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Brief Report: A Differential Transcriptomic Profile of Ex Vivo Expanded Adult Human Hematopoietic Stem Cells Empowers Them for Engraftment Better than Their Surface Phenotype

NIKOLETTA PSATHA [a], GRIGORIOS GEORGOLOPOULOS,[b] SUSAN PHELPS,[c] THALIA PAPAYANNOPOULOU[c]

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[a]Division of Medical Genetics, University of Washington, Seattle, Washington, USA, [b]Altius Institute for Biomedical Sciences, Seattle, Washington, USA; [c]Division of Hematology, University of Washington, Seattle, Washington, USA

Correspondence: Thalia Papayannopoulou, M.D., Department of Medicine/Hematology, University of Washington, 1705 NE Pacific, Box 357710, Seattle, Washington 98195-7710, USA. Telephone: 206-543-5756; Fax: 206-543-3050; e-mail: thalp@u.washington.edu

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ABSTRACT

Transplantation of small cord blood (CB) units, or of autologous ex vivo-genetically modified adult hematopoietic stem cells (HSC), face the common challenge of suboptimal HSC doses for infusion and impaired engraftment of the transplanted cells. Ex vivo expansion of HSCs, using either cell-based coculture approaches or especially small molecules have been successfully tested mainly in CB and in prolonged cultures. Here, we explored whether innovative combinations of small molecules can sufficiently, after short culture, expand adult HSCs while retaining their functionality in vivo. We found that 5-day cultured cells, in the presence of the small molecule combinations tested, achieved higher engraftment levels in NSG mice than both their uncultured and their cytokine only-cultured counterparts. Surprisingly, the engraftment levels were neither concordant to the numbers of phenotypically similar HSCs expanded under different small molecule combinations, nor explained by their distinct companion cells present. Transcriptomic comparative analysis of sorted, phenotypically similar, ex vivo generated HSCs transplanted in equal numbers, suggested that HSCs generated under expansion conditions that maintain low expression of the Rap1/Ras/PI3K-AKT pathway exhibit a superior functional profile in vivo. STEM CELLS TRANSLATIONAL MEDICINE *2017;6:1852–1858*

SIGNIFICANCE STATEMENT

Here, we document that the engraftment potential of ex vivo expanded phenotypically similar hematopoietic stem cells (HSCs) is dictated by the specific expansion conditions rather than their surface phenotype. The results of our study have important implications for all investigators studying the ex vivo expansion of adult HSCs.

INTRODUCTION

Successful hematopoietic stem cells (HSC) transplantation protocols have been associated with high numbers of donor cells. The need for large numbers of engraftable HSCs becomes particularly challenging in case of cord blood (CB)-transplantation because of the size of suitable grafts, and in adult HSC-gene therapy protocols, where the culture conditions may lead to impaired engraftment potential [1]. Even though long-term HSC cultures in cytokine supplemented medium can dramatically increase the numbers of immunophenotypically identified HSCs [2, 3], the numbers of engraftable HSCs under these conditions can at best be maintained, and their functionality is largely compromised [2]. Methods to improve maintenance as well as expansion of the genetically modified cells, would enhance hematologic recovery in myeloablated patients and increase long-term in vivo engraftment of the modified graft, greatly improving the short-term safety and long-term efficacy of such protocols [4].

Recently, high throughput screening of large chemical libraries identified a number of small molecules as potential new tools for HSC expansion. Some of the most promising molecules described, include the aryl hydrocarbon receptor (AHR) antagonist StemRegenin1 (SR1) [5], the p38-MAPK14 inhibitor LY2228820 (Ly) [6], and the pyrimidoindole derivative UM171 [7]. Expansion efforts using these molecules, however, have been focused on CB-HSCs, while their effects on adult HSCs have been less explored. In the present study, we compared these promising conditions, alone and in combinations, for expanding ex vivo, engraftable adult HSCs. To avoid loss of stemness of the expanded cells with prolonged cultures, we

