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Identification of a Human Heme Exporter that Is Essential for Erythropoiesis

John G. Quigley,[1,6,7] Zhantao Yang,[1,6] Mark T. Worthington,[3] John D. Phillips,[4] Kathleen M. Sabo,[1] Daniel E. Sabath,[2] Carl L. Berg,[3] Shigeru Sassa,[5] Brent L. Wood,[2] and Janis L. Abkowitz[1,*]

[1]Department of Medicine/Hematology
[2]Department of Laboratory Medicine
University of Washington
Seattle, Washington 98195
[3]Department of Medicine/Gastroenterology
University of Virginia
Charlottesville, Virginia 22908
[4]Department of Medicine
University of Utah
Salt Lake City, Utah 84132
[5]Laboratory of Biochemical Hematology
Rockefeller University
New York, New York 10021

Summary

FLVCR, a member of the major facilitator superfamily of transporter proteins, is the cell surface receptor for feline leukemia virus, subgroup C. Retroviral interference with FLVCR display results in a loss of erythroid progenitors (colony-forming units-erythroid, CFU-E) and severe anemia in cats. In this report, we demonstrate that human FLVCR exports cytoplasmic heme and hypothesize that human FLVCR is required on developing erythroid cells to protect them from heme toxicity. Inhibition of FLVCR in K562 cells decreases heme export, impairs their erythroid maturation and leads to apoptosis. FLVCR is upregulated on CFU-E, indicating that heme export is important in primary cells at this stage. Studies of FLVCR expression in cell lines suggest this exporter also impacts heme trafficking in intestine and liver. To our knowledge, this is the first description of a mammalian heme transporter.

Introduction

Heme, a complex of iron and protoporphyrin IX, is an important component of a diverse group of hemoproteins, including those involved in oxygen transport and storage (hemoglobin, myoglobin), electron transfer and drug metabolism (cytochromes), and signal transduction (nitric oxide synthases) (Ponka, 1997). Heme, however, is toxic, promoting oxidative cell membrane damage through lipid peroxidation, which necessitates tight regulation of heme's intracellular concentration (Ryter and Tyrrell, 2000). This regulation is thought to occur through a balance among heme biosynthesis, utilization for hemoproteins, and catabolism by the microsomal enzyme, heme oxygenase (HO) (Ponka, 1997), to generate carbon monoxide, iron, and biliverdin.

Red cells have a unique requirement for heme as the prosthetic group of hemoglobin, which in the mature erythrocyte comprises over 90% of cellular protein content. Thus, coordinate regulation of heme and globin synthesis is required to avoid heme toxicity yet preserve heme supply. During erythropoiesis as the erythroid progenitors, burst-forming units-erythroid (BFU-E), mature to colony-forming units-erythroid (CFU-E) and then erythroid precursor cells, there is an increase in intracellular iron levels (via transferrin-iron uptake), and heme biosynthesis initiates (Ponka, 1997; Wickrema et al., 1992). Previous studies have demonstrated that heme induces the erythroid differentiation of erythroid cell lines (e.g., K562 cells; Rutherford et al., 1979) in vitro. More recently it has been shown that intracellular heme regulates both the transcription and translation of globin mRNAs through interference with the DNA binding of Bach1, a transcriptional repressor of the globin genes, and inhibition of an erythroid-specific eIF-2α kinase (Ogawa et al., 2001; Rafie-Kolpin et al., 2000). Thus, it appears that increasing intracellular heme levels during early erythropoiesis triggers the onset of globin protein synthesis, which ultimately reduces the levels of uncommitted heme. The response of nonerythroid cells to any excess in intracellular heme includes the rapid induction of HO. However, as heme availability is critical for the continued development of erythroid progenitors, induction of this enzyme in erythroid cells may be inappropriate given its prolonged half-life (20 hr; Ibrahim et al., 1982), suggesting a stage-specific requirement for a more dynamic protection mechanism.

Previously, we and others cloned FLVCR (Quigley et al., 2000; Tailor et al., 1999), a cell surface protein that serves as the receptor for feline leukemia virus, subgroup C (FeLV-C). By homology, FLVCR is a member of the major facilitator superfamily (MFS; Pao et al., 1994) of secondary permeases, which transport small solutes (e.g., sugars, amino acids) across membranes in response to chemico-osmotic gradients. However, the transport function of this viral receptor was unknown.

Cats viremic with FeLV-C, a simple (nononcogene-containing) retrovirus, develop profound anemia (Abkowitz et al., 1987; Onions et al., 1982; Riedel et al., 1986, 1988; Wardrop et al., 1986); while BFU-E are present at normal frequency, there is a paucity of CFU-E and erythroid precursors, suggesting that erythropoiesis is arrested at the CFU-E/proerythroblast stage (Abkowitz et al., 1987). Studies indicate that in infected cells, the FeLV-C envelope surface unit protein acts as a dominant-negative protein to prevent the display and/or function of its cell surface receptor (feFLVCR) and cause this phenotype (Weiss and Tailor, 1995; Riedel et al., 1988; Rigby et al., 1992). Further investigations using viral envelope chimeras of FeLV-A, a related but nonpathogenic virus which also infects all feline bone marrow (BM) cells (Dean et al., 1992), demonstrate that replacement of as

*Correspondence: janabk@u.washington.edu
[6]These authors contributed equally to this work.
[7]Present address: Section of Hematology/Oncology (MC 734), Department of Medicine, University of Illinois at Chicago, Chicago, Illinois 60607.

few as 11 amino acids of FeLV-A envelope variable region 1 (VR1) with amino acids contained in the VR1 of FeLV-C alters both the host range and receptor use of FeLV-A to that of FeLV-C and results in anemia in viremic cats (Rigby et al., 1992). Thus, the genetic determinants of viral binding to feFLVCR and the impairment of erythroid differentiation map to VR1 of the FeLV-C envelope. Since feFLVCR expression is downregulated by intracellular envelope production in all infected BM cells (including BFU-E and CFU-GM; Abkowitz et al., 1987), the specific loss of CFU-E implies that this transporter serves a cellular function uniquely required for CFU-E differentiation or survival (a conclusion that is further supported by studies in which normal feline progenitors infected in vitro with FeLV-C/Sarma have deficient erythroid, but preserved myeloid, differentiation [K.M.S. and J.L.A., unpublished data]).

Here, we demonstrate that FLVCR functions as an exporter of cytoplasmic heme and provide evidence of FLVCR's importance for human erythroid cell differentiation.

Results

FLVCR Expression in Cells and Cell Lines

FLVCR is widely expressed as demonstrated by Northern blot analysis (Tailor et al., 1999; Z.Y., J.G.Q., and J.L.A., unpublished data) and by the broad range of tissues easily infected by FeLV-C in vivo and in vitro (Dean et al., 1992 and references therein). Thus, as an initial study to gain insight into FLVCR function, we quantitated human FLVCR mRNA and cell surface protein expression in cell lines and primary cells (Figure 1) using RT-PCR and a polyclonal antibody specific to FLVCR (α-FLVCR, Figure 2). These experiments demonstrate that mRNA and protein expression are particularly high in Caco-2 (small intestinal phenotype) and HepG2 (hepatic phenotype) cells—cell lines previously utilized for studies of heme transport (Galbraith, 1990; Worthington et al., 2001). In addition, as predicted from Unigene and FLVCR EST expression information (enter FLVCR as a search term at http://source.stanford.edu), FLVCR expression is high in a number of cancer cell lines, which may have increased heme utilization due to rapid cell turnover. Notably, mRNA and protein levels are high in mobilized peripheral blood (PB) $CD34^+$ stem/progenitor cells and in hematopoietic cell lines with erythroid features (e.g., K562 and HEL-DR), but cell surface protein expression is absent in a more mature erythroid cell line, HEL-R, with spontaneous hemoglobinization, which is derived from HEL-DR (Papayannopoulou et al., 1987). In some cells, there were disparities between protein and mRNA expression levels (Figure 1). These disparities may reflect posttranscriptional regulation of protein levels, for example, regulation of the rate of cycling of FLVCR protein to and from the cell surface, a control mechanism important in the cell surface expression of another MFS permease, the Glut-4 transporter (Bryant et al., 2002).

