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Self-Renewal of Leukemia Stem Cells in Friend Virus-Induced Erythroleukemia Requires Proviral Insertional Activation of Spi1 and Hedgehog Signaling but Not Mutation of p53

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Abstract

Friend virus induces erythroleukemia through a characteristic two-stage progression. The prevailing model proposes that during the initial, polyclonal stage of disease most of the infected cells terminally differentiate, resulting in acute erythrocytosis. In the late stage of disease, a clonal leukemia develops through the acquisition of new mutations—proviral insertional activation of Spi1/Pu.1 and mutation of *p53*. Previous work from our laboratory demonstrated that Friend virus activates the bone morphogenic protein 4 (BMP4)-dependent stress erythropoiesis pathway, which leads to the rapid expansion of stress erythroid progenitors, which are the targets for Friend virus in the spleen. We recently showed that stress erythroid progenitors have intrinsic self-renewal ability and therefore could function as leukemia stem cells (LSCs) when infected with Friend virus. Here, we show that the two stages of Friend virus-induced disease are caused by infection of distinct stress progenitor populations in the spleen. The development of leukemia relies on the ability of the virus to hijack the intrinsic self-renewal capability of stress erythroid progenitors leading to the generation of LSCs. Two signals are required for the self-renewal of Friend virus LSCs proviral insertional activation of Spi1/Pu.1 and Hedgehog-dependent signaling. Surprisingly, mutation of *p53* is not observed in LSCs. These data establish a new model for Friend virus-induced erythroleukemia and demonstrate the utility of Friend virus as a model system to study LSC self-renewal.

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Disclosure of Potential Conflicts of Interest

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Keywords

Acute leukemia; Adult stem Cells; Erythropoiesis; Stem cell microenvironment interactions; Self-renewal

Introduction

For more than 50 years, Friend erythroleukemia virus has been a model system for studying the multistage nature of leukemia progression and the mechanisms that regulate erythroid development [1, 2]. Friend virus induces a characteristic two-stage disease. In the first stage, a polyclonal infection of erythroid progenitors in the spleen results in terminal differentiation and acute erythrocytosis. In the second stage, a clonal leukemia develops. Analysis of cell lines generated from Friend virus-infected mice identified two consistent mutations. Proviral insertion upstream of the *Spi1/Pu.1* gene activates the expression of this gene in the transformed cells [3, 4]. Several laboratories have shown that overexpression of Spi1/Pu.1 inhibits erythroid differentiation, which is thought to allow the expansion of transformed cells [5, 6]. The role of Spi1/Pu.1 in the genesis of leukemia is further supported by the observation that Spi1/Pu.1 transgenic mice develops erythroleukemia [7, 8]. The second lesion is a mutation of *p53*, which is thought to lead to transformation of the infected cells [9–11].

Analysis of human acute myeloid leukemia (AML) showed that transformation of a critical population of stem cells, leukemia stem cells (LSCs), was responsible for the maintenance of the leukemia [12, 13]. AML LSCs exhibited extensive self-renewal potential as measured in serial transplantation assays using a xenograft model. The analysis of LSCs in mouse models of AML showed that the development of LSCs could be induced by the expression of fusion protein oncogenes. These experiments showed that certain oncogenes, for example, BCR-ABL, could only induce LSC development in cells that had an intrinsic self-renewal potential. In contrast, other oncogenes, for example, MLL-AF9, could induce LSCs even when expressed in committed progenitor cells, which suggests that these oncogenes are capable of activating regulatory networks required for self-renewal in committed progenitors [14].

Friend virus activates the BMP4-dependent stress erythropoiesis pathway. Following infection, infected bone marrow cells migrate to the spleen where they induce the expression of BMP4, which in turn promotes the expansion of stress erythroid progenitors that are the targets of the virus [15, 16]. Recent work from our laboratory characterizing the stress erythroid progenitors showed that these cells exhibit a novel combination of properties—erythroid restriction and in vivo self-renewal capability [17]. Given that Friend virus infects these cells [15, 16], the identification of a Friend virus target cell that is a self-renewing stress erythroid progenitor cell suggested to us that these cells may act as LSCs during the progression of Friend virus-induced erythroleukemia. In this report, we show that the difference between the early and late stages of the Friend virus-induced disease is due to the infection of two distinct progenitor populations. Infection of self-renewing stress erythroid progenitors leads to the development of LSCs, which are capable of causing erythroleukemia when serially transplanted into mice. Analysis of LSCs showed that proviral insertional activation of *Spi1/Pu.1* is a required feature of these cells but mutation of *p53* is not. In fact, mutation of *p53* leads to a loss of properties of LSC. In addition, we show that LSCs require Hedgehog (HH) signaling for self-renewal in vitro and in vivo. Based on these observations, we have developed a new model for Friend virus-induced erythroleukemia where infection of self-renewing stress erythroid progenitors results in the

development of LSCs, whose self-renewal is regulated by signals from the microenvironment.

Materials and Methods

Additional methods are presented in the supporting information methods section in the supporting information data.

