Using Eukaryotic Expression Systems to Generate Human α 1,3 Fucosyltransferases that Effectively Create Selectin-binding Glycans on Stem Cells

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Supporting Information Included: Methods, Figures S1-S3 and Tables SI and SII

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Highlights

- Human fucosyltransferases expressed and purified from both *Pichia pastoris* yeast and *Bombyx mori* silkworm display specific activities > 1000 pmol/min/μg that efficiently create sialyl Lewis X epitopes on primary human stem cells capable of binding E-selectin.
- Fusing BmApoLpIII to fucosyltransferases allows for the production of highly active human enzymes in large scale from *Bombyx mori* silkworm.

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3 **ABSTRACT**

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5 Recruitment of circulating cells towards target sites is primarily dependent on selectin/ligand adhesive

6 interactions. Glycosyltransferases are involved in the creation of selectin ligands on proteins and lipids. α 1,3-

7 fucosylation is imperative for the creation of selectin ligands and a number of fucosyltransferases (FTs) are

8 able to modify terminal lactosamines on cells to create these ligands. One FT, fucosyltransferase VI (FTVI)

9 adds a fucose in an α 1,3 configuration to N-acetylglucosamine to generate sialyl Lewis X (sLe^x) epitopes on

10 proteins of live cells and enhances their ability to bind E-selectin. Although a number of recombinant human

11 FTVIs have been purified, apart from limited commercial enzymes, they were not characterized for their

12 activity on live cells. Here we focused on establishing a robust method to produce FTVI that is active on living

13 cells (hematopoietic cells and mesenchymal stromal cells). To this end, we used two expression systems,

14 *Bombyx mori* (silkworm) and *Pichia pastoris* (yeast), to produce significant amounts of N-terminally tagged

15 FTVI and demonstrated that these enzymes have superior activity when compared to currently available

16 commercial enzymes that are produced from various expression systems. Overall, we outline a scheme to attain

17 large amounts of highly active FTVI that can be used for the applications of FTVI in enhancing the engraftment

18 of cells lacking the sLe^x epitopes.

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INTRODUCTION

The addition of fucose by α 1,3- or α 1,4-fucosyltransferases (FTs) to lactosamine structures completes the formation of sialyl Lewis X (sLe^x or also to its isomer sLe^a) epitopes, which are key post-translational modifications on proteins and lipids important for mediating the initial essential interactions between cells in flow and endothelial cells of tissues and organs ¹⁻⁵. The FT family of enzymes share a common structure characterized as type 2 transmembrane Golgi-anchored proteins containing an N-terminal cytoplasmic tail, a transmembrane region, and an extended stem region followed by a large globular C-terminal catalytic domain facing the Golgi lumen ⁶. Thirteen FT enzymes have been identified in the human genome and classified according to the type of linkage into α -1,2, α -1,3/4 and α -1,6 based on the site of fucose addition. α -1,3 FTs belong to the Carbohydrate Active Enzyme (CAZy, <http://www.cazy.org/>) glycosyltransferase (GT) family GT10. FTVI ([NCBI accession number NP_000141](#)) can synthesize three type 2 Lewis antigens, Le^x, Le^y and sLe^x, and the main acceptor substrates for FTVI are Gal β 1-4GlcNAc β 1-3-R and NeuAca2-3Gal β 1-4GlcNAc β 1-3-R ⁷. FTVI exhibits the strongest α -1,3 FT activity among the five α -1,3 FTs ⁸ thus its expression and purification can be highly beneficial.

Previously, recombinant human FTVI (rhFTVI) was expressed in many systems from prokaryotic to higher order eukaryotic systems including yeast ⁹, insect ⁷, and mammalian ¹⁰. However, none of the purified rhFTVI enzymes were tested for their activity on cells. The only tested rhFTVI enzymes on cells are of previous commercial sources as indicated in these references ¹¹⁻¹⁴. In an alternative strategy, a recent study illustrated that cells can be reprogrammed by transfection with modified mRNA encoding each of the human α (1,3)-FTs ¹⁵. In the current study, we describe a scheme to purify rhFTVI expressed in yeast or silkworm and to thoroughly validate its activity on cells. Moreover, we showed that these enzymes were superior to available commercial sources. Yeast and silkworm combine the ease, simplicity, yield and low costs sought for in protein expression systems. Yeast expression systems such as the *Pichia pastoris* strain have produced a number of GTs involved in the biosynthesis of N- and O-linked glycans including: β 1,4galactosyltransferase, α 2,6sialyltransferase (ST), α (2,3)-ST, α (1,3)-FTIII and VI, and α (1,2)-mannosyltransferase ¹⁶⁻¹⁸. Alternatively, advances in the *Bombyx mori* silkworm expression have resulted in a silkworm-baculovirus expression vector systems (BEVS) that can

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be used for mass production of mammalian-like post-translational modifications of proteins, limited proteolytical processing, proper folding and high level expression compared to those in other eukaryotic systems ^{19, 20}. In the current study, we outline a robust purification scheme to attain active rhFTVI in high yield from both of these expression systems especially in silkworm-BVES in which rhFTVI fused with BmApoLpII to gain stable active enzyme. Further, we demonstrate their activity through the creation of sLe^x epitopes able to bind E-selectin on human stem cells—mesenchymal stromal cells (MSCs) and hematopoietic stem/progenitor cells (HSPCs) derived from induced pluripotent stem cells (iPSs)—previously void of E-selectin ligands.

