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Experimental Hematology

Experimental Hematology 2017;■:■–■

HIF-1α-stabilizing agent FG-4497 rescues human CD34$^+$ cell mobilization in response to G-CSF in immunodeficient mice

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(Received 5 May 2017; revised 9 May 2017; accepted 10 May 2017)

Granulocyte colony-stimulating factor (G-CSF) is used routinely in the clinical setting to mobilize hematopoietic stem progenitor cells (HSPCs) into the patient's blood for collection and subsequent transplantation. However, a significant proportion of patients who have previously received chemotherapy or radiotherapy and require autologous HSPC transplantation cannot mobilize the minimal threshold of mobilized HSPCs to achieve rapid and successful hematopoietic reconstitution. Although several alternatives to the G-CSF regime have been tested, few are used in the clinical setting. We have shown previously in mice that administration of prolyl 4-hydroxylase domain enzyme (PHD) inhibitors, which stabilize hypoxia-inducible factor (HIF)-1α, synergize with G-CSF in vivo to enhance mouse HSPC mobilization into blood, leading to enhanced engraftment via an HSPC-intrinsic mechanism. To evaluate whether PHD inhibitors could be used to enhance mobilization of human HSPCs, we humanized nonobese, diabetic severe combined immune-deficient *Il2rg*$^{-/-}$ mice by transplanting them with human umbilical cord blood CD34$^+$ HSPCs and then treating them with G-CSF with and without co-administration of the PHD inhibitor FG-4497. We observed that combination treatment with G-CSF and FG-4497 resulted in significant mobilization of human lineage-negative (Lin$^-$) CD34$^+$ HSPCs and more primitive human Lin$^-$CD34$^+$CD38$^-$ HSPCs into blood and spleen, whereas mice treated with G-CSF alone did not mobilize human HSPCs significantly. These results suggest that the PHD inhibitor FG-4497 also increases human HSPC mobilization in a xenograft mouse model, suggesting the possibility of testing PHD inhibitors to boost HSPC mobilization in response to G-CSF in humans.

Granulocyte-stimulating factor (G-CSF) is used in the clinical setting to mobilize CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) into the blood for subsequent transplantation [1]. Autologous HSPC transplantations, which represent half of all HSC transplantations, increase survival rates of relapsed non-Hodgkin's lymphoma patients and multiple myeloma patients [2,3]. In the autologous transplantation setting, 20–60% of patients previously treated with repeated cycles of high-dose chemotherapy or radiotherapy fail to mobilize the minimum threshold of 2×10^6 CD34$^+$ cells/kg required for rapid hematopoietic reconstitution [4], precluding potentially curative autologous HSPC transplantation. The synthetic CXCR4 chemokine receptor antagonist AMD3100/Plerixafor synergizes with G-CSF to promote HSPC mobilization and salvages mobilization in about 70% of poor mobilizers [5,6]. Because Plerixafor is the only drug approved by the U.S. Food and Drug Administration that boosts HSPC mobilization, there remains considerable scope to identify alternative strategies to further improve HSPC mobilization in poor mobilizers [4].

Recently, we have demonstrated in mice that HSPC-autonomous stabilization of oxygen-labile transcription factor hypoxia-inducible factor (HIF)-1 is necessary for mobilization in response to G-CSF or Plerixafor [7]. In mice, pharmacological stabilization of HIF-1 with HIF

MRD and J-PL contributed equally to this work.

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Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.exphem.2017.05.004.

0301-472X/
http://dx.doi.org/10.1016/j.exphem.2017.05.004

prolyl 4-hydroxylase domain enzyme inhibitors, such as FG-4497, synergized with both G-CSF and Plerixafor to boost HSC mobilization into the blood [7]. To confirm the role of HIF-1α and its pharmacological stabilization in human HSPC mobilization, we tested the combination of G-CSF plus FG-4497 in human $CD34^+$ HSPC xenografts in immune-deficient mice.

Methods

Xenograft

Purified human cord blood $CD34^+$ cells were purchased from STEMCELL Technologies (Vancouver, Canada). Cell transplantations were completed as previously described [8]. In brief, female non-obese, diabetic severe combined immune-deficient *Il2rg*$^{-/-}$ (NSG) mice at 6–8 weeks of age were sublethally irradiated (2.5 Gy) 24 hours before transplantation. Mice were anesthetized with isoflurane inhalation, and 50×10^3 freshly thawed $CD34^+$ cells were injected intravenously retro-orbitally. At 6 and 8 weeks after transplantation, blood was collected from mice by submandibular bleed for flow cytometry analysis to measure human $CD45^+$ leukocyte engraftment. Based on the 8-week bleed, mice were distributed into groups with similar human leukocyte chimerism (Supplementary Figure E1, online only, available at www.exphem.org).

