a thin rim of cytoplasm and a highly condensed nucleus and expelled nuclei. These orthochromatophilic erythroblasts were scored as hemoglobin-positive cells. (E,F) Cumulative numbers of differentiating cells and hemoglobin-positive and hemoglobin-negative cells in cultures of TR $\alpha^{0/0}$  spleen (E) and bone marrow (F) cultures. (No statistical differences were observed in hemoglobin-positive cell numbers between -T3 and +T3 at the respective times;  $n = 3$  for each condition).

**Table 1. Phenotypic analysis of reconstituted spleens**

Donor	Recipient	Total number of cells per recipient spleen ( $\times 10^6$ )	Total nucleated HB <sup>+</sup> erythroblasts per recipient spleen ( $\times 10^6$ )	Total nucleated HB <sup>-</sup> erythroblasts per recipient spleen ( $\times 10^6$ )	Total CFU-Es per recipient spleen ( $\times 10^3$ )
Wild type (n=2)	Wild type (n=4)	70 $\pm$ 23	51 $\pm$ 22	11.5 $\pm$ 6	511 $\pm$ 57
TR $\alpha^{0/0}$ (n=2)	Wild type (n=4)	38.8 $\pm$ 8.7*	27 $\pm$ 9 <sup>†</sup>	10.2 $\pm$ 2.3	415 $\pm$ 72
Wild type (n=2)	TR $\alpha^{0/0}$ (n=2)	91 (70; 104)	53 (47; 81)	14.8 (14; 15.6)	595 (670; 520)

The total number of spleen cells of recipient mice was determined by counting Trypan Blue-excluding cells. Total numbers of hemoglobin-negative (HB<sup>-</sup>) and hemoglobin-positive (HB<sup>+</sup>) nucleated erythroblasts per spleen were calculated after determining the frequencies of these cells by counting up to 300 cells on cytopsin in randomly selected fields of the slide. Total numbers of CFU-Es were determined by multiplying the frequency of CFU-Es assessed in the in vitro colony assay by the spleen cellularity. Data represent mean $\pm$ s.d. except for the graft of wild type into TR $\alpha^{0/0}$  recipient mice, where only the mean is given, with the two individual values in parenthesis. (\* $P$ <0.05; <sup>†</sup> $P$ =0.05 in comparison with wild-type recipient grafted with wild-type donor cells). Data for each grafting combination were collected from two independent experiments.

recipient mice, grafted with TR $\alpha^{0/0}$  splenocytes, spleen cellularity was decreased by about 40% compared with wild-type mice grafted with wild-type cells. By contrast, no major defects were observed in spleen cellularity of recipient TR $\alpha^{0/0}$  mice grafted with wild-type splenocytes (Table 1). In agreement with this observation, histological examination on recipient spleen sections after hematoxylin and eosin coloration, showed significantly smaller hematopoietic colonies when wild-type mice had been reconstituted with TR $\alpha^{0/0}$  splenocytes (Fig. 6B); whereas no major difference was observed in TR $\alpha^{0/0}$  or wild-type recipient spleen grafted with wild-type splenocytes (Fig. 6A,C). These results indicate that TR $\alpha$  exerts a direct effect on erythrocytic cells and not on the spleen environment.