Infection of Mice with Friend Virus

BALB/c mice were infected with the polycythemia inducing strain of Friend virus as previously described [15, 16]. The conditional allele of *Spi1/Pu.1*, *Sfp1^{tm2Dgt}/J* [18], was purchased from JAX (Bar Harbor, ME, www.jax.org). These mice were then crossed with 129S.Cg.Tg(UBC-cre/ERT2)1Ejb/J [19] to generate *Sfp1^{tm2Dgt}/Tg(UBC-cre/ERT2)*. These mice were then crossed with BALB/c mice to generate BALB/c-*Sfp1^{tm2Dgt}/Tg(UBC-cre/ERT2)* mice that are sensitive to Friend virus infection. All experiments using mice were approved by the Institutional Animal Care and Use Committee (IACUC) of the Pennsylvania State University.

Sorting of Infected Cell Populations

Spleen cells were isolated on the indicated days after infection, and erythrocytes were lysed as previously described. Cells were labeled with anti-Kit, -m34 [20], -CD71, -Ter119, and -Sca1 antibodies. Cells were sorted using a Cytopeia Influx cell sorter. Antibodies used were (Alexa647)-anti-Kit and (phycoerythrin [PE]-Cy7)-anti-Sca1 (Biolegend, San Diego, CA, www.biolegend.com), (fluorescein isothiocyanate, FITC)-anti-CD71 and PE-anti-Ter119 (BD Biosciences San Jose, CA, www.bdbiosciences.com). M34 antibody was conjugated with Alexa660 and was a gift of K. Hasenkrug and used as previously described [15, 16].

Analysis of LSC Self-Renewal by In Vitro Serial Plating

m34+Kit+Sca1+ or Sca1- cells were sorted and plated in Methocult media lacking cytokines (Stem Cell Technologies, Vancouver, Canada, www.stemcell.com) supplemented with no growth factors (colony forming units-Friend virus [CFU-FV] media [21]), BMP4 (15 ng/ml) + stem cell factor (SCF) (50 ng/ml) + interleukin-3 (IL-3) (10 ng/ml; BMP4 media), or BMP4 (15 ng/ml) + SCF (50 ng/ml) + IL-3 (10 ng/ml) + Sonic HH, Shh (200 ng/ml; HH media). Cells were grown for 7 days at 37°C with 5% CO₂. BMP4 and Shh were obtained from R&D Systems, Minneapolis, MN, www.rndsystems.com, SCF and IL-3 from Peprotech, Rocky Hill, NJ, www.peprotech.com. Colonies were counted, and the cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) media and replated in the same Methocult media at a concentration of 10⁴ cells per milliliter. This procedure was repeated for each round of plating. Analysis of erythropoietin independent burst forming units erythroid (Epo^{ind} BFU-E) was done as previously described.

Analysis of Spi1/Pu.1 Proviral Insertion and Mutations in p53

DNA was isolated from individual colonies grown as described above in Methocult media. Polymerase chain reaction (PCR) analysis of proviral junction fragments was done as previously described [22]. SFFV-P3 primer: 5'-CACTAGAATACGAGCCACGATAAAT-3', Spi1-u primer: 5'-CTTTCACCTTGTGTAGTTGAAGATGG-3'. Mutation of p53 was analyzed by functional assay. Cells were suspended in DMEM media containing 5% FCS and irradiated with 1,000 rad. The cells were allowed to recover for 4 hours at 37°C, 5% CO₂. The cells were then fixed as previously described [23] and stained with FITC-anti-p21 (Santa Cruz

Biotechnology, Santa Cruz, CA, www.scbt.com). p21 labeling was analyzed using a Beckman Coulter FC500 Flow cytometer.

Analysis of LSC Self-Renewal In Vivo by Serial Transplants in *Stk-/-* Mice

LSCs grown in HH or CFU-FV media as described above were suspended in phosphate buffered saline (PBS). A total of 5×10^5 cells were injected into the retro-orbital sinus of BALB/c-*Stk-/-* mice [24]. At the indicated times, mice were sacrificed. WBC counts, spleen weights, and analysis of p53 function using the p21 induction assay were done to examine the extent of leukemia and to identify p53 mutations.

Analysis of the Role of Hedgehog Signaling

LSCs were expanded in HH media as described above. Cells were then plated in HH media $\pm 10 \mu\text{M}$ cyclopamine (LC Labs, Woburn, MA, www.lclabs.com). RNA was isolated from cells grown in CFU-FV, BMP4, or HH media as describe above using Trizol reagent (Invitrogen, Grand Island, NY, www.invitrogen.com). cDNA was synthesized using Superscript II (Invitrogen, Grand Island, NY, www.invitrogen.com). Analysis of *Spi1/Pu.1* expression was done using Taq-man probes for *Spi1/Pu.1* (Catalog# Mm01719550) and *Gapdh* (Catalog# Mm03302249-91, Applied Biosystems, Grand Island, NY, www.appliedbiosystems.com). The expression of *Spi1/Pu.1* is expressed relative to *Gapdh* expression.