Mobilization

Six different treatment groups (Supplementary Figure E2, online only, available at www.exphem.org) were used: saline alone for 4 days (S), FG-4497 alone for 3 days (F3), G-CSF alone for 2 (G2) or 4 days (G4), and 3 days of FG-4497 co-administrated with G-CSF for 2 (G2F3) or G-CSF for 4 days (G4F3).

Mice were administrated with recombinant human G-CSF (Filgrastim, Amgen) twice daily subcutaneously at 125 μg/kg or with saline for 2 or 4 days before harvest. FG-4497 20 mg/kg (Fibrogen, San Francisco, CA), or vehicle was injected intraperitoneally daily for the last 3 days before tissue harvest as previously described [7].

Tissue harvest and flow cytometry

For details of tissue harvest and flow cytometry, please see the Supplementary Methods and Supplementary Table E1 (online only, available at www.exphem.org).

Statistics

To account for the different levels of human hematopoietic engraftment between each recipient mouse, we normalized the numbers of mobilized human HSPCs (huCD45$^+$Lin$^-$ CD34$^+$ or huCD45$^+$Lin$^-$ CD34$^+$CD38$^-$) to the percentage of human chimerism in blood at 8 weeks after transplantation (before mobilization). This normalization was performed using the following equation:

$$\frac{\text{HSPC per mL blood or per spleen}}{\%\text{huCD45}^+ \text{ leukocytes in blood at week 8}}$$

All statistical analyses were performed using Prism software (GraphPad, La Jolla, CA). Sample groups were tested for outlying data using Prism. Data were analyzed using one-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons.

Results and discussion

To test whether a combination of the PHD inhibitor FG-4497 with G-CSF could mobilize human HSPCs, we transplanted 42 NSG mice with 50,000 thawed human cord blood $CD34^+$ cells. Eight weeks after transplantation, we measured human leukocyte engraftment, which ranged from 20% to 60% total blood leukocytes (Supplementary Figure E1). Considering this wide range of human chimerism, we distributed the 42 mice across six experimental groups to have similar averages and standard deviations in human leukocyte chimerism. Two weeks later, mice were injected with saline, FG-4497 alone, G-CSF alone, or the combination of G-CSF plus FG-4497 (Supplementary Figure E2).

At the endpoint of the experiment, blood and spleen content in human $CD45^+Lin^-CD34^+$ HSPCs and more primitive $CD45^+Lin^-CD34^+CD38^-$ HSPCs were measured by flow cytometry. Considering the highly variable levels of human leukocyte chimerism between mice from each experimental group (range: 9.3% to 61.0%; Supplementary Table E2, online only, available at www.exphem.org), the number of human HSPCs mobilized in each tissue was normalized relative to the percentage of human chimerism before treatment by dividing the number of mobilized HSPCs by the proportion of human $CD45^+$ leukocytes in the blood of each individual mouse before treatment. (At an equivalent level of HSPC mobilization, one would expect that a mouse with 60% human chimerism would mobilize sixfold more human HSPCs per milliliter of blood and spleen than a mouse with 10% human chimerism.)

After this normalization relative to human engraftment, G-CSF alone did not increase human $CD45^+Lin^-CD34^+$ HSPC cell mobilization in the blood of NSG mice at either time point compared with the saline-treated group ($p = 0.44$ G2 vs. S; $p = 0.47$ G4 vs. S by ANOVA; Fig. 1A–D). However, the combination of FG-4497 plus G-CSF for 2 days (G2F3) or 4 days (G4F3) significantly mobilized human $CD45^+Lin^-CD34^+$ HSPCs ($p = 0.0204$ G2F3 vs. saline; $p = 0.0059$ G4F3 vs. saline by ANOVA; Fig. 1A, 1F–H). Therefore, the addition of FG-4437 to G-CSF significantly mobilized human $CD45^+Lin^-CD34^+$ HSPCs into the blood of NSG mice, whereas G-CSF alone did not. More primitive $CD45^+Lin^-CD34^+CD38^-$ HSPCs were significantly mobilized at day 4 of G-CSF treatment, irrespective of FG-4497 treatment, which did not further increase their mobilization into the blood (Fig. 1E).

Because the spleen is a sieve in which mobilized HSPCs accumulate during mobilizing treatments, we measured the number of human HSPCs in this organ. G-CSF alone or in combination with FG-4497 did not cause significant accumulation of HSPCs into the spleen after 2 days of G-CSF treatment (Fig. 2A and 2E). However, at day 4 of G-CSF treatment, the combination with FG-4497 caused a significant increase in the number of $CD45^+$ Lin^- $CD34^+$ HSPCs