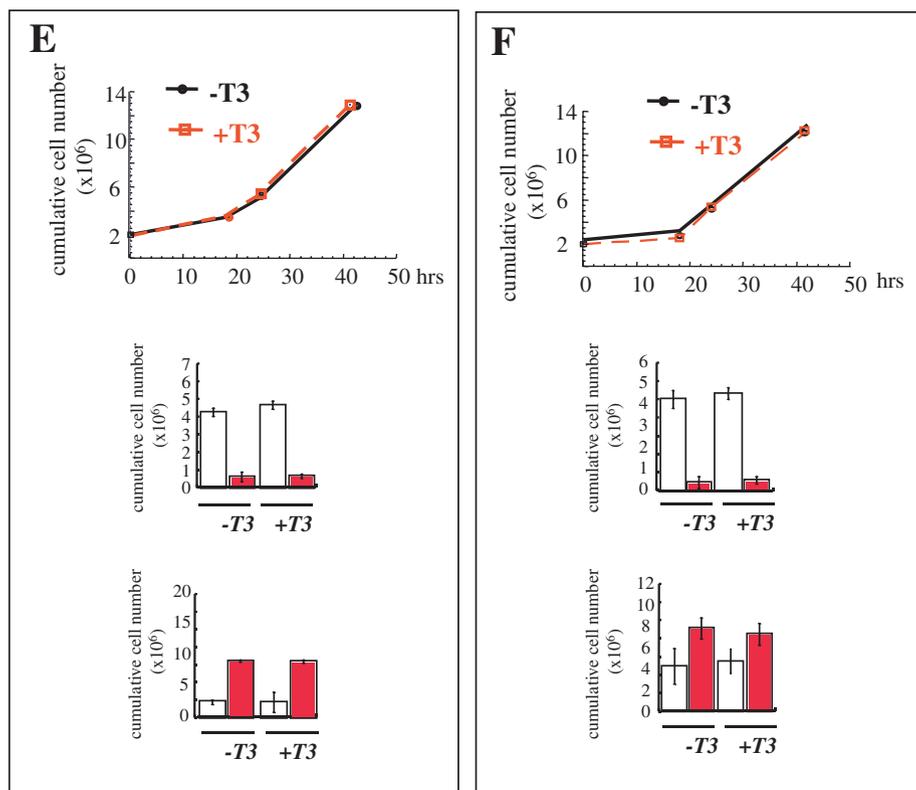
To further analyze erythrocytic cell production in recipient spleens, cytological analysis was performed on spleen smears. As presented in Fig. 6E and in Table 1, spleens of wild-type

mice grafted with TR $\alpha^{0/0}$  cells showed a significantly lower number of hemoglobin-positive erythroblasts compared with spleens of wild-type mice grafted with wild-type splenocytes (Fig. 6D), or to spleens of TR $\alpha^{0/0}$  mice grafted with wild-type splenocytes (Fig. 6F). Interestingly, the spleen of any of the recipients contained nearly the same number of early basophilic hemoglobin-negative erythroblasts. By contrast, spleens of the wild-type recipients grafted with TR $\alpha^{0/0}$  splenocytes contained less than half hemoglobin-positive erythroblasts, which were mostly basophilic, than spleens of wild-type recipients grafted with wild-type cells (Fig. 6D-E and Table 1). All these observations strongly support the view that in wild-type mice grafted with TR $\alpha^{0/0}$  spleen cells erythropoiesis in the spleen does not reach final differentiation. When the number of CFU-Es in recipient spleens was determined, no statistically significant differences were observed in any reconstituted situation, and the CFU-Es from

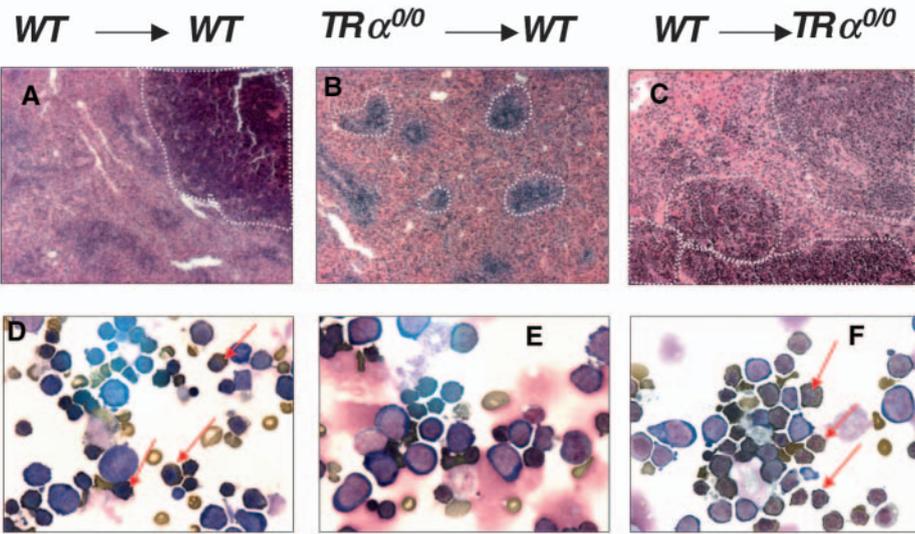
each origin gave rise ex vivo to colonies of similar sizes (data not shown), suggesting that the TR $\alpha^{0/0}$  CFU-Es undergo normal differentiation outside the spleen environment. These results support the conclusion that TR $\alpha^{0/0}$  erythrocytic progenitor cells are intrinsically impaired in their ability to undergo late steps of differentiation in the spleen environment in vivo.

