Reduced c-Myb activity compromises HSCs and leads to a myeloproliferation with a novel stem cell basis

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Murine haematopoietic stem cells (HSCs) are contained in the Kit⁺ Sca1⁺ Lin⁻ (KSL) population of bone marrow and are able to repopulate lethally irradiated mice. Myeloproliferative disorders (MPDs) are thought to be clonogenic diseases arising at the level of the HSC. Here, we show that mice expressing low levels of the transcription factor c-Myb, as the result of genetic knockdown, develop a transplantable myeloproliferative phenotype that closely resembles the human disease essential thrombocythaemia (ET). Unlike wild-type cells, the KSL population in c-myb knockdown bone marrow cannot repopulate irradiated mice and does not transfer the disease. Instead, cells positive for Kit and expressing low to medium levels of CD11b acquire self-renewing stem cell properties and are responsible for the perpetuation of the myeloproliferative phenotype.

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Introduction

At the top of the hierarchy of cells giving rise to the blood cell system sit a small number of haematopoietic stem cells (HSCs) that are able to keep supplying the system without themselves becoming depleted. It is essential that a single, identical cell replaces each HSC committing towards a haematopoietic progenitor fate—if this process of self-renewal is not achieved then the complement of HSCs will eventually be depleted. By contrast, an excess of self-renewal could result in over expansion potentially leading to leukaemia (Dick, 2003). The concept that haematological malignancies have a

stem cell component that is responsible for their aberrant self-renewal properties arose originally from studies on acute myeloid leukaemia (AML) in which it was found that a small subpopulation of the leukaemic cells was capable of initiating the leukaemia when transplanted into immunocompromised mice (Bonnet and Dick, 1997). Subsequently, evidence has emerged for a similar stem cell-like component in other haematological malignancies, including chronic myeloid leukaemia (CML) (Jamieson *et al*, 2004), acute lymphocytic leukaemia T-ALL (Cox *et al*, 2007) and myeloma (Matsui *et al*, 2004).

The regulation of gene transcription is recognised as a central component of the HSCs control, and a number of recent studies, involving gene knockout or overexpression in mice, have indicated a function for specific transcription factors (Lessard *et al*, 2004). In parallel with the emerging concept of the leukaemic stem cell, it is becoming clear that at least some leukaemias arise from transforming mutations in transcription factor genes that lead to aberrant self-renewal capacity occurring in either the HSC itself or in downstream progenitors (Huntly and Gilliland, 2005), whereas changes in the expression of certain crucial regulators can predispose mice to the development of specific leukaemias (Rosenbauer *et al*, 2005).

The c-Myb transcription factor, originally identified as the oncogenic component of two different avian leukaemia viruses (Lipsick and Wang, 1999), is the founding member of a family of proteins, characterised by a highly conserved DNA-binding domain (Frampton *et al*, 1989), which are implicated in regulatory decisions affecting cell proliferation, differentiation and apoptosis (Oh and Reddy, 1999).

The c-Myb has been shown to be crucial in the development of the haematopoietic system and in c-myb knockout mice dying of anemia at day 15 of gestation because of a failure to develop adult haematopoiesis in the foetal liver (Mucenski *et al*, 1991). Nevertheless, commitment to definitive haematopoiesis can occur in the absence of c-Myb (Allen *et al*, 1999; Sumner *et al*, 2000). Analysis of mice expressing a knockdown allele of c-myb, undergoing conditional gene deletion, or overexpressing miR-150, a micro-RNA (miRNA) that targets the c-myb transcript, showed that the level of c-Myb expression controls the differentiation along individual blood cell lineages (Allen *et al*, 1999; Emambokus *et al*, 2003; Bender *et al*, 2004; Thomas *et al*, 2005; Vegiopoulos *et al*, 2006; Xiao *et al*, 2007), but these studies did not investigate any direct influence of c-Myb on HSCs. More recently, examination of mice homozygous for a point mutation in the p300/CBP interacting domain of c-Myb (M303V) showed an increase in the relative number of HSCs, possibly through an influence on quiescence (Sandberg *et al*, 2005).

Reflecting the central importance of c-Myb in haematopoiesis, evidence has recently emerged for a role of c-Myb in leukaemia. Hence, c-myb gene duplications or translocations

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of the *c-myb* gene to the TCR β locus have been observed in a significant proportion of childhood T-ALLs (Clappier *et al*, 2007; Lahortiga *et al*, 2007), whereas a less direct involvement of c-Myb, as a downstream target gene of the t(11;19) translocation–fusion transcriptional regulator MLL–ENL in myeloid leukaemia, has also recently been shown (Hess *et al*, 2006).

Here, through studies using a knockdown allele of the *c-myb* gene and the generation of radiation chimeras, we show for the first time that c-Myb has a crucial role in the control of both the proliferation and differentiation fate of the HSC, and show that compromise of this control leads to the development of a myeloproliferative disease (MPD)-like phenotype. Downregulation of c-Myb leads to the appearance of a phenotypic KSL population that lacks self-renewal capacity and quiescence, presents an altered transcriptional program and loses the capacity of bone marrow repopulation after transplantation. More importantly, we show that the MPD is not maintained at the level of the KSL HSC but rather by a Kit⁺CD11b^{low/med} population that acquires self-renewal potential and the capacity to transfer the MPD-like phenotype to recipient animals. The perpetuation of atypical repopulating stem cells in mice transplanted with cells defective in *c-myb* expression suggests that such mice represent a model for the

study of the stem cell component of potentially related human MPDs and for factors that might influence progression towards leukaemia.

