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mRNA-mediated glycoengineering ameliorates deficient homing of human stem cell-derived hematopoietic progenitors

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Introduction
Hematopoietic stem and progenitor cell (HSPC) transplantation is the paradigmatic stem cell therapy, with ~50,000 transplants performed worldwide per year to treat a variety of blood disorders (1). Despite its curative potential, difficulties in obtaining sufficient numbers of HLA-matched HSPCs contribute to poor transplantation outcomes and limit broader applicability. Derivation of large quantities of HSPCs from human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and/or induced pluripotent stem cells (iPSCs), holds great promise to mitigate many HSPC transplantation-related limitations. However, despite much progress, generation of fully functional and engraftment-competent HSPCs from human pluripotent stem cells ex vivo has remained challenging (2).

“Bone marrow homing” refers to the process by which HSPCs transit from the bloodstream to the bone marrow (BM). This process, which is a prerequisite for functional hematopoiesis, involves two components: (1) trafficking of circulating HSPCs to specialized BM endothelial beds and (2) extravasation of HSPCs at those beds. Homing involves a multistep cascade that begins with the tethering and rolling of transplanted cells on discrete BM sinusoidal vessels that is mediated by interactions between E-selectin on endothelial cells and its ligands on HSPCs. Once cells have migrated to relevant sinusoids, extravasation ensues as integrins (principally VLA-4) become activated via chemokines by binding to their receptors (e.g., SDF-1 [also known as CXCL12] binding to CXCR4) to induce firm adherence of HSPCs to the endothelial wall. Finally, cells undergo transendothelial migration and parenchymal lodgment, processes modulated by chemokine gradients within the BM (3). Although BM homing is a critical aspect of HSPC biology, studies assessing the homing properties of HSPCs from human pluripotent stem cells are lacking (2).

In this study, we examined the expression and function of molecules that mediate HSPC homing to BM and identified a marked deficiency of E-selectin ligands on the surface of PSC-derived HSPCs. We also demonstrate a simple and potent strategy to create functional E-selectin ligands on the surface of iPSC-derived HSPCs using modified mRNA encoding the glycosyltransferase fucosyltransferase 6 (FUT6). The glycoengineered human iPSC-derived HSPCs exhibited markedly enhanced tethering and rolling inter-

Generation of functional hematopoietic stem and progenitor cells (HSPCs) from human pluripotent stem cells (PSCs) has been a long-sought-after goal for use in hematopoietic cell production, disease modeling, and eventually transplantation medicine. Homing of HSPCs from bloodstream to bone marrow (BM) is an important aspect of HSPC biology that has remained unaddressed in efforts to derive functional HSPCs from human PSCs. We have therefore examined the BM homing properties of human induced pluripotent stem cell–derived HSPCs (hiPSC–HSPCs). We found that they express molecular effectors of BM extravasation, such as the chemokine receptor CXCR4 and the integrin dimer VLA-4, but lack expression of E-selectin ligands that program HSPC trafficking to BM. To overcome this deficiency, we expressed human fucosyltransferase 6 using modified mRNA. Expression of fucosyltransferase 6 resulted in marked increases in levels of cell surface E-selectin ligands. The glycoengineered cells exhibited enhanced tethering and rolling interactions on E-selectin–bearing endothelium under flow conditions in vitro as well as increased BM trafficking and extravasation when transplanted into mice. However, glycoengineered hiPSC-HSPCs did not engraft long-term, indicating that additional functional deficiencies exist in these cells. Our results suggest that strategies toward increasing E-selectin ligand expression could be applicable as part of a multifaceted approach to optimize the production of HSPCs from human PSCs.

Authorship note: J. Lee and B. Dykstra contributed equally to this work.

Conflict of Interest: D.J. Rossi is a founder of Moderna Therapeutics, a Cambridge, Massachusetts, company that is developing modified mRNA therapeutics, and is also a co-founder of Magenta Therapeutics, which is focused on transplantation medicine.

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actions with endothelial cells under shear stress conditions in vitro and displayed increased homing and extravasation into the calvarial BM of immunocompromised mice in vivo.

Results and Discussion

Building upon previously reported protocols (4–6), we developed a serum-free, stromal cell–free differentiation protocol capable of generating high percentages of hematopoietic cells from a human iPSC line derived using modified mRNA (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI92030DS1) (7). By day 10 of differentiation, round, refractile, non-adherent hematopoietic cells were observed above the adherent cell layer (Supplemental Figure 1B) and corresponded with the expression of hematopoietic progenitor markers (Supplemental Figure 1, C and D). Hematopoietic differentiation was similarly highly efficient across multiple human PSC lines, including two ESC lines and iPSCs derived from a patient with Pearson’s syndrome (Supplemental Figure 1E). Consistent with their acquisition of primitive hematopoietic markers, the human iPSC-derived hematopoietic cells possessed robust progenitor activity (Supplemental Figure 1, F and G), and we termed these cells human iPSC-derived HSPCs (hiPS-HSPCs).

