Unsuspected role of the brain morphogenetic gene *Otx1* in hematopoiesis

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***Otx1* belongs to the paired class of homeobox genes and plays a pivotal role in brain development. Here, we show that *Otx1* is expressed in hematopoietic pluripotent and erythroid progenitor cells. Moreover, bone marrow cells from mice lacking *Otx1* exhibit a cell-autonomous impairment of the erythroid compartment. In agreement with these results, molecular analysis revealed decreased levels of erythroid genes that include the *SCL* and *GATA-1* transcription factors. Accordingly, a gain of function of SCL rescues the erythroid deficiency in *Otx1*$^{-/-}$ mice. Taken together, our findings indicate a function for *Otx1* in the regulation of blood cell production.**

There is growing evidence suggesting that common cellular and molecular mechanisms orchestrate differentiation in various tissues (1–3). Homeobox-containing genes seem to be strong candidate genes to regulate a number of developmental processes, including neurogenesis and hematopoiesis (4–8).

Members of the *Otx* family (*Otx1*, *Otx2*, *Otx3*, and *Crx*) are the vertebrate homologues of the *Drosophila* head gap gene *orthodenticle* and encode transcription factors containing a bicoid-like homeodomain (9, 10). They are temporally and spatially regulated during development and seem to be required for proper head and sense organ patterning. *Otx1*, *Otx2*, and *Otx3* show partially overlapping, but distinct expression patterns, and *Otx2*, the first to be activated during development, plays a major role in gastrulation and in the early specification of the anterior neural plate. In contrast, *Otx1* shows a later onset and is involved in corticogenesis, sense organ development, and pituitary function (9). Mice bearing targeted deletion of *Otx1* are affected by a permanent epileptic phenotype and show multiple brain abnormalities and morphological defects of the acoustic and visual sense organs (11). In addition, at the prepubescent stage, they exhibit transient dwarfism and hypogonadism because of low levels of pituitary hormones (12). In the present study, we have investigated whether *Otx1* also plays a role in blood cell production, as several homeobox genes of different families are involved in normal and/or malignant hematopoiesis (13–16).

Materials and Methods

Mice. *Otx1*$^{-/-}$ (C57BL/6) mice (11) were kept according to institutional regulations. *SCL* transgenic (C57BL/6) mice in which the *SCL* transgene encoding the p22 isoform was placed under the *Sil* promoter have been described (17). Mice were genotyped by PCR as described (11, 18). These mice were interbred to generate *Otx1*$^{-/-}$*SCL*^{tg} mice.

RT-PCR. RNA was prepared from hematopoietic tissues. Where indicated, *SCL*, *Otx1*, and *GAPDH* were analyzed by RT-PCR as reported (18). Primer sequences are shown separately. The identity of the amplified product was confirmed through hybridization with an internal oligonucleotide. For colony cells, as cell number was limiting, cDNA was first globally amplified with a method described in detail (19, 20), then subjected to a second run of targeted amplification with specific primers.

Slot blots from globally amplified cDNAs from adult hematopoietic progenitors have been described (19, 20). Probes used were designed to cover the 3′ UTR sequences. The sequence for the murine *Otx1* transcript available from the National Center for Biotechnology Information (accession no. AF424700) lacked a polyadenylation signal. It was extended *in silico* to a poly(A) signal 1,475 bases downstream by using ESTs in the corresponding Unigene cluster Mm.129663 and genomic sequence. The extent and validity of the extension is supported by mouse ESTs and orthologous human and rat data. The additional information is accessible now in RefSeq NM_011023. A probe spanning positions 2289–2476 was prepared by PCR. Ribosomal *L32* was a murine genomic 1.6-kb *Sac*I fragment encompassing the final exon (21). Hybridization signals on x-ray films were quantified by scanning densitometry.

Blood Parameters. Heparinized blood was collected immediately after the mice died. Blood cell counts were performed with a cell counter (model H10, SEAC, Calenzano, Italy).