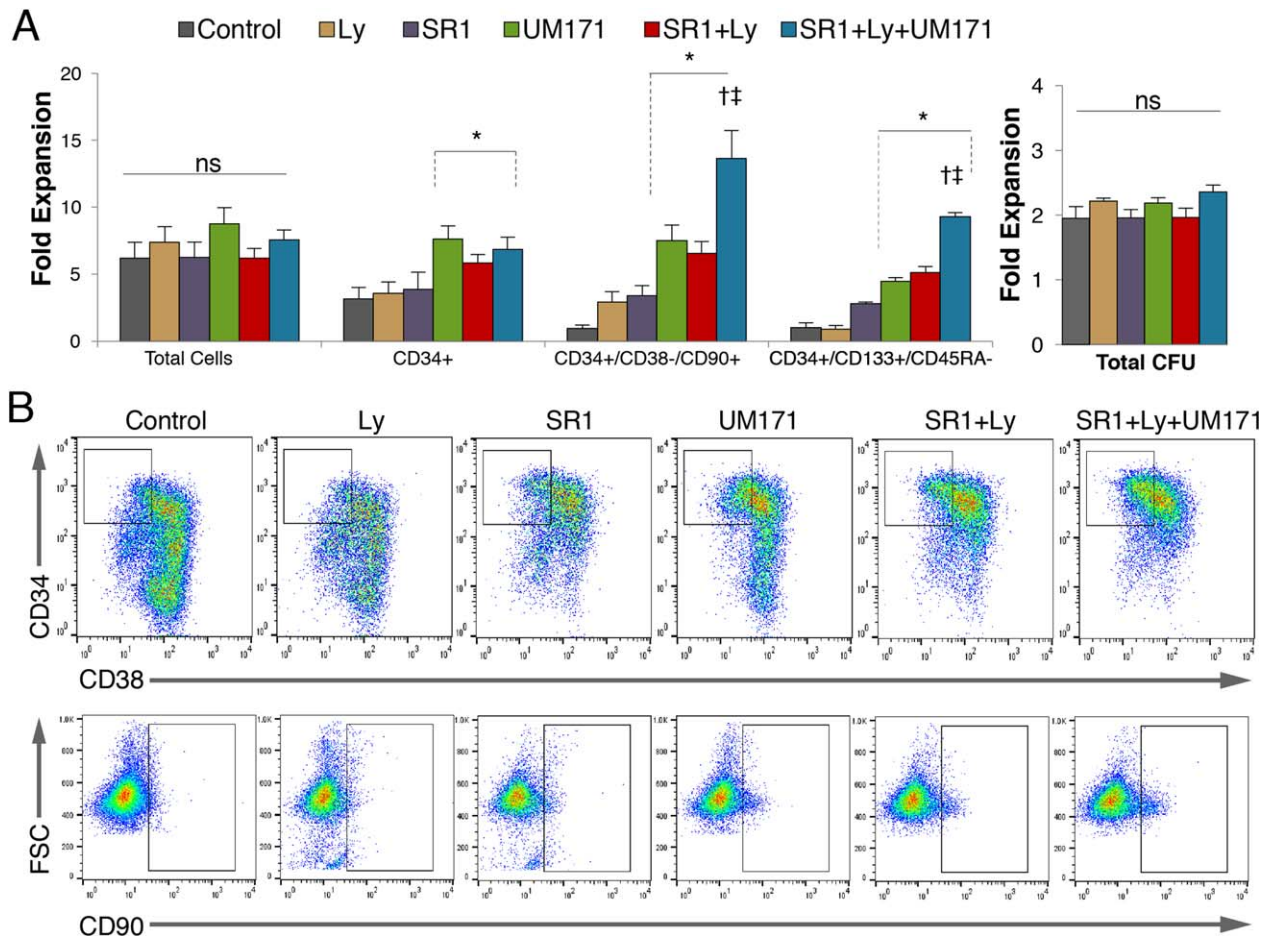

Figure 1. Hematopoietic stem and progenitor cell expansion after 5 days of culture. **(A):** Fold expansion of total nucleated cells and progenitor (CD34+, CFCs) and stem cell (CD34+/38–/90+, CD34+/133+/45RA−) subpopulations on day 5 compared to the input on day 0. Control: cells cultured in the presence of cytokines and vehicle only. **(B):** Representative flow cytomery plots of each condition, for CD34, CD38, and CD90 expression on day 5 of the culture. CD90+ cells are gated in within the CD34+/CD38− subpopulation. *, $p < .05$ versus control, †, $p < .05$ versus UM171 ‡, $p < .05$ versus SR1+Ly, ns: not significant. Abbreviations: CFU, colony forming units; FSC, forward scatter.

tested the different protocols in a short 5-day culture, which would likely be beneficial in a gene therapy setting.