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MATERIALS AND METHODS

Cells and Silkworm Larvae

K562 (human Erythroleukemia cell line), KG1a (human acute myelogenous leukemia cell line), THP1 (human acute monocytic leukemia), HL60 (human acute promyelocytic leukemia) (ATCC) and MSCs (primary Mesenchymal stromal cells) (AllCells) were cultured according to the manufacturer's instructions. hiPS clone SC-9N was obtained from Prof. Belmonte's lab (Salk Institute for Biological Sciences, USA) and were cultured and induced to differentiate towards hematopoietic lineage as described in Supporting Information. PichiaPink Strain 1 methylotrophic yeast was used as host for the expression of glycosyltransferases, which are *adenine* auxotrophs (Invitrogen). The d17 x p53 strain of silkworm larva was used in this study was provided by and reared at the Institute of Genetic Resources, Kyushu University Graduate School. The larvae were reared on fresh mulberry leaves at 25–27°C.

Construction FTVI recombinant vector and transformation to Pichia pastoris

Briefly, a pPink-αHC vector was used (Invitrogen) to integrate the human FTVI cDNA encoding amino acid 35-359 of the FTVI protein sequence ([with UniProt Accession ID: P51993](#)) that omits the cytoplasmic and transmembrane regions of full length human FTVI, and encompassed the entire catalytic domain of the enzyme. A human FTVI luminal domain sequence was codon-optimized with JCat software, synthesized, and amplified with the primers AGAGTTTCTCAAGACGACCCAAGTGTTC and TTGGTACCAGTGAACCAAGCAGCGATACCTCTAGT. The obtained fragment was digested with KpnI and ligated together with a pPink-αHC fragment digested with StuI and KpnI to construct pPink-αHC-hFTVIlum. In order to include 6XHistidine-tag on the N-terminus of the FTVI ORF, a PCR reaction was conducted with the primers GGCATCATCACCATCATCATGGTAGAGTTTCTCAAGACGACCCA and CTACCATGATGATGGTGATGATGCCTTTTCTCGAGAGATACCCCTTC. The vector was propagated in *E. coli* strain TOP 10F (Invitrogen). The recovered DNA was linearized with SpeI restriction enzyme and then the digested DNA was used to transform *Pichia Pastoris* strains according to the manufacturer's instructions (Invitrogen). Stable transformants were selected on minimal dextrose ²¹ medium agar plates without Adenine.

Following selection of these colonies, we analyzed the colonies for the integration of the plasmid by performing PCR using 5' and 3'AOX1 primers corresponding to the flanking sequences of the native promoter *AOX1* gene.

Construction of recombinant baculovirus and inoculation of silkworm larvae

Truncated human FTVI constructs were cloned into the modified pENTR11 (Invitrogen) vector to utilize Gateway™ cloning system for generating virus donor expression vector. Briefly, the amplified PCR product of human FTVI cDNA and silkworm apolipophorin III (BmApoLpIII: NCBI accession number AK383413) PCR fragment were inserted into a multiple cloning site of the modified pENTR11 (Invitrogen) containing silkworm 30K6G protein signal peptide and an N-terminal 8XHis-Strep-tag. The resulting construct was designated pEntr-30KdH8S-BmApoLpIII-rhFTVI.

The recombinant donor plasmid for generating baculoviruses was constructed by a Gateway LR reaction between the pEntr-30KdH8S-BmApoLpIII-rhFTVIn and the pDEST8 vector (Invitrogen). The resulting construct was named pDEST8-30KdH8S-BmApoLpIII-rhFTVIn. Using the pDEST8-30KdH8S-BmApoLpIII-rhFTVIn as a donor, the recombinant bacmid DNAs were generated by BmNPV/T3 bacmid system as described previously ²⁰. Generation and large-scale harvest of recombinant BmNPV/-BmApoLpIII-rhFTVI was carried out following the protocols recommended in the manufacturer's manual (Invitrogen). Recombinant viruses were carefully injected into hemocoel of silkworm larvae on day 2 of the 5th instar at a dose of 1 × 10⁵ pfu per larva.