Results

Reduced c-Myb levels lead to a myeloid disorder reminiscent of essential thrombocythemia

Earlier, we have shown in E14 embryos how homozygosity for a knockdown mutation of *c-myb* perturbs foetal liver haematopoiesis, most notably by reducing erythroid and granulocytic differentiation and increasing thrombopoiesis (Emambokus *et al*, 2003). Furthermore, others have shown that point mutations in c-Myb, which partially disable specific functional domains of the protein, or a transgene insertion in an enhancer located 77 kb upstream of *c-myb* gene cause an increase in platelet numbers (Carpinelli *et al*, 2004; Sandberg *et al*, 2005; Mukai *et al*, 2006). In order to determine how the more profound decrease in all c-Myb functions that are characteristic of our knockdown mutant affects adult haematopoiesis, we analysed peripheral blood and bone marrow of 4- to 6-week-old *c-myb* knockdown animals. In agreement with our earlier observations on foetal liver haematopoiesis, we were able to show a decrease in red cells and

Figure 1 The *c-myb* knockdown has profound effects on adult haematopoiesis. **(A)** Histograms comparing the numbers of red blood cells (RBC), platelets, and white blood cells (WBC) and the percentage of monocytes and neutrophils in the WBC fraction in wild-type (WT) and *c-myb* knockdown (KD) peripheral blood. **(B)** Diff-Quick staining from WT and KD blood smears. **(C)** Histogram comparing the total number of cells in WT and KD bone marrow after red cell lysis (mean $\pm$ s.d., $n = 7$). The micrographs on the right show hematoxylin and eosin-stained paraffin sections of WT and KD bone marrow, the arrows indicating megakaryocytes. **(D)** Comparative picture of the spleens from WT and KD mice. **(E)** Reticulin staining from WT and KD bone marrow.

neutrophils, contrasted by an increase in macrophage and platelets, in the blood of *c-myb* knockdown versus wild type adult mice (Figure 1A). We observed a decreased bone-marrow cellularity (>5-fold) in *c-myb* knockdown animals, whereas thrombocytosis, as seen in peripheral blood (Figure 1B), was reflected by a dramatic increase in bone marrow megakaryocytes, often arranged in clusters (Figure 1C). Paralleling the anemia, the Ter119/CD71 profiles showed that *c-myb* knockdown bone marrow showed an increased ratio of immature to mature erythroid cells (Supplementary Figure 1A), whereas the lower percentage of peripheral neutrophils and the reciprocal increase in macrophage was reflected by a relative loss of CD11b^{high}Gr1^{high} cells and an increase in CD11b^{med}Gr1^{low} cells in the bone marrow (Supplementary Figure 1B). The increase in bone marrow megakaryocytes was also apparent from the presence of a high proportion of c-Kit⁺CD41⁺ cells (Supplementary Figure 1C), suggestive of an expansion in the

number of megakaryocyte-lineage progenitors. Splenomegaly was also a characteristic of the *c-myb* knockdown animals (Figure 1D). The absence of reticulin staining in the bone marrow in the *c-myb* knockdowns, characteristic of myelofibrosis, suggests that the phenotype is very similar to that seen in the MPD essential thrombocytosis (ET) (Figure 1E).

Altered c-Myb activity also profoundly affects haematopoietic stem cells

As MPDs are thought to arise clonally at the level of the HSC or multipotential progenitors, we decided to study this compartment in our animal model. We compared the Kit⁺Sca1⁺Lin⁻ (KSL) (Pohlmann *et al*, 2001) and Kit⁺Sca1⁻Lin⁻ (committed progenitor) fraction of wild-type and *c-myb*-knockdown bone marrow. Mutant bone marrow contained approximately fivefold more progenitors Kit⁺Sca1⁻Lin⁻ (GMP, MEP, CMP; (Akashi *et al*, 2000)), as

Figure 2 The *c-myb* knockdown retains phenotypically defined HSCs, but these show changes in quiescence and stem cell phenotype and differentiation capacity. (A) Flow cytometric profiles of red-cell-depleted WT and KD bone marrow stem cells (KSL: Kit⁺Sca1⁺Lin⁻). The histogram shows a comparison of the number of KSL cells (right) and Kit⁺Sca1⁻Lin⁻ (left) in WT and KD as a percentage of the total number of bone marrow cells (mean ± s.d., *n* = 7). (B) Histogram representing a quantification of the relative proportions of KSL cells defined on the basis of their expression of Flt3 (mean ± s.d., *n* = 7). (C) Flow cytometric profiles comparing staining with Hoechst 33342 of WT and KD KSL cells. The side population cells are defined as regions R2 and R3. (D) Histograms representing a quantification of gated KSL cells in different cell-cycle phases staining with an antibody specific for the nuclear antigen Ki67 and with propidium iodide to determine the DNA content (mean ± s.d., *n* = 3). (E) WT and KD KSL cells subdivided for Flt3 expression were seeded in methylcellulose as described in Materials and methods. Colonies were counted and their morphology assessed after 6 days (mean ± s.d., *n* = 3). The May-Grünwald Giemsa-stained cells illustrate the phenotype of the predominant colony types seen in the KSL Flt3^{high} population isolated from WT (macrophage) and KD (megakaryocyte).

well as KSL cells; however, taking into account the reduced cellularity of the *c-myb* knockdown bone marrow, this meant that their absolute number was approximately equal in the wild-type and mutant mice (Figure 2A). Unlike Sandberg *et al* (2005), but paralleling our recent findings on erythroid precursors (Vegiopoulos *et al*, 2006), we found that c-Kit expression in the KSL population was ~2–4-fold lower in the *c-myb* knockdown, whereas other stem-cell surface markers such as CD31, CD34 and CD105 (endoglin) were not altered (data not shown). The KSL population can be further subdivided into functionally distinct cell types based on the expression of CD34 and Flt3 (Adolfsson *et al*, 2005). Hence, cells capable of long-term reconstitution (LT-HSC; CD34[−]Flt3[−]), short-term reconstitution (ST-HSC; CD34⁺Flt3[−]), or that are multipotent progenitors incapable of reconstitution (MPP; CD34⁺Flt3⁺) can be defined. Comparing the expression of Flt3 on KSL cells, we found that the proportion of Flt3^{high} cells was reduced from 28% in wild-type bone marrow to 6% in *c-myb* knockdown, with a corresponding increase in Flt3[−] cells (Figure 2B and Supplementary Figure 2A). This indicates that less MPP are present and therefore the increase seen in the number of KSL should correspond to LT-HSC and ST-HSC.

