

Expression of the fetal hematopoiesis regulator FEV indicates leukemias of prenatal origin

Short title: FEV-expression indicates leukemias of fetal origin

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Abstract

The origin of cancers is associated with etiology as well as therapeutics. Several studies reveal that malignancies in children can originate *in utero*. However a diagnostic approach to distinguish between cancers initiated pre- or postnatally is absent. Here we identified a transcriptional factor FEV that was expressed in fetal hematopoietic cells and became silent after birth. We characterized that FEV was essential for the self-renewal of hematopoietic stem cells (HSCs). We next found FEV was expressed in most infant leukemia samples, but not in adult samples, in accord with the known pre-natal origins of the former. We further determined the majority of pediatric acute lymphoid and myeloid leukemias (ALL and AML) were FEV-positive. Moreover FEV-knockdown markedly impaired the leukemia-propagating ability of leukemic stem cells. We therefore identified FEV is unique to fetal HSCs and stably expressed in leukemic cells of prenatal origin. It may also provide a tractable therapeutic target.

Introduction

Recognition of embryonal features in cancer cells presumes the possibility of a prenatal origin for cancer.¹⁻³ Since the first demonstrations that leukemogenic chromosome translocations can occur prenatally,⁴ increasing evidence supports that a substantial number of malignancies in children and adolescent can originate prenatally.⁵⁻¹³ However, there is currently no diagnostic test or biomarker that distinguishes cancers that are pre- versus post-natal in origin. FEV-expression may provide such a test. FEV (also known as PET1 in mammals) is an ETS (E26 transformation-specific) transcription factor.^{14, 15} We previously reported that this transcription factor is essential for embryonic development of hemogenic endothelium-based hematopoietic stem cells (HSCs) and for its functional maintenance in fetal hematopoiesis.¹⁶ Yet it remains to be confirmed whether FEV regulates the self-renewal in HSCs or plays other roles. In the current study, we systemically analyzed the developmental profile of FEV-expression and characterized its functional roles in HSCs. Further, we investigated FEV-expression in leukemic cells in terms of its correlation with origins of the disease and its effect on leukemia-propagation of leukemic stem cells (LSCs). We demonstrated that FEV is a specific regulator of self-renewal in fetal HSCs. FEV-expression is essential for leukemia-propagation of LSCs and is reflective of the common prenatal initiation of the disease.

Materials and Methods

Human samples

Umbilical vein cord blood (CB) from anonymized healthy donors was purchased from Shanghai Cord Blood Bank in accordance with local ethics procedures. Clinical Samples of fetal liver and BM from healthy donors and leukemia patients were obtained from Shanghai Jiaotong University and Fudan University-affiliated hospitals and the studies were approved by their Medical Ethical Committees and all human participants gave written informed consent. Detailed information of the clinical samples is described in Supplementary Tables S1–3.

Reverse-transcriptase quantitative polymerase-chain-reaction (RT-qPCR) detection of FEV-expression and the positivity definition

Total RNA was extracted by Trizol reagent according to the manufacturer's instructions (Invitrogen) and reversely transcribed. RT-qPCR was performed using SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. All experiments were performed in triplicate with Applied Biosystems 7900HT. Differences in cDNA input were normalized by ABL1 expression levels.¹⁷ Samples with ABL1 cycle-threshold values of 30 or more were excluded from the analysis. The positivity for FEV-expression is defined according to amplification in at least two of three replicates with cycle-threshold values of 40 or less (using a threshold setting of 0.2) and a single melt peak (FEV at 89.5 °C; ABL1 at 85.5 °C).¹⁸ The detail is described in Supplemental Methods.

Preparation of lentivirus and transduction

FEV interference oligonucleotides (cat. no. RHS4533) were purchased from Open Biosystems. Lentiviruses were packaged as previously described ¹⁹. Lin⁻CD34⁺ CD38⁻ cells or AML LSCs or ALL cells were infected with lentivirus (either iFEV or control virus) at an MOI of 100 for 18 h. Transduced cells were either transplanted into NOD/SCID mice or used for *in vitro* assays.

Colony-forming cell (CFC) assay

Assays were performed in a semi-solid methylcellulose medium as previously described. ²⁰ Cells were plated in 35 mm dishes containing methylcellulose. The colonies were scored under a microscope 13–14 days post-plating. Replating was carried out by pooling total cells from primary cultures and inoculating 10⁵ into fresh methylcellulose medium.

Long-term culture-initiating cell (LTC-IC) assay

Limiting dilution LTC-IC assays were performed in 96-well format in myelocult H5100 medium (Stem Cell Technologies) on a feeder layer comprising a 1:1 mixture of irradiated M2-10B4 and S1/S1 mouse fibroblasts. This assay was performed following the protocol provided with the products. ²⁰ LTC-IC frequency was calculated using L-Calc software (Stem Cell Technologies).

Advanced xenograft transplantation

NOD-SCID mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (SLAC). These mice were maintained and handled under sterile conditions at the animal facility in accordance with local regulations. 6–8 weeks old mice received 150 cGy of total body irradiation from a ^{137}Cs source and subsequently treated with 200 μg of CD122 antibody via intraperitoneal injection. Recipients were transplanted with 10^4 transduced HSCs, 10^4 – 10^5 flow-sorted AML LSCs or 10^6 ALL cells via intratibial injections. BM engraftment was assessed at 10–12 weeks post-transplantation for transduced cord blood HSCs and BM HSCs¹¹ or 12–16 weeks for AML LSCs or ALL cells.^{21, 22} All animal experiments were approved by the Experimental Animal Ethical Committee at Shanghai Jiao Tong University School of Medicine in accordance with the ARRIVE guidelines.

Immunophenotypic analysis of BM cells of mouse recipients

BM cells of recipients were flushed down with Iscove's modified Dulbecco's medium (IMDM; Gibco) supplemented with 1% BSA (Sigma). Cells were stained CD45-APC or CD45-PE-Cy7 for engraftment; CD34-PE-Cy7 for total stem/progenitor cells; and CD34-PE-Cy7 and CD38-PE for engrafted HSCs. Stained cells were analyzed or sorted using flow cytometer (Aria II; BD). All antibodies were obtained from BD Bioscience. The detail is described in Supplemental Methods.

Flow sorting of LSCs

LSCs were sorted as **previously** described²². LMPP-LSCs and GMP-LSCs were

isolated as CD34⁺CD38⁻CD45RA⁺CD90^{-/low} and CD34⁺CD38⁺CD123^{low/+}CD45RA⁺CD110^{-/low} respectively.