***In Vitro* Colony Assays.** The culture conditions have been described (22). Briefly, bone marrow cells were flushed from femurs and tibias in Iscove's modified Dulbecco's medium (IMDM) with 2% FCS and cultured at a maximum density of 10^5 per ml in IMDM containing α-thioglycerol, methyl cellulose, BSA, 5% FCS, iron-saturated transferrin, lecithin, oleic acid, cholesterol, 2 units/ml human recombinant erythropoietin, 10 ng/ml murine stem cell factor, and 10 ng/ml murine IL-3. Cultures were scored at 2, 7, and 12 days with an inverted microscope by using standard criteria.

Bone Marrow Transplantation. C57BL/6 female recipient mice, 8–12 weeks old, were lethally irradiated with 800 rads (x-ray source). Two hours after irradiation, they were i.v. injected with 10^6 bone marrow cells, derived from male donors, and resuspended in Iscove's modified Dulbecco's medium. Controls were injected with cell-free suspending medium. Engrafted animals were killed and analyzed 4–6 months after transplantation.

Results and Discussion

As a first step, we analyzed *Otx1* expression in different hematopoietic tissues, using RT-PCR. Results, illustrated in Fig. 1*a*,

Abbreviation: EB, embryoid bodies.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. NM_011023).

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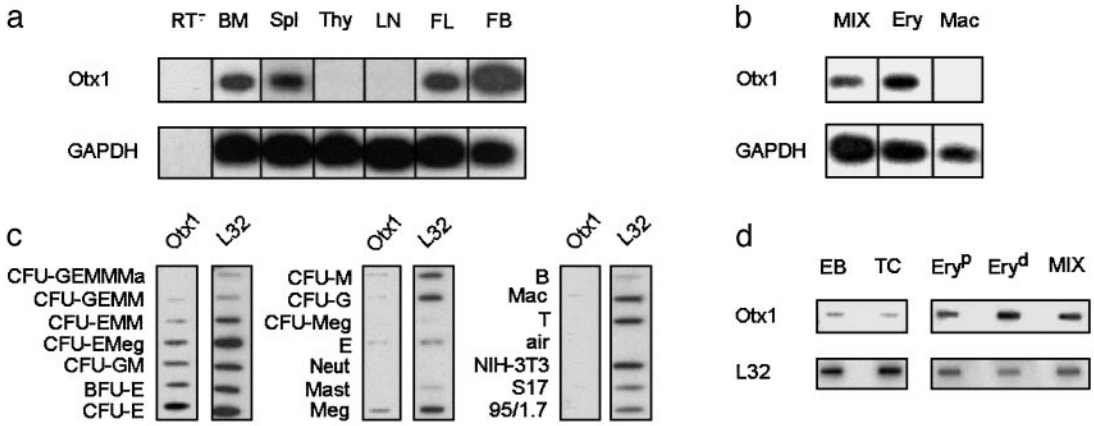

Fig. 1. Expression of *Otx1* in hematopoietic cells. (*a*) *Otx1* expression in hematopoietic tissues: bone marrow (BM), spleen (Spl), thymus (Thy), lymph nodes (LN), fetal liver (FL), and fetal brain (FB). RT^- represents the negative control. (*b*) Analysis of hematopoietic colonies *in vitro* from mouse bone marrow cultures: mixed (Mix), erythroid (Ery), and macrophage (Mac) colonies. Six colonies of each type, derived from three different mice, have been tested. (*c*) Slot blot of amplified cDNAs from adult hematopoietic pluripotent precursors (CFU-GEMMMa, CFU-GEMM, and CFU-EMM); bipotential precursors of erythroid-megakaryocytic (CFU-EMeg) and myelo-monocytic lineages (CFU-GM); erythroid early (BFU-E) and late (CFU-E) precursors, monocytic (CFU-M), granulocytic (CFU-G), and megakaryocytic (CFU-Meg) precursors; and terminally differentiated erythroid (E), myelo-monocytic (Neut, Mast, Mac), megakaryocytic (Meg), and lymphoid (B, T) cells. None, NIH 3T3, 95/1.7, and S17 represent control lanes containing primer elongation products and three fibroblastic cell lines. Respective sample arrangements are detailed in ref 20. (*d*) Slot blot of amplified cDNAs from embryonic stem cells induced into hematopoiesis: transitional colonies (TC), EB-derived primitive erythroid colonies (Ery^p), EB-derived definitive erythroid colonies (Ery^d), and EB-derived mixed colonies (MIX). *Otx1* was analyzed by RT-PCR with specific primers in *a* and *b* and by hybridization of globally amplified cDNA with a 3′ UTR *Otx1* probe. *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and *L32* probes were analyzed to normalize for the relative amount of cDNA present in each sample.