In order to further define and verify the effect of reduced c-Myb levels on HSC, we stained KSL cells with the dye Hoechst 33342 and determined by flow cytometry what proportion excluded the dye. Such so-called 'side population' (SP) cells, which represent predominantly immature, quiescent, niche-associated HSCs (Goodell *et al*, 1996), were considerably reduced in the *c-myb* knockdown bone marrow (Figure 2C). The apparent loss of quiescent cells from the KSL population was confirmed by staining of cells simultaneously for expression of the nuclear antigen Ki67 and for DNA content, which showed a relative reduction of cells in G₀ in the *c-myb* knockdown compared with wild type (Figure 2D). It would seem that the change in Flt3 expression described above did not simply reflect a shift towards more immature/quiescent cells as the *c-myb* knockdown KSL population showed a loss of SP cells, most of which fall in the Flt3^{−/low} fraction in the wild type (Supplementary Figure 2B).

To assess any difference in the differentiation potential of wild-type and *c-myb* knockdown stem cells, bone-marrow KSL cells sorted into Flt3[−], Flt3^{low} and Flt3^{high} fractions were compared for their ability to form colonies in methylcellulose containing growth factors compatible with multilineage haematopoietic differentiation (Figure 2E). These assays revealed that *c-myb* knockdown cells showed a dramatically higher potential for megakaryocyte differentiation and a corresponding decrease in macrophage/granulocyte colony-forming ability. Wild-type Flt3^{high} KSL cells, corresponding to the MPP, gave rise almost entirely to macrophage/granulocytes, whereas, strikingly, virtually all *c-myb* knockdown-derived colonies were mixed with a predominantly megakaryocytic content.

The myeloid disorder in *c-myb* knockdown mice has a self-renewing component

To determine the functional properties of HSCs and multipotent progenitors (i.e. the KSL population) with decreased levels of c-Myb, we carried out competitive repopulation assays (Szilvassy *et al*, 1990) by transplanting total wild-type or *c-myb* knockdown bone marrow cells (CD45.2) together with wild-type competitor cells (CD45.1/CD45.2)

into lethally irradiated mice (CD45.1). After transplantation the ratios of total donor to competitor cells were determined in peripheral blood every 4 weeks. The input donor test:competitor ratios were maintained throughout the experiment using either wild-type or *c-myb* knockdown donor cells (Figure 3A), implying that both were capable of short-term and long-term reconstitution.

A closer examination showed that the mutant cells adopted a distinct differentiated phenotype. A first indication of the behaviour of the *c-myb* knockdown cells came from blood samples taken 12 weeks after transplantation. The profiles of cells of wild-type origin were largely similar whether they were test donor or competitor cells; however, *c-myb* knockdown test donor cells seemed to give rise to differentiated cells that were predominantly CD11b⁺. Intriguingly, most of these cells also expressed moderate levels of CD8 (but lower than normally seen on CD8⁺ T-cells) and low levels of B220, and there was an almost complete absence of CD4⁺ T-cells or B-cells expressing high levels of B220 (Figure 3B).

Paralleling the phenotype observed in *c-myb* knockdown animals, blood smears from primary transplanted animals receiving *c-myb* knockdown cells showed the presence of large numbers of platelets and an increased prevalence of monocytic cells (Figure 3C), reminiscent of blood in patients with ET (Campbell and Green, 2006). To further investigate the atypical peripheral white-blood cells, we isolated buffy coat cells from animals 6 months post-transplantation and put them in culture in the presence of a cocktail of cytokines to support multilineage myeloid differentiation (Figure 3D). Cultures of blood cells derived from animals transplanted with wild-type test donor cells showed no signs of cellular expansion. By contrast, cells obtained from *c-myb* knockdown transplants proliferated extensively. Staining of the expanded cells with haematoxylin and for acetylcholine esterase (Figure 3E, left) and analysis of CD11b and CD41 expression (Figure 3E, right) confirmed the presence of macrophage and megakaryocytes. The continuing presence of proliferating blasts was also shown by replating the cultured cells in methylcellulose (Figure 3F). As anticipated from the peripheral blood results, examination of the bone marrow of the primary transplants at 6 months showed good reconstitution by both the test and competitor donor cells, the *c-myb* knockdown contribution being, if anything, greater than expected based on the input ratio (Figure 4A). Detailed examination of the CD45.2⁺ wild-type and *c-myb* knockdown test donor cells showed a profile of immature and differentiated cells reminiscent of that seen in the donor animals (Figure 4B). Hence, the *c-myb* knockdown donor-derived population contained an increased proportion of Kit⁺ cells, increased CD41⁺ cells and, among the myelomonocytic cells, a shift towards a monocytic phenotype (CD11b^{med}Gr1^{low}) at the expense of the neutrophil component (CD11b^{high}Gr1^{high}). Although aberrant in terms of the efficiency of differentiation, there was evidence for the presence of *c-myb* knockdown donor-derived lymphoid cells including small numbers of CD4⁺ cells (Supplementary Figure 3). Focusing on immature progenitors and stem cells, it was again apparent that *c-myb* knockdown cells adopt a profile similar to that seen in the donor animals in that the proportion of Lin[−] and KSL cells was higher (Figure 4C). Most interestingly, staining with Hoechst 33342 showed that although both wild-type and *c-myb* knockdown

Figure 3 Short-term engraftment by *c-myb* knockdown bone marrow. Irradiated hosts were injected with 2.5×10^6 test WT or KD total bone marrow cells together with 5×10^5 competitor WT from CD45.1/CD45.2 animals ($n = 3$). The percentages of the wild-type competitor and test donor populations are indicated. The ratios of wild-type competitor to test donor for all animals transplanted gave means and s.d. of 3 ± 0.28 , 6 ± 1.4 and 3.2 ± 0.15 for the peripheral blood (PB) from 4, 8 and 12 weeks, respectively. **(A)** Flow cytometric profiles of peripheral blood taken from primary engrafted animals 4, 8 and 12 weeks after transplantation with identifying host, competitor and KD donor cell populations based on CD45.1/CD45.2 staining. The competitor/donor ratio (C:D) is indicated in the top right corner of each histogram. **(B)** CD4, CD8, CD11b and B220 expression on peripheral blood cells of host, competitor and donor origin from primary engrafted animals 12 weeks after transplantation with WT or KD donor cells. **(C)** Blood smears from primary engrafted animals 6 months after transplantation with WT or KD donor cells. **(D)** Peripheral blood cells from primary engrafted animals 6 months after transplantation with WT or KD donor cells were cultured for 6 days. **(E)** Acetylcholine esterase staining (left panel) and analysis of CD41 and CD11b expression (right panel) in 6 day-cultured peripheral blood from a recipient transplanted with KD donor cells. **(F)** Methylcellulose culture of peripheral blood from a recipient transplanted with KD donor cells, showing a typical colony present after 2 weeks.

donor cells were able to reconstitute, only the wild-type KSL cells adopted side population characteristics (Figure 4C).