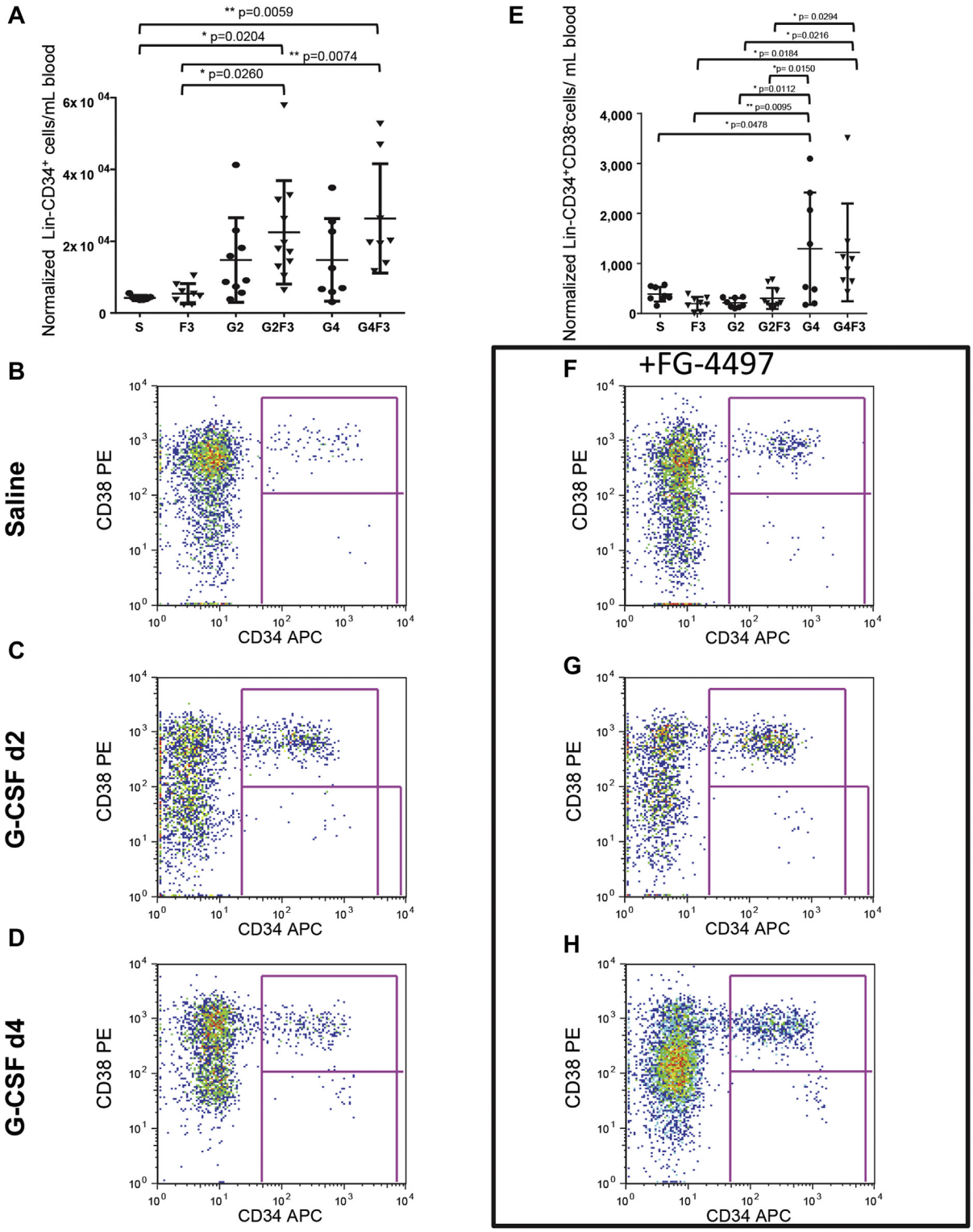

Figure 1. FG-4497 increases mobilization of human HSPCs into the blood in response to human G-CSF in transplanted NSG mice. (**A**) Human $CD45^+Lin^-CD34^+$ cells mobilized into peripheral blood normalized to human $CD45^+$ leukocyte engraftment at 8 weeks after transplantation. S = saline for 4 days; F3 = G-4497 for 3 days; G2 = G-CSF for 2 days; G4 = G-CSF for 4 days. Each dot represents an individual mouse from each group; average and standard deviations are shown for each group. *p* values were calculated by ANOVA with Tukey's correction for multiple comparisons. Shown are representative flow cytometry dot-plots for peripheral blood from mice treated with saline (**B**) or G-CSF alone for 2 days (**C**) or 4 days (**D**). Dot-plots show expression of human CD34 versus CD38 on blood cells within the live gate and human $CD45^+Lin^-$ gate. (**E**) FG-4497 increases the mobilization of more primitive human $CD45^+Lin^-CD34^+CD38^-$ HSPCs into the blood in response to human G-CSF in transplanted NSG mice. Representative flow cytometry dot-plots are shown for spleens from mice treated with FG-4497 alone (**F**), and within the box, FG-4497 plus G-CSF for 2 days (**G**) or 4 days (**H**).