Cellular Heme Content Is Dependent on FLVCR Cell Surface Expression

As an initial screen, to determine if FLVCR could have a role in heme trafficking or metabolism, the heme content of a rat renal epithelial cell line (NRK) engineered to express the feline ortholog of FLVCR, NRK/feFLVCR, was compared with that of control NRK cells. NRK cells are resistant to FeLV-C infection, implying that the rodent ortholog of FLVCR is poorly expressed or does not efficiently bind FeLV-C; however, once transduced with feFLVCR, the cells can be easily infected by FeLV-C (Quigley et al., 2000). The heme content of NRK/feFLVCR cells was significantly (though minimally) lower than that of control cells (10.71 vs. 11.93 pmol heme/10^6 cells, $p = 0.04$ by two-tailed Student's t test). We reasoned that compensatory events during the in vitro selection and expansion of the cell lines might account for the smallness of the difference in steady-state heme content.

We then examined the heme content of cell lines upon viral interference with FLVCR cell surface expression. Feline embryonic fibroblasts (FEA) are readily infected with both FeLV subgroups B and C (FeLV-B and FeLV-C) and thus express their respective receptors, fePiT-1 and feFLVCR (Quigley et al., 2000; Rudra-Ganguly et al.,

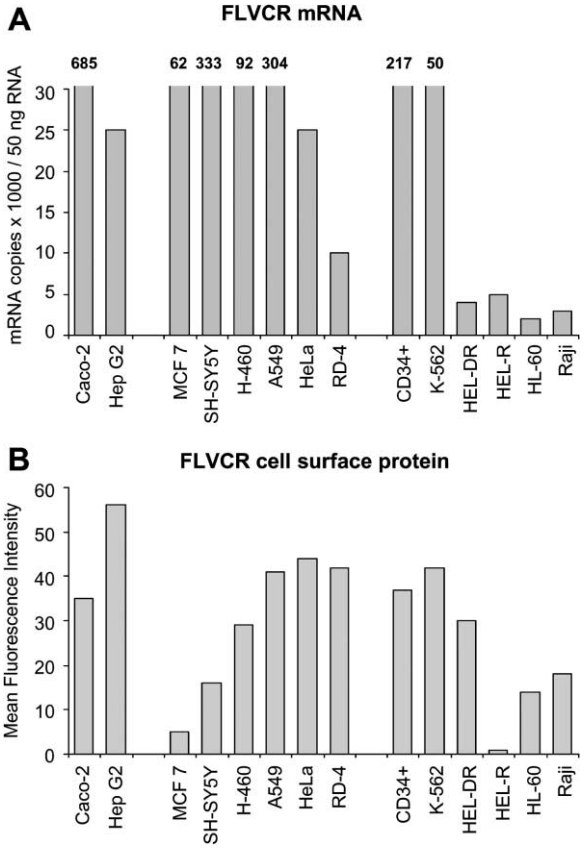


Figure 1. FLVCR Expression in Human Cells

Small intestinal (Caco-2) and hepatic phenotype (HepG2) cell lines have high levels of FLVCR mRNA (A) and protein (B), as measured by quantitative RT-PCR and flow cytometry with a polyclonal antibody specific to FLVCR, α-FLVCR, respectively. Studies of cancer cell lines of breast (MCF7), neuroblastoma (SH-SY5Y), lung (H-460 and A549), cervix (HeLa), rhabdosarcoma (RD-4), and hematologic origin are also shown. Mobilized PB $CD34^+$ cells and cell lines with an early erythroid phenotype (HEL-DR, K562) have high levels of FLVCR protein on their cell surface, while the more mature erythroid line, HEL-R, fails to express FLVCR protein.

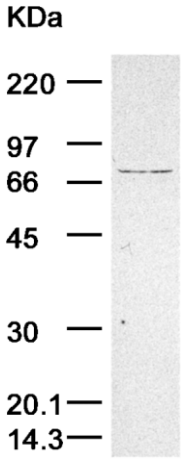


Figure 2. α-FLVCR Is Specific for Human FLVCR

Western blot analysis demonstrates the specificity of the rabbit polyclonal antibody, α-FLVCR. A single band is detected in this study of HepG2 cell lysates. Its location is in agreement with the predicted mass of FLVCR.

1998). Infection of FEA cells with either retrovirus impairs the surface expression of its specific cell surface receptor through binding of the viral envelope protein to receptor in the ER or Golgi apparatus. This viral interference prevents further infection of the cell by retroviruses of the same subgroup (Weiss and Tailor, 1995). Infection of FEA cells with FeLV-B (FEA/FeLV-B) has no effect on cell heme content. However, infection with FeLV-C, which impairs cell surface expression of FLVCR, results in a significant increase in the intracellular heme content of FEA cells (FEA, 5.91 pmol heme/10^6 cells; FEA/FeLV-B, 6.30 pmol heme/10^6 cells; FEA/FeLV-C, 10.96 pmol heme/10^6 cells; $p = 0.05$ FEA/FeLV-C versus FEA/FeLV-B cellular heme content).

Heme Is Exported by FLVCR

We next evaluated heme transport using zinc mesoporphyrin (ZnMP), a fluorescent heme analog previously validated in heme transport studies (Worthington et al., 2001). NRK cells transduced with human *FLVCR* (NRK/FLVCR) or empty vector alone (NRK/ev) were incubated in ZnMP for 30 min at 37°C, and quantitative fluorescence microscopy was performed. The mean fluorescent intensity (MFI) of NRK/FLVCR cells was 74.5% ± 10.9% SD of that of NRK/ev cells ($n = 5$ experiments, $p < 0.001$, two-tailed Student's t test). As ZnMP blocks its own catabolism within cells by inhibition of microsomal HO (Worthington et al., 2001), the reduced net intracellular accumulation (or loading) of ZnMP observed in NRK/FLVCR cells after 30 min must result from either increased export or impaired import of the mesoporphyrin.

To distinguish between these possibilities, we studied the washout of ZnMP. Cells were loaded with ZnMP, washed extensively, then placed in buffer alone for 90 min, and the percentage of ZnMP exported was determined by calculating the ratio of the cellular MFI after washout to the MFI after loading. The MFI of NRK/FLVCR cells markedly decreases during the washout period (MFI at baseline = 0, after the 30 min load = 74.9% ± 10.5%, and after 90 min of washout [120 min total time] = 36.5% ± 5.2%; $p < 0.001$), and thus 51.3% ± 7.1% of ZnMP was exported. However, the MFI of control cells did not change significantly and export was 5.3% ± 0.6% (p = NS) (Figure 3B). In addition, there was no change in the cellular ZnMP content of NRK/FLVCR cells when washout experiments were performed at 4°C, demonstrating that export is a temperature-dependent process (Figures 3A and 3B).

The uptake and washout studies were repeated using ^{55}Fe-hemin so that heme export could be directly assessed. These studies required preincubation with ZnMP to prevent the breakdown of ^{55}Fe-heme by the cytosolic enzyme HO and thus confirm that both ZnMP and ^{55}Fe-hemin were internalized. Cells were incubated with ^{55}Fe-hemin then placed in washout buffer, and the decrease in the cells' radioactivity was measured over time. As shown in Figure 3C, the results were comparable to those seen when the export of the mesoporphyrin was tracked. Forty-nine ± two percent of ^{55}Fe-heme was exported by NRK/FLVCR cells ($p < 0.01$), while the heme content of control NRK/ev cells and NRK/14q cells, cells engineered to overexpress an FLVCR paralog on chromosome 14q (Lipovich et al., 2002), did not decrease significantly during the washout period.

Quantification of heme in the supernatant confirmed these findings. Specifically, 10^7 NRK/FLVCR and NRK/ev cells were preincubated with ZnMP (to inhibit HO), loaded with heme over 30 min, then washed extensively and placed in washout buffer as described above. After 90 min, the amount of heme (and mesoporphyrin) exported into the washout buffer was determined by organic extraction of heme and porphyrins from the media, and their separation done by high-performance liquid chromatography (HPLC; Lübben and Morand, 1994). NRK/FLVCR cells exported 3292 ± 424 pmol of heme/10^7 cells into the washout buffer, while NRK/ev cells exported 1762 ± 106 pmol ($p < 0.004$). Consistent with these results, NRK/FLVCR cells also exported 1504 ± 10 pmol of ZnMP into the washout buffer, while NRK/ev cells exported 789 ± 25 pmol/10^7 cells ($p < 0.001$). Thus, despite reduced porphyrin loading of NRK/FLVCR cells due to continued export during the loading phase (see Figure 3B), there is an approximately 2-fold increase in both the heme and ZnMP content of the washout buffer, demonstrating that FLVCR exports heme (and ZnMP).