Collectively, the results from the analysis of the primary transplants implied that both wild-type and *c-myb* knockdown bone marrow cells are capable of long-term reconstitution but that, unlike the wild-type cells, those from the mutant animals have a distinct surface antigen phenotype, do not become established in a quiescent state and instead continue to proliferate extensively and give rise to mature cells largely of the monocyte/macrophage and megakaryocyte/platelet lineages. Intriguingly, these differentiated cells seem to derive from a population of proliferating progenitors, some of which are present in the peripheral circulation.

Next, we carried out secondary transplantations to determine whether *c-myb* knockdown long-term reconstituting stem cells retain the capacity for self-renewal and repopulation. However, at 3 weeks post-transplantation into secondary hosts, before it was possible to make an assessment of

long-term repopulation, we noticed that the animals receiving bone marrow from the primary *c-myb* knockdown transplants started to lose weight (Figure 5A). Although these animals were showing short-term repopulation on the basis of the presence of *c-myb* knockdown donor-derived cells (Figure 5B), this evidently was not sufficient or was disruptive of normal haematopoiesis and the animals had to be culled between 30–37 days after transplantation. As seen in the primary transplants, analysis of peripheral blood at 28 days showed increased monocytic cells and platelets in animals receiving *c-myb* knockdown cells (Figure 5C and D). Likewise, CD45.2⁺ *c-myb* knockdown donor cells in the bone marrow showed the characteristic features seen in *c-myb* knockdown animals; that is, increased KSL cells, decreased neutrophils paralleled by increased monocyte/macrophage, increased CD41⁺ cells (Figure 5E and data not shown) and a relative decrease in progenitors committed to the neutrophil lineage (GMPs—Figure 5F).

Figure 4 *c-myb* knockdown bone marrow is capable of long-term engraftment. **(A)** Flow cytometric profiles of CD45.1/CD45.2 expression in bone marrow (BM) and spleen (Sp) from primary engrafted animals described in Figures 3 and 6 months after transplantation. The percentages of the wild-type competitor and test donor populations are indicated. The ratios of wild-type competitor to test donor for all animals transplanted gave means and s.d. of 15 ± 1.8 , 12 ± 0.45 and 0.45 ± 0.2 for the KD BM, WT Sp and KD Sp, respectively. **(B, C)** Cells from donor origin (CD45.2) were gated and analysed for **(B)** Kit/CD41 or CD11b/Gr1, and **(C)** lineage antigens (Lin) versus cell size (FSC). Lin[−] cells (indicated by the gated region in the upper panels) were further analysed for expression of c-Kit and Sca1, the KSL population being defined as those cells in the gated region **(C, central panels)**. KSL cells were further analysed by staining with Hoechst 33342 **(C, lower panels)**, side population cells being indicated by the gated region.

KSL cells from *c-myb* knockdown animals do not have repopulation capacity and are not responsible for the myeloproliferative phenotype

As the first set of transplantations had been carried out using equal numbers of total donor bone marrow cells, which, from our analyses, we knew would have contained different proportions of immature progenitors, we injected either 500 or 2000 sorted KSL cells from wild-type or *c-myb* knockdown CD45.2 donors into irradiated hosts together with 1×10^6 CD45.1/CD45.2 wild-type total bone marrow cells. Analysis of peripheral blood of the transplanted animals after 4, 8 and 12 weeks indicated that wild-type competitor and test donor cells had yielded successful reconstitution, but, surprisingly, the *c-myb* knockdown KSL donor cells had not (Figure 6A). Similarly, when the bone marrow was analysed, no cells of *c-myb* knockdown donor origin were present (Figure 6B).

Next, we decided to investigate whether the failure of *c-myb* knockdown KSL cells to repopulate can be explained by an altered transcriptional program. We selected a variety of genes that have been related with HSC function. Semi-quantitative (Figure 6C) or quantitative (Figure 6D)

RT-PCR revealed that three genes known to be crucial in stem cells, namely *ikaros*, *c-mpl* and *flt-3*, were expressed at a much-reduced level in the mutant cells. In addition, among the family of cell-cycle inhibitors we observed that *p21*, *p27* and *p57* mRNAs were present in lower amounts in the *c-myb* knockdown KSL cells, correlating with their loss of quiescence and enhanced proliferation. Also correlating with an increased proliferative, yet still immature state, was an observed increase in the level of expression of *GATA-2* mRNA.