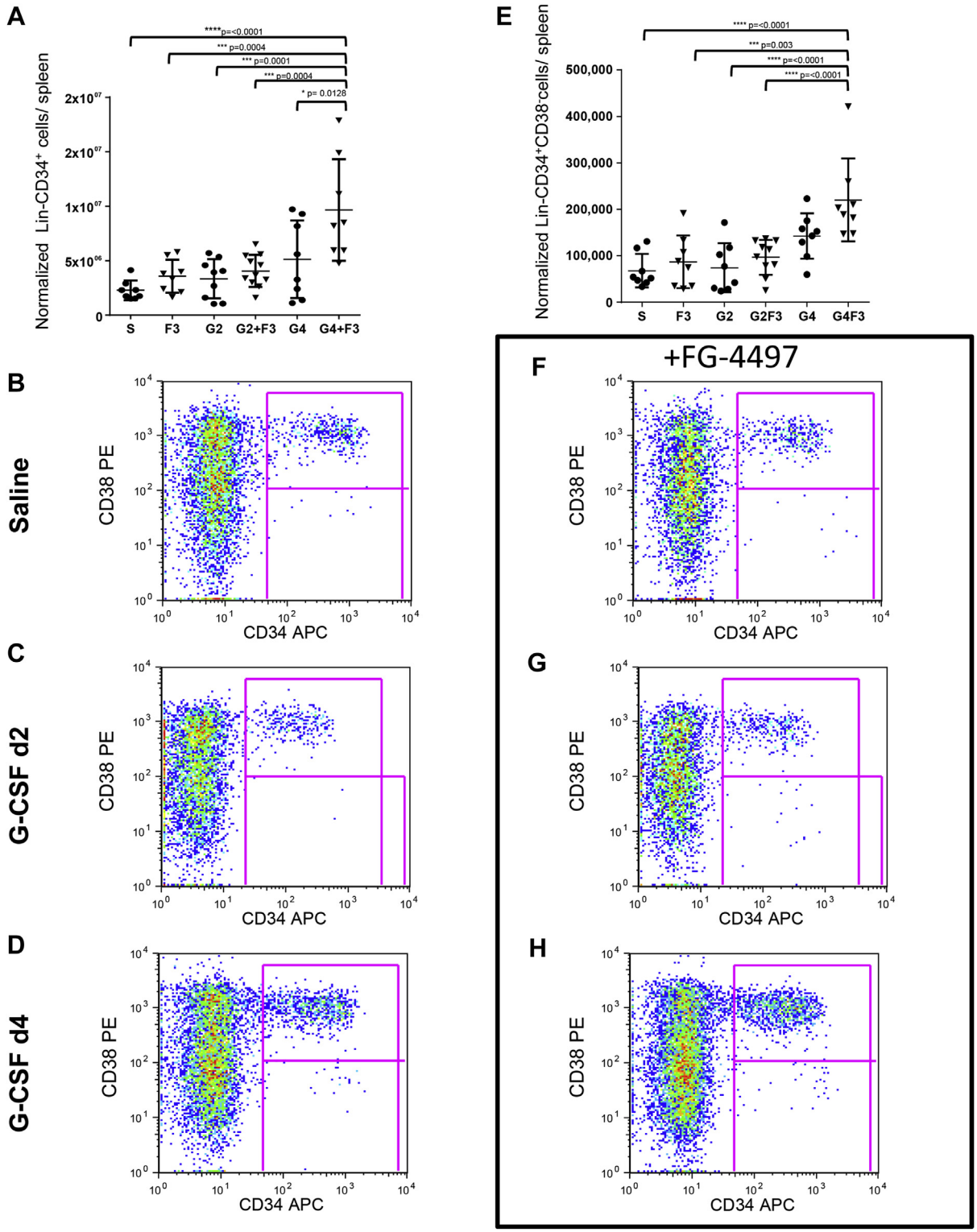

Figure 2. FG-4497 increases mobilization of human HSPCs into the spleen in response to human G-CSF in transplanted NSG mice. (**A**) Human $CD45^{+}Lin^{-}CD34^{+}$ cells mobilized to the spleen normalized to human $CD45^{+}$ leukocyte engraftment at 8 weeks after transplantation. S = saline for 4 days; F3=FG-4497 for 3 days; G2 = G-CSF for 2 days; G4 = G-CSF for 4 days. Each dot represents an individual mouse from each group; average and standard deviations are shown for each group. *p* values were calculated by ANOVA with Tukey's correction for multiple comparisons. Representative flow cytometry dot-plots are shown for peripheral blood from mice treated with saline (**B**) or G-CSF alone for 2 days (**C**) or 4 days (**D**). (**E**) FG-4497 increases mobilization of more primitive human $Lin^{-}CD34^{+}CD38^{-}$ HSPCs to the spleen in response to human G-CSF in transplanted NSG mice. Representative flow cytometry dot-plots are shown for spleens from mice treated with FG-4497 alone (**F**), and within the box, FG-4497 plus G-CSF for 2 days (**G**) or 4 days (**H**). Dot-plots show human CD34 versus CD38 within the live gate and human $CD45^{+}Lin^{-}$ gate.