Lastly, to demonstrate that FLVCR exports heme from human hematopoietic cells and is required for erythroid differentiation, we studied its cell surface expression and function in K562 cells. As shown in Figure 1 and Table 1, FLVCR is highly expressed in undifferentiated K562 cells. Since human FLVCR efficiently binds FeLV-C, these cells can be infected with FeLV-C (K562/FeLV-C). As expected, the cell surface expression of FLVCR decreases through viral interference (despite a compensatory increase in FLVCR mRNA levels). This results in a dramatic reduction in ^{55}Fe-heme export. K562/FeLV-C cells export only 3% ± 6% of accumulated ^{55}Fe-heme, while naive K562 cells export 54% ± 8%, and cells infected with FeLV-B, which decreases the cell surface expression of human Pit-1 (Takeuchi et al., 1992), export 54% ± 10%. Notably, there is a similar

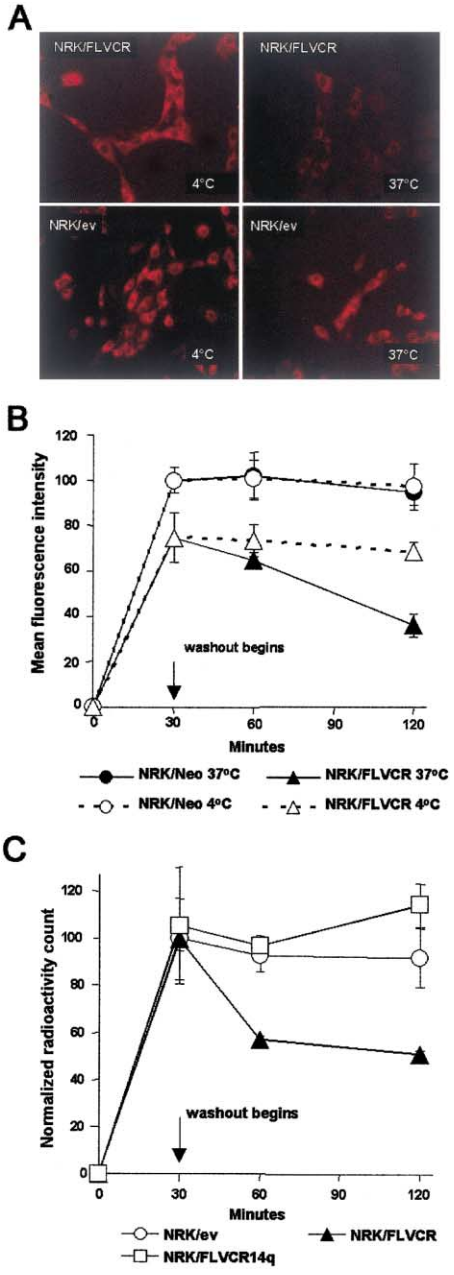


Figure 3. Overexpression of FLVCR Increases Export of the Heme Analog ZnMP and ^{55}Fe-Heme

Cells engineered to overexpress FLVCR (NRK/FLVCR) and control cells (NRK/ev) were incubated with ZnMP (5 μM) for 30 min at 37°C, then washed and incubated for 90 min in buffer alone at 37°C or 4°C. The relative fluorescence of NRK/FLVCR cells is reduced at 37°C (A, right panels). No effects are seen at 4°C (A, left panels). The analysis of ZnMP washout after 30 and 90 min (B) demonstrates a significant time-dependent reduction in NRK/FLVCR cell fluorescence at 37°C, but not at 4°C (n = 4 independent studies).

Similar studies of NRK/FLVCR and control cells were performed with ^{55}Fe-heme. After a brief incubation in ZnMP to inhibit HO activity, ^{55}Fe-hemin (0.9 μM) was added to the cell media for 30 min. Next, cells were washed and the cellular ^{55}Fe-heme content was quantified. Cells were then incubated in buffer alone for 90 min to observe ^{55}Fe-heme washout (n = 3). There is a significant decrease in the ^{55}Fe-heme content of NRK/FLVCR, but not NRK/ev cells or NRK/14q cells (cells engineered to overexpress a FLVCR paralog on chromosome 14q) (C). When the ZnMP pre-incubation step was omitted, ^{55}Fe-heme disappeared quickly and equivalently from NRK/FLVCR and control cells (data not shown).

decrease in heme export when the function of FLVCR is inhibited by incubation with α-FLVCR (0.25 mg/ml).

In addition, the inhibition of FLVCR cell surface expression (by FeLV-C) or FLVCR function (by α-FLVCR) significantly impairs the ability of these cells to undergo erythroid differentiation, as assessed by the frequency of benzidine-positive cells present after 3 days of exposure to hemin (50 μM) or imatinib (0.25 μM; Table 1; or butyrate [0.5 mM] or TGF-β [5 ng/ml]; K.M.S. and J.L.A., unpublished data]). As importantly, when FLVCR is inhibited and erythroid differentiation is induced, apoptosis initiates, as shown by an increase in annexin V binding (Table 2).

The Expression of FLVCR on the Surface of CFU-E Is Higher Than on Other Hematopoietic Progenitor Cells

The specific loss of CFU-E in cats infected with FeLV-C suggests that the function of FLVCR as a heme exporter is uniquely important at this stage of erythropoiesis. We therefore examined the expression of FLVCR on CFU-E derived from mobilized human PB CD34$^+$ stem/progenitor cells. CD34$^+$ cells were incubated in stem cell factor (SCF), interleukin 3 (IL3), and IL6 for 48 hr, and then in an erythroid differentiation cocktail containing SCF, IL3, and erythropoietin for 72 hr. Next, cells were sorted by FACS (using α-FLVCR) into two populations, FLVCR^{hi}, comprising the brightest 13%, and FLVCR^{lo}, the bottom 77%, and the prevalence of erythroid and myeloid progenitors (BFU-E, CFU-E, and CFU-GM) was quantitated using methylcellulose assays. The FLVCR^{hi} population contained 48.7% ± 9.2% of all CFU-E present in the initial samples, an enrichment of CFU-E content of 3.8-fold, but as expected, BFU-E and CFU-GM were distributed proportionally across both subpopulations with average enrichments of 1.1-fold (n = 4 experiments). A representative study is shown in Figure 4.

FLVCR Cell Surface Expression Decreases as Erythropoiesis Proceeds

An extension of our hypothesis is that FLVCR expression might decrease as CFU-E differentiate and hemoglobinization proceeds. To investigate this possibility, the FLVCR cell surface expression of normal human BM cells was examined by flow cytometry. Maturing erythroid forms (CD71^{hi}, CD34^{neg}) had consistently less FLVCR expression than did CD34$^+$ cells (1.98 ± 0.87 versus 4.71 ± 2.61 relative fluorescent units, $p < 0.01$, n = 10).

In additional experiments, we observed the FLVCR protein and mRNA expression of mobilized PB CD34+ cells undergoing erythroid differentiation during 10 days of culture (Figure 5). At day 0, the FLVCR cell surface protein expression was high, but by day 10, as hemoglobinized cells predominated in the culture, protein expression decreased to background levels. The downregulation of expression with erythroid maturation is also in agreement with the results from studies of the mature and immature human erythroid cell lines, HEL-R and HEL-DR (Figure 1).