Expression of rhFTVI in cultured Pichia pastoris and Bombyx mori

Pichia pastoris:

For rhFTVI expression, single colonies of *Pichia pastoris* yeast that express recombinant human FTVI were inoculated in 1-L baffled flasks containing 100-mL buffered complex glycerol or methanol medium (BMGY or BMMY), composed of 1% yeast extract (BD), 2% peptone (BD), 100-mM potassium phosphate, pH= 6.0 (Fisher Scientific), 1.34% YNB (Sigma), 0.0004% biotin (Sigma) and 1% glycerol (Sigma; BMGY medium) or 0.5% methanol (VWR; BMMY medium). Briefly, the cells were grown first in 100-mL of BMGY media for 1–2 days at 30°C in a shaking incubator set at 200-rpm. The cells were then transferred to 1-L of

BMGY medium and cultured for another day under the same conditions. The following day the cells were divided into five flasks of 2-L BMGY medium and cultured for two days at 30°C and 200-rpm. The approximate number of cells in a culture was determined with a spectrophotometer by measuring the optical density (OD) at 600-nm. The cells were then pelleted in a sterile centrifuge bottle at 3000×g for 15-min and re-suspended the cell in two/1-L of BMMY medium to induce expression and then cultured at 30°C in a shaking incubator for an additional seven days with the addition of 0.5% methanol daily. At day 8 of induction, cells were harvested by centrifugation at 3000-rpm for 10-min and re-suspended in 200-mL of lysis buffer [100-mM potassium phosphate (Fisher Scientific), 500-mM NaCl (Fisher Scientific), 10-mM MnCl₂ (Fisher Scientific), 2.5-mM imidazole, 1-mM PMSF (Alexis) and EDTA free protease inhibitor cocktail tablet (Roche, UK) pH 7.8.].

Bombyx mori: Four days after infection and rearing at 25°C, the silkworm larva was dissected, and fat body or hemolymph was collected. 10-mL of the hemolymph can be recovered from roughly 30 silkworm larvae. The hemolymph was collected into a 15-mL tube containing 20-mM 1-phenyl-2-thiourea and subsequently centrifuged at 10,000×g for 30-min at 4°C to remove cell debris. The resulting supernatant contained the secreted protein. The fat bodies were dissolved in lysis buffer [20-mM Tris-HCl (pH 7.4), 500-mM NaCl, 0.5% CHAPS, 1-mM PMSF] and homogenized. Then the homogenates were centrifuged at 52,500g for 30-min at 4 °C to remove debris. The resulting supernatant contained the intracellular protein.

Purification of rhFTVI from Pichia pastoris cells and Bombyx mori Larvae

Pichia pastoris: Cells in lysis buffer were disrupted using French Press at 40,000-Kpsi to achieve complete lysis and cell debris was removed by centrifugation step (15000-rpm, 30-min, 4°C). The supernatant was incubated with 2-mL of Ni-NTA agarose resin (Thermo Scientific) pre-equilibrated with binding buffer [20-mM Tris-HCl (pH 7.8), 500-mM NaCl, 10%-glycerol, 2-mM MnCl₂ and 2.5-mM imidazole] for 2-h at 4°C (batch procedure). After incubation, the resin was collected by low speed centrifugation and loaded onto a pre-equilibrated Polypropylene Column (Qiagen). The column containing rhFTVI bound to resin was then washed with 50-mL washing buffer [20-mM Tris-HCl (pH 7.8), 500-mM NaCl, 10% glycerol, 2-mM MnCl₂ and 5-

mM imidazole]. The bound protein was eluted by 15-mL of elution buffer [20-mM Tris-HCl (pH-7.8), 150-mM NaCl, 10% glycerol, 2-mM MnCl₂ and 400-mM imidazole]. The elution fraction was then concentrated to 0.25-mL by using Amicon concentrator (10-kDa) (PALL) and dialyzed with buffer containing 20-mM Tris-HCl (pH-7.8), 150-mM NaCl, and 5% glycerol. All fractions including the crude extract, the flow through, the washing and the elution fractions were confirmed for rhFTVI expression using 4-20% SDS-PAGE (Criterion Bio-Rad). rhFTVI was detected in the elution fraction only.

Bombyx mori: 10-mL of hemolymph or fat body lysate was diluted in a binding buffer [20-mM Tris-HCl (pH 7.4), 500-mM NaCl, 1-mM PMSF] and pelleted down by centrifugation at 20,000×g for 30-min at 4°C. The supernatant was filtered through a 0.45-μm filter (Millipore, USA). A two-step purification of rhFTVI was processed through the same procedure as previously published protocol ²⁰. Briefly, the supernatant was loaded onto a nickel affinity chromatography with 5-mL HisTrap excel column (GE Healthcare Bioscience, USA) followed by StrepTrap HP column (GEHealthcare Bioscience, USA).