mobilized into the spleen compared with saline-treated mice ($p < 10^{-4}$ by ANOVA), whereas mice treated with G-CSF alone did not ($p = 0.26$ by ANOVA; Fig. 2A–D). Likewise, $CD45^+$ $Lin^-CD34^+CD38^-$ HSPCs were also significantly increased in the spleen at day 4 of G-CSF plus FG-4497 compared with the saline-treated group ($p < 10^{-4}$ by ANOVA), whereas they were not increased in mice treated with G-CSF alone for 4 days ($p = 0.10$ by ANOVA; Fig. 2E–H). There was significantly higher accumulation of $CD45^+Lin^-CD34^+$ HSPCs in the spleen of animals treated with G-CSF for 4 days plus FG-4497 than in those treated with G-CSF alone ($p = 0.0128$, ANOVA), whereas there was a trend of higher accumulation of $CD45^+Lin^-CD34^+$ HSPCs in the spleen after G4F3 treatment versus G4 ($p = 0.079$, ANOVA).

The poor efficacy of human G-CSF to mobilize human HSPCs in NSG mice in our experiments is consistent with previous publications [9,10]. Poor human HSPC mobilization in humanized NSG or NOD/SCID mice has been linked to lack of immunoglobulins that promote the activation of complement proteins C3 and C5 in response to G-CSF [10–12] and the formation of the membrane attack complex that lyses blood erythrocytes, releasing the chemoattractant shingossine-1-phosphate into the blood [13,14]. In addition, irradiation of mice before transplantation could further account for poor HSPC mobilization [4,15]. Indeed, total body irradiation and cytotoxic drugs damage the BM stroma in humans [4,16,17] and mice [18,19] in a similar manner and decrease subsequent HSPC mobilization.

Despite these limitations of humanized NSG and NOD/SCID mice [9,10] as models of HSPC mobilization, we observed significant human HSPC mobilization in response to G-CSF plus FG-4497 combination in the blood as early as day 2 of G-CSF and in the spleen at day 4 of G-CSF, whereas NSG mice did not mobilize efficiently in response to G-CSF alone. These results are consistent with the mobilization profile of mouse HSPCs in immune-competent mice, in which HSPC mobilization plateaus in the peripheral blood at day 4 of G-CSF administration and starts accumulating in the spleen from day 4 onward [20,21]. Therefore, our results suggest that additional pharmacological stabilization of FG-4497 rescues the mobilization defect in response to G-CSF in NSG mice.

We have demonstrated previously that the effect of FG-4497 on mouse HSPC mobilization in response to G-CSF is mostly HSPC autonomous, because conditional deletion of the *Hif1a* gene in mouse HSPC abolishes the effect of FG-4497 on HSPC mobilization [7]. Therefore, by inference, the enhancing effect of FG-4497 on human HSPC mobilization in response to G-CSF in humanized NSG mice may be caused by the cell-autonomous effects of HIF-1 stabilization, which can partly rescue the HSPC extrinsic mechanism defect in NSG mice.

In conclusion, we have found that the PHD inhibitor FG-4497 rescues human HSPC mobilization in G-CSF–treated NSG mice transplanted with human umbilical cord $CD34^+$ cells. The related PHD inhibitor FG-4592 is well tolerated in humans and is already in clinical trials for correcting the anemia associated with chronic kidney disease [22], opening the way for testing the use of PHD inhibitors in combination with G-CSF to enhance HSPC mobilization in humans.

Acknowledgments

This work was supported by Project Grant APP1061333 from the National Health and Medical Research Council of Australia (NHMRC), (Canberra, Australia). J-PL is supported by Research Fellowship APP1044091 from the NHMRC. MD is funded by Cure Cancer Australia (Sydney, Australia) and Cancer Council Australia (Sydney, Australia) and Inner Wheel Australia (Ballarat, Australia).

We would also like to thank the TRI-BRF and TRI Flow Facility for assistance in this project.

Conflict of interest disclosure

GW and LEF are employees of FibroGen, Inc., which owns the commercial rights of FG-4497. J-PL is an inventor of a pending U.S. patent application related to this work. The remaining authors declare no competing financial interests.