Analysis of the Role of *Spi1/Pu.1*

LSCs derived from BALB/c-*Sfp1^{tm2Dgt/J};CreERTM* were expanded in HH media. Cells were then treated with $10 \mu\text{M}$ 4-hydroxytamoxifen (Sigma, St. Louis, MO, www.sigmaaldrich.com) for 24 hours. The cells were plated in Methocult for LSC colonies (HH media) or for *Epo^{ind}* BFU-E as described above.

Results

Friend Virus Infection of Distinct Target Cell Populations Leads to Either Erythrocytosis or Erythroleukemia

Based on their expression of Kit, Sca1, CD71, and Ter119, we recently identified three distinct populations of stress erythroid progenitors that expand in the spleen during the recovery from bone marrow transplant [17]. Using a monoclonal antibody (m34) that recognizes the Friend virus envelope protein [20], gp55, we determined whether these spleen stress progenitors were infected at early (day 7) or late (day 14) times after infection. Flow cytometry analysis shows that m34+ cells were found in the Kit+CD71+Ter119^{lo} population of cells, which overlaps our previously characterized stress progenitor populations I and II [17]. A subset of population I and II cells are Sca1+. When we examined the m34+Kit+CD71+Ter119^{lo} cells, we found that 72% were Sca1+ at day 14 (Fig. 1A, 1B).

Friend virus infection in the spleen causes two functional outcomes that can be measured in colony assays. Some of the infected cells will form BFU-E colonies that form in the absence of added erythropoietin (we will refer to these progenitors as erythropoietin independent, *Epo^{ind}* BFU-E) [25–28], while late in infection other cells will form transformed CFU-FV colonies [21], which are characterized by their ability to form compact colonies in methylcellulose media lacking added growth factors. We isolated m34+Kit+CD71+Ter119^{lo} sorted into Sca1+ and Sca1– populations by fluorescence activated cell sorting (FACS) from the spleens of Friend virus-infected mice on days 7 and 14 after infection. The sorted cells were plated in methylcellulose media to assay for *Epo^{ind}* BFU-E or CFU-FV. On day 7 after infection, only *Epo^{ind}* BFU-E were observed and only the Sca1– population generated

colonies. At day 14 after infection, we observed both Epo^{ind} BFU-E and CFU-FV; however, the CFU-FV were restricted to the Sca1⁺ population, while the Epo^{ind} BFU-E were observed only in the Sca1[−] population (Fig. 1C). Furthermore, the m34+Kit+CD71+Ter119^{lo}Sca1^{+/−} population contained all the Epo^{ind} BFU-E and CFU-FV present in the spleen at day 14 after infection (data not shown). (Note: Subsequent analysis showed that the use of CD71 and Ter119 in the FACS protocol did not significantly enrich the isolation of Epo^{ind} BFU-E or CFU-FV when compared to FACS using only m34, Kit, and Sca1 antibodies. Therefore, we routinely used m34+Kit+Sca1[−] to sort Epo^{ind} BFU-E and m34+Kit+Sca1⁺ to sort CFU-FV.) These data suggest that the target cells that give rise to Epo^{ind} BFU-E are distinct from the target cells that give rise to transformed CFU-FV. As a control, bone marrow cells isolated from infected mice were plated in methylcellulose media. As shown previously, infected bone marrow contains Epo^{ind} BFU-E. However, we did not observe CFU-FV (data not shown). These data support the early observations and our previous work that induction of erythroleukemia by Friend virus requires both target cells and microenvironmental signals present in the spleen [15, 16, 29, 30].

Friend Virus-Infected Kit⁺ Sca1⁺ Cells Can Self-Renew In Vitro

The CFU-FV assay was designed to identify transformed cells. It selects for factor-independent growth. However, in vivo, many growth factors are expressed in the spleen, which would affect the growth of Friend virus-infected cells. Our previous work on stress erythropoiesis and the initial stage of Friend virus infection showed that SCF, IL-3, BMP4, and HH all play an essential role in the development and expansion of Friend virus target cells and stress erythroid progenitors [15, 16, 31, 32]. We hypothesized that including these factors in the media would allow the expansion of nontransformed CFU-FV, which could function as LSCs. On day 14 after infection, m34+kit⁺ cells were sorted into Sca1⁺ or Sca1[−] populations and plated in methylcellulose media containing no growth factors (CFU-FV conditions), SCF+IL-3+BMP4 (BMP4 conditions), or SCF+IL-3+BMP4+Sonic HH, Shh (HH conditions). After 7 days in culture, colonies were counted. For the Sca1⁺ cells, each culture condition led to the formation of large colonies, >1,000 cells (Fig. 2A). Overall, the colonies varied in size with colonies formed in HH media > BMP4 media > CFU-FV media. Furthermore, HH conditions promoted the greatest colony formation, while the CFU-FV conditions resulted in the fewest colonies. The cells were resuspended and replated in the same media and this process was repeated for four serial platings. Using this regimen, the number of colonies observed in the HH and BMP4 conditions significantly increased each week, with the cells grown in the HH media expanding significantly faster than those grown in BMP4 conditions at each of the rounds of replating. The number of colonies increased in the CFU-FV conditions, but a significant difference in colony number was only observed when week 1 was compared to week 4. In contrast, the Sca1[−] cells formed significantly fewer colonies on initial plating and the number of colonies decreased each week. No colonies were observed after the fourth plating (Fig. 2B). These data demonstrate that in vitro, m34+Kit+Sca1⁺ cells can self-renew in culture, and HH media significantly improves the self-renewal of these cells. Although we could readily identify self-renewing m34+Kit+Sca1⁺ cells at 14 days after infection, when we tested the ability of m34+Kit+Sca1⁺ cells isolated on day 7 after infection, these cells formed fewer colonies on initial plating and the colonies failed to self-renew on replating (data not shown). These observations demonstrate that the development of self-renewing LSCs is associated with later stage disease.