Statistical analysis

Differential FEV-expression in infant and adult leukemias was analyzed by using chi-square test. Correlation of FEV-expression levels with leukemia lineage distribution, gender, and cytogenetics were analyzed by spearman's test, Correlation of FEV-expression levels with age were analyzed by Pearson's test. Differential FEV-expression in ALL and AML were analyzed by t test. Results of the functional assays were compared between Ctr.V, NSC and iFEV groups by t tests. Statistical analysis was evaluated by using SPSS 18.0 software (SPSS Inc.). Two-sided P values less than 0.05 were considered statistically significant.

Results

FEV is expressed only in fetal hematopoietic cells

Expression of FEV in hematopoietic cells was initially detected at different developmental stages of zebrafish.¹⁶ Results of whole-mount in situ hybridization (WISH) showed that fev-expression was detected in the lateral late mesoderm at 10-somite stage. fev-expression continued in the aorta-gonad-mesonephros (AGM) at 24 hours postfertilization (hpf) in zebrafish (Supplementary Figure S1a, left and middle panels). This expression was not detected in the kidney marrow that is equivalent to bone marrow (BM) of mammals of 4 dpf zebrafish (Supplementary

Figure S1a, right panel). In line with this result, the transgenic line using the *fev* promoter (*fev*:GFP) showed bright GFP fluorescence in the AGM at 24 hpf; slight expression was detected in the pancreas but not in the kidney marrow of zebrafish at 3 months (Supplementary Figure S1b).

To determine whether FEV-expression profile is conservative in mammals, we flow sorted the primitive lineage-negative (Lin^-) and mature lineage-positive (Lin^+) murine hematopoietic cells with high purities from the fetal liver (14.5 dpf) and BM of 1–4 and 8 weeks old (Supplementary Figure S2a). *Fev*-expression in these cells was detected by RT-qPCR.¹⁸ Consistent with the expression in zebrafish, murine *Fev* was expressed in Lin^- and not in Lin^+ hematopoietic cells of the fetal liver. However, *Fev*-expression was undetected in both Lin^- and Lin^+ cells of the BM (Supplementary Figure S2b and c). Taken together, *Fev* was only expressed in fetal cells and became silent after birth.

Analysis of FEV-expression was further assessed in human hematopoietic cells of fetal liver, neonatal umbilical vein cord blood (CB), and postnatal BM of infants (4 to 12 months old) children (3 to 14 years old) and adults (31 to 45 years old). CD45-expressing hematopoietic cells were flow sorted into primitive and more mature cell populations based on CD34 expression, high purities were achieved (Supplementary Figure S3a). FEV was expressed in fetal and neonatal cells, mainly in primitive CD34⁺ cells, but not in infant, child and adult BM cells of both CD34⁺ and CD34⁻ populations as determined by RT-qPCR (Figure 1a and b). The RT-qPCR products of CD34⁺ cells were cloned and sequencing confirmed FEV (Supplementary

Figure S3b).

These results show that FEV is conservatively expressed in embryonic and fetal hematopoietic cells and becomes silent after birth in different species from zebrafish to human, suggesting that FEV may be a specific regulator of fetal hematopoiesis.

FEV-expression is required for the self-renewal of HSCs

We next systemically characterized FEV functional roles in $\text{Lin}^- \text{CD34}^+ \text{CD38}^-$ HSCs. Human CB cells were selected for the assays because of their availability, neonatal nature, and FEV-expression profile (Figure 1a and b). After Lin^- cells were enriched, $\text{CD34}^+ \text{CD38}^-$ stem cells and multipotent progenitor cells and $\text{CD34}^+ \text{CD38}^+$ committed progenitor cells were flow sorted with high purities (Supplementary Figure S3c), and FEV-expression was analyzed using RT-PCR (Supplementary Figure S3d). $\text{Lin}^- \text{CD34}^+ \text{CD38}^-$ cells were used for functional assays of FEV-expression in HSCs. FEV-knockdown was achieved by a lentiviral vector-driven interference short-hairpin RNA (iFEV; Figure 2a)¹⁶. Transduction efficiencies of the control vector (Ctr.V), non-silencing control (NSC) and iFEV were detected 3 days after viral infection (Figure 2b, top panels), and the transduced cells (GFP^+) were flow sorted with high purities (Figure 2b, bottom panels). FEV-knockdown was determined by RT-PCR (Figure 2c). The quantitative limiting dilution assay was used to analyze the frequency of LTC-ICs, the most primitive human hematopoietic cells assessable *in vitro*. FEV-knockdown markedly reduced LTC-IC readout compared with that in Ctr.V and NSC settings (Figure 2d). There is no significant difference between Ctr.V and NSC

(Figure 2d). In line with this result, loss of FEV significantly reduced replatable CFCs as assessed by plating (Figure 2e) and replating (Figure 2f) CFC assays.

The cultured cells (Figure 2b) were analyzed to determine whether reduction of CFCs and LTC-ICs in iFEV cells could also reflect differences in cell cycle kinetics or cell survival. Results of apoptosis assay (Supplementary Figure S4a and b) and cell cycle analysis (Supplementary Figure S4c and d) showed no difference between iFEV and Ctr.V cells. These results suggest that FEV is an obligatory regulator of HSC self-renewal.

To further determine the role of FEV in regulating self-renewal in HSCs, total unsorted GFP⁺ and GFP⁻ cells after transduction were transplanted into immunodeficient NOD-SCID mice treated with CD122 antibody (NS122 mice)^{21, 23, 24}. After 12 weeks post-transplantation, flow cytometry was conducted to analyze human cell engraftments by using human CD45-specific antibody. The contribution of iFEV cells (GFP⁺) to the engraftment was considerably reduced compared with that of Ctr.V-transduced cells as shown in the representative flow-cytometric plots (Figure 3a) and the statistic diagram of mice tested in more than three separate experiments (Figure 3b). Within the engrafted GFP⁺ cells of iFEV and Ctr.V, proportions of total CD34⁺ (Figure 3c and d) and primitive CD34⁺CD38⁻ (Figure 3e and f) were analyzed. The populations of progenitor and stem cells were lower in iFEV than that in the control. This result indicates that the lower engraftment contribution of iFEV cells may be attributed to impaired self-renewal by FEV knockdown. To consolidate this result, the engrafted GFP⁺ cells from the primary iFEV and Ctr.V recipient mice were

flow sorted and transplanted into secondary recipients. After 10–12 weeks post-transplantation, human cell engraftments were analyzed. The details are shown in Table 1. The iFEV cells failed to reconstitute in secondary recipients, whereas the Ctr.V cells engrafted well, with comparable numbers of cells transplanted (Figure 3g and h).