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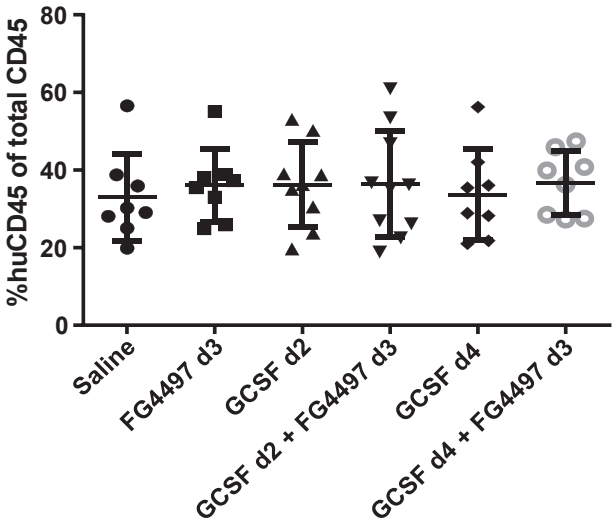
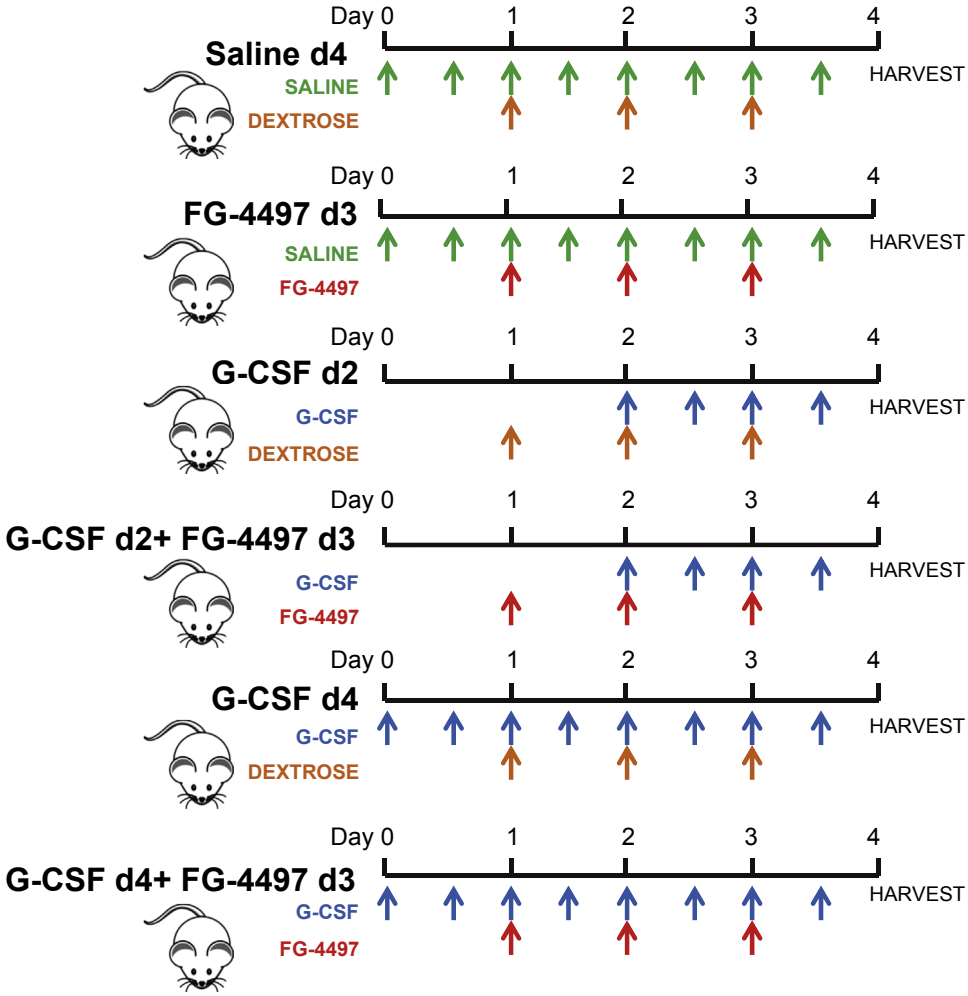
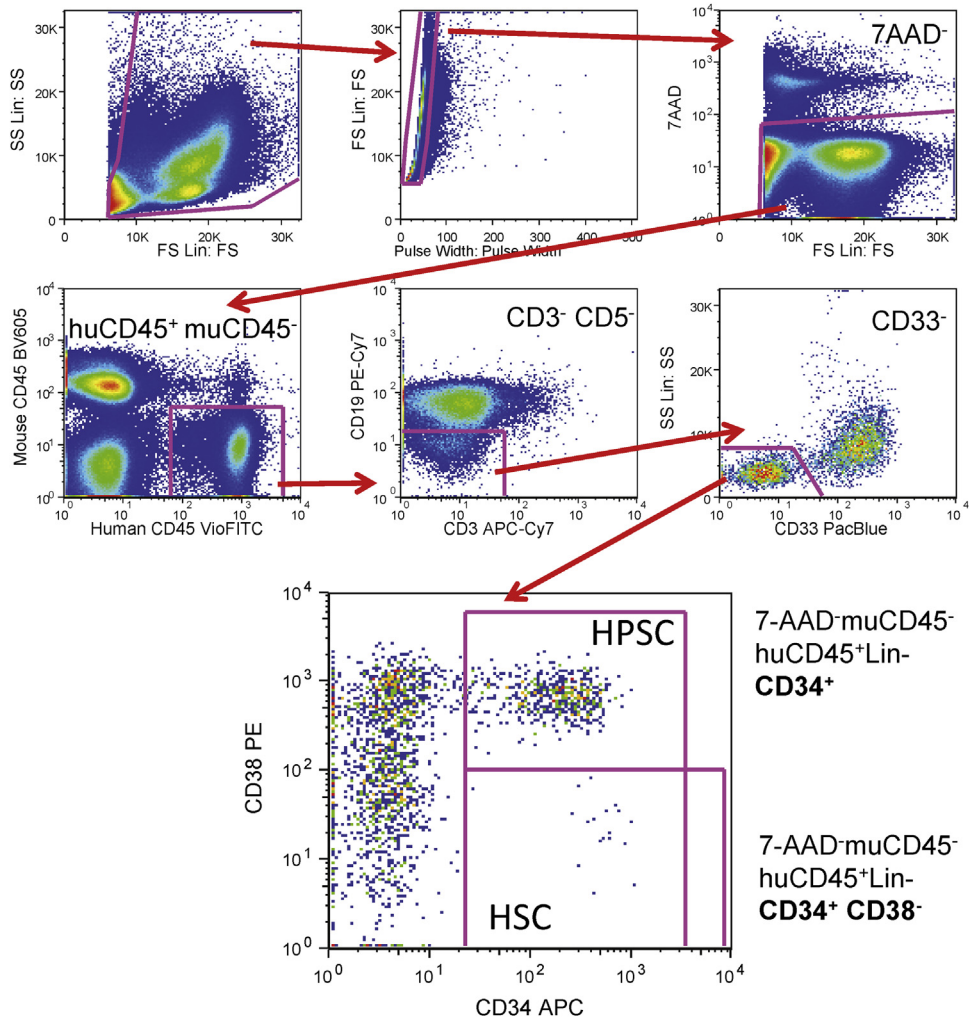