We then proceeded to examine on hiPS-HSPCs the molecules known to mediate BM extravasation, and marrow lodgment, of human HSPCs. First, we assessed expression of the SDF-1 receptor CXCR4 and the hyaluronan receptor CD44 (8) and observed that hiPS-HSPCs expressed moderate to high levels of these molecules (Figure 1A). Function of CXCR4 was confirmed by transwell migration assays, where hiPS-HSPCs demonstrated significantly increased transmigration activity toward an SDF-1 gradient, an activity that was completely blocked by the CXCR4 antagonist AMD3100 (Figure 1B). We next assessed expression of the integrin subunits that constitute VLA-4 and VLA-5, as VLA-4 is critical for HSPC extravasation, and both VLA-4 and VLA-5 mediate binding to fibronectin, a key mediator of HSPC lodgment. hiPS-HSPCs expressed robust levels of the VLA-4 integrin subunits α4 and β1, and moderate expression of α5 (Figure 1C), which together with β1, constitutes VLA-5 (9). Integrin dimers are known to exist in three distinct conformations: bent-closed (inactive), extended-closed (primed), and extended-open (active) (10). Activation of integrins, canonically by CXCR4 engagement (SDF-1–induced signaling), is critical to enabling their function as mediators of homing and marrow lodgment (11). To assess the integrin activation status of hiPS-HSPCs, we used activation-specific antibodies to the integrin β1 subunit, which is common to both VLA-4 and VLA-5 (Figure 1D). This analysis revealed that hiPS-HSPCs natively display β1 integrins in an extended-closed (primed) conformation. Furthermore, the β1 integrins were converted to the extended-open (active) conformation via SDF-1–induced signaling, to levels comparable to that of cells exposed to manganese, a strong signaling-independent integrin activator (Figure 1D). This analysis revealed that hiPS-HSPCs naturally display β1 integrins in an extended-closed (primed) conformation. Furthermore, the β1 integrins were converted to the extended-open (active) conformation via SDF-1–induced signaling, to levels comparable to that of cells exposed to manganese, a strong signaling-independent integrin activator (Figure 1D). Collectively, these data indicate that hiPS-HSPCs express appreciable levels of CD44, CXCR4, VLA-4, and VLA-5, and that these mediators of extravasation and lodgment function normally in these cells.

Next, we analyzed E-selectin ligand expression using HECA452, an antibody that detects a tetrasaccharide glycan known as sialyl Lewis X (sLeX), the canonical E-selectin ligand binding determinant (12). Strikingly, the hiPS-HSPCs had very low sLeX expression compared with control peripheral blood...
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Since sLeX-bearing glycoproteins are specifically known to play a critical role in cellular trafficking, FUT6 modified mRNA was utilized for all further experiments. FUT6 modified mRNA transfection consistently and robustly increased sLex expression of hiPS-HSPCs in multiple independent experiments (Supplemental Figure 2E). Time course analysis of FUT6 modified mRNA–transfected hiPS-HSPCs showed that sLex expression peaked between 24 and 72 hours after transfection and decreased thereafter (Supplemental Figure 2F). No detrimental effects on hematopoietic differentiation or colony-forming activity were observed (Supplemental Figure 2, G–I). Collectively, these data indicate that FUT6 modified mRNA transfection is an effective strategy for generating E-selectin ligands on hiPS-HSPCs.

To determine whether the increased sLeX and E-selectin–Ig reactivity corresponds to functional E-selectin binding activity, we tested the ability of FUT6 modified mRNA–transfected hiPS-HSPCs to tether and roll under fluid shear conditions on TNF-α–activated HUVECs (Figure 2D and Supplemental Video 1). Furthermore, rolling velocities were significantly lower in FUT6 modified mRNA–transfected hiPS-HSPCs, indicative of increased E-selectin ligand binding (Figure 2E). Blocking E-selectin or