show that *Otx1* mRNA is present in the bone marrow and spleen of adult mice and in the fetal liver at 12 days of development. In contrast, *Otx1* signal was not detected in the thymus and lymph nodes. Fetal brain represents a positive control, as it is known that *Otx1* is highly expressed during brain morphogenesis (9). Because of the heterogeneity of bone marrow cells, we aimed at identifying the cell populations that express *Otx1*. Subsequently, we tested *Otx1* expression by RT-PCR in cells isolated from individual colonies grown in methyl cellulose medium after 4–7 days of culture. As illustrated in Fig. 1*b*, *Otx1* is expressed in erythroid cells (BFU-E-derived colony) and cells isolated from a colony containing multiple cell types (CFU-MIX-derived colony). In contrast, *Otx1* is undetectable in macrophage colonies. We next investigated *Otx1* expression in hematopoietic precursors, defined by their developmental potentials, i.e., their capacities to give rise to colonies containing distinct cell types. To this end, we used an experimental strategy based on sib analysis, involving single-cell RT-PCR and *in vitro* clonogenic assays of siblings that were separated at the initiation of culture (19, 20). Results shown in Fig. 1*c* indicate that *Otx1* expression is detectable at low levels in pluripotent precursors and persists in the bipotential progenitors of the erythroid-megakaryocytic and granulocytic-macrophage lineages. Furthermore, it is present in erythroid precursors and can still be detected in mature cells of the erythroid and megakaryocytic pathway.

During development, erythropoiesis first takes place in the yolk sac to produce a wave of primitive erythropoiesis, then moves to in the fetal liver, starting at embryonic day 12.5, where definitive erythropoiesis is initiated (23). At this point, the fetal liver contains both primitive and definitive erythroid cells. These two waves are recapitulated in culture, in differentiating embryonic stem cells. In addition, distinct steps of embryonic stem cell *in vitro* differentiation have been characterized, providing a model of the early events occurring *in vivo*. The initial stages involve the formation of embryoid bodies (EB), formed by progenitor cells of different tissues, followed by the appearance of transitional colonies containing hematopoietic and endothelial precursors (24). Finally, primitive erythroid precursors and subsequently definitive erythroid cells are produced. As illustrated in Fig. 1*d*, *Otx1* transcripts can be already detected in EB and transitional colonies. Furthermore, *Otx1* mRNA is present, apparently at even higher levels, in primitive and definitive erythroid progenitors and in pluripotent cells. These results indicate that the *Otx1* locus is transcriptionally active in various hematopoietic sites and is differentially expressed in bone marrow cells, especially in cells of the erythroid lineage.

To gain insights into the functional importance of Otx1 in blood cell production *in vivo*, we studied the effects of loss of Otx1 function in the hematopoietic system of *Otx1* null mice in which the gene has been disrupted by homologous recombination (11). Mature and progenitor cells of WT, $Otx1^{+/-}$, and $Otx1^{-/-}$ adult mice have been analyzed by using peripheral blood cell counts and *in vitro* clonogenic assays, respectively. *Otx1*-deficient mice show a decreased number of red blood cells, as compared with heterozygous and WT animals, and they exhibit a marked reduction of early (BFU-E) and late (CFU-E) erythroid progenitors (Fig. 2*a Left*). In contrast, we did not observe significant variations in the number of circulating leukocytes and myelo-monocytic precursors (Fig. 2*b Left*).