Supplementary Figure E1. Engraftment of human $CD45^+$ leukocytes in blood of NSG mice transplanted with human cord blood $CD34^+$ cells. Human $CD45^+$ leukocyte chimerism was measured by flow cytometry 8 weeks post-transplantation. Following measurement in each individual mouse, mice were allocated in the indicated experimental groups in order to have similar mean and standard deviation between all the groups. Dots represent values for each mouse. Error bars are average ± standard deviation for each allocated experimental group.



Supplementary Figure E2. Injection schedule of $CD34^{+}$ transplanted NSG mice. Recombinant human G-CSF was injected subcutaneously twice daily at 125 μg kg^{-1} for 2 to 4 days before harvest. FG-4497 was injected intraperitoneally daily at 20 mg kg^{-1} 3 days before harvest as previously described. For control groups, equivalent volumes of saline and dextrose vehicle were used in place of G-CSF and FG-4497 respectively.



Supplementary Figure E3. Gating strategy for analysis of human CD34$^+$ HSPC mobilization in NSG mice transplanted with human cord blood CD34$^+$ cells. Sample shown is blood from a FG4497+G-CSF day2 mouse treated mouse.

Supplementary Table E1. Antibodies and dilutions used

	Target	Antibody	Clone	Source	Dilution
bleed	mouse	CD16/32 (FcBlock)	2.4G2	BD Bioscience	1/200
	mouse	CD45-APC	30-F11	Biolegend	1/200
	human	CD45-VioBright FITC	5B1	Miltenyi Bioscience	1/60
	human	CD33-BV421	WM53	Biolegend	1/20
	human	CD19-PECy7	HIB19	Biolegend	1/50
	human	CD3-APCCy7	HIT3a	Biolegend	1/50
	human	CD15-PE	H198	Miltenyi Bioscience	1/50
harvest	mouse	CD16/32 (FcBlock)	2.4G2	BD Bioscience	1/200
	mouse	CD45-BV605	30-F11	Biolegend	1/200
	human	CD45-VioBright FITC	5B1	Miltenyi Bioscience	1/60
	human	CD33-BV421	WM53	Biolegend	1/20
	human	CD19-PECy7	HIB19	Biolegend	1/50
	human	CD3-APCCy7	HIT3a	Biolegend	1/50
	human	CD34-APC	AC136	Miltenyi Bioscience	1/30
	human	CD38-PE	1B6	Miltenyi Bioscience	1/40

Supplementary Table E2. Levels of Engraftment per mouse at week 8 post-transplant and human CD34^{+} cells mobilized into blood and spleen