Materials and Methods

Mobilized peripheral blood CD34+ cells from healthy donors were cultured for 5 days in serum free medium, supplemented with Fms related tyrosine kinase 3 (Flt-3), stem cell factor (SCF), and thrombopoietin (TPO) and in the presence of the small molecules SR1, Ly, and UM171, alone or in combinations; SR1+Ly, SR1+Ly+UM171. Hematopoietic stem and progenitor cell (HSPC) expansion, evaluated by flow cytometry and clonogenic assays was compared to either the input HSPCs (Day 0) or the expansion culture without small molecules. For the in vivo experiments, NSG/IL2γnull mice were transplanted with the uncultured inoculum or the total cell output after culture, unless otherwise stated. For more details, see supplemental Methods.

Results and Discussion

To optimize ex vivo expansion of adult HSCs we tested three modalities (SR1, Ly, and UM171) alone or in certain combinations, but only for a short time in culture to preserve stemness. UM171 alone yielded more phenotypically characterized HSCs compared to the other two small molecules tested alone (Fig. 1A, 1B). Then, we subsequently combined SR1 and Ly hoping to uncover synergistic and/or additive effects, as inhibition of p38/MAPK is also AHR dependent [8]. This combination achieved similar expansion of HSCs to that of UM171 alone and combination of all three molecules appeared to have an additive effect.

Of interest, under the different protocols apart from the effects on HSC numbers, distinct downstream lineages were enhanced. In UM171 cultures, mainly progenitors with a myeloid bias were enhanced (Fig. 2A). By contrast, a high number of megakaryocytic progenitors (Fig. 2B, 2C), was present in all SR1-containing cultures along with more mature megakaryocytes, even in the absence of TPO and these were completely absent from the UM171 cultures (Fig. 2D–2F). Although aryl hydrocarbon pathway repression has been previously shown to promote megakaryocytic specification [9, 10], the mechanism through which both the megakaryocytic and erythroid differentiation is suppressed by UM171, requires further studies.

Consistent with the phenotypic and CFU culture data (Fig. 2A–2F), transcriptomic analysis of cultured cells also uncovered high expression of granulocytic/monocytic genes, and suppression