Fucosyltransferase activity assay

The specific activity of the purified rhFTVI enzyme was determined by using the Glycosyltransferase Activity Kit (R&D), as per the manufacturer’s instructions. Briefly, 0.5-μg of recombinant enzymes (purified from silk worm, purified from yeast, or commercial enzymes [yeast, mammalian and baculovirus Cat NO.: MBS952976, MyBioSource]) was added to 50-μL of reaction buffer (25-mM HEPES (pH 7.5), 10-mM MnCl₂ and HBSS) containing 0.08-mM GDP-Fucose (Sigma) and 0.3-mM of N-acetyl-D-lactosamine (Sigma). To determine non-specific signals, we included negative control wells: 1) reaction that includes all components (reaction buffer) but lacking the rhFTVI enzyme and; 2) reaction that includes an unrelated enzyme, i.e. ST3GALIV, a kind gift from the Kusakabe lab. The reactions were then incubated at 37°C for 20-min. Following this incubation, reactions were terminated by the addition of 30-μL of Malachite Green Reagent A and 100-μL of water. Color was developed by the addition of 30-μL of Malachite Green Reagent B and incubation at room temperature for 20-min. The plate was read at 620-nm using a multi-well plate reader (xMark microplate reader (Bio-Rad)). The optical density reading correlates to phosphate concentrations that are determined using a phosphate

standard curve. All measurements were performed in triplicates. The specific activity of the rhFTVI enzyme was calculated using the phosphate standard curve (**Figure S1A and B**) as pmol of phosphate produced/min/μg.

rhFTVI treatment

For treatment of cells (K562, MSCs, iPS-HSPCs) with rhFTVI enzyme, cells were harvested, washed 2x with Hank's Balanced Salt Solution (HBSS), and resuspended at a density of 1×10^6 cells/mL in FTVI reaction buffer [25-mM HEPES (pH 7.5) (Gibco Invitrogen), 0.1% human serum albumin (Sigma-Aldrich), 0.5-mM GDP-fucose (Sigma), 5-mM MnCl_2] and 1-μg purified rhFTVI enzyme in HBSS. Cells were incubated at 37°C for 30-min. Buffer only controls excluding the rhFTVI enzyme were used as a negative control. After the reaction, the cells were washed 2X with HBSS and 10-mM EDTA and used immediately for downstream experiments.

Statistical analysis

All experiments were carried out in triplicates. Data obtained were plotted and statistically analyzed using the software package GraphPad Prism version 5.0 for Windows. Data are represented as the mean $\pm$ standard deviation (SD). Statistical analysis was performed using unpaired Student t-test. P values <0.05 were considered significant.

Online Supporting Information

Detailed Methods, Figures and Tables are available in Supporting Information online.

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3 **RESULTS**

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6 *Expression and purification of rhFTVI from Pichia pastoris yeast*

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8 We designed an expression construct that spans the catalytic domain of human FTVI (35-359-aa; **Figure 1A**,

9 *top*) with a *Pichia pastoris* secretion signal, the α -mating factor, to induce secretion (**Figure 1A**, *middle*). In

10 addition, the vector includes a 6XHistidine (His)-tag added to the N-terminus of rhFTVI to help in its

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12 purification. rhFTVI was produced by *Pichia pastoris* according to standard protocols (Materials and Methods).

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14 Briefly, electroporation was used to transform cells prior to metabolic phenotype selection. White colonies

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16 indicating higher expression of rhFTVI gene were picked and the presence of functional expression units was

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18 confirmed by small-scale cultures induced with 0.5% methanol. Lysates of the *Pichia pastoris* cells were then

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20 prepared and tested for their expression of rhFTVI enzyme. rhFTVI enzyme was purified using a single

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22 purification step by immobilized metal affinity chromatography (IMAC) with a Ni-NTA agarose resin.

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24 Interestingly, the rhFTVI enzyme was localized within the *Pichia pastoris* cells and was not secreted into the

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Figure 1: Construction, expression and purification of functional domains of rhFTVI from *Pichia pastoris* and *Bombyx mori*. (A) The human FTVI gene encodes for a 359-amino acid (aa) protein that includes an N-terminal region composed of a cytoplasmic tail and a signal-anchor for type II membrane sequence while the C-terminal region consists of a luminal sequence containing a catalytic domain (325-aa) and a stem domain that is adjacent to a transmembrane domain (TM). The N-terminal region is not required for activity, and thereby can be deleted without adverse effects on enzyme activity while changes in the C-terminal catalytic domain results in inactivity²² (top). The carboxyl terminal (COOH) region including the stem and catalytic

domains are typically found in the lumen of the Golgi. The vector construction for expression of the mature form of the rhFTVI enzyme in *Pichia pastoris* (yeast) is shown for the luminal FTVI human enzyme containing 6XHistidine (His-tag) at the N-terminus of the protein (*middle*). The pPink- α HC vector was used, which contains the alcohol oxidase (AOX1) promotor and the open reading frame of FTVI downstream of the α -mating factor secretion signal. The vector construction for expression of the mature form of the luminal FTVI enzyme in *Bombyx mori* (silkworm) is shown (*bottom*). The construct was used which has the polyhedrin (PH) promotor followed by the silkworm signal peptide 30k protein (30K). An 8XHistidine (His-tag) and a Strep (STREP-tag) was added upstream of *B. mori* apolipophorin-III and sortase cleavage site/tease cleavage site. B1 and B2 represent the recombination sites for Gateway cloning and the SV40 is the polyadenylation signal. **(B)** Schematic representation of the expression of recombinant GT enzymes in eukaryotic organisms, *Bombyx mori* and *Pichia pastoris*, (*top left*) along with their purification scheme (*bottom left*). rhFTVI will be used for cell surface glycan engineering to create sLe^x on glycoproteins by transferring the fucose from GDP-fucose to N-acetylglucosamine. *Figure 1B was created by Heno Hwang, scientific illustrator at KAUST.* **(C)** Western-blot analysis of purified rhFTVI enzyme from yeast cultures and silkworm fat body. The concentrated eluate following purification was run on an SDS-PAGE gel and transferred to a PVDF membrane prior to blotting with anti-FTVI antibody (1:1000; Abcam). To detect the primary antibodies, incubation with goat anti-rabbit horseradish peroxidase (1:20000) secondary antibody was used. A blot incubated with secondary antibody alone was used as a control. This represents $n = 3$ independent experiments.