## Discussion

The role of TH and TRs in erythropoiesis has been so far suggested only by indirect observations. In chickens, the *erbA* oncogene, a dominant negative form of TR $\alpha$ , blocks erythrocytic differentiation at the CFU-E stage (Gandrillon et al., 1989), yet in-vitro development of CFU-Es appears only slightly activated by T3 (Dainiak et al., 1986). In humans, hypothyroidism is frequently associated with anemia (Green and Ng, 1986). However, adult mice deficient in TH production or TRs expression did not show overt signs of erythropoiesis defects at the adult stage (Gauthier et



Donor → Recipient



**Fig. 6.** Erythropoietic reconstitution in lethally irradiated mice. Donor spleen cells from 10-day-old wild-type or  $TR\alpha^{0/0}$  mice were injected into irradiated adult mice. Eleven days after the grafting the adult recipient mice (wild-type or  $TR\alpha^{0/0}$ ) reconstituted with wild-type or  $TR\alpha^{0/0}$  cells, as indicated, were sacrificed and the hematopoietic reconstitution in the spleen was analyzed by examination of spleen paraffin sections after hematoxylin and eosin coloration to show the size of the hematopoietic colonies (colonies are delineated by white dotted lines in A–C), and by examination of cytopsin cells after neutral benzidine and MGG staining. Arrows in D and F point to hemoglobin-positive erythroblasts.

al., 1999; Gothe et al., 1999). TH plays a major role in the development of tissues at the transition between fetal and adult life in the mouse (Billon et al., 2001; Plateroti et al., 2001; Flamant et al., 2002; Harvey et al., 2002; Morte et al., 2002). Therefore, we wondered whether TH signaling might be necessary for the neonatal erythropoiesis in the spleen soon after birth in the mouse.

In mouse, erythropoiesis is regulated timely throughout life in three different organs (reviewed by Godin and Cumano, 2002). It starts in the yolk sac at embryonic day (E) 7.5 to give rise to primitive erythropoiesis, then in fetal liver to produce definitive erythrocytic cells. By E12, the fetal liver, colonized by hematopoietic stem cells (HSCs), becomes the main erythropoietic organ until birth (Delassus and Cumano, 1996; Robb, 1997; Dzierzak et al., 1998; Keller et al., 1999; Wolber et al., 2002). The bone marrow initiates production of red blood cells at birth and then constantly raises its production until adult age. The spleen is colonized at E12 by HSCs originating in the aorta-gonad-mesonephros area (Godin et al., 1999), but its erythropoietic potential is confined to the first two postnatal weeks. During this period, the spleen contains a number of early erythrocytic progenitors comparable to bone marrow, but its contribution to blood cell production might not be as much as bone marrow, and it is considered as an additional erythropoietic organ for stress conditions (Wolber et al., 2002). Even if BFU-Es are still found in constant numbers in the spleen between the pup and adult stages, the bone marrow remains by far the major erythropoietic organ during adult life (Wolber et al., 2002). The physiological mediators of the switch of erythropoietic activity from one organ to another are not yet known.