Kit⁺ CD11^{low/med} cells have the capacity to transfer the *c-myb* knockdown myeloproliferative phenotype

Collectively, the phenotypic and functional data for the *c-myb* knockdown KSL cells indicate that they have lost all capacity for stem cell behaviour, in which case the question remains as to the nature of the cells that are capable of transferring and perpetuating the mutant phenotype. In order to address this, we carried out similar transplantations, subdividing the bone marrow into four different fractions: Kit[−]Lin[−], Kit[−]Lin⁺, Kit⁺Lin[−] and Kit⁺Lin⁺ cells (Supplementary Figure 4). Successful engraftment and perpetuation of the

Figure 5 Primary and secondary transplanted animals receiving *c-myb* knockdown bone marrow cells develop a myeloproliferative disorder. Irradiated hosts were injected with 2×10^6 ($n=3$) bone marrow cells derived from primary transplants that had received WT or KD total bone marrow cells. **(A)** Weight curve of secondary engrafted animals. **(B)** CD45.1/CD45.2 staining of peripheral blood taken from secondary engrafted animals 4 weeks after transplantation with donor cells from either a WT competitor/WT test (WT:WT primary) or WT competitor/KD test (WT:KD primary) primary transplant. The percentages of the wild-type competitor and test donor populations are indicated. The ratios of wild-type competitor to test donor for all animals transplanted gave means and s.d. of 12 ± 6.3 and 29.6 ± 7.2 WT:WT primary and WT:KD primary, respectively). **(C, D)** Blood smears (C) and blood cell counts (D) for secondary engrafted animals described in (B). **(E)** Staining for CD11b/Gr1 expression in bone marrow cells of test donor origin (CD45.2⁺) from secondary engrafted animals as described in (C). **(F)** Analysis of multipotent progenitors (GMP, CMP and MEP) of test donor (CD45.2⁺) origin, depicting FcγRII/III/CD34 expression on the gated $\text{Kit}^+ \text{Sca1}^- \text{Lin}^- \text{IL-7R}^-$ population. Staining boundaries for the relevant isotype controls are indicated by the dashed lines.

c-myb knockdown myeloproliferative phenotype was achieved with the $\text{Kit}^+ \text{Lin}^+$ fraction (Figure 7A). As observed in both *c-myb* knockdown animals and mice engrafted with total *c-myb*-knockdown bone marrow, the bone marrow of animals transplanted with $\text{Kit}^+ \text{Lin}^+$ cells contained an expanded population of $\text{CD11b}^{\text{low/med}} \text{Gr1}^{\text{low}}$ cells (Figure 7B). Surprisingly, although *c-myb* knockdown KSL cells failed to engraft, mutant $\text{Kit}^+ \text{Lin}^+$ cells gave rise to a population of cells in the bone marrow of engrafted animals that were $\text{Kit}^+ \text{Sca}^+$ and had lost lineage markers, thus being phenotypically KSL-like (Figure 7C).

As the $\text{Kit}^+ \text{Lin}^+$ fraction is a heterogeneous population, we decided to define more precisely which cells act as the stem cell component of the myeloproliferative phenotype. On the basis of the predominance of $\text{Kit}^+ \text{CD11b}^{\text{low/med}}$ in the bone marrow of *c-myb* knockdown animals and recipients transplanted with *c-myb* knockdown cells, we wanted to determine whether these or a specific subfraction were responsible for the transplantable phenotype. We first examined the heterogeneity of the $\text{Kit}^+ \text{CD11b}^{\text{low/med}}$ population with respect to other lineage-associated antigens. This showed that both wild-type and *c-myb* knockdown $\text{Kit}^+ \text{CD11b}^{\text{low/med}}$ cells co-expressed low to medium levels of a range of myeloid and lymphoid markers (Supplementary Figure 5A), the only significant difference being that the wild-type cells could be subdivided into two populations on the basis of low/medium or high Gr1 expression whereas *c-myb* knockdown $\text{Kit}^+ \text{CD11b}^{\text{low/med}}$ cells consistently expressed low/medium

levels of the antigen. Both the wild-type and *c-myb* knockdown $\text{Kit}^+ \text{CD11b}^{\text{low/med}}$ populations were found to be heterogeneous for Sca1 expression, although the distribution of Sca1^+ and Sca1^- cells was different, there being proportionately about twice as many Sca1^- cells in the case of the *c-myb* knockdown (Supplementary Figure 5B). Taking into account the results of this analysis, we sorted $\text{Kit}^+ \text{CD11b}^{\text{low/med}}$ cells into Lin^- and Lin^{low} fractions and then further subdivided these on the basis of Sca1 expression (Supplementary Figure 6). For each of the sorted subpopulations, 500 cells (CD45.2) were injected together with 1×10^6 wild-type (CD45.1/CD45.2) total bone-marrow cells into lethally irradiated hosts (CD45.1). Peripheral blood was collected and analysed after 8 weeks in order to determine the relative efficiency of engraftment. Both wild-type and *c-myb* knockdown $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^- \text{Sca1}^+$ cells resulted in efficient engraftment, whereas the $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^- \text{Sca1}^-$ cells did not (Figure 8A). Likewise, wild-type and *c-myb* knockdown $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^{\text{low}} \text{Sca1}^+$ cells engrafted and wild-type $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^{\text{low}} \text{Sca1}^-$ failed to. However, the equivalent *c-myb* knockdown Sca1^- population successfully engrafted (Figure 8A). Staining of peripheral blood test donor-derived cells (CD45.2) for markers of lymphoid and myeloid lineages indicated that wild-type $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^- \text{Sca1}^+$ cells gave rise to CD4^+ and CD8^+ T-cells and $\text{CD11b}^+ \text{Gr1}^{\text{med/high}}$ myelomonocytic cells (Figure 8B and C), presumably because inclusion of low-level expression of Lin antigens (including CD11b) retains

Figure 6 Engrafting cells from *c-myb* knockdown bone marrow are not in the KSL population. Irradiated hosts were injected with 500 ($n=6$) or 2×10^3 ($n=6$) WT or KD sorted KSL cells together with 1×10^6 CD45.1/CD45.2 WT competitor cells. **(A)** Peripheral blood and **(B)** bone marrow taken from animals engrafted with KSL cells 12 weeks after transplantation, distinguishing host and donor cells based on CD45.1/CD45.2 staining as described in Figure 3. The percentages of the wild-type competitor and test donor populations are indicated. The ratios of wild-type competitor to test donor for all animals transplanted gave means and s.d. of 0.15 ± 0.05 and 0.17 ± 0.02 for WT in the peripheral blood and the bone marrow, respectively. **(C)** Semi-quantitative RT-PCR of KSL-cell RNA from WT versus KD animals using primers corresponding to the indicated gene (see Supplementary Table 2). Each reaction was sampled at three-cycle intervals in the exponential range of the amplification. **(D)** Histogram representing the results of quantitative RT-PCR of KSL-cell RNA from WT versus KD animals using primers corresponding to the indicated genes (see Supplementary Table 3). The data are normalised against $\beta 2$ microglobulin expression and the wild-type values set as 1.