Expression and purification of rhFTVI from Bombyx mori silkworm fat body

To investigate whether active rhFTVI can be expressed in silkworm, we constructed a 30K6G secretion signal at the N-terminus of human FTVI (35-359 aa), which is a signal peptide of silkworm 30-kDa lipoprotein that induces effective secretion. Furthermore, tandem His-Strep tags were added behind the secretion signal to improve purification efficiency **Figure 1A** (*bottom*). Additionally, we employed the silkworm apolipophorin III protein ([BmApoLpIII: NCBI accession number AK383413](#)) as a fusion tag to facilitate the solubility and stability. In fact, when expressed and purified with the above construct without the fusion BmApoLpIII, the yield of purified rhFTVI could not be determined due to protein aggregation during the IMAC purification step (data not shown). Using this BmApoLpIII-fusion hFTVI construct, we successfully generated BmNPV/-BmApoLpIII-rhFTVI and the expression of rhFTVI was detected in virus-infected silkworm cells at 4-days post-inoculation. Subsequently, to examine the amount of purification and recovery, both secreted rhFTVI and intracellular rhFTVI were purified from silkworm hemolymph or fat body by His- and Strep-tag affinity chromatography, respectively. This suggests that the rhFTVI enzyme could be more efficiently produced from the fat body of silkworm larvae than from the hemolymph.

The illustration in **Figure 1B** outlines the steps to produce functional rhFTVI. The enzymes were purified mainly in one step using Ni-NTA agarose with an additional StrepTrap HP column in the case of silkworm. The yield was approximate >10-mg/L. The purified rhFTVI enzymes transfer fucose from the GDP-fucose substrate to N –acetylglucosamine of the sialylated lactosamine on the cells of interest (cell line or primary stem cells) during a 30-min reaction at 37°C to create the sLe^x epitope (**Figure 1B**).

Molecular Characterization of rhFTVI enzymes

In order to characterize the identity of the purified enzymes, we first performed a Western blot analysis using antibodies specific to the human FTVI enzyme. As evident in the *Pichia pastoris* expressed rhFTVI (**Figure 1C, left**), the washing step before eluting the protein off the Ni-NTA column influenced the molecular weight of the eluted rhFTVI. When the column was washed with 1-M NaCl and 20-mM imidazole (high salt), the predominant elution band had the expected molecular weight of ~38-kDa based on the aa sequence, while when it was washed with lower concentrations of NaCl (500-mM) and imidazole (5-mM) (low salt), the predominant elution bands were of higher molecular weights (~50-kDa, likely corresponding to FTVI and the secretion signal, and ~70-kDa). These higher molecular weight bands were not detected in the secondary alone controls (data not shown) suggesting they also represented rhFTVI protein. A previous study reported that the secreted protein from *Pichia pastoris* was ~38-kDa while the non-secreted intracellular form was ~76-kDa¹⁰. It is likely that, at least, our ~70-kDa band is due to differential posttranslational modifications of the four potential N-linked glycosylation sites on rhFTVI. On the other hand, the purified BmApoLpIII-fused rhFTVI from *Bombyx mori* fat body, exhibited one major band around 60-kDa that corresponds to the expected 60-kDa of FTVI and the BmApoLpIII-fused tag (**Figure 1C, right**).

To decipher which band(s) represents rhFTVI from the *Pichia Pastoris* yeast system, we used a mass spectrometry approach to confirm the purification of the rhFTVI enzyme as well as to confirm the results obtained from the Western blot analysis. To this end, samples were separated on an SDS-PAGE gel and stained using SimplyBlue Safe Stain. Bands within each molecular weight range as outlined in Supporting Methods (i.e. ~70-kDa, ~50-kDa, and ~38-kDa) were cut and prepared for MS analysis as described in Supporting

information. As shown in **Table I**, rhFTVI from *Pichia pastoris* was identified at ~50-kDa. The rhFTVI enzyme was also identified at ~70 and ~38-kDa bands (**Table I**). Overall, the MS analysis was consistent with the Western blots (**Figure 1C**) and confirm that all detected bands contained rhFTVI enzyme. Human FTVI has four potential N-glycosylation sites and a shift of at least 2 kDa per site can be expected ²³. We compared rhFTVI treated with Peptide:N-glycosidase F (PNGaseF) enzyme, which removes N-linked glycans on proteins, to untreated rhFTVI (**Figure S1A**). The predominant bands at 38, 50 and 70 kDa in untreated sample appear to have disappeared in the PNGaseF treated sample suggesting that some of N-glycan sites have been removed from the enzyme. Although less variability in molecular weights was detected, we did not observe that through removal of the N-glycans a more homogenous population, i.e. a single molecular weight species, remained.