Figure 2. FUT6 modified mRNA–mediated glycoengineering enhances tethering and rolling of hiPS-HSPCs on endothelial cells under shear conditions. (A) Representative histograms showing expression of sLeX on hiPS-HSPCs and PBMC control cells measured by HECA452 antibody. (B) Representative histograms showing expression of sLeX on hiPS-HSPCs 24 hours after transfection with modified mRNAs encoding FUT3, FUT6, and FUT7. (C) Western blot with an E-selectin–Ig chimera or β-actin (loading control) on lysates of hiPS-HSPCs cells mock transfected or transfected with the indicated modified mRNAs 2 days earlier. (D) Quantitation of E-selectin–mediated rolling of control or FUT6 modified mRNA–transfected hiPS-HSPCs on TNF-α–activated HUVECs under increasing shear stress. E-selectin blocking antibody reduces rolling to baseline, while no interaction was observed on HUVECs not activated with TNF-α. n = 3. (E) FUT6 modified mRNA–transfected hiPS-HSPCs exhibit reduced rolling velocities on TNF-α–activated HUVECs. n = 3. Error bars indicate SEM. *P < 0.05, **P < 0.01 by Student’s t test.
selecting 24 hours after transplant (Figure 3D). These studies revealed that FUT6 modified mRNA-transfected hiPS-HSPCs had significantly increased extravasation frequency compared with co-transplanted control-treated cells (Figure 3E). To establish the absolute homing efficiency of FUT6 modified mRNA-transfected hiPS-HSPCs, we performed short-term BM homing experiments, comparing FUT6 modified mRNA-transfected hiPS-HSPCs with CD34+ cells from mobilized peripheral blood (CD34+ mPB cells) (Supplemental Figure 4, A and B). Despite the improved homing and transmigration engendered by the creation of functional E-selectin ligands, FUT6 modified mRNA-transfected hiPS-HSPCs still showed 5-fold lower BM homing efficiency compared with CD34+ mPB cells (Supplemental Figure 4, A and B). To determine whether the increased BM homing of the modified cells was sufficient to enable hematopoietic engraftment, we injected control or FUT6 modified mRNA-transfected hiPS-HSPCs into NSG mice. Mice transplanted with control-transfected hiPS-HSPCs did not show detectable human engraftment (Figure 3, F and G). In contrast, a subset of mice receiving transplant of FUT6 modified mRNA-transfected hiPS-HSPCs exhibited low human chimerism in the peripheral blood at 2 weeks after transplant (Figure 3, F and G).
G). However, human chimerism decreased over time and was no longer detectable 8 weeks after transplant (Figure 3, F and G).

In the present study, we focused on BM homing, a previously unaddressed aspect for deriving engraftment-competent hiPS-HSPCs. We report that molecular determinants of extravasation are intact in hiPS-HSPCs, but hiPS-HSPCs are deficient in expression of E-selectin ligands. To overcome this deficiency, we developed a simple modified mRNA-based approach to temporally increase the E-selectin ligands on these cells, resulting in increased tethering/rolling in vitro, and improved calvarial homing and extravasation in vivo. We anticipate that strategies to increase E-selectin ligand expression could be applicable as part of a multifaceted approach to optimize the production of HSPCs from human PSCs. As an example of the power of glycoengineering to enhance E-selectin ligand expression, fucosylation of human cord blood using recombinant fucosyltransferase has recently been reported to accelerate engraftment in xenotransplant models (15, 16) and has also shown promising results in early-stage clinical trials (17).

It should be noted that in this study, increasing homing and extravasation by FUT6 modified mRNA was not sufficient to generate long-term engraftable hiPS-HSPCs. Additional functional deficiencies (unrelated to homing) likely exist and must be overcome before fully engraftable HSPCs from human PSCs can be generated. One promising strategy that we are pursuing is the use of modified mRNA to transiently increase expression of relevant transcription factors. Since several modified mRNAs encoding different proteins can be introduced simultaneously (7), a combinatorial modified mRNA transfection approach could be a powerful strategy to generate fully functional human PSC-derived HSPCs. Importantly, since modified mRNA is non-permanent and non-genome-integrative, it is particularly amenable to clinical use. In conclusion, we have identified E-selectin ligand deficiency as an important factor limiting the BM homing ability of hiPS-HSPCs and provide evidence that a modified mRNA-based glycoengineering strategy can overcome this deficiency. Importantly, our findings suggest that glycoengineering to enhance E-selectin ligand expression could be considered within the context of future efforts focused on generating functional HSPCs from human PSCs.

Methods
Details about experimental procedures are provided in Supplemental Methods.

Statistics
P values were calculated using a 1-way ANOVA with Tukey’s honest significant difference (HSD) or Student’s t test as indicated in the figure legends. Student’s t test was 2-tailed. A P value less than 0.05 considered significant, and all data are reported as mean ± SEM.

Study approval
All animal use was in accordance with the guidelines of the Animal Care and Use Committee of the University of Massachusetts Medical School and The Jackson Laboratory. Human cord blood and PBMCs from peripheral blood draws were collected in heparin from healthy volunteers under signed informed consent in accordance with the Declaration of Helsinki and with approval from the Institutional Review Board of University of Massachusetts Medical School or Brigham and Women’s Hospital, respectively.

Author contributions
JML, BD, JS, RS, and DJR designed experiments; JML, BD, JS, and LLK performed experiments and analyzed data; and JML, BD, JS, LLK, DLP, LDS, MAB, CPL, RS, and DJR wrote the manuscript.

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