To show that single LSCs could self-renew, we followed the expansion of LSCs derived from a single colony. In Figure 2C, a single colony on average gives rise to 12 new LSC colonies in the first round of plating; in subsequent rounds of plating, the number of LSC colonies increased approximately threefold to fourfold each round. These data show that a

single LSC can expand and self-renew in vitro. We also measured the frequency of LSCs in cultures containing HH media by limiting dilution. This analysis showed that 1 in 920 cells were able to form LSC colonies (1:752–1:1,125 upper and lower limits).

Proviral Insertion into the *Spi1/Pu.1* Locus Is a Common Feature of Self-Renewing m34+Kit+Sca1+ Cells

Friend erythroleukemia cell lines exhibit two characteristic mutations, proviral insertional activation of *Spi1/Pu.1* expression and mutation of *p53*, that are thought to be required for the progression to leukemia [1, 2]. As these lesions were characterized in Friend erythroleukemia cell lines grown under CFU-FV conditions, we compared m34+Kit+Sca1+ cells grown in CFU-FV conditions with cells grown using the other two conditions (BMP4 or HH) for proviral insertions into the *Spi1/Pu.1* locus and mutations in *p53*. More than 95% of the Friend erythroleukemia cell lines characterized have insertions into the 5' end of the *Spi1/Pu.1* locus [22]. We tested individual LSC colonies grown for 1 week in CFU-FV, BMP4, or HH media for proviral insertion into the *Spi1/Pu.1* locus by PCR of genomic DNA using one primer specific to the spleen focus forming virus (SFFV) long terminal repeat (LTR) and a second primer in the 5'-end of the *Spi1/Pu.1*. By analyzing seven independent infections and 15–30 colonies for each culture condition, we observed that 100% of the colonies showed an insertion into the 5'-end of *Spi1/Pu.1*. However, when we examined m34+Kit+Sca1– cells, we did not observe proviral insertions into *Spi1/Pu.1*; furthermore, m34+Kit+Sca1+ cells isolated on day 7 after infection, which do not form CFU-FV or self-renewing colonies, also did not exhibit proviral insertions into *Spi1/Pu.1* (Fig. 3A).

Mutation of *p53* Is Not Required for the LSC Development or Self-Renewal

The second lesion associated with Friend erythroleukemia cells is mutation of *p53* [9]. All manner of *p53* mutations have been identified in Friend leukemia cell lines [10]. To examine the role of *p53* mutations in the development of Friend virus LSCs, we used two strategies. We directly sequenced cDNA clones of *p53* amplified from independent LSC colonies. In addition, we used a functional assay, which measured the *p53*-dependent upregulation of the cyclin-dependent kinase inhibitor p21 that occurs in response to treatment with ionizing radiation [33]. Using direct sequencing, we analyzed multiple PCR clones from 12 independent LSC clones. We did not find any mutations in any of the clones (data not shown). In addition, we examined *p53* protein levels in LSC clones and compared expression to normal spleen cells from uninfected mice and CFU-FV clones. *p53* protein expression in LSC clones was indistinguishable from control spleen cells. In contrast, CFU-FV exhibited lower or no *p53* protein expression (Fig. 3B). These assays relied on the analysis of LSC clones; however, to analyze bulk populations of LSCs, we turned to the functional assay where we analyzed p21 expression by flow cytometry 4 hours after treatment with ionizing radiation. In response to ionizing radiation, 100% of control spleen cells (*p53*^{+/+}) upregulated p21 protein expression, while murine erythroleukemia (MEL) cells which are derived from CFU-FV and are *p53*^{–/–} could not upregulate p21 expression (Fig. 3C). We examined the induction of p21 in cells grown using CFU-FV conditions and self-renewing cells grown in BMP4 or HH media. Similar to what we observed with MEL cells, the CFU-FV cells failed to up-regulate p21 expression and exhibited staining that for the most part overlapped with unirradiated cells. In contrast, 85%–90% of the self-renewing cells grown in HH media were p21+ and therefore had functional *p53* (Fig. 3D). The level of p21 activation was less in cells grown in BMP4 media. Loss of functional *p53* was only consistently observed in the cells grown under CFU-FV conditions, which suggested that this event was due to selection caused by the severe growth conditions of the CFU-FV media.

