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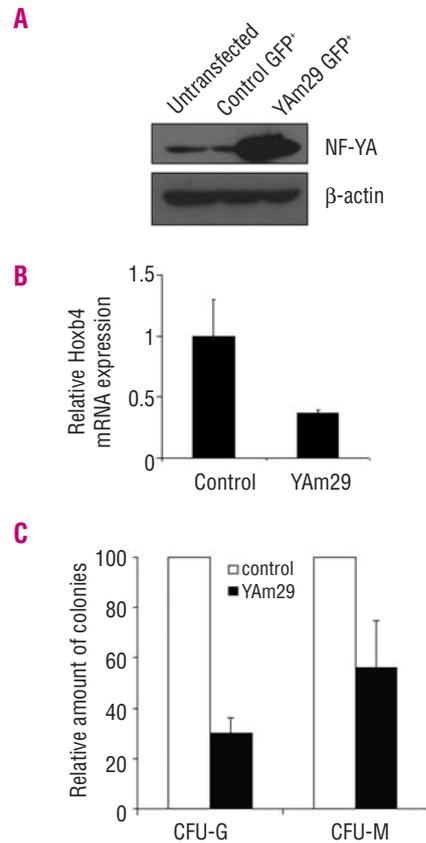
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### The transcription factor nuclear factor Y regulates the proliferation of myeloid progenitor cells

The transcription factor nuclear factor Y (NF-Y) consists of three subunits; NF-YA, -B and -C, and this complex binds DNA and the basal transcription machinery through binding of TBP.<sup>1</sup> NF-Y targets include cell cycle related genes and hematopoietic (stem) cell genes such as *cd34* and *hoxb4*.<sup>2</sup> Deletion of both *nf-ya* alleles in mice results in embryonic lethality. Inactivation of NF-YA in mouse embryonic fibroblasts results in a block in proliferation, which is followed by apoptosis.<sup>3</sup> A few studies have been performed addressing the role of NF-Y in hematopoiesis. Mouse bone marrow (BM) cells overexpressing NF-YA are biased towards primitive hematopoiesis and show increased repopulating ability after bone marrow transplantation.<sup>2</sup> During macrophage differentiation NF-Y shows increasing binding activity to several promoters<sup>4</sup> and NF-Y has been shown to mediate the IL-6 response in BM cells, which results in induction of macrophage differentiation.<sup>5</sup>