rhFTVI expressed in both expression systems were active

To determine whether the isolated rhFTVI enzymes were active, an *in vitro* enzymatic activity assay was performed (Materials and Methods) (**Figure S1A and B**). As a negative control, recombinant human ST3GALIV was utilized. We compared the activity of our purified rhFTVI enzymes to those available commercially (35-359-aa; MyBioSource) that were expressed in yeast, baculovirus or mammalian cells. The specific activity was determined to be 1760 ± 70 pmol/min/ μ g (for the *Pichia pastoris* FTVI eluted after washing the column with low salt and imidazole), 1330 ± 65 pmol/min/ μ g (for the *Pichia pastoris* FTVI eluted after washing the column with high salt and imidazole) and 1910 ± 220 pmol/min/ μ g (for the *Bombyx mori* FTVI). These results demonstrated that all the forms of our purified rhFTVI enzymes have similar activity. Interestingly, our purified rhFTVI enzymes were significantly more active than the commercial ones (105 ± 25 pmol/min/ μ g for FTVI purified from yeast, 135 ± 5 pmol/min/ μ g for FTVI purified from mammalian cells and 210 ± 20 pmol/min/ μ g for FTVI purified from baculovirus; $n = 3$ independent experiments, p -value < 0.0001; **Figure 2A**). In order to determine the enzymatic activity on glycans on the surface of live cells, we treated K562 cells, a cell line that has limited expression of HECA452 reactive epitopes, with our purified rhFTVI enzymes as well as commercial enzymes for 30-min. If the enzyme is active and able to transfer the fucose and

create the sLe^{x/a} epitope on K562 cells, the HECA-452 mAb will stain the cells. Interestingly, while flow cytometric analysis showed that treatment of live cells with our purified enzymes illustrated activity on live K562 cells, the commercial enzymes did not, even when titrated to high concentrations (**Figure 2B**). It is possible that the reason for this lack of activity is due to the storage conditions of the commercial enzymes; these enzymes were stored in a buffer lacking salt [10-mM Tris-HCl (pH-8) and 10% glycerol] and salt has a dramatic effect on preserving protein folding and enzyme catalysis⁶⁷.

Figure 2: rhFTVI enzymes from both yeast and silkworm expression systems are active. Using the Glycosyltransferase Activity Kit (R&D), the activity of rhFTVI enzymes was determined. A phosphate standard curve was first prepared with a concentration range (0-100-μM) in a total volume of 50-μL per reaction. **(A)** Recombinant human FTVI enzymes were then titrated separately in 50-μL in Glycosyltransferase reaction buffer (25-mM HEPES, 10-mM MnCl₂, pH 7.5 and HBSS) in a 96-well plate in the presence 0.08-mM GDP-fucose. The specific activity of 0.5-μg purified (*Pichia pastoris* and *Bombyx mori*) eluted at high (i.e. 1-M NaCl and 20-mM imidazole to elute ~38-kDa form) and low (i.e. 500-mM NaCl and 5-mM imidazole to elute higher MWs of ~50-kDa and ~70-kDa) salt as well as the commercial enzymes (Yeast, Mammalian and Baculovirus) were calculated based on the amount of phosphate produced in pmol/min/μg as pmol/min/μg. Each data point represents mean ± SD ($n = 3$ independent experiments). **(B)** sLe^x expression was measured after each treatment with 0.5-μg of rhFTVI using flow cytometry analysis with HECA-452 antibody. The graph represents one experiment from $n = 2$ independent experiments.

rhFTVI creates functional sLe^x positive E-selectin binding epitopes on selectin ligand negative cells

We next tested the activity of our rhFTVI enzymes on live cells. A number of different leukemic cell lines were first tested for their native sLe^x expression and E-selectin binding [E-selectin-hIg chimera; E-Ig] by flow

cytometry. As shown in **Figure 3A**, all cell lines (HL-60, THP1 and KG1a), with the exception of K562 cells, expressed sLe^x. The expression of sLe^x correlated with the ability of cells to bind E-Ig (**Figure 3A**). K562 cells were therefore chosen as a model cell line to determine whether the activity of the rhFTVI enzymes leads to the creation of sLe^x structures on live cells. Biosynthesis of sLe^x involves α 2,3-STs (encoded by ST3GAL genes ST3GALIII and ST3GALIV) and α 1,3-FTs (encoded by FT genes FTIII, FTIV, FTV, FTVI, FTVII) ²⁴. To better understand the expression of the GTs, FTs and ST3GALs, necessary for the terminal monosaccharide additions of fucose and sialic acid in the biosynthesis of sLe^x, we performed an mRNA expression analysis of these genes in K562 cells by real-time semi-quantitative PCR (**Figure 3B**) using the primers list in **Table SII**. Our results revealed that ST3GALIII and ST3GALIV transcripts were relatively highly expressed in K562 cells. The expression of the α 1,3-FTs, i.e. FTIII, FTIV, FTV, FTVI and FTVII ⁶ were found to be expressed at very low levels or absent in K562 cells compared to the α 1,2-FTs, FTI and FTII, which are responsible for H blood group antigen ²⁵. Interestingly, the α 1,6-FT, FTVIII, was also expressed in these cells. FTIV and FTVII are the main human FTs expressed in leukocytes responsible for the creation of functional selectin ligands ²⁶ but both of these enzymes were found to be expressed at low levels in the K562 cells (**Figure 3B**).