Self-Renewing m34+Kit+Sca1+Cells Expanded in HH Media Are Self-Renewing Leukemia Stem Cells In Vivo

The serial plating assay demonstrated that m34+Kit+Sca1+ cells grown in HH or BMP4 media can self-renew in vitro, which is consistent with these cells being LSCs. However, to rigorously test this idea, these cells must be able to cause leukemia when transplanted into mice. One complication to transplant experiments is that these cells are infected with Friend virus (m34+) and make infectious virus. If we transplanted them into sensitive animals, we would be unable to distinguish leukemia caused by the expansion of transplanted LSCs from leukemia caused by Friend virus infection of endogenous target cells. To circumvent this problem, we used syngeneic recipients that were mutated at the *Stk* locus (BALB/c-*Stk*^{-/-}) [24]. *Stk* mutant mice do not develop erythroleukemia because they lack the expression of Short-form Stk, which is required for the development of Friend erythroleukemia [34]. LSCs were expanded through four serial platings in HH media. Our limiting dilution analysis showed that approximately 1 in 920 cells were LSCs. We transplanted 1,000, 5,000, 10,000, and 100,000 cells into unirradiated BALB/c-*Stk*^{-/-} recipients. After 5 weeks, all mice exhibited signs of leukemia, splenomegaly, and slightly increased hematocrit. Donor LSCs were isolated from primary transplants and transplanted into secondary recipients at the same cell dose. The secondary recipients exhibited a significant increase in spleen size and peripheral blood white blood cell (WBC) counts were significantly increased as was observed in the primary transplants (Fig. 4A). These data show that transplanting approximately one LSC is sufficient to induce erythroleukemia and LSCs self-renew in vivo. In contrast, recipient mice transplanted with 500,000 CFU-FV failed to develop leukemia as their spleen size and WBC counts were indistinguishable from untransplanted controls, which is consistent with previous work (supporting information Figure S1). Although our sequencing and in vitro analysis of p53 function showed that nearly all the cells expanded in HH media exhibited functional p53, it is possible that during the serial transplants a clone of *p53*^{-/-} cells could develop into a dominant clone, which would be responsible for inducing leukemia. We tested this possibility by serially transplanting LSCs at a dose of 500,000 cells into primary, secondary, and tertiary recipients. Donor-derived m34+Kit+Sca1+ were sorted from secondary and tertiary transplants and tested for their ability to upregulate p21 expression following treatment with ionizing radiation as analyzed by flow cytometry. In both secondary and tertiary transplants, more than 90% of the cells were capable of upregulating p21 in response to ionizing radiation (Fig. 4B, 4C). These data demonstrate that mutation of *p53* is not required for the in vivo self-renewal of Friend virus LSCs and does not contribute to the progression of disease (Fig. 4C).

Hedgehog Signaling Inhibits Differentiation and Promotes Self-Renewal of LSCs

AML LSCs give rise to non-LSC progeny which generate “bulk” leukemia cells [35]. Previously, we showed that immature stress erythroid progenitors gave rise to more mature stress erythroid progenitors when cultured in vitro [17]. m34+Kit+Sca1+ cells freshly sorted from infected mice do not form Epo^{ind} BFU-E. However, when LSCs were cultured in BMP4 or HH media for 4 weeks, they do generate progenitor cells capable of forming Epo^{ind} BFU-E. In fact, Epo^{ind} BFU-E were observed after 7 days of culture (data not shown). Interestingly, the Epo^{ind} BFU-E were still Kit+Sca1+ (data not shown). HH media generate significantly fewer Epo^{ind} BFU-E than cells cultured in BMP4 media. As expected, cells cultured in CFU-FV media were no Epo^{ind} BFU-E colonies. These data demonstrate that HH signaling promotes the self-renewal of LSCs and inhibits the generation of non-LSC progeny.

Previous work in the field showed that proviral insertional activation of *Spi1/Pu.1* expression inhibits erythroid differentiation by binding to Gata1 and blocking its function [5, 6]. We tested the expression of Spi1/Pu.1 in cells grown in HH, BMP4, or CFU-FV media

by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Cells grown in HH media expressed approximately fivefold more *Spi1/Pu.1* than cells grown in BMP4 media and almost 10-fold more than cells grown CFU-FV media (Fig. 5B). Although this observation is consistent with the idea that high *Spi1/Pu.1* levels would inhibit Gata1-dependent erythroid differentiation, analysis of Gata factor expression in LSCs cultured in HH media showed that Gata2 is the primary Gata factor expressed, which suggests that the situation may be more complex than the simple model of *Spi1/Pu.1* inhibiting Gata1 (data not shown). To determine the role of *Spi1/Pu.1* in the self-renewal of LSCs, we used a conditional allele of *Spi1/Pu.1* [18] and a tamoxifen-inducible Cre recombinase transgene [19] to delete *Spi1/Pu.1* in LSCs grown in HH media. We tested whether the *Spi1/Pu.1*^{Δ/Δ} LSCs could self-renew in vitro and whether deletion of *Spi1/Pu.1* promoted erythroid differentiation by measuring Epo^{ind} BFU-E (Fig. 5C, 5D). Deletion of *Spi1/Pu.1* led to a significant decrease in LSC colonies; however, the number of Epo^{ind} BFU-E was unaffected by the deletion of *Spi1/Pu.1*. These data support the idea that proviral insertional activation of *Spi1/Pu.1* is required for the self-renewal of Friend virus LSCs and its function is not limited to inhibit erythroid differentiation.