To molecularly confirm the regulatory role of FEV in the self-renewal of fetal HSCs, we comparatively analyzed the differential expression of those genes were reported to be essential for the maintenance of fetal HSCs^{25,26} by using RT-qPCR in iFEV and Ctr.V cells (Figure 2b). With FEV knockdown, the expression of genes SCL, MLL, IKZF1, BMI1, and MEIS1 were significantly reduced (Supplementary Figure S5), further supporting that FEV is a crucial regulator of HSCs.

FEV-expression in leukemic cells is indicative of a prenatal origin

We next investigated the FEV-expression in leukemic cells by using the same RT-qPCR. First, we measured FEV-expression in infant leukemias (0-12 months old) that are almost all prenatal in origin.^{13,27} Fifty three infant samples were obtained and qualified for the detection of RT-qPCR and data analysis (Table 2 and Supplementary Table S1). The results showed that most infant samples (51 in 53) were FEV positive (FEV⁺) at a comparable level as in CB CD34⁺ cells (Figure 4 and Table 2). We then screened for FEV-expression in adult leukemias (older than 40 years old) that are presumed to rise postnatally. Eleven adult samples were tested for FEV-expression by RT-qPCR and data analysis (Table 2 and Supplementary Table S2). The results

showed that most adult samples (10 in 11) were FEV negative (FEV⁻) similarly as detected in normal BM cells (Figure 4 and Table 2). Together the dramatic difference between infant and adult samples suggests that FEV-expression in leukemic cells is indicative of a prenatal origin.

To extend the detection in wider spectrum of pediatric leukemias, we measure the FEV-expression in 193 samples of children (1–13 years old), 15 teenagers (13–20 years old), and 3 young adults (20–40 years old) with AML or ALL (Supplementary Table S3). The results **are** summarized in Figure 4. The expression pattern in children is similar to that in infants, most samples **being** FEV⁺ (Table 2). Surprisingly all the tested samples of teenagers and young adults were FEV⁺. The results of FEV positivity suggested that the majority of pediatric leukemias are possibly prenatal in origin.

Considering there was a range of expression levels in the FEV⁺ samples, we took the pediatric samples collected in one hospital (Shanghai Medical Center for Children) for further analysis to determine whether there was any correlation of FEV-expression levels with diagnostic factors. A significant correlation between FEV-expression levels and leukemia subtype (lymphoid or myeloid) was identified ($r = -0.253$, $P < 0.001$); FEV-expression in ALL samples was significantly higher than that in AML samples (Supplementary Figure S6a). There **was** no significant correlation of FEV-expression levels with **age** (Supplementary Figure S6b, $r = -0.122$, $P = 0.052$), **karyotype** ($r = 0.090$, $P = 0.229$) and **gender** ($r = -0.036$, $P = 0.570$).

FEV-knockdown markedly impairs the leukemia-propagating of LSCs

We then asked whether FEV played any functional role in leukemic cells and thus could be a potential therapeutic target. FEV function in LSCs of the BM aspirate samples of pediatric patients was thus studied. In CD34⁺ AML samples, two populations, namely, lymphoid-primed multipotential progenitors (LMPP)-like (CD34⁺CD38⁻CD45RA⁺ CD90^{-/low}) and granulocyte-macrophage progenitors (GMP)-like (CD34⁺ CD38⁺ CD123^{low}/ ⁺ CD45RA⁺ CD110^{-/low}) were previously characterized as containing LSCs.²² Similar immunophenotypes in the FEV⁺ samples tested in this study were observed (Supplementary Figure S7a, left and middle panels). LMPP-LSCs and GMP-LSCs populations were flow sorted with high purities (Supplementary Figure S7a, right panel).

LMPP-LSCs and GMP-LSCs were infected with iFEV and Ctr.V viruses and subjected to leukemic reconstitution in the NS122 mice model. Infection efficiencies were calculated, and the infected cells (GFP⁺) were flow sorted to measure FEV-expression (Supplementary Figure S7b). RT-PCR results showed that FEV-expression in iFEV-transduced cells was efficiently knocked down (Supplementary Figure S7c). Total unsorted GFP⁺ and GFP⁻ cells were transplanted into NS122 mice model. After 16 weeks post-transplantation the leukemic reconstitution in the mouse BM was analyzed by detecting human CD45.^{22, 24} Ctr.V-transduced cells propagated well, whereas iFEV-LMPP and iFEV-GMP cells failed to reconstitute in the BM as shown in the representative flow-cytometric plots (Figure 5a and b) and the statistical diagram of the total samples and mice studied (Figure 5c and d). The results indicate

that iFEV impaired the leukemia-propagating capacity of LSCs.

A similar functional assay was also performed on FEV⁺ ALL cells. Previous studies showed that cell fractions containing LSCs with a wide spectrum of immunophenotypes can propagate leukemia.^{11, 19, 28-30} The total leukemic cells of ALL samples in the current study were applied to assess the effect of iFEV on leukemia-propagating ability in NS122 mice.²¹ Similar results were obtained; the knockdown of FEV-expression in ALL cells markedly impaired the leukemia-propagating capacity in recipient mice (Figure 5e and f).

The normal human HSCs (Lin⁻CD34⁺CD38⁻) isolated from donor's BM at transplantation were used as a control in the xenotransplantation experiments to determine whether or not iFEV transduction affects hematopoietic reconstitution of normal BM HSCs. The results showed no significant difference in engraftment in mice between Ctr.V and iFEV cells, indicating iFEV treatment did not affect the function of normal HSCs (Supplementary Figure S8a and b).

Taken together, FEV is expressed in childhood leukemic cells of both AML and ALL, and FEV-expression is functionally essential for the leukemia-propagating capacity of LSCs. Treatments such as iFEV transduction may selectively impair LSCs and spare normal BM HSCs.