Figure 3: K562 cells lack E-selectin binding due to the absence of fucosylated epitopes. (A) Flow cytometric analysis of sLe^x expression was assayed in various cell lines, HL60, KG1a, K562 and THP1, by staining with HECA-452-mAb and CD15s-mAb. Isotype control (rat IgM and mouse IgM) are shown in blue for HECA-452 and CD15s respectively. E-selectin binding was determined by incubating the cells with E-Ig followed by PE anti-human IgG Fc. 10-mM EDTA (blue) was added to abrogate binding of cells to E-selectin. (B) The expression of GTs in K562 cells was determined using real-time semi-quantitative PCR. cDNA was prepared from mRNA isolated from K562 cells. The expression of GT genes relative to the level of expression of the mRNA of GAPDH housekeeping genes was determined. Each data point represents mean $\pm$ SEM ($n = 3$ independent experiments). (C) Flow cytometric analysis shows that K562 cells express PSGL-1, CD43 and CD44 glycoproteins (light gray). Isotype controls (mouse IgG and mouse IgG2a) are shown in black for each specific ligand. (D) K562 cells were treated with either the appropriate concentration of purified rhFTVI in buffer (HBSS, 0.1% human serum albumin, 0.5-mM GDP-Fucose, 5-mM MnCl₂ and 25-mM HEPES pH=7.5; treated) or in buffer alone (untreated) and incubated for 30-min at 37°C. Following treatment, flow cytometric analysis for sLe^x expression (HECA-452, CD15s) and E-Ig binding was determined (left panel) and the average of percentage expression for each staining is represented in the right panel. This is representative of $n=4$ independent experiments.

We next sought to characterize which potential E-selectin protein ligands were expressed on K562 cells by staining cells with antibodies directed against known glycoprotein ligands, namely, CD44, CD43 and P-selectin glycoprotein ligand 1 (PSGL-1) ²⁷, and analyzed their expression by flow cytometry. As shown in **Figure 3C**, K562 cells expressed PSGL-1, CD43 and CD44. It should be noted that expression of these glycoproteins does not equate to expression of sLe^x or to functional E-selectin binding (**Figure 3A**, **Figure 3D** and **Figure 4A**). To this end, K562 cells were incubated with the purified rhFTVI enzymes (**Figure 3D**). Following treatment with either of the rhFTVI enzymes (*Pichia pastoris* and *Bombyx mori*), the cells were stained using antibodies that recognize the sLe^x as well as with E-Ig, and analyzed by flow cytometry. As illustrated in **Figure 3D**, following treatment, sLe^x expression was increased. Furthermore, rhFTVI treatment allowed the cells to bind to E-Ig. The specificity of binding of E-Ig to selectin ligands on the cells was demonstrated through the addition of EDTA that chelated cations (i.e. Ca²⁺) essential for mediating binding of E-selectin to its ligands ²⁸. Overall, this shows that the rhFTVI produced from both yeast and silkworm were able to add fucose to ligands in order to create sLe^x and allow for E-selectin to bind cells that previously did not bind. It is interesting to note that there does seem to be a higher ability of the yeast preparation to create sLe^x epitopes as depicted by the higher level of CD15s, however the overall ability of both enzyme preparations to create sLe^x and sLe^a binding epitopes capable of E-selectin binding is equal (see HECA452 and E-selectin binding graphs in **Figure 3D**).

Figure 4: rhFTVI treatment generated E-selectin ligands on cells. (A) K562 cells were treated with rhFTVI (+) or with buffer alone (-) and then cell lysates were immunoprecipitated for CD44, CD43 and PSGL-1. The immunopurified proteins were prepared for Western blot analysis and blotted for E-Ig (*top*) or sLe^{x/a} using HECA-452 (*middle*) or directly for each protein (*bottom*). (B) The Stamper Woodruff adhesion assay was performed on K562 cells that were either treated with rhFTVI enzymes or buffer alone (untreated). Following rotation for 30-min, the cells were viewed under light microscopy using an ocular grid under 20X magnification (7 field per slide; *n* = 3 independent experiments). KG1a cells were used as a positive control and EDTA was used to chelate the Ca²⁺ out of the buffer in order to show specificity of binding between E-selectin and its ligands on both KG1a cells and K562 treated cells. (C) Flow cytometric analysis of human MSCs markers, CD105 (clone 43A3) and CD73 (clone AD2), are shown (black line). Mouse IgG isotype control is shown as a gray line. (D) MSCs were either treated (+) with purified rhFTVI (HBSS, 0.1% human serum albumin, 0.5-mM GDP-Fucose, 5-mM MnCl₂ and 25-mM HEPES pH=7.5; treated) or in buffer alone (-) and incubated for 30-min at 37°C. The cells were then lysed and prepared for Western blot analysis for HECA-452 or E-Ig. (E) Following MSCs treatment with rhFTVI, flow cytometric analysis for sLe^x expression (HECA-452, CD15s) and E-Ig binding was determined. Red: untreated MSCs incubated with buffer only without rhFTVI; Blue: MSCs treated with rhFTVI in the presence of 2-mM Ca²⁺; Green: MSCs treated with rhFTVI in the presence of 10-mM EDTA.