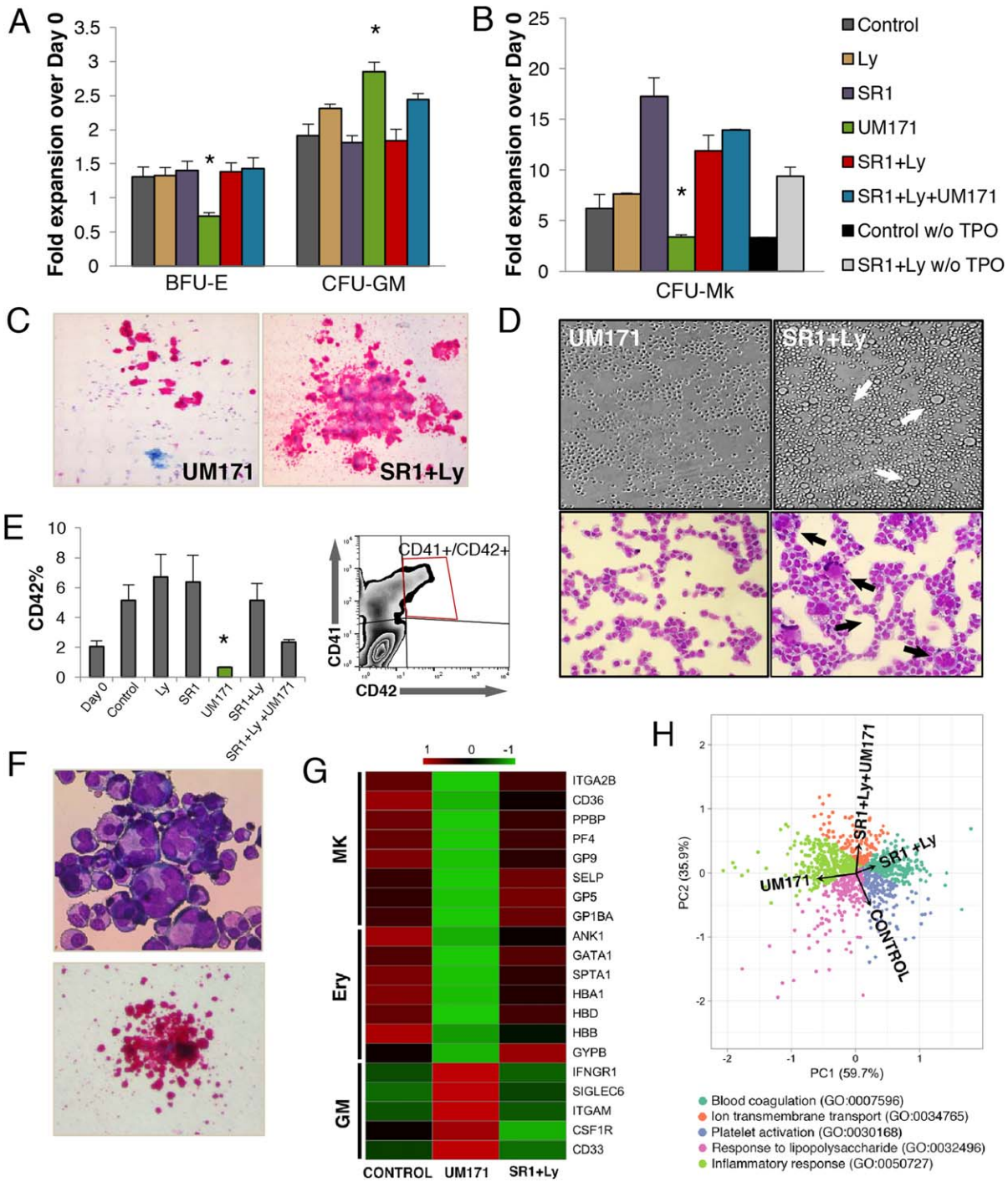

Figure 2. Distinct lineage enhancement within the SR1+Ly and UM171 cultures. Fold expansion of total numbers of BFU-E and CFU-GM **(A)** and CFU-Mk **(B)**, over the day 0-uncultured cell inoculum. **(C):** Representative morphology of CFU-Mk, derived from UM171 (left) and SR1+Ly (right) cultured cells. Colonies from UM171 cultures were smaller in size and consisted of a few scattered cells. (×40 objective) **(D):** Morphologically recognizable mature megakaryocytes present in SR1-containing suspension cultures, and absent from the UM171-cultures. Preparations were stained with Giemsa and pictures were taken with a ×40 objective. **(E):** Frequency of CD41+/CD42+ cells among the different conditions, and contour plot for CD41+/CD42+ cells in SR1+Ly cultures. **(F):** Sorted CD41+/CD42+ cells were cytospun and stained with Giemsa or plated in Megacult medium to test production of proplatelets. (×80 magnification). **(G):** Z-scored expression of megakaryocytic, erythroid, and granulocytic marker genes from bulk cultures. Control: 5-day cultured cells w/o small molecules **(H)** Biplot of the 1,000 top variable genes. Cells cultured under UM171 have a diametrically opposite profile to the cells cultured with SR1+Ly. Cells cultured under the SR1+Ly+UM171 combination exhibited a comparable transcriptomic profile to that of the SR1+Ly-cells, implying that the SR1+Ly combination is dominant over UM171. Genes are represented as dots and treatments as the loadings vectors. Colors represent K-means clusters (K = 5) and the most significant ($p < .05$) gene-ontology term of the corresponding cluster is annotated. Error bars represent SEM from three independent experiments. *, $p < .05$ versus all other conditions. Abbreviations: BFU-E, burst forming units-erythroid; CFU-GM, colony forming units-granulocytic/monocytic; CFU-Mk, colony forming units-megakaryocytic.