Table 1. Impairment of FLVCR Expression or Function in K562 Cells Inhibits Heme Export and Erythroid Differentiation

Cell Lines	FLVCR Expression		Heme Export	Ability to Differentiate	
				Benzidine-Positive After 3 days of Incubation in (%):	
	Protein (MFI)	mRNA (copies × 1000/50 ng RNA)	^{55}Fe-Heme Exported (%)	a) Hemin	b) Imatinib
K562	34.3 ± 10.4	51.4 ± 2.2	54 ± 8	51.2 ± 4.1	60.7 ± 0.9
K562/FeLV-B	26.8 ± 3.2	61.4 ± 11.7	54 ± 10	51.5 ± 2.0	61.8 ± 0.6
K562/FeLV-C	18.4 ± 3.3	88.4 ± 6.2	3 ± 6	20.7 ± 5.5	31.4 ± 0.7
K562 + control IgG	ND	ND	44 ± 4	50.6 ± 5.2	59.5 ± 1.7
K562 + α-FLVCR	ND	ND	6 ± 4	28.2 ± 1.2	24.4 ± 1.5
p values					
K562/FeLV-C versus K562/FeLV-B	$p < 0.05$	$p < 0.05$	$p < 0.01$	$p < 0.05$	$p < 0.01$
K562/FeLV-C versus K562	$p < 0.05$	$p < 0.01$	$p < 0.01$	$p < 0.05$	$p < 0.01$
K562 + α-FLVCR versus K562 + control IgG	ND	ND	$p < 0.01$	$p < 0.05$	$p < 0.01$

K562 cells were differentiated utilizing hemin (50 μM) or imatinib (0.25 μM). Results represent the mean ± SD of 4–6 independent studies. p values were derived with two-tailed Student's t-tests. As a further control, α-FLVCR IgG was incubated with NRK/FLVCR cells (to adsorb the α-FLVCR activity) prior to incubation with K562 cells. Adsorption abrogated the α-FLVCR-induced impairment of K562 erythroid differentiation.

Discussion

Numerous reports, beginning in the 1950s, allude to the presence of a heme binding protein on the surface of mammalian cells (enterocytes, hepatocytes, and hematopoietic cell lines) (Galbraith, 1990; Worthington et al., 2001; reviewed in Uzel and Conrad, 1998). However, these putative mammalian heme importers were not identified. Here, we have performed a series of experiments which indicate that the human ortholog of feFLVCR, FLVCR, exports cytoplasmic heme and is important for erythroid differentiation.

Identification of FLVCR as a Mammalian Heme Exporter

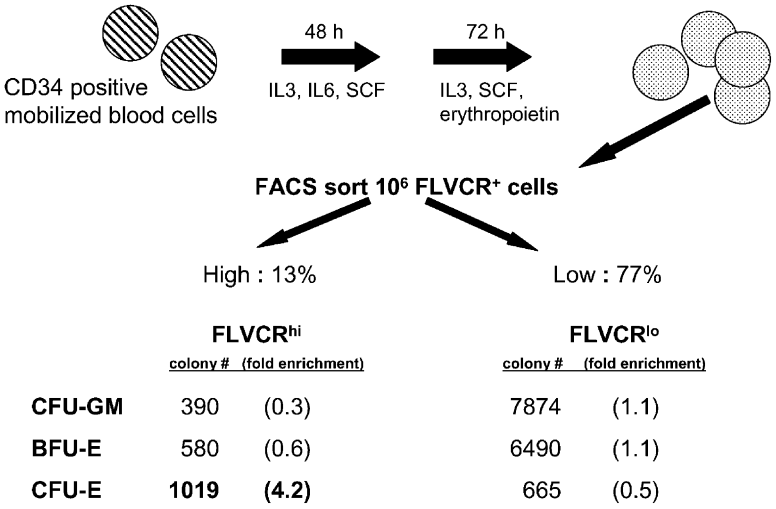
FLVCR mRNA and protein expression levels are increased in cell lines derived from tissues that either transport heme (e.g., intestinal and hepatic cell lines) or have increased heme synthesis (e.g., erythroid cell lines). In addition, cell lines engineered to overexpress feFLVCR have a significant decrease in cellular heme content, while specific (FeLV-C) interference with feFLVCR expression or function significantly increases cellular heme content.

In order to directly demonstrate the role of FLVCR in heme export, we performed quantitative fluorescent microscopy studies on cell lines overexpressing human FLVCR, utilizing a fluorescent heme analog, zinc mesoporphyrin (ZnMP). ZnMP has been used to examine heme transport in intestinal and hepatic cell lines (Worthington et al., 2001) and yeast (M.T.W., unpublished data). Due to its potent inhibition of HO, changes in cellular fluorescence reflect mesoporphyrin trafficking and not modification of heme or ZnMP catabolism (Worthington et al., 2001). Our studies demonstrate a significant reduction in ZnMP accumulation in cells overexpressing FLVCR as compared to control cells under steady-state conditions. More importantly, washout studies show a dramatic time-dependent decrease in the fluorescence of NRK/FLVCR but not control cells

Table 2. Impairment of FLVCR Expression or Function in K562 Cells Is Associated with Apoptosis

Cell Lines	Induction by Hemin		Induction by Imatinib	
	Annexin V Binding (%)	PI Positive (%)	Annexin V Binding (%)	PI Positive (%)
K562	9.6 ± 0.1	0.01 ± 0.01	12.9 ± 0.4	0.04 ± 0.05
K562/FeLV-B	8.7 ± 1.2	0.01 ± 0.01	11.3 ± 0.5	0.02 ± 0.03
K562/FeLV-C	23.6 ± 0.8	0.02 ± 0.02	21.4 ± 3.8	0.32 ± 0.14
K562 + control IgG	10.6 ± 1.3	0.02 ± 0.01	10.9 ± 0.4	0.01 ± 0.01
K562 + α-FLVCR	25.3 ± 0.6	0.02 ± 0.02	21.9 ± 0.9	0.02 ± 0.01
p values				
K562/FeLV-C versus K562/FeLV-B	$p < 0.005$	$p = NS$	$p < 0.07$	$p = NS$
K562/FeLV-C versus K562	$p < 0.002$	$p = NS$	$p < 0.09$	$p = NS$
K562 + α-FLVCR versus K562 + control IgG	$p < 0.005$	$p = NS$	$p < 0.03$	$p = NS$

K562 cells were differentiated utilizing hemin (50 μM) or imatinib (0.25 μM) for 24 hr. Early and late apoptosis was assessed by binding of annexin V and staining with propidium iodide (PI), respectively. Results represent the mean ± SD of two independent studies. p values were derived with two-tailed Student's t-tests.



	FLVCR^{hi} colony #	(fold enrichment)	FLVCR^{lo} colony #	(fold enrichment)
CFU-GM	390	(0.3)	7874	(1.1)
BFU-E	580	(0.6)	6490	(1.1)
CFU-E	**1019**	**(4.2)**	665	(0.5)

Figure 4. FLVCR Expression Is High on CFU-E Progenitor Cells

Mobilized PB CD34$^+$ cells were exposed to an erythroid differentiation cocktail for 72 hr. FLVCR$^+$ cells were then sorted into FLVCR^{hi} (13% with highest fluorescence) and FLVCR^{lo} (bottom 77%) subpopulations. The number of progenitors derived from sorting 10^6 cells is shown. In this representative study, the FLVCR^{hi} population contains 54% of all FLVCR$^+$ CFU-E, when 13% is expected, a 4.2-fold enrichment, while the early erythroid progenitors, BFU-E, and granulocyte-macrophage progenitors, CFU-GM, are distributed normally. Similar results were observed in three independent experiments.

indicating that FLVCR exports heme. Similar fluorescence methodologies and experimental design have been utilized to demonstrate the role of other secondary transporters (e.g., Bmr, Neyfakh et al., 1991 and QacA, Mitchell et al., 1999) in multidrug export from bacteria.

In confirmatory studies, the ^{55}Fe-heme content of NRK/FLVCR cells and NRK/ev cells was quantified after ^{55}Fe-hemin exposure and washout conditions—studies performed in the presence of ZnMP to competitively inhibit HO-mediated catabolism of ^{55}Fe-heme. Identical experiments with NRK cells overexpressing a paralog of FLVCR from chromosome 14q (60% amino acid identity; Lipovich et al., 2002) demonstrate that heme export is a specific function of FLVCR (Figure 3C). Furthermore, quantification of porphyrins in the washout supernatants of NRK/FLVCR cells (with HPLC) proves that heme (and ZnMP) are exported as intact molecules across the cell surface membrane.

Finally, ^{55}Fe-heme washout studies in K562 cells, a human erythroid cell line, demonstrate that specific interference with either the cell surface expression of FLVCR (by FeLV-C) or its membrane transport function (by α-FLVCR) significantly reduces the export of heme. As heme can stick indiscriminately to phospholipid-containing cell membranes, experiments where FLVCR is selectively modulated are critical. The α-FLVCR experiments are especially informative as they demonstrate that targeted (and nonretroviral-mediated) cell surface inhibition of FLVCR function impairs heme export.