Decreased erythropoiesis could be caused by an intrinsic defect of $Otx1^{-/-}$ progenitors or an impaired microenvironment. Alternatively, erythropoiesis could also be defective as a consequence of endocrine dysregulation caused by the lack of Otx1. To distinguish between these possibilities, we compared the long-term reconstitution ability of $Otx1^{+/+}$, $^{+/-}$, and $^{-/-}$ bone marrow cells after transplantation into lethally irradiated recipients (25). Grafted mice were killed 4–6 months later and tested for circulating blood cells and bone marrow progenitors. Results, summarized in Fig. 2 *Right*, show that the animals reconstituted with *Otx1*-deficient cells exhibit a significantly lower number of red blood cells, as compared with mice that received WT marrow. Moreover, the number of CFU-E and BFU-E is reduced by 4- to 5-fold in the $Otx1^{-/-}$ reconstituted mice, as compared with control animals. In contrast, no significant difference among the three groups of mice was detected in the number of mature or progenitor cells of the myelo-monocytic lineage, as previously observed in untreated animals (Fig. 2*b*). Interestingly, mice grafted with null cells displayed a more severe

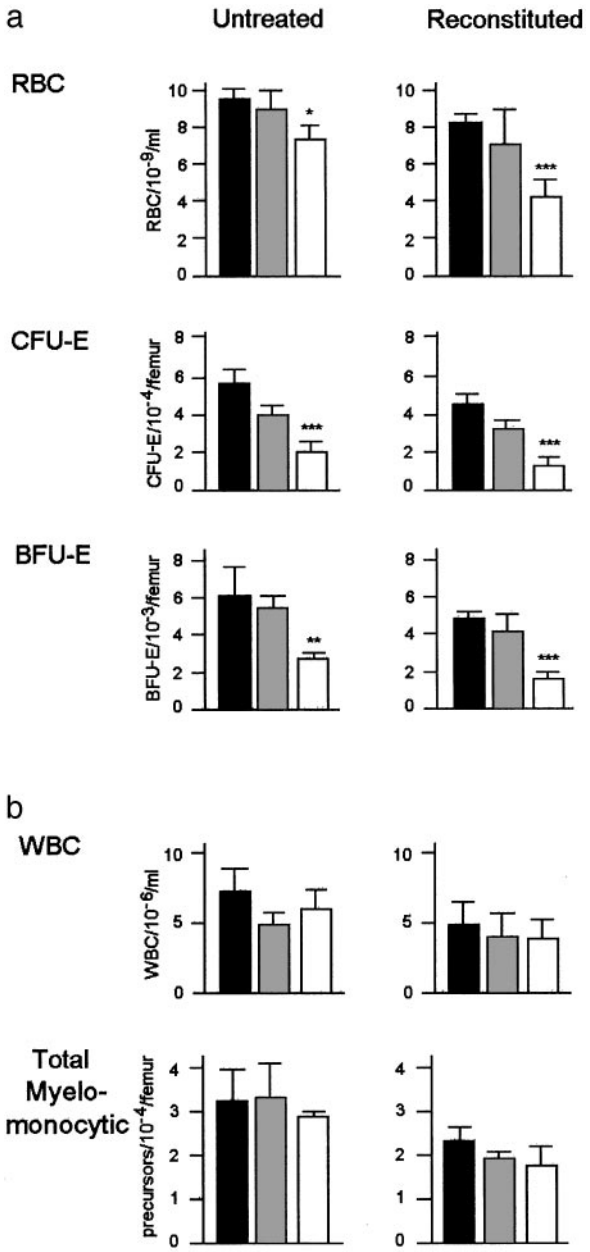

Fig. 2. Peripheral blood and bone marrow progenitors in WT (■), *Otx1*$^{+/-}$ (▨), and *Otx1*$^{-/-}$ (□) mice (Untreated, *Left*) and in animals transplanted with WT (■), *Otx1*$^{+/-}$ (▨), and *Otx1*$^{-/-}$ (□) bone marrow cells (Reconstituted, *Right*). Hematopoietic progenitors were evaluated by using clonogenic assays in methyl cellulose medium in the presence of stem cell factor, IL-3, and erythropoietin. Results show the average of duplicate determinations per bone marrow sample and are representative of a minimum of 10 mice for each group. Asterisks indicate significant variations from WT animals: *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$.

anemic phenotype than the untreated homozygous mutants, in which compensatory mechanisms possibly account for the limited reduction of erythrocytes as compared with the more significant drop of erythroid progenitors (Fig. 2*a*). Such mechanisms may not be sufficient to compensate the robust differentiation demand in the transplanted animals. Taken together, these data indicate that loss of Otx1 function is associated with impaired erythropoiesis, and the defect is cell autonomous.