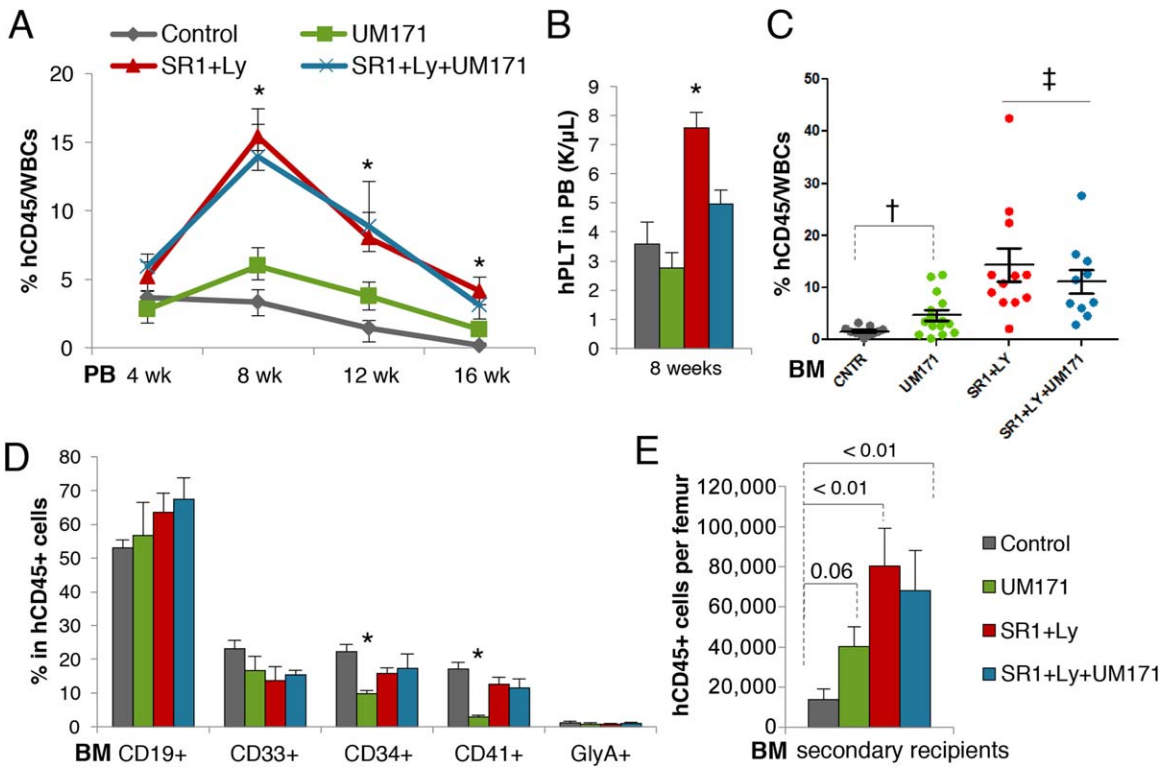

Figure 3. Functional differences of ex vivo expanded cells. **(A):** Human cell chimerism in PB of NSG xenotransplanted mice. 4×10^5 cells were either transplanted uncultured (CNTR) in sublethally irradiated NSG mice, or were cultured for 5 days under different conditions. On day 5 the entire culture output was transplanted in NSG mice in the same manner. **(B):** Total number of hPLT in peripheral blood, 8 weeks post transplantation. **(C):** Human cell frequency in BM 16 weeks post transplantation. **(D):** Multilineage reconstitution based on the frequency of the annotated subpopulations within the human CD45 cell population. **(E):** Total human cells per femur in secondary recipients, 16 weeks post transplantation. For these experiments, 16 weeks after the primary transplantations, the human CD45+ cells engrafted in the primary mice were enriched, and the cell number was normalized so that each secondary mouse receive the same number of human cells. *, $p < .05$ versus all other conditions; †, $p < .05$ versus control; ‡, $p < .05$ versus control and UM171, ns: not significant. Abbreviations: BM, bone marrow; CNTR transplanted uncultured; hPLT, human platelets; PB, peripheral blood.

of megakaryocytic/erythroid marker genes in the UM171 cultures; in contrast, cells cultured in the presence of SR1+Ly displayed enhanced erythroid/megakaryocytic but suppressed granulocytic/monocytic gene signature (Fig. 2G). Overall, populations expanded under UM171 or SR1+Ly seem to be distinguished by diametrically opposed expression profiles (Fig. 2H).

The in vivo functional potential of HSCs expanded under different small molecule combinations (UM171, SR1+Ly, and SR1+Ly+UM171), was tested after transplantation in NSG mice. These mice were transplanted with the entire cultured output generated from the same starting inoculum of CD34+ cells. Higher levels of human chimerism were observed in the peripheral blood of mice receiving small molecule-expanded cells compared to the uncultured cells (Fig. 3A), 4–16 weeks post transplantation, suggesting that a brief 5-day culture period in the presence of small molecules is beneficial. Cells cultured for the same time period only with cytokines, achieved lower engraftment compared to the uncultured cells (Supporting Information Fig. S1), as previously reported [11]. However, cells cultured in the presence of SR1+Ly exhibited superior engraftment compared to similar total cell numbers of UM171-cells transplanted (Figs. 1A, 3A). SR1+Ly-cells also yielded higher platelet numbers in peripheral blood than all other conditions, 8-weeks post-transplantation, possibly due to the higher numbers of mature megakaryocytes in the inoculum (Fig. 3B), a feature that could potentially be associated with faster platelet recovery in a transplantation setting. The SR1+Ly+UM171 combination, did not further improve the engraftment of the SR1+Ly graft (Fig. 3A), even though contained the highest number of HSCs (Fig. 1A). Bone marrow studies at week 16, confirmed the robust engraftment of human cells in the SR1+Ly (±UM171)-recipients (Fig. 3C). Multilineage engraftment was detected in every condition. Of note, a significantly lower percentage of CD34+ and CD41+ cells was detected in the UM171-recipients (Fig. 3D). Furthermore, the engraftment difference between SR1+Ly and UM171 was maintained in secondary recipients (Fig. 3E).