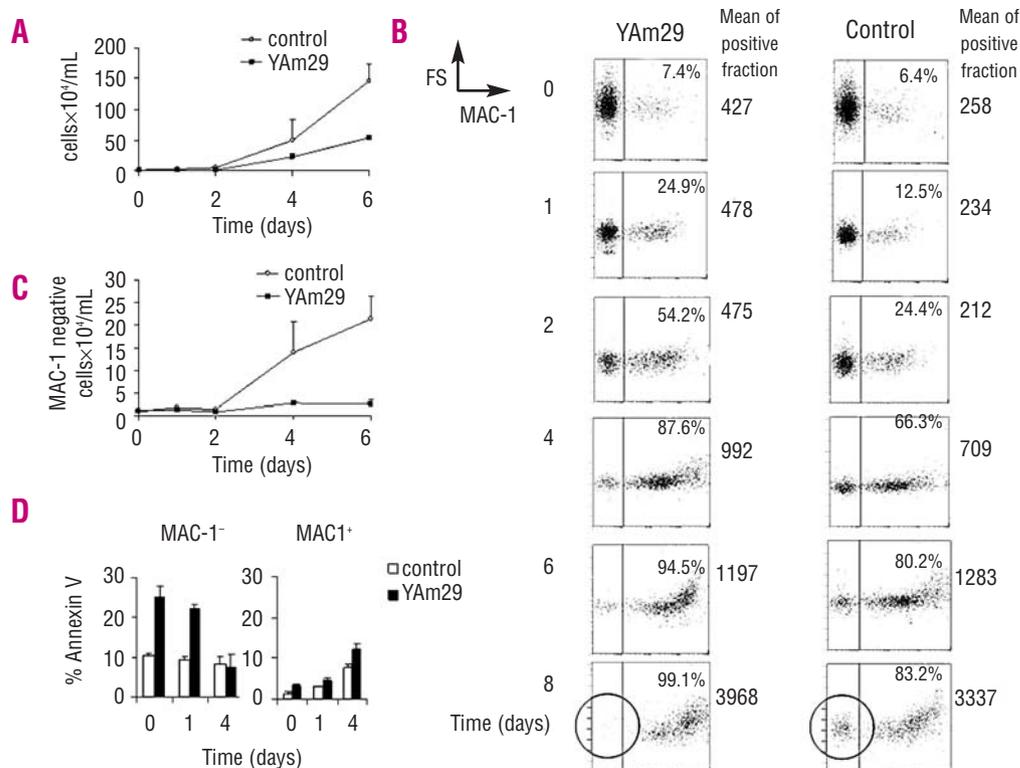
Mantovani *et al.* constructed a mutant form of NF-YA (YAm29), defective in DNA-binding and shown to have a dominant-negative effect on transcription regulation by wild type NF-Y.<sup>6,7</sup> To determine the requirement of NF-Y in myelopoiesis, we cloned the YAm29 cDNA in the LZRS vector,<sup>8</sup> containing an IRES-GFP expression cassette. Mouse BM cells were transduced with YAm29 (efficiency of 23±8.9%, 3 independent experiments) as described earlier.<sup>9</sup> As a control we transduced cells with an empty vector (efficiency of 14.5±8.1%). One day after transduction, GFP<sup>+</sup> cells were sorted by flow cytometry and analyzed. Endogenous NF-YA expression was detected by Western blot analysis in the control cells. Clear overexpression of YAm29 was detected in the GFP<sup>+</sup> fraction of the YAm29 transduced cells (Figure 1A). To determine whether expression of YAm29 repressed the expression of NF-Y target genes we measured the levels of Hoxb4 mRNA by qPCR, performed as previously described.<sup>7</sup> Hoxb4 mRNA levels were significantly down-regulated in cells transduced with YAm29 compared to cells transduced with empty vector, indicating that YAm29 effectively represses NF-Y function (Figure 1B). To determine the effect of YAm29 expression on colony formation, GFP<sup>+</sup> cells were plated in methylcellulose and colonies were counted after 5-7 days. Transduction with YAm29 resulted in a reduction of granulocytic (CFU-G) and monocytic (CFU-M) colony formation by 70±6% ( $p<0.01$ ) and 44±19% ( $p<0.1$ ) respectively, compared to cells transduced with empty vector (Figure 1C). In addition, transduction with YAm29 resulted in a reduction of the size of both colony types (*data not shown*). The data suggest that NF-Y is required for the proliferation of myeloid progenitor cells.

To quantify the inhibitory effect of YAm29 on proliferation, cells were grown in liquid culture. The empty vector transduced population expanded faster than the YAm29 transduced population, resulting in a three-fold difference in cell numbers on day 6 (Figure 2A). To investigate the effect of YAm29 on maturation, the differentiation marker MAC-1 was used. MAC-1 is expressed on maturing myeloid cells, beyond the colony-forming unit (CFU) stage.<sup>11</sup> The percentage of MAC-1 positive cells increased more rapidly within the YAm29 transduced



**Figure 1.** YAm29 inhibits clonogenic growth of mouse bone marrow cells. Bone marrow cells were transduced with YAm29 or empty vector and after 24 hours transduction cells were sorted for GFP positivity by flow cytometry. (A) Complete cell lysates were resolved on SDS-PAGE and stained using NF-YA specific antibody (C-18, Santa Cruz) and β-Actin (A-5441, Sigma) specific antibody. (B) Quantitative PCR for HoxB4 (Mm01307004\_mH, Applied Biosystems) and 18S rRNA (cat no. 4310893E, AB). Experiments were repeated three times and *p* values were determined with a *t*-test. The expression of HoxB4 was significantly lower in YAm29 expressing cells ( $p<0.05$ ) (C) For the semi-solid culture 5000 cells were plated per mL methylcellulose medium. Granulocyte colony-forming units (CFU-G) and monocyte colony-forming units (CFU-M) were counted five seven days later using an inverted microscope. The amount of colonies is shown after transduction with YAm29 as a percentage relative to the amount of colonies after transduction with empty vector. Experiments were repeated three times independently. There was a statistically significant difference in the amount of colonies (CFU-G;  $p<0.05$ , CFU-M;  $p<0.1$ ) between the two treatments.