The pattern of expression of *Otx1* is reminiscent of that of the basic helix–loop–helix transcription factor *SCL/TAL1* (26), which is also present in transitional colonies (24) and multipotent progenitors (19). Furthermore, *SCL* expression is highest in erythroid progenitors (BFU-E, CFU-E), and functional studies indicate that SCL is essential for erythroid cell differentiation (26, 27). Hence, we compared *SCL* expression in bone marrow cells derived from WT, heterozygous, and null mutant mice by semiquantitative RT-PCR. Fig. 3*a* shows that *Otx1*$^{-/-}$ bone marrow cells express low levels of *SCL* as compared with their normal counterparts. Quantitation of *SCL* expression levels by densitometry revealed a 2-fold reduction in *Otx1*-deficient cells, whereas *SCL* levels in cells harboring one functional *Otx1* allele were unaffected. Although densitometry provides a nonlinear quantitation of transcript levels, this molecular analysis is consistent with cellular analysis indicating a 2- or 3-fold decrease in CFU-E and BFU-E, as shown in Fig. 2. Because the erythroid cell impairment observed in *Otx1*-deficient mice was more severe in animals grafted with null cells, we next compared *SCL* expression in hematopoietic cells of mice transplanted with *Otx1*$^{+/-}$ or *Otx1*$^{-/-}$ cells. Results, shown in Fig. 3 *b* and *c*, indicate that *SCL* is expressed at significantly lower levels in bone marrow cells of mice reconstituted with homozygous mutant cells. Reduced *SCL* levels could be a direct consequence of the lack of Otx1 or an indirect effect of an imbalance in cell populations in the bone marrow. We took two approaches to address this question. First, *SCL* expression was measured in marrow populations containing comparable numbers of erythroid cells, as evaluated by β major globin expression (Fig. 3*b*). Moreover, a similar analysis was carried out in erythroid colonies generated *in vitro* by *Otx1*$^{+/-}$ or *Otx1*$^{-/-}$ reconstituted marrows (data not shown). In both cases, *SCL* was reduced in the erythroid populations of mice grafted with null cells, consistent with the view that loss of Otx1 function is associated with a down-regulation of SCL in erythroid cells.