We have no evidence to suggest that FLVCR is a bidirectional heme transporter. The observations demonstrating increased accumulation of ZnMP in NRK/ev compared with NRK/FLVCR cells after a 30 min loading phase speak against an import function (Figures 3A and 3B). Also, studies following the overnight incubation of these cells with the heme synthesis inhibitor succinylacetone (Ponka et al., 1982; Worthington et al., 2001) suggest that FLVCR does not reverse function even when intracellular heme is depleted (M.T.W., unpublished data).

Prokaryotic heme import systems consisting of an outer membrane TonB-dependent hemoreceptor (e.g., HemR), a periplasmic heme binding protein, and an ATP binding permease that delivers heme to the cytoplasm have been described in gram-negative bacteria (see Wandersman and Stojiljkovic, 2000 for review). Detailed analysis of the hemoreceptors has revealed a conserved receptor domain containing invariant histidine residues, FRAP and NPNL amino acid boxes, suggesting their importance in prokaryotic heme binding and/or transport. In addition, a number of mammalian intracellular heme binding proteins (e.g., HBP23; Iwahara et al., 1995) and more general tetrapyrrole binding proteins (e.g., p22HBP; Jacob Blackmon et al., 2002; Taketani et al., 1998) have been identified. Sequence comparisons, however, show no similarity between FLVCR and these proteins or HemR.

Heme Export Is of Critical Importance in Erythropoiesis

Heme is essential to oxidation-reduction reactions in all aerobic cells, thus it is not surprising that FLVCR orthologs are present in bacterial, plant, and animal genomes (Lipovich et al., 2002; see also http://www.ncbi.nlm.nih.gov/sutils/blink.cgi?pid=7661708). However, in mammals, heme has a unique role in oxygen binding and transport as the prosthetic group of hemoglobin in erythrocytes. At, or just after, the CFU-E stage of erythroid differentiation, heme synthesis increases, as documented by ^{59}Fe incorporation into heme (Wickrema et al., 1992). We propose that this results in a unique requirement for FLVCR to control intracellular heme content. In agreement with this hypothesis, these progenitors have increased levels of FLVCR on their cell surface compared to levels on the less-mature progenitor, BFU-E (Figure 4). Also, a region of the *FLVCR* promoter, between −370 and −1030 nucleotides relative to the translation initiation site, contains four potential STAT5a binding sites, as well as consensus GATA-1, GATA-2, c-myb and NF-E2 binding sites, providing potential mechanisms for the upregulation of *FLVCR* transcription during early erythroid commitment and differentiation.

An extension of our hypothesis is that FLVCR expression may be downregulated when globin protein levels increase and hemoglobinization proceeds. The analyses of the erythroid cell lines HEL-DR and HEL-R, data from

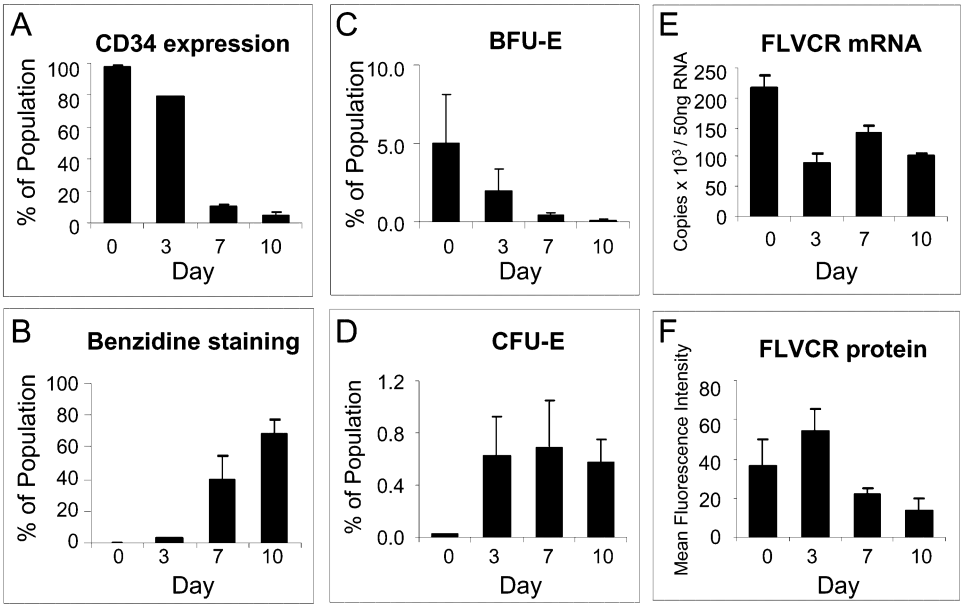


Figure 5. FLVCR mRNA and Cell Surface Protein Expression Decrease as CD34$^+$ Cells Differentiate

FLVCR mRNA and protein expression were observed during 10 days of erythroid differentiation. The number of CD34$^+$ cells decreases (A), CD71$^+$ cells increase (not shown), and hemoglobinization occurs (as measured by benzidine staining [B]) during this time. Initially there are high frequencies of CFU-GM (not shown) and BFU-E (C) but few CFU-E. The number of CFU-E increases throughout the study; however, their frequency remains constant due to an increase in the number of maturing erythroid cells (D). The FLVCR expression in this heterogeneous population of cells is initially high but decreases with erythroid differentiation (E–F). The results reflect the mean (±SD) of three independent experiments plus two additional studies observing days 0–3.

normal BM CD34$^+$ versus maturing (bright CD71$^+$) erythroid cells, and the studies of FLVCR mRNA and protein expression during the in vitro differentiation of mobilized human peripheral blood CD34$^+$ cells (and K562 cells; Z.Y. and J.L.A., unpublished data) all support this premise.

The studies of K562 cells are also in accordance with the concept that FLVCR is required for erythroid differentiation. Retroviral interference with FLVCR expression or specific antibody-mediated inhibition of FLVCR function impairs both heme export and the subsequent hemoglobinization of these cells (Table 1). Also, there is increased apoptosis (Table 2). It is possible that heme excess (e.g., through oxidative cell membrane damage) induces apoptosis and, in turn, the failure of erythroid differentiation. Alternatively, heme excess might dysregulate erythropoiesis (e.g., by prematurely initiating globin translation) and cause apoptosis.

The effects of FeLV-C viremia on feline erythropoiesis, where there is normal development of BFU-E but severe anemia due to a block of CFU-E and further erythroid maturation, attest to the importance of FLVCR function in vivo. As the viral envelope interferes with the expression/function of its receptor (FLVCR) in all infected cells (including BFU-E and CFU-GM progenitors; Abkowitz et al., 1987), the specific loss of cells at the CFU-E stage of differentiation argues for a unique requirement for heme export at this time.

FLVCR Functions as an Overflow Valve

Previous studies demonstrate that exogenous heme induces the erythroid differentiation of cell lines such as K562 cells and improves the differentiation of erythroid progenitors (BFU-E) in vitro (Holden et al., 1983; Rutherford et al., 1979). Heme upregulates the erythroid heme biosynthetic pathway, possibly through inhibition of the transcriptional repressor, Bach1, and also contributes to optimal transferrin receptor expression (Ponka, 1997). Furthermore, erythroid differentiation is arrested upon disruption of the murine erythroid-specific 5-aminolevulinate synthase gene (ALAS-2), the critical initial enzyme in erythroid intracellular heme synthesis (Nakajima et al., 1999). Thus, the results presented here, demonstrating that FLVCR-mediated heme export is required during early erythroid development, may appear counterintuitive. Heme, however, is also toxic, necessitating redundant mechanisms to stringently control its intracellular concentration. Thus, we hypothesize that FLVCR functions as a cell membrane export channel (overflow valve), providing a safety mechanism that is uniquely important at the CFU-E stage of differentiation.