population than in the control population. After eight days, all YAm29 transduced cells were MAC-1 positive (Figure 2B), while in the control population around 17% of the cells were still MAC-1 negative. This suggested that the immature cells in the control population still proliferated and in the YAm29 transduced population proliferation of immature cells was inhibited. The expansion of the MAC-1 negative cell fraction was calculated by multiplying the total amount of cells with the percentage of MAC-1 negative cells. This revealed that proliferation of the YAm29 transduced immature MAC-1 negative population was completely impaired, whereas the MAC-1 negative population in the empty vector transduced cells



**Figure 2.** YAm29 inhibits proliferation of MAC-1 negative cells. For the liquid cultures bone marrow cells were grown in the same manner as used for the colony assays, but lacking methylcellulose. Cells were grown in 96 well plates. (A) Bone marrow cells were transduced with control or YAm29 retrovirus ( $t = -1$ ) and GFP<sup>+</sup> cells were grown in liquid medium. Cells were counted on a flowcytometer by adding a constant amount of beads to each sample (flow-check beads, Beckman coulter). There was a statistically significant difference in the amount of cells on day 6 ( $p < 0.05$ ) between the two treatments. (B) Transduced cells were incubated with MAC-1 antibody (1:100, Pharmingen, the Netherlands, Alphen a/d Rijn) to discriminate between immature and mature myeloid cells. The flowcytometer dot plots are shown, forward scatter (FS) against MAC-1 expression, from day 1 to day 8. Also shown is the mean intensity of the MAC-1 expression (C) The expansion of MAC-1 negative cells was calculated by multiplying the percentage of MAC-1 negative cells, from panel B, with the total amount of cells, from panel A. There was a statistically significant difference in the amount of cells on day 6 ( $p < 0.05$ ) between the two treatments (D) Cells were double stained with MAC-1 antibody and annexin V and analyzed by flowcytometry to assess the percentage of apoptotic cells.<sup>11</sup> The increase in annexin V in the YAm29 transduced cells in the MAC-1 negative fraction was significantly higher on day 0 ( $p < 0.05$ ) and day 1 ( $p < 0.05$ ).

still expanded (Figure 2C). The intensity of the MAC-1 marker, determined by mean fluorescence (Figure 2B), which correlates with the maturation stage of the cells, increased at an almost comparable rate for both populations. This suggested that YAm29 expression did not severely affect differentiation.

DNA histograms of the YAm29 transduced and empty vector transduced cells showed no difference in the amount of cells in the different phases of the cell cycle (*data not shown*). To investigate whether the loss of expansion in YAm29 transduced BM cells was accompanied by increased apoptosis, we stained the BM cells with annexin V. Within the MAC-1 negative population, 9-10% annexin V positive cells were found the first two days in the culture of cells transduced with the empty vector, whereas 23-25% of annexin V positive cells were found the first two days in the culture of cells transduced with YAm29 (Figure 2D). This higher percentage of annexin V positivity was not found in the YAm29 transduced MAC-1 positive population. These data suggest that loss of NF-Y function resulted in increased apoptosis, specifically in MAC-1 negative myeloid progenitor cells.

To summarize, we show that blocking the function of NF-Y in BM cells results in a significant reduction of CFU-G and CFU-M formation both in size and number. BM cells transduced with YAm29 were still able to become MAC-1 positive at a comparable pace as cells transduced with empty vector, suggesting that maturation was not severely affected. However, YAm29 transduction completely inhibited the expansion of the immature, MAC-1 negative population. This suggests that YAm29 inhibits expansion of the myeloid progenitor cells, explaining the overall decrease in myeloid cells in the liquid and colony assays upon YAm29 expression and indicating that functional NF-Y is needed for expansion of the myeloid progenitor fraction.