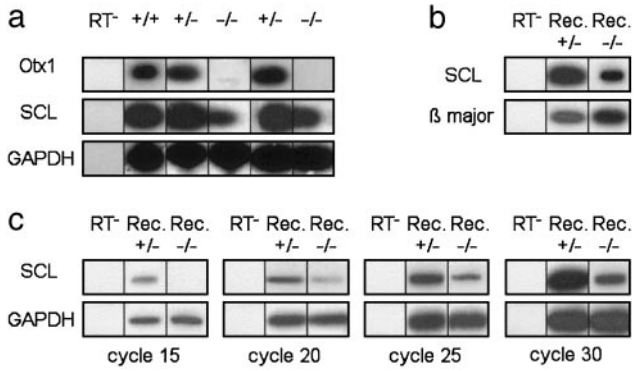

Fig. 3. Expression of *SCL* in bone marrow cells of untreated (*wt*, *Otx1*$^{+/-}$, and *Otx1*$^{-/-}$) and reconstituted mice (with *Otx1*$^{+/-}$ and *Otx1*$^{-/-}$ bone marrow cells). (*a*) *Otx1*, *SCL*, and *GAPDH* mRNAs were analyzed in *wt*, *Otx1*$^{+/-}$, and *Otx1*$^{-/-}$ bone marrow cells by RT-PCR with specific primers (18). (*b*) Expression of *SCL* and β major globin (24) in bone marrow cells of mice transplanted with *Otx1*$^{+/-}$ and *Otx1*$^{-/-}$ bone marrow cells; data are typical of three mice for each genotype. (*c*) Amplification kinetics of cDNA from bone marrow cells of *Otx1*$^{+/-}$ and *Otx1*$^{-/-}$ reconstituted animals. Hybridization of the PCR products shows a constant decrease of *SCL* expression in *Otx1*$^{-/-}$ reconstituted mice.

Second, because SCL drives erythroid differentiation, we asked the question whether the hematopoietic phenotype of *Otx1* null mice could be functionally rescued by *in vivo* overexpression of *SCL*. Heterozygous mice for *Otx1* were crossed with transgenic mice that constitutively express *SCL* under the control of the *Sil* (*SCL* interrupting locus) promoter (17). These mice were interbred to generate *Otx1*-deficient mice that constitutively express *SCL* (*Otx1*$^{-/-}$*SCL*^{tg}). At 1 month of age, littermates of the different genotypes were compared, using clonogenic assays. The rationale for using very young animals was that *Otx1*-deficient mice at the prepubescent stage display a more severe hematopoietic phenotype than older animals. Indeed, prepubescent null mice exhibit also more frequent and severe epileptic seizures and reduced size and

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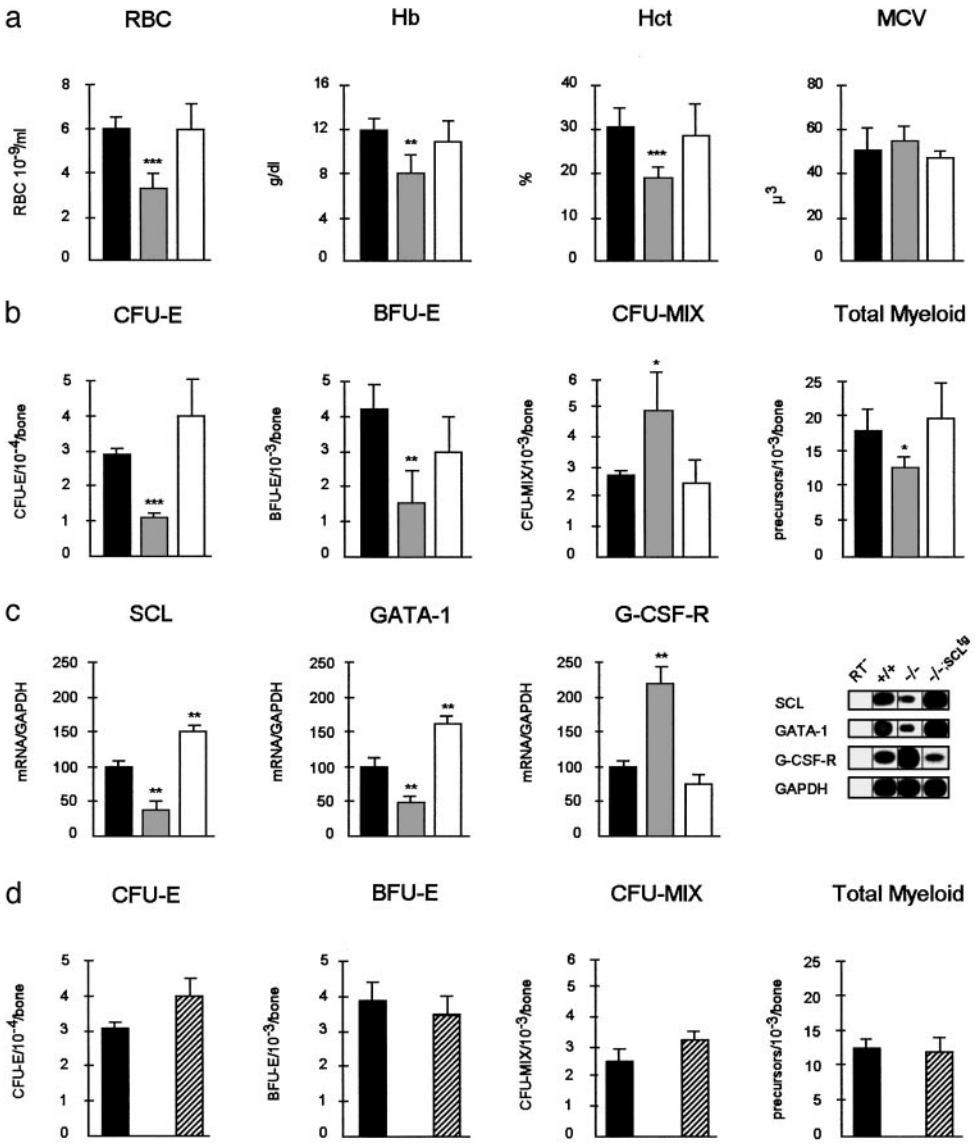