Discussion

Since the first evidence was reported that leukemogenic mutations occurred *in utero*,⁴ several approaches have been developed to track down the prenatal origin of

childhood leukemia. For example, genetic analysis of concordant leukemia of twins^{5, 31} and detection of genetic lesions of the neonatal blood in archived Guthrie cards^{5, 32-34}. These studies revealed that more than fifty percent of cases of childhood leukemia are prenatal in origin, although these approaches might technically underestimate the frequency. However, due to lack of a feasible diagnostic approach to determine the pre- or post-natal origin of leukemias, the exact frequency and biologic and medical implications of determining prenatal origination remains unknown in pediatric leukemias. Here, we have addressed these issues by identifying and characterizing FEV molecule.

FEV-expression profiles are highly conservative in zebrafish, mouse, and human. FEV is active in fetal and neonatal HSCs and silent in postnatal HSCs. We demonstrated that FEV-expression is functionally essential to the self-renewal of HSCs. In line with our previous finding,¹⁶ FEV regulates HSC emergence in embryonic development of hematopoietic system and functionally maintains the self-renewal capacity of HSCs in fetal development. We identified FEV as a unique regulator of fetal hematopoiesis. Understanding the how the fetal self-renewal machinery in HSCs switches to postnatal one when HSCs migrate to the BM after birth is expected to elucidate the mechanism of how leukemia develops.³⁵

More interestingly therefore, FEV-expression in leukemic cells was positive in most infant patients' samples, whereas rarely positive in adult patients, indicating that FEV-expressed leukemia may be prenatal in origin. By detecting and modifying FEV-expression, we investigated the frequency of prenatal origination and explored

the potential therapeutic implication in pediatric leukemias. Consistent with the prediction by previous studies,^{5-7, 32, 33} most cases of ~~the~~ pediatric leukemias were FEV-expression positive, in accord with a prenatal origin. Moreover FEV was positive in all the tested samples of teenagers and young adults, suggesting that leukemias in these patients who are routinely admitted **to adult wards and receive treatment as adult patients may** actually **be** similar to childhood leukemias in terms of etiology and biologic basis and thus therapeutics.

Further analyses of the correlation of FEV-expression levels with diagnostic factors in pediatric samples were performed. The results showed that the FEV-expression level in ALL was significantly higher than that in AML. Yet the relevance of the difference remains to be determined, **and** may be associated with distinct cellular origins or different leukemia-propagating capacity of leukemic cells. **Further studies** are required to **define changes in FEV expression by** leukemia subtype of genetic lesions including MLL fusions, which may contribute ~~much~~ to our understanding of leukemogenesis **both** in terms of the precise period in which the initiating events occur and the covert latency of the disease.^{36, 37}

More importantly the results of our xenotransplantation experiments showed that iFEV treatment significantly impaired the leukemia-propagating capacity of LSCs, **without affecting** hematopoietic reconstitution of normal HSCs. **This suggests** that FEV may provide a tractable target for therapy in childhood leukemias, killing LSCs and sparing normal HSCs. Therefore, our findings and conceptual views have implications in both etiology and therapeutics of cancer, particularly in childhood and

adolescent patients.

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Authorship

T.-H.L. designed and performed the experiments and analyze the results. Y.-J.T. and T.-H.L. collected and analyzed the clinical data. L.W. and F.L. performed zebrafish experiments. Y.H. and Q.-J.Y. collected human fetal liver and BM cells. X.-L.G., Y.Z., L.C., H.Z., X.L. and L.-H.Z. collected human CB cells. J.-Y.T., B.-S.L., J.-Q.M., and L.-G.L. collected patient samples and related clinical information. A.F. detected FEV expression and analyzed data. T.E., F.L. and G.-Q.C. discussed the data and contributed to the writing of the manuscript. D.-L.H. supervised the project, designed

the experiments, and wrote the paper.

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Figures:

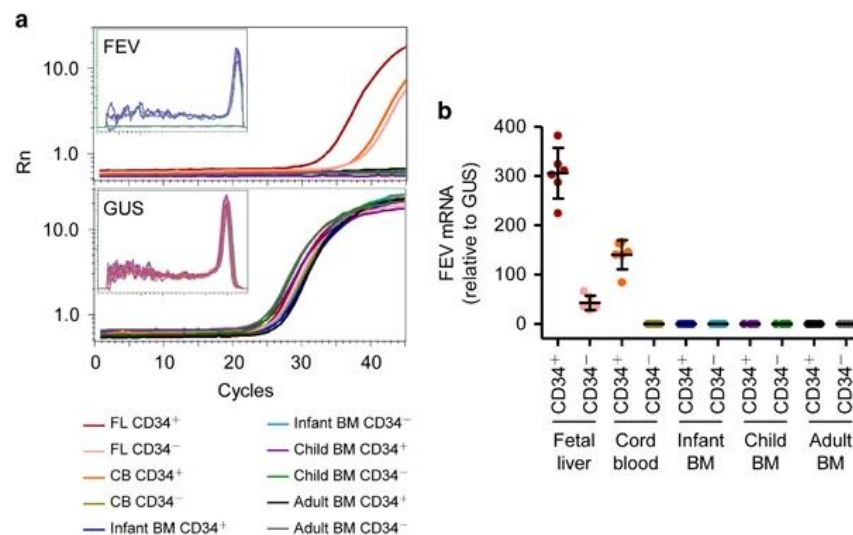


Figure 1. FEV-expression profiles in human hematopoietic cells in development. **(a)** Representative RT-qPCR amplification and dissociation curves of FEV and ABL1 in the human cells as labelled. **(b)** The results of all the samples tested are summarized. Data are mean \pm SD in triplicates in an independent experiment, which was repeated at least for three times with the same results.