Inhibition of HH Signaling Blocks Self-Renewal In Vitro and Stops the Progression of Leukemia In Vivo

In vitro, HH signaling supports the self-renewal of LSCs as shown in the serial plating assay (Fig. 2A). We next tested the effect of inhibiting HH signaling on the self-renewal of Friend virus LSCs in vitro and in vivo. LSCs were cultured in media containing the HH inhibitor, cyclopamine, and the number of LSC colonies generated was scored. In Figure 6A, the data show that cyclopamine completely inhibits LSC colony formation. Compared to control, treatment with cyclopamine did not increase apoptosis, but we did observe that the cells changed morphology on treatment. Cyclopamine-treated cells exhibited a flattened morphology more reminiscent of macrophages than erythroleukemia cells. Indeed, flow cytometry analysis of cyclopamine-treated LSCs showed that the treated cells were F4/80 positive and given their morphology, it appears that inhibition of HH signaling in Friend virus-induced LSCs results in their development into macrophages (data not shown). The overexpression of *Spi1/Pu.1* caused by the proviral insertional activation may contribute to this response [36].

We next tested whether inhibiting HH signaling in vivo would affect the progression to leukemia. LSCs expanded in vitro were transplanted into *Stk-/-* recipient mice. Using this protocol, we were able to bypass any effects that cyclopamine might have on Friend virus infection. After 6 weeks of transplantation, the mice developed leukemia as measured by high WBC counts and palpable splenomegaly. Transplanted mice were treated daily for 7 days with cyclopamine or vehicle control. Analysis of treated mice showed that their WBC counts had returned to normal values and spleen weights were indistinguishable from untransplanted control mice. Analysis of spleen cells by flow cytometry showed that the percentage of m34+ donor LSCs had diminished from approximately 87% to 0.5% of total spleen cells (Fig. 6B, 6C). The mice showed no sign of relapse 8–10 weeks after treatment with cyclopamine. Taken together, these data demonstrate that the HH signaling is required in vitro and in vivo for the self-renewal of LSCs.

Friend Virus LSCs Are Kit+Sca1+CD133+ Cells That Divide Infrequently

The analysis of LSC cultures showed that the frequency of LSCs was approximately 1 in 920 cells. We next sought to identify markers that would allow for the purification of LSCs. Previous work has suggested that cancer stem cells could be enriched by selecting for cells that did not divide as frequently as bulk cancer cells [37]. We stained LSC cultures grown in HH media with the lipophilic dye PKH26. The cells were grown for 7 days in culture, and

PKH26^{hi} cells (slow dividing) and PKH26^{lo} cells (rapidly dividing cells) were isolated by FACS and plated in methylcellulose media. PKH26^{hi} cells generated primarily LSC colonies and few Epo^{ind} BFU-E, while PKH26^{lo} cells produced primarily Epo^{ind} BFU-E and few LSC colonies (supporting information Fig. S2A). Analysis of PKH26^{hi} (LSCs) and PKH26^{lo} (Epo^{ind} BFU-E) cells showed that the LSCs expressed stem cell markers such as *Nanog*, *Musashi2*, and *Bmi1*, while Epo^{ind} BFU-E expressed *Numb*, a gene associated with progenitor cells (supporting information Fig. S2B). CD133 is another marker associated with cancer stem cells [38]. Analysis of CD133 expression showed that CD133+ cells were highly enriched in LSCs while cells that gave rise to Epo^{ind} BFU-E were CD133+ (supporting information Fig. S2C). Combining the markers, we found that CD133+PKH26^{hi} fraction contained almost the entire population of LSC colony forming cells, while the CD133-PKH26^{lo} population contained all of the Epo^{ind} BFU-E. Interestingly, the CD133+PKH26^{lo} cells produced essentially the entire population of CFU-FV in the culture, which demonstrates that LSCs and CFU-FV are distinct populations of cells (Fig. 6D).

Discussion

Previous work on the progression of Friend virus-induced erythroleukemia suggested that the development of leukemia relied on the accumulation of new mutations, which inhibited differentiation and promoted transformation [1, 2]. Based on our data, we propose a new model where infection of a mature stress erythroid progenitor (Kit+Sca1⁻) leads to terminal differentiation and erythrocytosis, while infection of a more immature population (Kit+Sca1⁺) leads to the development of LSCs and erythroleukemia. Two factors were necessary for LSC development and self-renewal. Proviral insertional activation of *Spi1/Pu.1* expression was a common and required feature of these cells in that deletion of *Spi1/Pu.1* blocked LSC self-renewal. This requirement is most likely the cause of the delay in the appearance of LSCs until 14 days after infection. In addition, HH signaling was required both in vitro and in vivo. Surprisingly, mutation of p53 was not observed in LSCs and not required for leukemia progression. Based on these data, we propose a new model for the progression of Friend virus-induced erythroleukemia (Fig. 7).