some degree of overlap with the KSL population. A similar analysis of the recipients of *c-myb* knockdown cells showed a subtle difference in the contribution of different subpopulations to the myeloproliferative phenotype. Hence, $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^- \text{Sca1}^+$ cells gave rise to small numbers of peripheral blood CD4^+ cells and a majority of $\text{CD11b}^{\text{low/med}} \text{Gr1}^{\text{low/med}}$ myelomonocytic cells characteristic of the *c-myb* knockdown phenotype (Figure 8B and C). By contrast, *c-myb* knockdown $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^{\text{low}}$ cells, whether Sca1^+ or Sca1^- , gave rise to cells in the peripheral blood that were also characteristically $\text{CD11b}^{\text{low/med}} \text{Gr1}^{\text{low/med}}$ but also largely co-expressed CD8 at a level somewhat lower than normally seen on mature T-cells (Figure 8B and C).

Collectively, the transplantation studies on sorted bone marrow cells indicated that normal lymphoid and myeloid engraftment potential of wild-type cells can be shown for the expected KSL population and also for cells that express low levels of lineage-associated antigens, including CD11b. By contrast, reduced c-Myb expression in the knockdown mutant abolishes the capacity of KSL cells to engraft but at the same time confers myeloproliferative stem-cell characteristics upon a spectrum of Kit^+ cells that express low to medium levels of myeloid and lymphoid antigens, but that

can be positive or negative for the stem-cell antigen Sca1 (Figure 8D).

Discussion

Here we have shown that reduction of c-Myb expression leads to a myeloproliferative phenotype, with an overabundance of platelets and monocytes in peripheral blood and an increase in megakaryocytes and a distinct $\text{CD11b}^{\text{low/med}} \text{Gr1}^{\text{low/med}}$ population in the bone marrow. In combination with the absence of myelofibrosis and presence of splenomegaly, the phenotype overall resembles the MPD essential thrombocythemia (ET). Most interestingly, the phenotype is transplantable and seems to have an underlying stem-cell component that can be defined as $\text{Kit}^+ \text{CD11b}^{\text{low/med}} \text{Lin}^- / \text{low}$, with no dependence on Sca1 expression. Human MPDs are believed to arise clonally at the level of HSC/multilineage progenitors (Campbell and Green, 2006) and the results of our transplantation experiments were consistent with a stem-cell component maintaining the aberrant myeloproliferative phenotype. As the myeloproliferative phenotype is clearly a direct consequence of the *c-myb* knockdown mutation, this means that this does not represent a model for the evolution of

Figure 7 Engrafting cells from *c-myb* knockdown bone marrow are in a lineage⁺ fraction that is expanded relative to the wild type. Irradiated hosts were injected with WT or KD subpopulations sorted on the basis of Kit and Lin expression (see Materials and Methods for the numbers used) together with 1×10^6 CD45.1/CD45.2 WT competitor cells (for each cell type $n=3$). (A) Flow cytometric profiles of bone marrow taken from animals engrafted with Kit⁺Lin⁺ cells seven weeks after transplantation identifying host, competitor and donor cell populations based on CD45.1/CD45.2 expression. The percentages of the wild-type competitor and test donor populations are indicated. The ratios of wild-type competitor to test donor for all animals transplanted gave means and s.d. of 0.89 ± 0.55 and 27 ± 13 for WT and KD test donors, respectively. Staining for CD11b/Gr1 (B) and KSL cells (C) in bone marrow of test donor origin (CD45.2⁺) from animals engrafted with Kit⁺Lin⁺ cells 7 weeks after transplantation.

MPD from stem cells or progenitors but, however, it does provide a basis to study the mechanisms MPD maintenance and possibly even for investigation into factors that can promote its progression towards leukaemia.

Similar to the situation observed in mice carrying the M3030V mutation in *c-Myb* (Sandberg *et al*, 2005) our knock-down mutant animals showed an increase in the number of phenotypically KSL cells showing increased proliferation. However, although we, like Sandberg *et al* (2005), were able to show the capacity of *c-myb* knockdown bone marrow to be transplanted into irradiated hosts, we recognised some features that indicated that normal stem-cell properties had been lost. First, repopulation by *c-myb* knockdown cells required co-transplanted wild-type cells in order for all lineages to be successfully reconstituted. Second, we observed early lethality of recipient animals after secondary transplant. No lethality in primary recipients was found even 10 months post-transplantation, invalidating the explanation

of a rapid death because of a development of a leukaemia-like disease. We consider that the most likely explanation for the death of these animals is the greatly reduced proportion of HSC of competitor wild-type origin in total bone marrow of the primary transplanted animals due to the extensive overproliferation of *c-myb* knockdown Kit⁺ cells. Third, and most significantly, phenotypically KSL cells derived from our *c-myb* knockdown animals showed no capacity to reconstitute irradiated animals. This result contrasts with the conclusions of Sandberg *et al*. (2005); however, they did not specifically isolate KSL cells for use in their assay but instead used the total bone marrow. The simplest explanation for these differences is that the *c-myb* M303V mutation is effectively a less potent hypomorph relative to our knockdown allele, either because it only partially disrupts a specific aspect of c-Myb function or because the presumed loss of interaction with p300/CBP has a limited effect on all, or affects only a subset of c-Myb targets.

A possible explanation, at the molecular level, of why *c-myb* knockdown KSL cells do not behave as normal KSL came from our preliminary studies of gene expression. We found that the mutant KSL cells have an altered transcriptional program. For example, the changes in the expression of crucial genes such as *ikaros* (Lopez *et al*, 2002; Yoshida *et al*, 2006), *c-mpl* and *flt-3* might explain why *c-myb* knockdown KSL cells commit towards myelo-megakaryocytic lineages, whereas the low levels of the cell-cycle inhibitors p21, p27 and p57 could explain the shift of KSL HSCs towards an aberrant proliferative progenitor state. Indeed, p27 down-regulation has been described to increase haematopoietic progenitor proliferation and p27-deficient HSCs generate progenitors that eventually dominate blood cell production (Cheng *et al*, 2000).