Regulation of intracellular heme levels differs markedly between erythroid and nonerythroid cells. In hepatocytes, for example, the heme pool is regulated through a combination of synthetic and degradative mechanisms (through heme-mediated negative feedback control of ALAS-1 and heme induction of HO). In contrast, in erythroid cells, the erythroid-specific isozyme ALAS-2 is not regulated by heme, but rather by iron supply (reviewed in Ponka, 1997). In addition, there is no evidence that HO is induced by endogenous heme; studies of the murine erythroid cell line MEL have shown that differentiation is in fact associated with a reduction in HO mRNA levels (Fujita and Sassa, 1989). Thus, apart from the

ability to upregulate globin protein synthesis, there is a paucity of identified heme control mechanisms in erythroid cells, presumably related to their requirement for maximal heme synthesis. We conclude that expression of a heme exporter on the cell membrane is therefore required during early erythropoiesis to allow for a rapid cellular response to fluctuating cytosolic heme levels, thus complementing the more static role of induction of globin in preventing heme toxicity.

Although FLVCR is downregulated with terminal erythroid differentiation, we suspect that FLVCR does not significantly interfere with heme binding to globin. Overexpression of FLVCR in K562 cells via retroviral gene transfer does not impede erythroid differentiation (Z.Y., J.G.Q., and J.L.A., unpublished data). These observations are consistent with a safety mechanism or overflow valve hypothesis.

The Function of FLVCR in Other Tissues

FLVCR is highly expressed in cell lines with a small intestinal or hepatic phenotype (Figure 1). As two-thirds of body iron derives from dietary heme iron (Carpenter and Mahoney, 1992), there must be significant heme trafficking into the small intestine. The liver has the second highest rate of heme biosynthesis (for cytochrome P450) and serves as a destination for heme linked to hemopexin or albumin. Thus, FLVCR might facilitate heme transport or, more likely, complement the role of HO induction in the control of cellular heme content at these sites. FLVCR is also expressed in hematopoietic stem cells (Z.Y. and J.L.A., unpublished data; and see Figures 1 and 5), which presumably require stringent protection from heme toxicity to assure genetic integrity. Although these cells are relatively quiescent, heme synthesis is needed for oxidative metabolism. FLVCR may function in their protection from perturbations in endogenous heme levels or from extracellular heme in the BM microenvironment (e.g., hemolysis in the BM is a consequence of ineffective erythropoiesis). Notably, recent studies demonstrate that the ABC transporter, ABCG2, which protects stem cells from environmental xenobiotics (Abbott, 2003), also functions as a cell membrane export pump for protoporphyrin IX, the direct precursor of heme in the heme synthesis pathway (Jonker et al., 2002; Krishnamurthy et al., 2004). These data lend further support for the concept that stem cells require redundant mechanisms for regulation of heme content.

Experimental Procedures

Cell Lines

HEL-DR and HEL-R cells were a gift from T. Papayannopoulou, MCF7 cells were obtained from M.-C. King, RD-4 was a gift of M. Hentze, and all other cell lines were obtained from the American Type Culture Collection. NRK/FLVCR and NRK/ev cells were generated by transduction of NRK-52E cells with the retroviral vector MSCVneo (BD Biosciences Clontech) containing the human *FLVCR* cDNA (amplified from a human kidney cDNA library [BD Biosciences Clontech], using primers specific to the human *FLVCR* cDNA [GenBank accession number: AF118637]) or no additional cDNA, respectively. Cells were selected in G418. The generation of NRK/feFLVCR has been described previously (Quigley et al., 2000). The cDNA encoding FLVCRL14q (I.M.A.G.E. clone 4866427) was inserted into MSCVneo, and NRK/14q cells generated in a similar fashion.

Heme Content of NRK Cell Lines

Heme content (reported as pmol heme/10^6 cells) was determined as described previously (Sassa, 1976).

ZnMP Uptake and Washout Studies

NRK/FLVCR and NRK/ev cell lines were grown on chamber slides in standard media, with parallel 30 min uptake studies performed at 37°C using 5 μM ZnMP (Frontier Scientific, Logan, Utah) dissolved in cell culture-grade DMSO to a concentration of 4 mM, followed by dilution in washout buffer (25 mM HEPES, [pH 7.4], 130 mM NaCl, 10 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$) and 2.5 μM BSA as previously described (Worthington et al., 2001). For washout experiments, the cells were washed 3 times and then incubated in working buffer and 5% BSA at 37°C or 4°C for 90 min.

Microscopy

Fluorescence microscopic imaging and quantitative digital microscopy measurement of cellular ZnMP uptake were performed as described (Worthington et al., 2001). All measurements utilized triplicate images for each data point. Digital images were analyzed as described previously (Worthington et al., 2001). The MFI of cellular ZnMP in the control NRK/ev cells after 30 min was considered 100% in each study.

^{55}Fe-Hemin Uptake and Washout Studies

The procedures and buffers were the same as for the ZnMP studies. After a brief rinse with working buffer, the cells (10^5 cells/well in 12-well dishes) were incubated with 5 μM of ZnMP in washout buffer and 2.5 μM BSA for 15 min, and then ^{55}Fe-hemin (RI Consultants LLC, Hudson, New Hampshire) was added (to a final concentration of 0.9 μM) for 30 additional minutes. The cells were then washed three times, and placed in washout conditions (washout buffer and 5% BSA) for 90 min at 37°C. To measure cellular radioactivity, the cells were rinsed twice with washout buffer, detached from the plates, mixed with liquid scintillation cocktail (ICN), and placed in a liquid scintillation counter. Counts were normalized by total cell protein content. The maximum scintillation count of control NRK/ev cells after the 30 min uptake of ^{55}Fe-heme was considered 100% in each study.

Measurement of Heme and ZnMP Export

The procedures and buffers were the same as described for the ZnMP and ^{55}Fe-hemin uptake and washout studies, except that cell number was increased to 10^7 cells in T-75 flasks. After preincubation with 5 μM ZnMP in working buffer, hemin was added to a final concentration of 50 μM for 30 min (loading). The cells were then washed and placed in washout conditions as above. After 90 min, the washout supernatant (30 ml) was collected and kept at −80°C until analysis. The extraction of porphyrins was adapted from previously described methods (Smith, 1975). Ten milliliter fractions were extracted in parallel three times with 10 ml of ethyl acetate/acetic acid 2:1, then washed twice with 0.5 volumes of saturated sodium acetate. The sodium acetate washings were also extracted with fresh ethyl acetate and combined with the original extract, which was then washed once with 0.1 volumes of 3% sodium acetate and dried down with argon. Samples were dissolved in buffer and the heme and mesoporphyrin separated by HPLC, as described (Lübben and Morand, 1994). Standards of heme, porphyrins and ZnMP (Frontier Scientific) were used for peak identification and quantification.

Western Blot Analysis

Western blots of HepG2 lysates were probed with rabbit anti-FLVCR (1 μg/ml), or preimmune serum (data not shown), followed by a 1:2000 dilution of HRP-labeled goat anti-rabbit IgG (PharMingen). Detection used a chemiluminescence kit (Supersignal West Pico Chemiluminescent kit, Pierce).

RNA Isolation, Quantitative RT-PCR, and Northern Blot Analysis

Cells were pelleted, and total RNA isolated using Trizol (Invitrogen), as suggested by the manufacturer. Total RNA was reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen). Quantitative RT-PCR was performed using TaqMan chem-

istry and the ABI PRISM 7700 sequence detection system (Applied Biosystems). The results are expressed as copies × 1000/50 ng total RNA. GADPH and β2-microglobulin were used for the internal normalization of $CD34^+$ cell differentiation and cell line experiments, respectively. PCR primers and probes (available on request) were designed using the Primer Express program (Applied Biosystems) and synthesized at Integrated DNA Technologies, Inc. (Coralville, Iowa). All forward and reverse primer pairs span an intron. To confirm our RT-PCR results, Northern blot analyses were performed according to standard protocols, using a 360 bp midsection fragment of human *FLVCR* cDNA that does not crossreact with *FLVCR* paralogs in the human genome (Lipovich et al., 2002; Z.Y., J.G.Q., and J.L.A., unpublished data).