NF-Y is a complex of three proteins. Zhu *et al.*<sup>2</sup> showed that overexpression of only the NF-YA subunit promotes the expansion of hematopoietic stem cells. Here we show that inhibiting the function of the trimeric NF-Y complex, with a dominant negative protein, reduces the expansion of progenitor BM cells. Together, these data suggest that the increased expansion of immature BM cells upon overexpression of only the NF-YA subunit is through

increased activity of the trimeric NF-Y complex. Therefore, the size of the pool of immature hematopoietic cells may be regulated through (de-)activating NF-Y.

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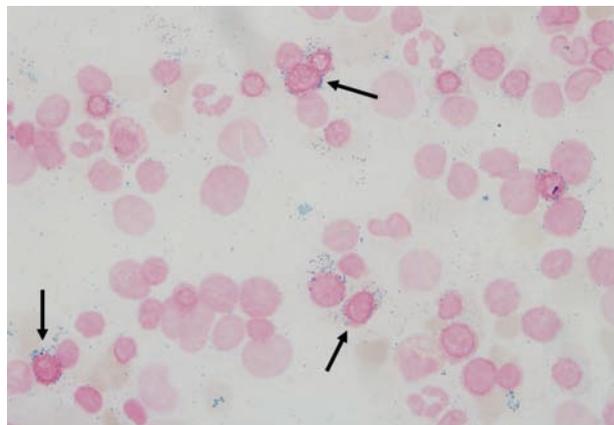
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## Congenital sideroblastic anemia associated with germline polymorphisms reducing expression of *FECH*

The sideroblastic anemias (SAs) are disorders of ineffective erythropoiesis, collectively characterized by abnormal Prussian blue-positive granules (i.e., iron-stuffed mitochondria) that encircle marrow erythroblast nuclei to form ringed sideroblast cells.<sup>1</sup> SAs are usually acquired, but occasionally congenital. While the causes of the common acquired forms of SA remain largely unknown, the molecular genetics of several of the inherited forms of SA is now well understood.<sup>2,3</sup> For instance, X-linked SA is often associated with germline mutations in the erythroid-specific isoform of 5-aminolevulinic synthase (*ALAS2*), and several mitochondrial metabolic defects have also been linked to inherited SAs. However, there are still many congenital SA cases of unknown molecular origin.

The precise relationship between SA and erythropoietic protoporphyria (EPP, MIM #177000) is unclear. A substantial fraction of patients with EPP have anemia (48% of women and 33% of men in the largest series), which is usually mild and associated with diminished iron stores.<sup>4</sup> Ferrochelatase, the enzyme deficient in EPP, is encoded by the *FECH* locus at 18q21.3 and catalyzes the final step in heme biosynthesis: addition of ferrous iron to the protoporphyrin ring.<sup>5</sup> In one analysis of 9 EPP patients, scattered ringed sideroblasts were observed by light microscopy in the bone marrows of 7 patients, while mitochondrial electron energy-loss spectroscopy (EELS) indicated SA-like iron compounds in all 9 samples.<sup>6</sup> Additionally, a 1973 report described a case of EPP with fatal liver disease associated with SA-like features.<sup>7</sup> Despite these observations, most idiopathic acquired SA cases do not have *FECH* mutations, even though modest elevations of erythrocyte protoporphyrin levels are common in this group.<sup>3,8</sup>

Here we describe a child who presented with congenital SA of unclear etiology, in whom we detected marked-



**Figure 1.** Iron stain of bone marrow aspirate demonstrating ringed sideroblasts. Numerous ringed sideroblasts (arrows) comprising 70-80% of the bone marrow erythroid cells are evident. (Prussian blue reaction, 400X, obtained with Olympus BX 40 microscope (Olympus, Tokyo, Japan) equipped with an Uplan 100 x/1.30 NA oil apochromatic lens and Olympus Q-color 3 CCD camera. Image processed for color balance using Adobe Photoshop CS2 (Adobe Systems, San José, CA, USA).