The results presented here demonstrate that TH via  $TR\alpha$  is required specifically for normal spleen, and not bone marrow, erythropoiesis during early postnatal development. Both mice deficient in TH production ( $Pax8^{-/-}$  congenital hypothyroid), and mice devoid of  $TR\alpha$  receptors ( $TR\alpha^{0/0}$ ), show a defect in spleen erythropoiesis soon after birth, which could be quickly rescued in  $Pax8^{-/-}$  mice upon injection of TH. Interestingly, the

defect in red cell production takes place at a late stage of erythrocytic differentiation. Indeed,  $Pax8^{-/-}$  and  $TR\alpha^{0/0}$  mice showed only minor changes in the relative frequencies of BFU-Es, CFU-Es and early erythroblasts, but a strong decrease in late basophilic erythroblasts. This population was also the one that increased sharply upon TH injection, as a consequence of enhanced proliferation. Yet, there was no major effect on the population at the next developmental stage, the orthochromatophilic erythroblasts. Several hypotheses may be put forward. First, this population is close to the terminal differentiation step and might have a longer turn over time. Second, many of these cells might be circulating cells coming from the bone marrow, thus masking a decreased production from the spleen. In agreement with this hypothesis, the defect in spleen erythropoiesis did not result in major anemia in mutant animals because of bone marrow erythropoiesis compensation.

One major question about the role of TH signaling on spleen erythropoiesis was to know whether it results from a direct effect on erythrocytic progenitors or from an indirect effect on the splenic environment. Two approaches showed that TH and  $TR\alpha$  work directly on erythrocytic cells. First, when cultured *ex vivo*, wild-type spleen erythroblasts showed enhanced proliferation and differentiation in the presence of T3, while T3 had no effect on splenic erythroblasts derived from  $TR\alpha^{0/0}$  mice. Second, when  $TR\alpha^{0/0}$  spleen cell progenitors were grafted into a wild-type spleen environment, the production of mature red cells was impaired, whereas the reverse grafting of wild-type progenitors into a  $TR\alpha^{0/0}$  spleen environment gave rise to normal erythropoietic production. These grafting experiments demonstrate that the defect is intrinsic to  $TR\alpha$ -expressing erythrocytic progenitors. This conclusion is consistent with the observation that spleen erythroblasts do express  $TR\alpha$  mRNAs and presumably the receptor (not shown).

We ignore at present the reasons for the specificity of TH on spleen erythrocytic cells, as  $TR\alpha$  mRNA expression was also detected in bone marrow erythroblasts (not shown). It is possible that  $TR\alpha$  is active only in splenic erythroblasts

because of the presence of specific transcription co-activators or co-repressors, themselves under the control of specific splenic signals. Alternatively, different progenitors may exist in the spleen and in the bone marrow. Proof of the presence of a specific erythroblast precursor in the spleen has been obtained so far only under conditions of stress-induced erythropoiesis in adult anemic mice (Bauer et al., 1999). However, it is not clear if this precursor is already present in the spleen during early postnatal development and if it is under TH control. Finally, it is possible that erythrocytic differentiation normally depends on interactions with the spleen environment and that this interaction is defective in TH/TR $\alpha$  mutant mice. Our data support this last possibility. Indeed, whereas TR $\alpha^{0/0}$  erythrocytic progenitors cannot completely achieve differentiation within the wild-type spleen environment, they can undergo full red cell differentiation when explanted in the in-vitro CFU-E assay (see Table 1), suggesting that the mutant CFU-Es do not recognize the wild-type spleen environment necessary for differentiation.

Whereas in neonatal spleen endogenous TR $\alpha^{0/0}$  progenitors show a limited differentiation, they show a higher extent of erythrocytic differentiation when grafted into irradiated wild-type recipient spleens. One explanation could be that the splenic environment of irradiated adult recipient animals might allow some differentiation of TR $\alpha^{0/0}$  erythrocytic progenitors in contrast to the normal newborn spleen environment.