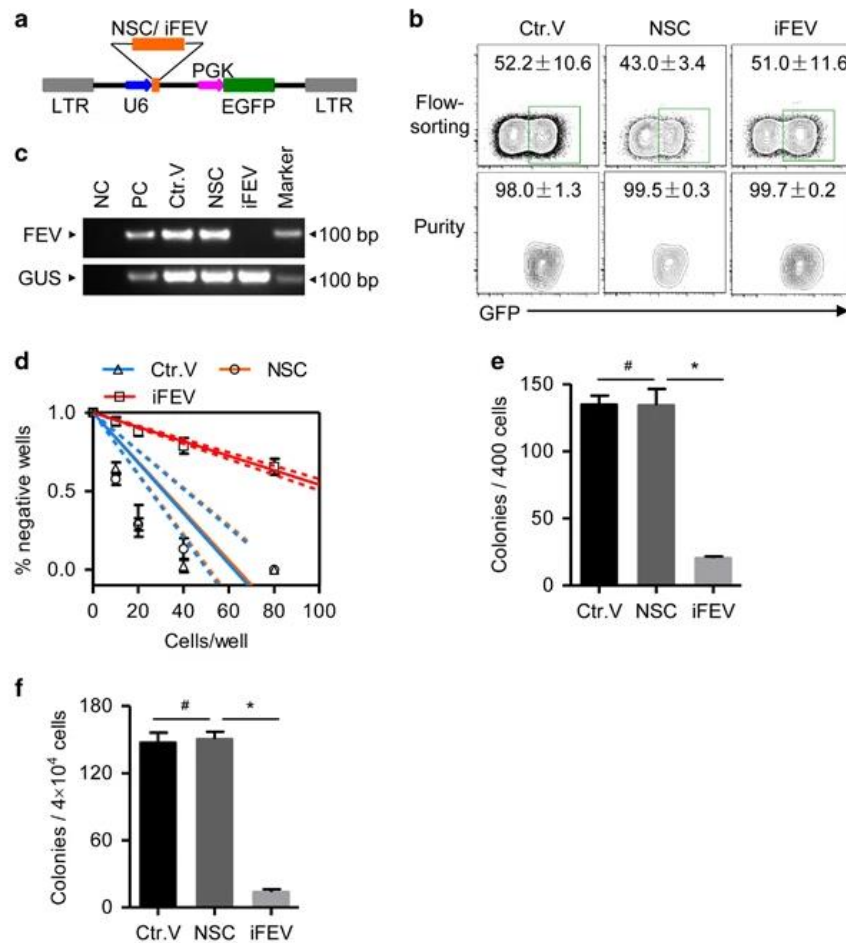


Figure 2. Functional assays of FEV in human primitive hematopoietic cells *in vitro*.

(a) Short-hairpin oligos of FEV interference (iFEV) and non-silencing oligos (NSC) were cloned into the lentiviral vector driven by U6 promotor. (b) Flow cytometric analysis of infection efficiencies of Lin⁻CD34⁺CD38⁻ HSCs by the virus of Ctr.V, NSC or iFEV 3 days post-infection. GFP⁺ cells were flow sorted (top panel), and the purities were detected (bottom panel). (c) RT-PCR detection of FEV-expression in the flow-sorted cells of **b**. PC, positive control using 293T cells permanently expressing human FEV. NC, non-template control. (d) LTC-IC assay by limiting dilution, the estimated LTC-IC frequencies (solid lines) for Ctr.V, NSC and iFEV cells were 1/16, 1/17 and 1/163, respectively. Dotted lines, 95% confidence intervals. (e) CFC assay,

iFEV cells had a 6.6-fold decrease in the total colonies compared with NSC and Ctr.V cells (20.4 ± 1.1 vs. 134.2 ± 10.0 vs. 134.7 ± 5.6). (f) Replating CFC assay of the primary colony cells of e. iFEV led to an 11.0-fold decrease in the total colonies compared with NSC and Ctr.V cells (13.6 ± 2.5 vs. 150.50 ± 5.4 vs. 147.3 ± 7.1). $n = 3$ separate experiments (e–g). $^{\#}P > 0.5$, $^{*}P < 0.001$ (Student's t test). All Data are presented as mean \pm SD.

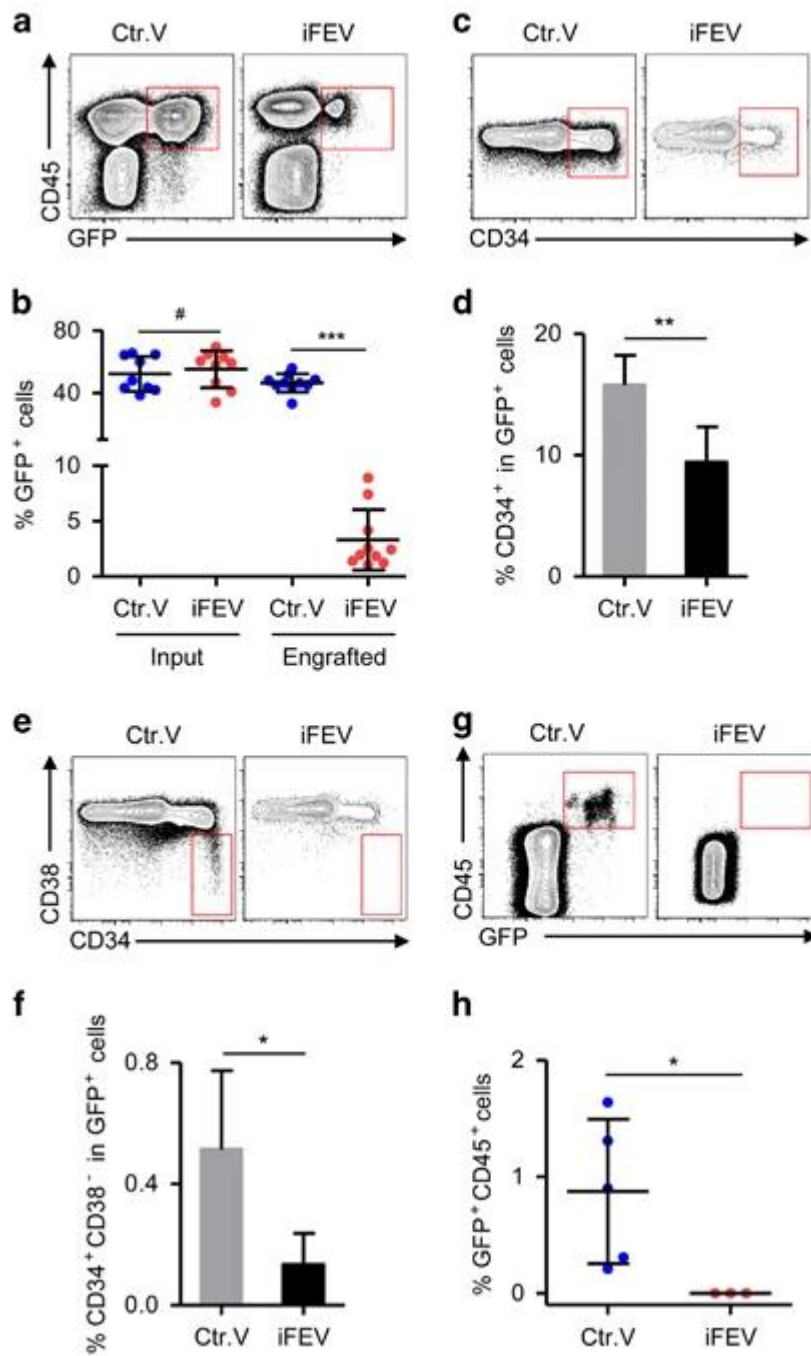


Figure 3. FEV-knockdown impaired the self-renewal ability of HSCs *in vivo*. (**a** and **b**) Flow-cytometric analysis of engraftment and contribution of GFP⁺ cells in primary mice and infection efficiency before transplantation (input), representative plots (**a**) and statistical summary (**b**). iFEV led to a 13.6-fold decrease ($45.1\% \pm 6.8\%$ vs. $3.3\% \pm 2.6\%$). $n = 10$ mice per group from nine separate experiments. (**c** and **d**)