Our in vivo data so far show that in contrast to expectations the number of ex vivo expanded phenotypic-HSCs under different expansion conditions is not predictive of the engraftment outcome. To explain the different engraftment outcomes, we hypothesized that there either are intrinsic differences among the expanded HSCs, or that the non-HSC, companion cells are responsible. For example, megakaryocytes, that are abound in the SR1+Ly cultures, have been implicated in affecting HSC engraftment potential as well as

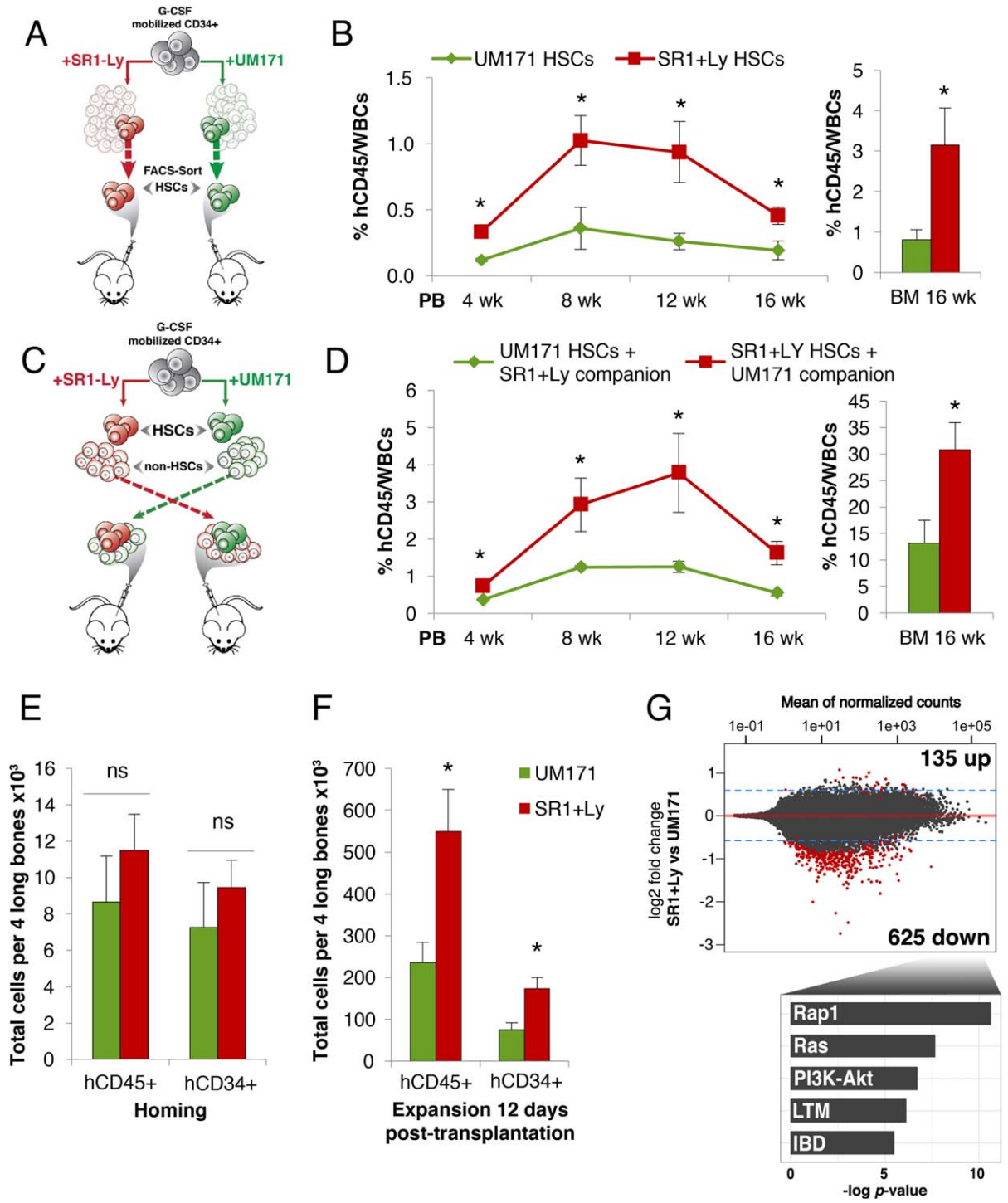

Figure 4. Functional differences and transcriptomic profile of ex vivo expanded adult HSCs. **(A):** Equal numbers of FACS sorted CD34+/CD38–/CD90+ cells, from SR1+Ly or UM171 cultures at day 5, were transplanted in NSG mice. **(B):** Equal numbers of FACS sorted CD34+/CD38–/CD90+ cells from SR1+Ly or UM171 cultures, were transplanted in NSG mice, along with the same number of their alternate non-HSC companion cells. **(C):** Engraftment (human CD45+ cells) was assessed in PB and BM 4–16 weeks post transplantation. **(D):** Human cell engraftment in PB and BM 4–16 weeks post transplantation. The companion cells enhanced the overall engraftment of both HSC populations, however without changing the outcome. **(E):** For the in vivo homing experiments, 5×10^5 sorted CD34+/CD38–/CD90+ cells from SR1+Ly or UM171 cultures, were transplanted in NSG recipients. The mice were sacrificed 48 hours post transplantation. **(F):** Total human and CD34+ cells in vivo expansion, 12 days post transplantation. 5×10^5 sorted CD34+/CD38–/CD90+ cells from SR1+Ly or UM171 cultures, were transplanted. **(G):** MA plot of the shrunken values of the differentially expressed genes (1.5-fold change, $p = .05$) in SR1+Ly versus UM171 treated CD34+/CD38–/CD90+ sorted cells and gene set enrichment analysis within the SR1+Ly downregulated genes showing the top five ranking KEGG pathway terms. Error bars represent SEM from three independent experiments, with 4 mice per group in each experiment. *, $p < .05$ versus all other groups, ns: not significant. Abbreviations: BM, bone marrow; IBD, inflammatory bowel disease; FACS, fluorescence-activated cell sorting; HSC, hematopoietic stem cells; LTM, leukocyte transendothelial migration; PB, peripheral blood.

cell cycle, proliferation and differentiation post ablation and transplantation [12–14].