Generation and Characterization of α-FLVCR

Polyclonal rabbit antisera were raised against NRK/FLVCR cell surface membranes isolated as described (Kinne-Saffran and Kinne, 1989) (performed at R&R Rabbitry, Stanwood, Washington). Preparation of acetone precipitates of NRK/ev cells was performed as previously described (Harlow and Lane, 1988). To remove antibodies to normal rat proteins, 1 ml of anti-FLVCR rabbit serum was incubated with 20 mg of NRK/ev cell acetone precipitate overnight at 4°C, centrifuged, and the supernatant applied to a protein A cartridge connected to a desalting cartridge (protein A antibody purification kit, Sigma) to purify the anti-FLVCR rabbit IgG, termed α-FLVCR.

Quantification of Cell Surface Expression of FLVCR

Cells (4×10^5) were suspended in 0.5 ml of X-VIVO15 media (Biowhittaker), blocked by 1% donkey serum and 1% BSA, and labeled with α-FLVCR (20 μg/ml) followed by R-phycoerythrin (PE)-conjugated donkey anti-rabbit IgG (5 μg/ml, Jackson ImmunoResearch Laboratories). The cells were washed with X-VIVO15 between incubations, fixed, and kept at 4°C until analysis. MFI unit measurements were calculated by subtraction of the fluorescence of cells labeled concurrently with preimmune rabbit IgG. The mean fluorescence of cells labeled with control rabbit IgG was set between 5 and 10 fluorescent units.

Studies of FLVCR Function in K562 Cells

Undifferentiated K562 cells (2×10^5/ml) were pre-incubated with α-FLVCR (0.25 mg/ml) or control IgG for 16–18 hr prior to study of ^{55}Fe-Heme washout. For differentiation studies, this preincubation period was followed by exposure to hemin (50 μM; Benz et al., 1980), or imatinib (0.25 μM; Druker et al., 1996; or Butyrate, 0.5 mM; or TGF-β, 5 ng/ml [K.M.S. and J.L.A., unpublished data]) for 3 days in the continued presence of antibody. Differentiation was assessed by the frequency of benzidine-positive cells (Borsook et al., 1969) present after 3 days of exposure to the inducing agent.

In Vitro Differentiation of Mobilized PB $CD34^+$ Cells

Mobilized human PB $CD34^+$ cells (from the Program in Excellence for Gene Therapy, Hematopoietic Cell Processing Core, Fred Hutchinson Cancer Research Center) were thawed, then incubated in IMDM containing rhSCF (100 ng/ml, gift from Amgen), rhIL3 (50 U/ml, PeproTech), and rhIL6 (50 ng/ml, PeproTech) for 48 hr (day −2 to 0), and then resuspended at 5×10^5/ml in an erythroid differentiation cocktail consisting of X-VIVO15, containing rhSCF (100 ng/ml), rhIL3 (50 u/ml), and rhEp (2 u/ml) for 10 days (termed days 0–10). Fresh medium was added at 3–4 day intervals to maintain cells at densities less than 4×10^6/ml during differentiation. At the time points indicated, aliquots of cells were removed from the culture. These aliquots were analyzed for (1) CD34, CD71, and FLVCR cell surface expression; (2) the degree of hemoglobinization (using benzidine staining); (3) *FLVCR* mRNA expression using quantitative RT-PCR; and (4), the frequency of BFU-E, CFU-E, and CFU-GM progenitors using the methylcellulose culture assay (a modification of previous methods; Charles et al., 1996). Erythroid colonies (cluster of 8–50 hemoglobin-containing cells from CFU-E) were quantitated at day 7 and erythroid bursts (>250 hemoglobin-containing cells from BFU-E) and GM colonies (>50 cells, from CFU-GM) at day 14.

Sorting of Mobilized Human PB $CD34^+$ Cells

For sorting experiments, cells were thawed and differentiated as above. At day +3, cells were pelleted and resuspended at 5×10^6/ml in X-VIVO15 containing 1% BSA, and blocked with 1% donkey serum, then labeled either with α-FLVCR or with rabbit IgG, followed by R-PE-conjugated donkey anti-rabbit IgG. Cells were washed between incubations with X-VIVO15 and 1% BSA. Labeled cells were resuspended at 5×10^6/ml in X-VIVO15 and 1% BSA and sorted using FACSVantage SE (BD Biosciences). From a dot plot of live cells, the highest expressing 13% were sorted as $FLVCR^{hi}$ and a distinct population as $FLVCR^{lo}$. The sorted subpopulations were then assayed for the prevalence of BFU-E, CFU-E, and CFU-GM progenitors using the methylcellulose culture assay described above.

Flow Cytometric Analysis of Normal Human Bone Marrow Cells

BM was aspirated from ten volunteers, after informed consent, in accordance with institutional guidelines. Cells were labeled with anti-CD71 FITC (clone YDJ1.2.2, Beckman Coulter), anti-CD34 PE-Cy5 (Beckman Coulter, clone 581), either polyclonal rabbit IgG or α-FLVCR, and an R-PE-conjugated anti-rabbit IgG as a secondary antibody prior to flow cytometric analysis using a Coulter-XL (Beckman Coulter). $CD34^+$ cells were identified by high CD34 expression and low sidescatter while maturing erythroid forms were identified by high CD71 expression without CD34 expression. For these studies, FLVCR expression was defined as the ratio of the mean fluorescence of cells labeled with FLVCR to the mean fluorescence of cells labeled with the IgG control.

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References

Abbott, B.L. (2003). ABCG2 (BCRP) expression in normal and malignant hematopoietic cells. Hematol. Oncol. *21*, 115–130.

Abkowitz, J.L., Holly, R.D., and Grant, C.K. (1987). Retrovirus-induced feline pure red cell aplasia: Hematopoietic progenitors are infected with feline leukemia virus and erythroid burst-forming cells are uniquely sensitive to heterologous complement. J. Clin. Invest. *80*, 1056–1063.

Benz, E.J., Jr., Murnane, M.J., Tonkonow, B.L., Berman, B.W., Mazur, E.M., Cavallesco, C., Jenko, T., Snyder, E.L., Forget, B.G., and Hoffman, R. (1980). Embryonic-fetal erythroid characteristics of a human leukemic cell line. Proc. Natl. Acad. Sci. USA *77*, 3509–3513.

Borsook, H., Ratner, K., and Tattrie, B. (1969). Studies on erythropoiesis. II. A method of segregating immature from mature adult rabbit erythroblasts. Blood *34*, 32–41.

Bryant, N.J., Govers, R., and James, D.E. (2002). Regulated transport of the glucose transporter GLUT4. Nat. Rev. Mol. Cell. Biol. *3*, 267–276.

Carpenter, C.E., and Mahoney, A.W. (1992). Contributions of heme and non-heme iron to human nutrition. Crit. Rev. Food Sci. Nutr. *31*, 333–367.

Charles, R.J., Sabo, K.M., Kidd, P.G., and Abkowitz, J.L. (1996). The pathophysiology of pure red cell aplasia: Implications for therapy. Blood *87*, 4831–4838.

Dean, G.A., Groshek, P.M., Mullins, J.I., and Hoover, E.A. (1992). Hematopoietic target cells of anemogenic subgroup C versus non-anemogenic subgroup A feline leukemia virus. J. Virol. *66*, 5561–5568.

Druker, B.J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G.M., Fanning, S., Zimmermann, J., and Lydon, N.B. (1996). Effects of a

selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat. Med. *2*, 561–566.

Fujita, H., and Sassa, S. (1989). The rapid and decremental change in haem oxygenase mRNA during erythroid differentiation of murine erythroleukaemia cells. Br. J. Haematol. *73*, 557–560.

Galbraith, R.A. (1990). Heme binding to Hep G2 human hepatoma cells. J. Hepatol. *10*, 305–310.

Harlow, E., and Lane, D. (1988). Antibodies, A Laboratory Manual (Cold Springs Harbor, NY: Cold Spring Harbor Laboratory Press).

Holden, S.A., Steinberg, H.N., Matzinger, E.A., and Monette, F.C. (1983). Further characterization of the hemin-induced enhancement of primitive erythroid progenitor cell growth in vitro. Exp. Hematol. *11*, 953–960.

Ibrahim, N.G., Lutton, J.D., and Levere, R.D. (1982). The role of haem biosynthetic and degradative enzymes in erythroid colony development: The effect of haemin. Br. J. Haematol. *50*, 17–28.