Flow cytometric analysis of total progenitors (CD34⁺) in the BM of primary Ctr.V and iFEV mice, representative plots (c) and statistical summary (d). (e and f) Flow cytometric analysis of more primitive CD34⁺CD38⁻ cells in the BM of primary Ctr.V and iFEV mice, representative plots (e) and statistical summary (f). $n = 9$ (Ctr.V) and $n = 7$ (iFEV) mice. (g and h) Engraftment contribution of Ctr.V or iFEV cells in secondary recipients by representative flow-cytometric plots (g) and statistical summary (h). Note: iFEV cells failed to reconstitute in secondary recipients. $n = 5$ (Ctr.V) and $n = 3$ (iFEV) mice. $^{\#}P > 0.5$, $^*P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$ (student's t test). All Data are presented as mean \pm SD.

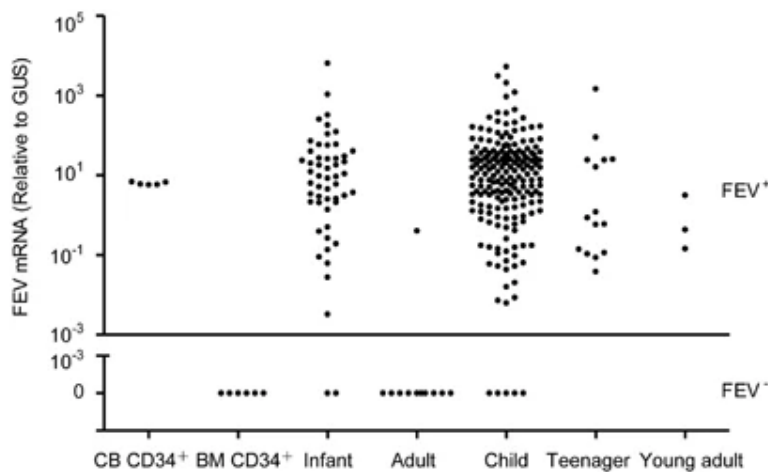


Figure 4. Differential FEV-expression in leukemic cells of infant, childhood, teenager, young adult and adult patients. FEV-expression in leukemic cells was measured by RT-qPCR. The results are presented in comparison with that in CB CD34⁺ cells (FEV⁺) and BM CD34⁺ cells (FEV⁻). SEM. $^*P < 0.05$, $^{**}P < 0.005$ (Student's t test).

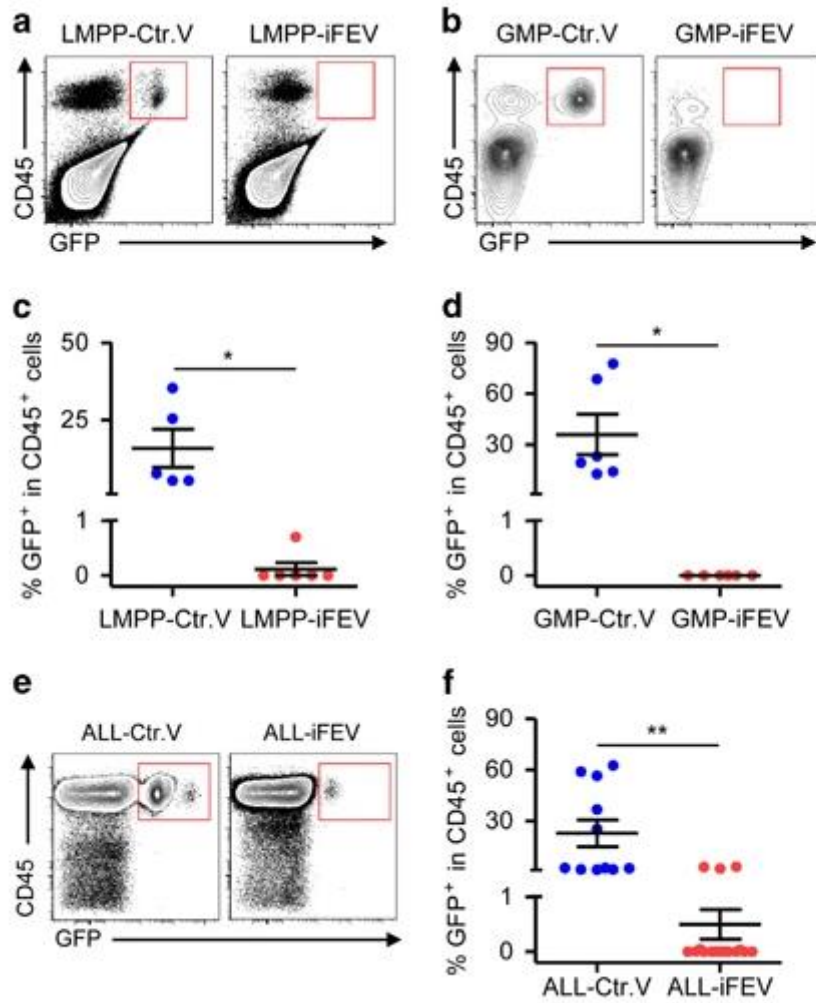


Figure 5. FEV-knockdown impaired the leukemia-propagating of LSCs. (a and b) Representative flow cytometric analysis of leukemic propagation of LMPP-like and GMP-like LSCs transduced with Ctr.V or iFEV in the mouse BM. (c and d) Statistical summary of engraft contribution of GFP⁺ cells in the recipients transplanted with LMPP-like and GMP-like LSCs. $n = 5$ or 6 (Ctr.V) and $n = 6$ (iFEV) mice from three patients. (e) Representative flow cytometric analysis of leukemic propagation of ALL cells transduced with Ctr.V or iFEV in the mouse BM. (f) Statistical summary of engraft contribution of GFP⁺ cells in the recipients transplanted with ALL cells. $n = 11$ (Ctr.V) and $n = 16$ (iFEV) mice from four patients. All data are presented as mean \pm