Fig. 4. Genetic complementation of erythroid deficiency in *Otx1*$^{-/-}$ mice by *SCL*. One-month-old mice of the different genotypes were analyzed: WT (■), *Otx1*$^{-/-}$ (▨), and *Otx1*$^{-/-}$*SCL*^{tg} (□). (*a*) Hematological values: red blood cells (RBC), hemoglobin (Hb), hematocrit (Hct), and mean corpuscular volume (MCV). (*b*) Number of early (BFU-E) and late (CFU-E) erythroid progenitors, pluripotent (CFU-MIX) and myelo-monocytic precursors, as determined by clonogenic assays. (*c*) Expression of *SCL*, *GATA-1*, and *G-CSF-R* in bone marrow cells from the same groups of mice. (*d*) Comparison of bone marrow progenitors in *SCL*^{tg} (▨) versus WT (■) mice. A minimum of 10 mice were studied in each group. Asterisks indicate significant variations from WT animals: *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.001$.

weight (11, 12). It is possible that the low levels of pituitary hormones observed in young mutants may affect multiple tissues, including the hematopoietic system and/or that compensatory mechanisms may be activated at later stages. As shown in Fig. 4, 1-month-old *Otx1*$^{-/-}$ mice exhibit severe erythroid deficiency, with significant reductions of red blood cells, hemoglobin, and hematocrit, as well as a 3-fold decrease in CFU-E and BFU-E when compared with WT littermates. However, no differences were observed in red cell shape and size. Furthermore, preliminary evidence indicates that the number of platelets is also diminished (20%) in the homozygous mutants (data not shown). Strikingly, the hematological values and the number of erythroid progenitors were restored to normal levels by the *SCL* transgene (Fig. 4 *a* and *b*). In comparison with erythroid progenitors, there was some degree of fluctuations in myelo-monocytic progenitors, which were not highly significant. Moreover, bone marrow cells of the three groups of animals were analyzed for the expression of two erythroid genes, *SCL* and *GATA-1* (28, 29), as well as for expression of a myeloid gene, encoding the receptor for the granulocyte colony-stimulating factor (*G-CSF-R*) (30). Consistent with the restoration of erythropoiesis, high levels of *SCL* and *GATA1* were detected in *SCL* transgenic animals, as opposed to low levels observed in null mutants (Fig. 4*c*). In contrast, *G-CSF-R* is present at high levels in *Otx1*$^{-/-}$ mice and normal levels in the double transgenics. Therefore, forced expression of *SCL* in *Otx1*$^{-/-}$ mutants can fully rescue the hematopoietic phenotype of *Otx1* null mice.