Iwahara, S., Satoh, H., Song, D.X., Webb, J., Burlingame, A.L., Nagae, Y., and Muller-Eberhard, U. (1995). Purification, characterization, and cloning of a heme-binding protein (23 kDa) in rat liver cytosol. Biochemistry *34*, 13398–13406.

Jacob Blackmon, B., Dailey, T.A., Lianchun, X., and Dailey, H.A. (2002). Characterization of a human and mouse tetrapyrrole-binding protein. Arch. Biochem. Biophys. *407*, 196–201.

Jonker, J.W., Buitelaar, M., Wagenaar, E., Van Der Valk, M.A., Scheffer, G.L., Scheper, R.J., Plosch, T., Kuipers, F., Elferink, R.P., Rosing, H., et al. (2002). The breast cancer resistance protein protects against a major chlorophyll-derived dietary phototoxin and protoporphyria. Proc. Natl. Acad. Sci. USA *99*, 15649–15654.

Kinne-Saffran, E., and Kinne, R.K.H. (1989). Membrane isolation: Strategy, techniques, markers. Methods Enzymol. *172*, 3–17.

Krishnamurthy, P., Ross, D.D., Nakanishi, T., Bailey-Dell, K., Zhou, S., Mercer, K.E., Sarkadi, B., Sorrentino, B.P., and Schuetz, J.D. (2004). The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. J. Biol. Chem. *279*, 24218–24225.

Lipovich, L., Hughes, A.L., King, M.C., Abkowitz, J.L., and Quigley, J.G. (2002). Genomic structure and evolutionary context of the human feline leukemia virus subgroup C receptor (hFLVCR) gene: Evidence for block duplications and de novo gene formation within duplicons of the hFLVCR locus. Gene *286*, 203–213.

Lübben, M., and Morand, K. (1994). Novel prenylated hemes as cofactors of cytochrome oxidases: Archea have modified hemes A and O. J. Biol. Chem. *269*, 21473–21479.

Mitchell, B.A., Paulsen, I.T., Brown, M.H., and Skurray, R.A. (1999). Bioenergetics of the Staphylococcal multidrug export protein QacA; identification of distinct binding sites for monovalent and divalent cations. J. Biol. Chem. *274*, 3541–3548.

Nakajima, O., Takahashi, S., Harigae, H., Furuyama, K., Hayashi, N., Sassa, S., and Yamamoto, M. (1999). Heme deficiency in erythroid lineage causes differentiation arrest and cytoplasmic iron overload. EMBO J. *18*, 6282–6289.

Neyfakh, A.A., Bidnenko, V.E., and Chen, L.B. (1991). Efflux-mediated multi-drug resistance in *Bacillus subtilis*: Similarities and dissimilarities with the mammalian system. Proc. Natl. Acad. Sci. USA *88*, 4781–4785.

Ogawa, K., Sun, J., Taketani, S., Nakajima, O., Nishitani, C., Sassa, S., Hayashi, N., Yamamoto, M., Shibahara, S., Fujita, H., and Igarashi, K. (2001). Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. EMBO J. *20*, 2835–2843.

Onions, D., Jarrett, O., Testa, N., Frassoni, F., and Toth, S. (1982). Selective effect of feline leukaemia virus on early erythroid precursors. Nature *296*, 156–158.

Pao, S.S., Paulsen, I.T., and Saier, M.H., Jr. (1994). Major facilitator superfamily. Microbiol. Mol. Biol. Rev. *62*, 1–34.

Papayannopoulou, T., Nakamoto, B., Kurachi, S., and Nelson, R. (1987). Analysis of the erythroid phenotype of HEL cells: Clonal variation and the effect of inducers. Blood *70*, 1764–1772.

Ponka, P. (1997). Tissue-specific regulation of iron metabolism and heme synthesis: Distinct control mechanisms in erythroid cells. Blood *89*, 1–25.

Ponka, P., Wilczynska, A., and Schulman, H.M. (1982). Iron utilization in rabbit reticulocytes. A study using succinylacetone as an inhibitor of heme synthesis. Biochim. Biophys. Acta *720*, 96–105.

Quigley, J.G., Burns, C.C., Anderson, M.M., Lynch, E.D., Sabo, K.M., Overbaugh, J., and Abkowitz, J.L. (2000). Cloning of the cellular receptor for feline leukemia virus subgroup C, a retrovirus that induces red cell aplasia. Blood *95*, 1093–1099.

Rafie-Kolpin, M., Chefalo, P.J., Hussain, Z., Hahn, J., Uma, S., Matts, R.L., and Chen, J.J. (2000). Two heme-binding domains of heme-regulated eukaryotic initiation factor-2αkinase. J. Biol. Chem. *275*, 5171–5178.

Riedel, N., Hoover, E.A., Gasper, P.W., Nicolson, M.O., and Mullins, J.I. (1986). Molecular analysis and pathogenesis of the feline aplastic anemia retrovirus, feline leukemia virus C-Sarma. J. Virol. *60*, 242–250.

Riedel, N., Hoover, E.A., Dornsife, R.E., and Mullins, J.I. (1988). Pathogenic and host range determinants of the feline aplastic anemia retrovirus. Proc. Natl. Acad. Sci. USA *85*, 2758–2762.

Rigby, M.A., Rojko, J.L., Stewart, M.A., Kociba, G.J., Cheney, C.M., Rezanka, L.J., Mathes, L.E., Hartke, J.R., Jarrett, O., and Neil, J.C. (1992). The dissociation of subgroup C phenotype and in vivo behaviour in feline leukaemia viruses with chimeric envelope genes. J. Gen. Virol. *73*, 2839–2847.

Rudra-Ganguly, N., Ghosh, A.K., and Roy-Burman, P. (1998). Retrovirus receptor PiT-1 of the Felis catus. Biochim. Biophys. Acta *1443*, 407–413.

Rutherford, T.R., Clegg, J.B., and Weatherall, D.J. (1979). K562 human leukaemic cells synthesize embryonic haemoglobin in response to haemin. Nature *280*, 164–165.

Ryter, S.W., and Tyrrell, R.M. (2000). The heme synthesis and degradation pathways: Role in oxidant sensitivity. Free Radic. Biol. Med. *28*, 289–309.

Sassa, S. (1976). Sequential induction of heme pathway enzymes during erythroid differentiation of mouse Friend leukemia virus-infected cells. J. Exp. Med. *143*, 305–315.

Smith, K.M. (1975). Porphyrins and Metalloporphyrins (Amsterdam: Elsevier Scientific).

Tailor, C.S., Willett, B.J., and Kabat, D. (1999). A putative cell surface receptor for anemia-inducing feline leukemia virus subgroup C is a member of a transporter superfamily. J. Virol. *73*, 6500–6505.

Taketani, S., Adachi, Y., Kohno, H., Ikehara, S., Tokunaga, R., and Ishii, T. (1998). Molecular characterization of a new heme-binding protein induced during differentiation of urine erythroleukemia cells. J. Biol. Chem. *273*, 31388–31394.

Takeuchi, Y., Vile, R.G., Simpson, G., O'Hara, B., Collins, M.K., and Weiss, R.A. (1992). Feline leukemia virus subgroup B uses the same cell surface receptor as gibbon ape leukemia virus. J. Virol. *66*, 1219–1222.

Uzel, C., and Conrad, M.E. (1998). Absorption of heme iron. Semin. Hematol. *35*, 27–34.

Wandersman, C., and Stojiljkovic, I. (2000). Bacterial heme sources: The role of heme, hemoprotein receptors and hemophores. Curr. Opin. Microbiol. *3*, 215–220.

Wardrop, K.J., Kramer, J.W., Abkowitz, J.L., Clemons, G., and Adamson, J.W. (1986). Quantitative studies of erythropoiesis in the clinically normal, phlebotomized, and feline leukemia virus-infected cat. Am. J. Vet. Res. *47*, 2274–2277.

Weiss, R.A., and Tailor, C.S. (1995). Retrovirus receptors. Cell *82*, 531–533.

Wickrema, A., Krantz, S.B., Winkelmann, J.C., and Bondurant, M.C. (1992). Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells. Blood *80*, 1940–1949.

Worthington, M.T., Cohn, S.M., Miller, S.K., Luo, R.Q., and Berg, C.L. (2001). Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines. Am. J. Physiol. Gastrointest. Liver Physiol. *280*, G1172–G1177.