To explore the two aforementioned possibilities, we designed a new set of in vivo experiments. We transplanted the same number of sorted CD34+/CD38–/CD90+ cells, expanded under SR1+Ly or UM171, either alone (Fig. 4A) or in combination with their switched-over non-HSC companion cells (Fig. 4B). Once again, the SR1+Ly-isolated HSCs achieved significantly higher engraftment levels than their numerically and phenotypically similar counterparts under UM171 (Fig. 4C). Upon addition of alternate companion cells, engraftment differences were maintained suggesting HSC-intrinsic rather than extrinsic effects (Fig. 4D), although, the theoretical possibility of an indirect effect exerted by the companion cells, shaping intrinsic properties of the HSCs during the 5-day culture, cannot be excluded.

To account for engraftment differences of sorted CD34+/CD38–/CD90+ expanded cells, we explored the homing potential of these donor populations. Surface molecule expression revealed no significant differences in any of the adhesion molecules tested (Supporting Information Fig. S2). Furthermore, in vivo homing experiments could not uncover a superior homing potential of the SR1+Ly HSCs (Fig. 4E). Instead, a faster and superior in vivo bone marrow expansion potential was documented 12 days post transplantation (Fig. 4F).

To further explore whether the in vivo functional advantage of SR+Ly versus UM171 expanded HSCs could be associated with their intrinsic transcriptional profile, we performed RNA sequencing of sorted populations (CD34+/CD38–/CD90+) expanded under the two different protocols. Surveying the differentially expressed genes, we found that genes involved in the Rap1, Ras, and PI3K-Akt pathways, were significantly downregulated in SR1+Ly over UM171 HSCs (Fig. 4F; Supporting Information Figs. S3–S5). These three closely regulated pathways play central role in regulating proliferation, apoptosis, differentiation, cell adhesion and metabolism [15–18] with both complementary and antagonistic cell-context dependent effects in hematopoiesis [19]. Highly active Rap1 and Ras pathways trigger PI3K and activated PI3K-Akt signaling has been associated with HSC exhaustion and depletion [20–22] as well as thrombocytopenia [20], whereas silencing enhances cell engraftment [23]. This latter outcome is consistent with our results with SR1+Ly versus UM171 reflecting the enhanced engraftment, of the former.