The rescue of erythroid progenitors by the *SCL* transgene in *Otx1*-deficient cells could be caused by a restoration of an Otx1-dependent pathway or complementation by an independent pathway. We therefore assessed the effect of the *SCL* transgene in WT mice, in which Otx1 function is normal. As shown in Fig. 4*d*, the *SCL* transgene did not affect the overall number of CFU-Es per femur. Other types of progenitors were also assessed and did not significantly differ among the various genotypes (Fig. 4*d*). Therefore, SCL rescues the erythroid deficiency caused by loss of Otx1 function without affecting this

lineage when Otx1 is operational. Combined, our results are consistent with the view that SCL and Otx1 may operate within the same pathway to specify erythroid differentiation.

In hematopoietic progenitors, *SCL* has a similar expression pattern as *Otx1* and plays a critical role in erythroid differentiation (26, 31, 32). Our study raises the possibility that *Otx1* might be required to maintain normal levels of *SCL* gene expression and, consequently, normal numbers of erythroid cells. It remains to be determined whether *SCL* may be a direct Otx1 molecular target. Strikingly, *SCL* and *SCL*-related genes, as well as GATA factors, are also expressed in the developing brain (33, 34), the major site of *Otx1* expression, and it is known that transcription factors form multimeric complexes that may undergo changes in composition and thus acquire new functions (35). These observations further support the view that a small group of regulators may control different cell lineages or even diverse cell systems.

A number of *paired* genes of different subfamilies have been implicated in hematopoiesis, such as *Mix1*, involved in the initial phases of blood development (36), and members of the *Pax* family playing pivotal roles in lymphoid development and differentiation (37, 38). Our findings provide evidence that the *Otx* subclass of homeobox genes contributes to the regulation of blood cell production and suggest that *Otx1* is important in erythropoiesis. There is evidence that >15 homeobox genes, including multiple members of the *HOX* family, the *distal-less* homolog *DLX7* and the LIM *Lhx2* gene, are expressed within the erythroid lineage; however, their mutual relations and specific functions have not yet been defined (8, 39). Our results indicate that *Otx1* is not redundant with these factors, as *Otx1* null mice develop anemia. It is possible, however, that ablation of more than one of these regulators is needed to completely block the erythroid lineage.

An intriguing observation is that transcription factors of the Ptx and Otx subfamilies recognize similar DNA sequences, are expressed in the brain, in the pituitary gland, and in hematopoietic progenitors, and, in *Xenopus laevis*, are involved in the control of cement gland formation (40–42). Although their relation is yet unknown, these *bicoid*-related genes might act as cofactors or within a genetic hierarchy. Furthermore, it is noteworthy that *Pitx2*, expressed in primitive hematopoietic stem/progenitor cells (43), has been hypothesized to play a role in leukemogenesis as a target of *ALL1*, the human homolog of *Drosophila trithorax* that frequently undergoes chromosomal translocations in human acute leukemias (44). Moreover, multiple homeobox genes of different classes are involved in the pathogenesis of leukemias and lymphomas (13–15), and *Otx* genes have recently been indicated as molecular markers for specific types of neural tumors. These observations raise the possibility that dysregulation of *Otx1* might also be associated with hematological diseases.

In conclusion, our findings indicate that *Otx1*, which plays a crucial role in brain morphogenesis, is also involved in the control of blood cell production, and our findings provide further support that common molecular mechanisms orchestrate differentiation of various tissues.

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