One of the surprising results of our study was that proviral insertional activation of *Spi1/Pu.1* was consistently observed, but mutation of p53 was not a common lesion in LSCs. In addition to its role in B cell and macrophage development [36, 39], *Spi1/Pu.1* plays a key role in the development and expansion of fetal liver BFU-E and in the self-renewal of immature erythroid progenitors [40, 41]. Our observation that LSCs are Friend virus-infected stress erythroid progenitors fits with the idea that *Spi1/Pu.1* functions to maintain an undifferentiated population of erythroid progenitors so that they can be expanded during times of erythropoietic need—fetal liver erythropoiesis and in response to acute anemia [17, 42]. Although the prevailing model for *Spi1/Pu.1* action is to inhibit Gata1 function [5, 6], recent work has also demonstrated that *Spi1/Pu.1* regulates the expression of Cdk6, which promotes the expansion MEL cells [43]. Our data support the idea that *Spi1/Pu.1* does more than just inhibit Gata1. We observed that deletion of *Spi1/Pu.1* does not result in an increase in Epo^{ind} BFU-E, which suggests that *Spi1/Pu.1* plays a more direct role in regulating the self-renewal of LSCs.

HH signaling has been implicated in number of human cancers including breast cancer [44] and medulloblastoma [45, 46]. Recent work has demonstrated that the expansion of LSCs in a murine model of chronic myelogenous leukemia (CML) requires HH signaling [47, 48]. Our observation that HH signaling is also required for the self-renewal of Friend virus LSCs suggests that microenvironmental signals like HH could be a general mechanism by which LSC self-renewal is maintained. Thus therapies that target these signals would be effective in reducing LSCs.

Summary

The pathology associated with Friend virus infection includes the development of erythrocytosis and the eventual development of erythroleukemia. Previous work suggested that this progression resulted from an accumulation of mutations, proviral insertional activation of Spi1/Pu.1 expression, and mutation of p53, resulting in a clonal leukemia. Our data here show that erythrocytosis and the development of erythroleukemia are caused by the infection of distinct populations of stress erythroid progenitor cells. Friend virus infection induces the development of LSCs, which are characterized by proviral insertion into Spi1/Pu.1. Increased expression of Spi1/Pu.1 is absolutely required for the self-renewal of LSCs but mutation of p53 is not. In addition to these intrinsic events, microenvironmental signals also play a key role in self-renewal of LSCs. HH signaling plays an essential role in the self-renewal as shown by experiments in which blocking HH signaling in vitro and in vivo leads to a loss of LSCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Friend virus-infected Kit+CD71+Ter119^{lo} cells form Epo^{ind} BFU-E and CFU-FV. **(A):** Flow cytometry analysis of Friend virus-infected spleen cells on days 7 and 14 after infection. Cells are gated on m34+Kit+ cells and the expression of CD71 and Ter119 is shown. **(B):** Analysis of Sca1 expression by m34+Kit+ cells on day 14 after infection. **(C):** Analysis of Epo^{ind} BFU-E (left) and CFU-FV (right) colony forming ability of m34+Kit+Sca1+ and m34+Kit+Sca1- cells at days 7 and 14 after infection. Abbreviations: BFU-E, burst forming units-erythroid; CFU-FV, colony forming units-Friend virus.

Figure 2.

Analysis of in vitro self-renewal of Friend virus-infected Kit+Sca1+/- spleen cells. Serial plating of (A) m34+Kit+Sca1+ cells or (B) m34+Kit+Sca1- cells isolated from the spleen on day 14 after infection. The cells were plated in media containing no added growth factors (CFU-FV), BMP4+SCF+IL-3 (BMP4), or BMP4+SCF+IL-3+Shh (BMP4+Shh). *, $p < .05$ when the number of colonies observed in one round was compared with the number of colonies in the preceding round. **, $p < .001$ when each round of a plating in given media is compared to the same round of plating in another media. ND, none detected. (C): A single m34+Kit+Sca1+ colony grown in BMP4+Shh methylcellulose media was isolated and replated in the same media and grown for 1 week. All the new colonies were isolated and replated. For each round of serial plating, the number of colonies isolated and replated is indicated on the X-axis, the total number of new colonies derived is shown on the Y-axis. Abbreviations: BMP4, bone morphogenic protein 4; CFU-FV, colony forming units-Friend virus; IL, interleukin; Shh, Sonic Hedgehog; SCF, stem cell factor.

Figure 3.