Together, these data lead us to propose the following model for the role of TH/TR $\alpha$  signaling in erythropoiesis of the mouse. In the bone marrow, TH/TR $\alpha$  signaling is not necessary for the pluripotent hematopoietic stem cells to achieve erythrocytic differentiation. On the contrary, in the spleen the stem cells give rise to erythrocytic progenitors (BFU-Es and CFU-Es) whose final differentiation depends on functional interactions with the spleen microenvironment. T3 via TR $\alpha$  would induce in red cell progenitors the synthesis of TR $\alpha$ -target gene products that would mediate this environmental interaction. Integrins are probable candidates, as homing of hematopoietic progenitors in different organs is regulated by integrin expression, as for example expression of  $\alpha 4/\beta 1$  integrins and hematopoietic progenitors homing in the bone marrow (Scott et al., 2003). Thus, TR $\alpha$  may act by modulating the expression of integrins in erythrocytic progenitors that home and differentiate specifically in the spleen. Interestingly, in chicken erythrocytic progenitors blocked by the ErbA oncoprotein, we have shown that the oncoprotein abrogates the expression of the gene encoding the  $\alpha 2/\beta 1$  integrin expression (Mey et al., 2002). However, we did not observe any inhibition in the expression of this integrin in the spleen erythrocytic cells of TR $\alpha^{0/0}$  mice (data not shown), suggesting that in mouse a different set of integrins or proteins interacting with the extracellular matrix might be involved. Alternatively, one could also imagine that the wild-type spleen environment expresses compounds that inhibit final differentiation of erythrocytic progenitors and that wild-type progenitors but not TR $\alpha^{0/0}$  progenitors have an intact signaling mechanism to overcome or abrogate this inhibition.

The fact that spleen-derived erythroblasts explanted in liquid cultures could be stimulated by T3 suggests that besides controlling recognition of the homing environment, T3/TR $\alpha$  signaling activates other physiological functions in spleen erythrocytic cells. It should be noticed that this direct effect of

T3 seen in vitro is not a determinant for the final differentiation under these experimental conditions.

The observation that alteration of spleen erythropoiesis is more severe in the Pax8<sup>-/-</sup> hypothyroid mutant than in the TR $\alpha^{0/0}$  mice is consistent with the hypothesis that in the hypothyroid mutant erythroblasts, these target genes are strongly repressed by the TR $\alpha$  apo-receptors. This assumption is supported by the fact that the spleen defect phenotype in Pax8<sup>-/-</sup> mice is partially overcome by inactivation of the TR $\alpha$  gene in these same mutants (Flamant et al., 2002).

This model in which T3/TR $\alpha$  signaling controls spleen erythropoiesis through spleen environment recognition would explain why spleen erythropoiesis develops at birth in the mouse. Indeed, at this time the level of T3 in the body of the pups sharply increases, peaking at the second week (Hadj-Sahraoui et al., 2000). This level then decreases to reach the adult basal level. The role of T3 would then be to support the development of a transient erythropoietic activity between the declining fetal liver erythropoiesis and the starting bone marrow erythropoiesis. Thus, while it is clear that T3 is not absolutely required for general erythropoiesis, our results show that it plays an important role in accelerating red blood production at an important changing state during development. Our data and the model we propose add one more example after intestine (Plateroti et al., 2001), heart (Mai et al., 2004) and brain (Morte et al., 2002), showing that TR $\alpha$  plays a major role in mouse ontogeny by managing the transition between fetal and postnatal life. Therefore, the early postnatal period represents a physiologic hyperthyroid situation that aims presumably at switching on, via the transcription factor TR $\alpha$ , gene expression programs involved in the maturation of many developmental processes and physiological functions in an overall mechanism that recalls TH/TR $\alpha$ -dependent amphibian metamorphosis (Shi, 1999; Sachs et al., 2002; Buchholz et al., 2003).

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