Tables:

| <i>Primary</i> | | <i>Secondary</i> | | |
|-------------------------------|------------------------|-------------------------------|-----------------------------|------------------------|
| <i>Recipients^a</i> | <i>Engraftment (%)</i> | <i>Recipients^b</i> | <i>Cell number injected</i> | <i>Engraftment (%)</i> |
| Ctr.V1 | 41.1 | Ctr.V1-1 | 3.37×10^5 | 0.9 |
| | | Ctr.V1-2 | 3.00×10^6 | 0.01 |
| Ctr.V2 | 35.3 | Ctr.V2-1 | 3.07×10^5 | 1.64 |
| | | Ctr.V2-2 | 2.76×10^6 | 3.05 |
| Ctr.V3 | 33.4 | Ctr.V3-1 | 2.00×10^5 | 0.31 |
| | | Ctr.V3-2 | 2.80×10^6 | 4.57 |
| Ctr.V4 | 25.2 | Ctr.V4-1 | 2.00×10^5 | 0.03 |
| | | Ctr.V4-2 | 2.80×10^6 | Dead |
| Ctr.V5 | 42.5 | Ctr.V5-1 | 3.80×10^5 | 1.31 |
| Ctr.V6 | 31.4 | Ctr.V6-1 | 2.00×10^5 | 0.21 |
| iFEV1 | 2.59 | iFEV1-1 | 2.10×10^5 | 0 |
| iFEV2 | 2.87 | iFEV2-1 | 3.38×10^5 | 0 |
| iFEV3 | 1.29 | iFEV3-1 | 2.15×10^5 | Dead |
| iFEV4 | 0.91 | iFEV4-1 | 2.60×10^5 | 0 |
| iFEV5 | 1.02 | | | |
| iFEV6 | 0.81 | iFEV6-1 | 2.12×10^5 | Dead |
| iFEV7 | 0.85 | | | |

Abbreviations: CB cord blood; Ctr.V control vector.

^a 2×10^4 Lin⁻CD34⁺CD38⁻ cells transduced with Ctr.V or iFEV were injected into primary recipients.

^bCD45⁺GFP⁺ cells sorted from primary recipients were injected into secondary recipients.

Table 1. Primary and secondary transplantation of CB Lin⁻CD34⁺CD38⁻ cells transduced with Ctr.V or iFEV

| Characteristics | Infant leukemias | Child leukemias | Adult leukemias | P-value |
|---------------------------------------|-------------------------|------------------------|------------------------|---|
| Total, <i>n</i> | 53 | 193 | 11 | |
| <i>Leukemia lineage</i> ^a | | | | 0.747 ^b /1.000 ^c |
| Acute lymphoid leukemia, <i>n</i> (%) | 25 (47.2) | 89 (46.1) | 4 (36.4) | |
| Acute myeloid leukemia, <i>n</i> (%) | 28 (52.8) | 103 (53.4) | 7 (63.6) | |
| Mixed lineage leukemia, <i>n</i> (%) | 0 (0) | 1 (0.5) | 0 (0.0) | |
| <i>Gender</i> ^a | | | | 0.747 ^b /0.204 ^c |
| Female, <i>n</i> (%) | 25 (47.2) | 71 (36.8) | 4 (36.4) | |
| Male, <i>n</i> (%) | 28 (52.8) | 122 (63.2) | 7 (63.6) | |
| <i>Cytogenetic</i> ^{a,d} | | | | 1.000 ^b /0.090 ^c |
| Normal, <i>n</i> (%) | 7 (30.4) | 74 (48.1) | 3 (33.3) | |
| Simple, <i>n</i> (%) | 12 (52.2) | 45 (29.2) | 4 (44.4) | |
| Complex, <i>n</i> (%) | 4 (17.4) | 35 (22.7) | 2 (22.2) | |
| <i>Age</i> ^e | | | | <0.001 ^b / ^c 0.001 ^c |
| Median | 8 Months | 4.7 Years | 59 Years | |
| Range | 2–12 Months | 1.1–13.0 Years | 50–85 Years | |
| <i>FEV expression</i> ^a | | | | <0.001 ^b /1.000 ^c |
| FEV ⁺ , <i>n</i> (%) | 2 (3.8) | 5 (2.6) | 10 (90.9) | |
| FEV ⁺ , <i>n</i> (%) | 51 (96.2) | 188 (97.4) | 1 (9.1) | |

Abbreviation: FEV, fifth Ewing variant.

^aPearson's χ^2 test.

^bP-value of infant vs adult.

^cP-value of child vs infant.

^dCytogenetics of 71 samples were unknown, including 30 infant leukemias, 39 child leukemias and 2 adult leukemias.
Mann–Whitney U-test.

Table 2. Summary of the patients' information and FEV positivity

Supplementary Methods

Isolation of hematopoietic stem and progenitor cells from human umbilical cord blood

Lin⁻ or CD34-enriched cells were achieved by Ficoll gradient centrifugation and magnetic-bead separation as previously described.^{1, 2} Lin⁻ or CD34-enriched cells were stained with lineage-APC, CD34-PE-cy7, and CD38-PE (BD Bioscience) at 4 °C for 30 min in PBS containing 0.5% bovine serum albumin (BSA; Sigma). Subsequently, Lin⁻CD34⁺CD38⁻ and CD38⁺ cells were flow sorted by using a flow cytometer (Aria II; BD).

RT-qPCR detection of FEV-expression

Total RNA was extracted by Trizol reagent according to the manufacturer's instructions (Invitrogen) and reversely transcribed. RT-qPCR was performed using SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. All experiments were performed in triplicate with Applied Biosystems 7900HT. PCR reaction was conducted as follows: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15 s, 59 °C for 20 s, and 72 °C for 20 s. And 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s for dissociation curve. Differences in cDNA input were normalized by ABL1 expression levels. Primer sequences are summarized in Supplementary Table S4.