Proviral insertion into *Spi1/Pu.1* is required for LSC self-renewal but mutation of p53 is not observed. **(A):** PCR analysis of DNA from individual colonies isolated on the indicated days and grown in the indicated conditions. **(B):** p53 protein expression in cell lysates derived from representative LSC or CFU-FV colonies. Spleen cells from uninfected animals are included as controls. β -Actin is shown as a loading control. **(C):** Analysis of radiation-induced p21 expression in control spleen (p53+/+) (left) and MEL (p53-/-) (right) cells. **(D):** Analysis of radiation-induced p21 expression in m34+Kit+Sca1+ spleen cells grown in the indicated media for four serial platings. Abbreviations: BMP4, bone morphogenic protein 4; CFU-FV, colony forming units-Friend virus; FITC, fluorescein isothiocyanate; HH, Hedgehog; IL, Interleukin; LSC, leukemia stem cell; MEL, murine erythroleukemia; PCR, polymerase chain reaction.

Figure 4.

Analysis of in vivo self-renewal of Friend virus LSCs expanded in vitro. **(A):** LSCs were expanded in culture and transplanted into *Stk-/-* recipients. For the indicated number of cells transplanted into primary (black bars) or secondary (gray bars) recipients, spleen weight (left) and WBC counts (right) were analyzed at 5 weeks after transplant. The increase in spleen weight and WBC count was significantly greater than control for all doses of transplanted LSCs. **(B):** Analysis of spleen weight 9 weeks after transplant in primary, secondary, and tertiary recipients transplanted with 5×10^5 LSCs (left). Analysis of WBC counts 9 weeks after transplant (right). **p* value indicates a significant difference in pairwise comparisons between control and transplanted mice. **(C):** Analysis of radiation-induced p21 expression of m34+Kit+ cells isolated from secondary (left) or tertiary (right) transplants. Abbreviations: LSC, leukemia stem cell; WBC, white blood cell.

Figure 5.

Hedgehog signaling decreases the ability of LSCs to differentiate and Spi1/Pu.1 expression is necessary for self-renewal. **(A):** m34+Kit+Sca1+ cells were plated through four serial passages in the indicated media and then plated in media containing IL-3 and SCF for Epo^{ind} BFU-E. **(B):** Real time PCR analysis of Spi1/Pu.1 expression in m34+Kit+Sca1+ cells that were plated through four serial passages in the indicated media. Expression is expressed relative to Gapdh expression. ND, none detected. **(C):** Analysis of LSC self-renewal when Pu.1 is deleted. Pu.1^{Δ/Δ} refers to Pu.1^{fx/fx};Cre treated with Tamoxifen and Pu.1^{+/+} refers to Pu.1^{fx/fx} LSCs treated with Tamoxifen. (Left) Pu.1^{Δ/Δ} LSCs and control LSCs were plated in Hedgehog (HH) media, and LSC colonies were scored or (right) plated in media containing IL-3 and SCF and Epo^{ind} BFU-E were scored. **(D):** Analysis of Spi1/Pu.1 mRNA (top) and protein (bottom) expression in Pu.1^{fx/fx};Cre (Pu.1^{Δ/Δ}) or Pu.1^{fx/fx} (Pu.1^{+/+}) LSCs treated with Tamoxifen. Abbreviations: BFU-E, burst forming units-erythroid; BMP4, bone morphogenic protein 4; CFU-FV, colony forming units-Friend virus; IL, interleukin; LSC, leukemia stem cell; SCF, stem cell factor; Shh, Sonic Hedgehog.

Figure 6.

Inhibition of HH signaling blocks self-renewal of LSCs in vitro and in vivo. **(A)**: m34+Kit+Sca1+ cells were grown for four serial passages and then cultured for 7 days in the indicated media. ND, none detected. **(B)**: Scheme for in vivo treatment of transplanted mice with cyclopamine or vehicle control. **(C)**: Analysis of WBC count (left), spleen weight (middle), or percentage of m34+ cells in the spleen 1 week after treatment with cyclopamine or vehicle control. **(D)**: Enrichment for LSCs using CD133 and PKH26 staining. (Left) Flow analysis of LSCs stained with PKH26 and cultured 1 week in HH media and sorted for PKH26 staining and CD133 expression. (Right) Analysis of colony forming ability of the three indicated populations. Abbreviations: BMP4, bone morphogenic protein 4; BFU-E, burst forming units-erythroid; CFU-FV, colony forming units-Friend virus; FITC, fluorescein isothiocyanate; HH, Hedgehog; LSC, leukemia stem cell; Shh, Sonic Hedgehog; WBC, white blood cell.

Figure 7.

Model for Friend virus-induced erythroleukemia. In the initial stage of disease, m34+Kit+Sca1- cells, which lack proviral insertion in to Spi1/Pu.1 expand and differentiate resulting in erythrocytosis (red cells). Later during this initial stage, m34+Kit+Sca1+ cells are infected. Infected cells in this population that do not have proviral insertion into Spi1/Pu.1 fail to progress (blue cells), while those clones that have an insertion into Spi1/Pu.1 develop into LSCs (black cells). In the late stage of disease, the LSCs self-renew and expand leading to acute erythroleukemia and anemia. During this stage, a population of LSCs that acquire p53 mutations develops. These cells give rise to CFU-FV (gray cells) but do not function as LSCs. Abbreviations: BFU-E, burst forming units-erythroid; CFU-FV, colony forming units-Friend virus; LSC, leukemia stem cell.