Conclusion

In summary, our data show that adult HSPCs cultured for 5 days in the presence of SR1+Ly, achieve post transplantation significantly higher and long-term sustained in vivo engraftment levels compared to cells cultured in the presence of UM171-alone. The transcriptomic profile of ex vivo expanded phenotypically similar HSCs, under different small molecule combinations, uncovers specific functional differences that likely impact their engraftment potential.

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Author Contributions

N.P. collection and assembly of data, data analysis and interpretation, manuscript writing; G.G. collection and assembly of data, data analysis and interpretation; S.P. experiment conduction; T.P. conception and design, data interpretation, manuscript writing and final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

References

1 Psatha N, Karponi G, Yannaki E. Optimizing autologous cell grafts to improve stem cell gene therapy. Exp Hematol 2016;44:528–539.

2 Magnusson M, Sierra MI, Sasidharan R et al. Expansion on stromal cells preserves the undifferentiated state of human hematopoietic stem cells despite compromised reconstitution ability. PLoS One 2013;8: e53912.

3 Nikiforow S, Ritz J. Dramatic expansion of HSCs: New possibilities for hsc transplants? Cell Stem Cell 2016;18:10–12.

4 Naldini L. Gene therapy returns to centre stage. Nature 2015;526:351–360.

5 Boitano AE, Wang J, Romeo R et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 2010;329:1345–1348.

6 Baudet AA, Karlsson C, Talkhoncheh MS et al. RNAi screen identifies MAPK14 as a druggable suppressor of human hematopoietic stem cell expansion. Blood 2012;119: 6255–6258.

7 Fares I, Chagraoui J, Gareau Y et al. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. Science 2014;345:1509.

8 Pombo M, Lamé MW, Walker NJ et al. TCDD and omeprazole prime platelets through the aryl hydrocarbon receptor (AhR) nongenomic pathway. Toxicol Lett 2015;235:28–36.

9 Smith BW, Rozelle SS, Leung A et al. The aryl hydrocarbon receptor directs hematopoietic progenitor cell expansion and differentiation. Blood 2013;122:376–385.

10 Strassel C, Brouard N, Mallo L et al. Aryl hydrocarbon receptor–dependent enrichment of a megakaryocytic precursor with a high potential to produce proplatelets. Blood 2016; 127:2231–2240.

11 Danet GH, Lee HW, Luongo JL et al. Dissociation between stem cell phenotype and NOD/SCID repopulating activity in human peripheral blood CD34+ cells after ex vivo expansion. Exp Hematol 2001;29:1465–1473.

12 Zhao M, Perry JM, Marshall H et al. Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat Med 2014;20:1321–1326.

13 Olson TS, Caselli A, Otsuru S et al. Megakaryocytes promote murine osteoblastic HSC niche expansion and stem cell engraftment after radioablative conditioning. Blood 2013; 121:5238–5249.

14 Heazlewood SY, Neaves RJ, Williams B et al. Megakaryocytes co-localise with hemopoietic stem cells and release cytokines that up-regulate stem cell proliferation. Stem Cell Res 2013;11:782–792.

15 Aksamitiene E, Kiyatkin A, Kholodenko BN. Cross-talk between mitogenic Ras/MAPK and survival PI3K/Akt pathways: A fine balance. Biochem Soc Trans 2012;40.

16 Steelman LS, Pohnert SC, Shelton JG et al. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia 2004;18:189–218.

17 Bos JL, de Rooij J, Reedquist KA. Rap1 signalling: Adhering to new models. Nat Rev Mol Cell Biol 2001;2:369–377.

18 Knapp DJHF, Hammond CA, Aghaeepour N et al. Distinct signaling programs control human hematopoietic stem cell survival and proliferation. Blood 2017; 129:307–318.

19 Stork PJS, Dillon TJ. Multiple roles of Rap1 in hematopoietic cells: Complementary versus antagonistic functions. Blood 2005; 106:2952–2961.

20 Kharas MG, Okabe R, Ganis JJ et al. Constitutively active AKT depletes hematopoietic stem cells and induces leukemia in mice. Blood 2010;115:1406–1415.

21 Zhang J, Grindley JC, Yin T et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. Nature 2006;441:518–522.

22 Yilmaz ÖH, Valdez R, Theisen BK et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 2006;441:475–482.

23 Chen S, Gao R, Kobayashi M et al. Pharmacological inhibition of AKT activity in human CD34+ cells enhances their ability to engraft immunodeficient mice. Exp Hematol 2017;45:74–84.

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