In order to determine which specific cell population is responsible for the transplantable myeloproliferative phenotype, we subdivided the bone marrow and carried out competitive repopulation experiments. We found that the cells capable of transferring the *c-myb* knockdown myeloproliferative phenotype were contained in the Kit⁺Lin⁺ fraction, suggesting that when c-Myb expression is lower either cells with stem cell-like properties derive from normal stem cells or more mature cells acquire stem cell properties. The fact that we observe lymphoid cells in the transplanted animals, albeit at low levels or only partly differentiated, argues that a modified multipotential stem cell underlies the phenotype. Further subfractionation of bone marrow Kit⁺ cells allowed us to define the transplantable component as Kit⁺CD11b^{low/med}Lin^{-/low}Sca1^{-/+}. The presence of Sca1 on one of the active subfractions also supports the notion that the transplantable *c-myb* knockdown cells derive from immature cells. CD11b is known to be expressed on foetal liver HSCs (Morrison *et al*, 1995), and this might suggest that c-Myb is required for the developmental transition to adult HSC, which are CD11b⁻, and, furthermore, that the cells giving rise to the myeloproliferative phenotype are in fact foetal liver HSCs. However, we were unable to detect expression of cells co-expressing AA4.1 and CD11b or of Sox17 RNA (data not shown), both of which have been shown to be characteristic of foetal HSCs (Kim *et al*, 2007). Interestingly, it was reported recently that a Kit⁺CD11b⁺ population acquires stem-cell characteristics in AML arising as the result of point mutations in C/EBP α (Kirstetter *et al*, 2008).

Figure 8 Engrafting cells from *c-myb* knockdown bone marrow express low to medium levels of CD11b. Irradiated hosts were injected with 500 WT or KD Kit⁺CD11b^{low/med} cell subpopulations that had been sorted on the basis of Lin (CD5, CD8a, B220, Gr-1, Ter119) and Sca1 expression together with 1×10^6 CD45.1/CD45.2 WT competitor cells (for each cell type $n = 3$). (**A**) Flow cytometric profiles of bone marrow taken from animals engrafted with Kit⁺CD11b^{low/med} cells 8 weeks after transplantation identifying host, competitor and donor cell populations based on CD45.1/CD45.2 expression. The percentages of the wild-type competitor and test donor populations are indicated. The means and s.d. of the ratios of wild-type competitor to test donor for all animals transplanted are indicated in the top left-hand corner of each profile (note: only one animal receiving transplantation of WT Kit⁺CD11b^{low/med}Lin^{low}Sca1⁺ cells survived to be analysed). Staining for CD4/CD8 (**B**) and CD11b/Gr1 (**C**) in bone marrow of test donor origin (CD45.2⁺) from animals engrafted with Kit⁺CD11b^{low/med} cells 8 weeks after transplantation. (**D**) Schematic representation of the engraftment achieved with fractionated WT and KD populations. The antigens expressed are indicated and the shading depicts whether transplanted cells gave full reconstitution (black), no reconstitution (white) or reconstitution of the myeloproliferative phenotype with either a limited myelomonocytic profile (white dotted) or the characteristic KD phenotype involving co-expression of lymphoid and myelomonocytic markers (black dotted).

Surprisingly, although *c-myb* knockdown cells that are phenotypically KSL-like do not act as stem cells, Kit⁺CD11b^{low/med}Lin^{-/low} cells that can transfer the phenotype generated a proliferating population that equates with KSL on the basis of surface antigen expression. Contamination of KSL cells in the transplanted Kit⁺CD11b^{low/med}Lin^{-/low} fraction cannot be an explanation for the presence of KSL cells after the transplantation as we have shown that *c-myb* knockdown KSL cells are not able to reconstitute. At present we can only speculate on the nature of these KSL-like cells ultimately deriving from the Kit⁺CD11b^{low/med}Lin^{-/low} cells. As the properties of the *c-myb* knockdown KSL-like cells are so distinct from those of wild-type KSL, we favour the view that the Kit⁺CD11b^{low/med}Lin^{-/low} cells are able to give rise to a population that express the same markers as KSL cells, but in all other regards are not true HSCs.

In summary, we have shown that when c-Myb is tipped towards an abnormally low level of expression, then a myeloproliferative phenotype with skewed lineage differen-

tiation can result (Figure 8). This adds to other recently described instances where the loss of normal control of c-Myb expression can lead to haematological disorders. It is becoming clear that miRNAs can regulate c-Myb levels and might underlie both normal and disease-related control of haematopoiesis (García and Frampton, 2008). The miRNA miR-150 has been shown recently to affect, presumably also through an influence of c-Myb, both normal and MPD-related lineage choice in the erythroid/megakaryocytic axis (Bruchova *et al*, 2007; Lu *et al*, 2008). Another instance of loss of normal control of c-Myb expression that leads to haematological disruption seems to underlie T-ALL in which the *c-myb* gene has been duplicated or translocated to the TCR β locus (Clappier *et al*, 2007; Lahortiga *et al*, 2007). A similar specific alteration to *c-myb* expression caused by new signalling from a disease-associated translocation also seems to be a crucial component of the leukaemogenic process in MLL-ENL induced AML, potentially through the intermediate action of HoxA9 and Meis1 (Hess *et al*, 2006). Furthermore, in each of these situations, the cells arising

Figure 9 Correct control of c-Myb levels is required to maintain the haematopoietic hierarchy. The diagram summarises the ways in which disrupted loss of normal (A) control of c-Myb expression has been shown to lead to expansion of cells with stem-cell characteristics at different points in the haematopoietic hierarchy, resulting in (B) myeloid and (C) lymphoid leukaemia or, as described here, myeloproliferation (D). The highly simplified representation of haematopoiesis is divided on the basis of cells being in the KSL stem cell compartment (purple) or committed towards myeloid (green) or lymphoid (blue) differentiation. The approximate range of expression of crucial markers is indicated at the bottom (SP, side population). Circular arrows are used to depict self-renewal, the extent of which is related to the size. Vertical bars indicate blocks to further differentiation. The dotted arrow and cell in (D) represent a putative new pathway of dedifferentiation seen in the *c-myb* knock-down.

from altered c-Myb expression have properties that are stem cell-like (Figure 9).