	8wk tx	Blood			Spleen		
Groups	% huCD45+	huCD34^{+}/mL x10^{3}	Average	St Dev	huCD34^{+}/spleen x10^{6}	Average	St Dev
Saline d4	38.1%	2.127	1.524	0.443	1.580	0.756	0.388
	24.9%	0.895			0.378		
	35.4%	1.580			0.820		
	25.9%	1.131			0.455		
	33.0%	1.222			0.760		
	55.0%	2.127			0.961		
	38.9%	1.533			0.601		
	16.9%	1.580			0.492		
FG-4497 d3	40.8%	4.274	1.953	1.138	0.865	1.285	0.527
	27.5%	2.217			1.096		
	28.5%	1.542			1.651		
	39.9%	0.965			0.667		
	45.9%	1.083			1.754		
	36.2%	1.336			1.997		
	47.4%	2.903			1.589		
	27.2%	1.304			0.658		
G-CSF d2	26.2%	6.020	4.303	2.543	1.201	1.002	0.519
	46.8%	2.668			0.486		
	27.0%	4.295			0.726		
	22.6%	9.302			1.208		
	36.2%	6.578			2.062		
	35.3%	3.066			1.432		
	18.9%	1.399			0.744		
	36.7%	3.344			0.589		
	53.5%	2.056			0.574		
G-CSF d2+ FG-4497 d3	9.3%	2.935	7.313	4.651	0.342	1.369	0.565
	34.9%	6.252			0.920		
	23.7%	13.709			1.541		
	30.5%	5.967			1.035		
	38.8%	12.758			2.172		
	36.3%	3.759			1.667		
	53.0%	6.895			1.729		
	19.7%	3.350			1.075		
	50.1%	3.214			0.807		
	39.0%	5.611			1.926		
	61.0%	15.998			1.841		
G-CSF d4	29.0%	1.714	4.246	3.042	0.404	1.516	1.027
	35.9%	4.503			0.867		
	30.2%	10.540			2.804		
	25.0%	6.363			1.399		
	19.9%	4.532			1.932		
	56.6%	1.766			0.637		
	38.8%	2.581			3.173		
	28.1%	1.968			0.916		
G-CSF d4+ FG-4497 d3	21.8%	11.506	8.100	3.345	3.244	3.008	1.247
	21.0%	4.117			2.334		
	56.2%	7.859			4.775		
	35.4%	9.348			2.102		
	28.2%	13.214			5.030		
	36.0%	7.256			1.715		
	42.0%	8.132			2.499		
	28.9%	3.371			2.368		

Tissue Harvest

Mice were anesthetized with isoflurane and blood collected with heparin-EDTA by cardiac puncture, and then euthanized to collect femurs and spleens. Bone marrow (BM) was flushed from the femur using a 23 gauge needle with 1mL PBS with 2% fetal bovine serum. Spleens were disassociated into single cell suspension using the GentleMACS Dissociator (Miltenyi, Bergisch Gladbach, Germany) in a C type gentleMACS tube (Miltenyi). Red blood cells were lysed in blood samples as previously described [9]. Cell counts were completed on a Coulter AcT Diff Analyzer (Beckman Coulter), reported as cells per fraction for analysis.

Flow Cytometry

Human chimerism levels: Red Blood cells were lysed by adding 4ml of red cell lysis buffer: 150mM NH_4Cl, 10mM Tris-HCl, 1mM EDTA pH=7.4 and a 6 minute incubation at room temperature with gently rocking before centrifugation at 4*C to eliminate cell debris. Lysed blood cells were washed twice with PBS containing 2% fetal bovine serum, then incubated with purified rat anti-mouse (mu) CD16/CD32 antibody (FcBlock) (BD Bioscience) and stained with fluorescence-conjugated antibodies as outlined in supplementary table 1. 7-amino-actinomycin D (7-AAD) (Invitrogen) was used for live dead discrimination. Human leukocytes were numerated as $muCD45^-huCD45^+$ cells and mouse leukocytes as $muCD45^+huCD45^-$ cells. For each individual mouse, human $CD45^+$ chimerism percentage was calculated as the ratio of $huCD45^+$ leukocytes over total number of human and mouse $CD45^+$ leukocytes in the blood at 8 weeks post-transplantation.

For measurements of human cell mobilization, $5x10^6$ cells from blood, BM and spleen from each mouse were blocked with muFcBlock and then stained with fluorescence-conjugated antibody cocktail antibodies as outlined in supplementary table 1. Cells were analyzed on a CyAn flow cytometer (Beckman Coulter) and post-acquisition compensated and analyzed with FlowJo software (Ashland, OR). Human HPSC were defined as $muCD45^-$, $huCD45^+$, lineage (CD33, CD19 and CD3) negative (Lin^-), and $huCD34^+$. Primitive HSPC were defined as $muCD45^-huCD45^+Lin^-CD34^+CD38^-$ (Supplementary figure 3).