Immunophenotypic analysis of BM cells of mouse recipients

BM cells of recipients were flushed down with Iscove's modified Dulbecco's medium

(IMDM; Gibco) supplemented with 1% BSA (Sigma). Afterward, the cells were treated with ACK solution (150 mM NH₄Cl, 1 mM KHCO₃, and 0.1 mM EDTA, reagents from Sigma) at room temperature for 5–10 min to lyse red blood cells. The cells were immediately washed and then resuspended in PBS supplemented with 0.5% BSA for antibody staining at 4 °C for 30 min in the dark: CD45-APC or CD45-PE-Cy7 for engraftment; CD34-PE-Cy7 for total stem/progenitor cells; and CD34-PE-Cy7 and CD38-PE for engrafted HSCs. Stained cells were analyzed or sorted using flow cytometer (Aria II; BD). All antibodies were obtained from BD Bioscience.

Cell cycle analysis

Cord blood Lin⁻CD34⁺CD38⁻ cells transduced with Ctr.V or iFEV were cultured in stem cell-maintaining medium for 3 days. Cells were collected, fixed and permeablized according to the manufacturer's instructions (BD Bioscience). Subsequently, the cells were stained with ki-67-PE and DAPI. Cells were analyzed using flow cytometry (LSRFortessa II; BD).

Cell apoptosis analysis

Cord blood Lin⁻CD34⁺CD38⁻ cells transduced with Ctr.V or iFEV were cultured in stem cell maintaining medium for 3 days. The cell viability was determined by flow cytometry staining with annexin-V and propidium iodide (BD Bioscience) according to the manufacturer's instructions.

Generation of *fev*:GFP transgenic line

We obtained a fish line through a large-scale Tol2 transposon-mediated enhancer-trap screen (unpublished data). Sequence analysis of the insertion site by linker-mediated PCR revealed that this line trapped gene *fev* at approximately 400 base pairs (bp) upstream of its putative transcription start site.

Whole mount in situ hybridization

Whole mount in situ hybridization with zebrafish embryos was performed using a ZF-A4 in situ hybridization machine (Zfand, China) with *fev* probes as previously described³.

Supplemental References

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3. Wang L, Liu T, Xu L, Gao Y, Wei Y, Duan C, *et al.* Fev regulates hematopoietic stem cell development via ERK signaling. *Blood* 2013; **122**: 367-375.

Supplemental Tables (See excel files)

Table S1. Clinical information of infant leukemia patients and FEV positivity

Table S2. Clinical information of adult leukemia patients and FEV positivity

Table S3. Clinical information of child, teenager and young adult leukemia patients and FEV positivity

Table S5. Primers for RT-qPCR

Supplementary Figure Legends

Supplementary Figure S1. *fev*-expression profile in zebrafish hematopoietic cells. **(a)**

Whole-mount in situ hybridization (WISH) showed that *fev* was expressed in the lateral plate mesoderm at 10-somite stage (left panel, arrowheads) and aorta-gonad-mesonephros (AGM) at 24 hpf (middle panel, arrowhead) but not in the kidney marrow at 4 dpf (right panel, dashed circle). **(b)** In the transgenic line using the *fev* promoter (*fev*:GFP), GFP fluorescence was observed in the AGM region at 24 hpf (left panel) and in pancreas but not in the adult kidney of adult zebrafish at 3 months (middle and right panels).

Supplementary Figure S2. FEV-expression profile in mouse hematopoietic cells in development. **(a)** Flow sorting and purity detection of lineage-negative (Lin^-) and

lineage-positive (Lin^+) cells of mouse 14.5 dpf fetal liver (FL), 1–4 weeks (wks) and 8 wks old bone marrow (BM). **(b)** Representative amplification and dissociation

curves of *Fev* and *ABL1* in the mouse cells labeled. **(c)** The results of all the samples

tested are summarized. Only FL Lin⁻ cells are positive in Fev-expression. Four independent experiments were conducted.

Supplementary Figure S3. Flow sorting and FEV-expression of human hematopoietic stem and progenitor cells. **(a)** CD34⁺ and CD34⁻ hematopoietic cells (expressing CD45) from the tissues labelled were flow-sorted with high purities. **(b)** The RT-qPCR products of CD34⁺ cells were cloned and sequencing confirmed FEV. **(c)** Flow sorting and purity detection of Lin⁻CD34⁺CD38⁻ and Lin⁻CD34⁺CD38⁺ fractions from CD34-enriched CB cells. **(d)** RT-PCR analysis of FEV-expression in the cell fractions of **c**.

Supplementary Figure S4. iFEV effect on apoptosis and cell cycle of CB HSCs. **(a)** and **(b)** Apoptotic analysis in the transduced cells by representative flow-cytometric plots **(a)** and statistical summary **(b)**. **(c and d)** Cell cycle in the in the transduced cells by representative flow-cytometric plots **(c)** and statistical summary **(d)**. $n = 3$ separate experiments. $^{\#}P > 0.5$ (Student's t test). All Data are presented as mean \pm SD.

Supplementary Figure S5. RT-qPCR detection of the expression of the genes labeled in the cells flow-sorted in Figure 2b. Expression of MLL, SCL, IKZF1, BMI-1, and MEIS1 was significantly reduced in iFEV cells. $n = 4$ separate experiments. $^*P < 0.05$ (student's t test). All Data are presented as mean \pm SD.

Supplementary Figure S6. The correlation of FEV-expression levels with leukemia subtype and age in FEV⁺ pediatric samples. **(a)** Differential FEV-expression levels in ALL and AML. **(b)** Correlation of FEV-expression levels with age in leukemic samples. Note, there is no significant correlation of FEV-expression levels with age. * $P < 0.001$ (student's t test).

Supplementary Figure S7. Flow sorting of AML LSCs and FEV-expression in the transduced LSCs. **(a)** Flow cytometric analysis, sorting and purity detection of LMPP- and GMP-like LSCs from FEV⁺ AML cells. **(b)** Flow cytometric analysis of infection efficiencies of LMPP- and GMP-like LSCs by iFEV or Ctr.V virus. GFP⁺ cells were flow-sorted. **(c)** RT-PCR analysis of FEV-expression in the flow-sorted cells of **b**. PC, positive control using 293T cells permanently expressing human FEV. NC, non-template control.

Supplementary Figure S8. Effect of iFEV on reconstitution of human BM HSCs in xenograft mice. **(a)** Flow-cytometric analysis of engraftment and contribution of GFP⁺ cells in recipients. **(b)** Statistical summary of infection efficiency before transplantation (input) and contribution of GFP⁺ cells in recipients (output). $n = 6$ mice in 3 separate experiments. # $P > 0.5$ (Student's t test). All Data are presented as mean \pm SD.