We hypothesise that c-Myb is a crucial component in a network of regulatory factors that control the balance between self-renewal and differentiation in normal HSCs and more mature cell types, and that its role in this network is crucially dependent on the fine tuning of its expression, both in terms of overall level and the specific timing, and when this control is compromised haematological defects, both leukaemic and myeloproliferative, can result.

Materials and methods

Mice and genotyping

All animal experiments were carried out under an animal project licence in accordance with UK legislation. The *c-myb* knockdown mutation corresponds to the *c-myb^{loxP}* allele as described earlier (Emambokus *et al*, 2003). Following their original derivation, *c-myb* knockdown mice were backcrossed against the C57/BL6 strain for at least 10 generations and were thereafter maintained on this

genetic background. Mice were genotyped by PCR analysis. Mice between 4–6 weeks of age were used as a source of bone marrow cells for analysis or transplantation.

Blood counts

Adult mice were bled in ACD solution (citric acid 6.8 mM, trisodium citrate 11.2 mM, glucose 24 mM) and blood counts were obtained with an ABX Pentra 60 (ABX Diagnostics) automatic blood counter. Five pairs of age-matched animals were analysed. The mean and standard error of the mean (s.e.m.) were calculated.

Bone marrow sections

Histological analysis of paraffin sections of femurs was carried out using standard methods. Hematoxylin and eosin-stained sections were analysed under bright-field microscopy.

Culture of primary cells

Sorted KSL cells were plated in Iscove's modified Eagle's medium (IMDM) containing 10% horse serum, SCF (20 ng/ml), FL (10 ng/ml) and TPO (5 ng/ml). To assess individual cell proliferation and differentiation capacity, 500 sorted KSL cells were plated in 1.5 ml 1% methylcellulose medium (Methocult M3434, Stem Cell Technologies, Vancouver) containing TPO (25 ng/ml). Colony numbers were assessed after 6 days. All cultures were incubated at 37 °C in a fully humidified 5% CO₂ in air atmosphere.

Peripheral blood cells were first treated with ACK buffer to deplete red cells before culturing in RPMI, 10% FCS, 5 ng/ml IL-3, IL-6, GM-CSF and 50 ng/ml SCF.

Competitive repopulation assays of HSC function

Transplantations to assess competitive repopulation potential were carried out by injecting 5×10^5 competitor wild-type ACK-treated bone marrow cells (B6 $\times$ B6.SJL F1, CD45.1/CD45.2) together with 2.5×10^6 donor cells from either wild-type or *c-myb* knockdown mice (B6, CD45.2/CD45.2) into the tail vein of host animals (B6.SJL, CD45.1/CD45.1) that had been lethally irradiated (975 Gy). Peripheral blood was analysed every 4 weeks following transplantation using antibodies to distinguish CD45.1 and CD45.2 and a range of lineage-specific markers. For secondary transplantation experiments, 2×10^6 ACK-treated total bone marrow from animals showing successful engraftment at 24 weeks were injected into lethally irradiated B6.SJL hosts. After transplantation, the animals were scored for engraftment by immunofluorescent flow cytometry as above.

Transplantations involving specific populations of cells employed 10^6 wild-type competitor cells and the following numbers of sorted cells: Kit⁺Lin⁺, 1.7×10^5 ; Kit⁺Lin⁺, 3×10^5 ; Kit⁺Lin⁺, 2×10^4 ; Kit⁺Lin⁺, 2×10^4 ; KSL, 500 or 2×10^3 ; Kit⁺CD11b^{low/med}Lin^{low}Sca^{+/+}, 500.

Antibodies, flow cytometry and cell sorting

Single-cell suspensions of bone marrow were prepared by standard techniques. Red cells were depleted, when required, by selective lysis and non-specific binding of antibodies to Fc receptors was prevented by use of anti-CD16/CD32 Fc block (BD Pharmingen). The lineage cocktail contained the following antibodies: CD5, CD8a, Gr-1, CD11b, Ter119 and B220. In experiments where the expression of CD11b was assayed, this antibody was removed from the lineage cocktail. Antibodies used are described in Supplementary Table 1. Stained cells were analysed on a CyAn flow cytometer using Summit software (Dako).

For cell sorting, red cells were ACK-lysed and after staining with the required conjugated monoclonal antibodies, samples were filtered through a 70- μ m strainer and sorted on a Cytomation MoFlo machine. Live cells were selected by forward/side scatter gating and doublet discrimination.

Side population and immunofluorescence

The procedures for SP and immunofluorescent staining were carried out as described earlier (Goodell *et al*, 1996).

Semi-quantitative RT-PCR determination of mRNA levels

Total RNA from sorted KSL cells was isolated using TRIzol (Invitrogen) and contaminating genomic DNA removed by treatment with RNase-free DNaseI (Pharmacia). Single-stranded cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen) with first-strand synthesis primed using oligo (dT). PCR was

carried out using ReddyMix PCR master mix (ABgene) in 50 µl containing appropriate concentration of gene-specific oligonucleotides, designed to span at least one intron (see Supplementary Table 2). HPRT was used as a control to standardise samples. For a given PCR reaction, samples were removed at intervals of three cycles to enable comparison of the linear phases of amplification. PCR products were analysed by agarose gel electrophoresis. The experiment was carried out in duplicates.

Quantitative RT-PCR determination of mRNA levels

Real-time PCR was carried out using TaqMan PCR master mix with 1 µl of predesigned TaqMan PCR primers (Applied Biosystems, see Supplementary Table 3). 1 µl of cDNA dilutions was used in a 20-µl reaction. The reactions were carried out in a Stratagene MX3000P machine. At least two separate reactions with three replicates per

sample were run. Relative gene expression was calculated as $2^{-\Delta\Delta C_t}$ values with β_2 microglobulin used as a control.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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