To establish which glycoproteins act as E-selectin ligands following rhFTVI treatment of K562 cells, we immuno-purified equal amounts of each ligand, CD43, CD44 and PSGL-1, before and after treatment (eg. *Pichia pastoris* FTVI) and assessed sLe^x expression and E-Ig binding activity by Western blot. As shown in **Figure 4A**, CD43 was the main E-selectin ligand created but CD44 and PSGL-1 also showed a low level of sLe^x expression and E-Ig binding. To establish if the ligands created following rhFTVI treatment on K562 cells were able to support binding in a freely rotating solution, the Stamper Woodruff assay was performed ^{28, 29}. Slides were coated with E-Ig and then both untreated K562 cells and rhFTVI treated-K562 cells (using both rhFTVI enzymes) were added onto slides and allowed to rotate for 30-min at 80-rpm. Following incubation, the numbers of bound cells/mm² in seven distinct fields of view were counted. As illustrated in **Figure 4B**, these results revealed that rhFTVI-treated cells supported E-Ig adherence (110±5 bound cells per field; 7 fields counted, *n* = 3 independent experiments) for cells treated with *Pichia pastoris* yeast rhFTVI and (70±10 bound cells per field; 7 fields counted, *n* = 3 independent experiments) for cells treated with *Bombyx mori* silkworm rhFTVI, whereas no binding was observed when EDTA was added. KG1a cells were used as a positive control due to their high expression of E-selectin ligands ^{27, 28, 30, 31}. Overall, K562 cells treated with either rhFTVI produced from *Pichia pastoris* or *Bombyx mori* bound E-Ig to a much greater degree than untreated cells.

Lastly, to assess the effect of rhFTVI treatment on K562 cell viability, we analyzed cell counts and apoptosis via Annexin-V/7-AAD staining of cells that were either treated with rhFTVI or with buffer alone over a 48-h period. As indicated in **Figure S2A and B**, treatment with either rhFTVI enzyme had no detrimental effect on cell counts or on apoptosis induction. The sLe^x epitope created by either rhFTVI enzyme was stable over a 24-h period as determined by flow cytometric analysis (**Figure S2C**).

Mesenchymal stromal cells (MSCs) and hematopoietic stem/progenitor cells derived from induced pluripotent stem cells gain selectin ligands following ex vivo treatment with rhFTVI

Often adult stem cell populations are inadequately α1,3-fucosylated and so exhibit homing defects and previous work has shown that using purified FTs *ex vivo* to add fucose to sialylated precursors on these cells successfully improves migration *in vivo* ^{13, 14, 32-36} To test the ability of rhFTVI to create sLe^x structures on primary MSCs,

we treated human MSCs with the rhFTVI enzyme from the silkworm system and measured sLe^x formation and E-selectin binding by flow cytometry. As evident in **Figure 4C**, the human MSCs displayed characteristic surface markers, CD105 and CD73³⁶. Following treatment with rhFTVI, the MSCs were lysed and assessed for E-Ig binding and sLe^x expression by Western blot. The major band observed was at ~80-kDa following treatment (**Figure 4D**), which is in agreement with previous studies indicating that the major glycoprotein E-selectin ligand on these cells is the standard glycoform of CD44, hematopoietic cell E- and/or L-selectin ligand^{13, 36}. Flow cytometric analysis also confirmed the gain in expression of sLe^x structures as well as E-selectin binding following treatment with rhFTVI (**Figure 4E**). This is consistent with previous work illustrating that the rhFTVI enzyme was able to create functional E-selectin ligands on human MSCs.

Hematopoietic stem cell transplantation is the most common cell-based therapy currently used to treat patients with life-threatening blood disorders/malignancies. Currently, the sources for transplantable HSPCs are bone marrow, umbilical cord blood (CB), or mobilized peripheral blood^{37, 38} but these are limited in supply and only one-third of the patients find HLA-matched donor cells³⁹⁻⁴¹. Direct differentiation of HSPCs from pluripotent sources such as induced pluripotent stem cells⁴² theoretically offer an unlimited source of allo-/auto-logous HSPCs for transplantation therapies. *In vitro* studies have shown that iPS-derived HSPCs (iPS-HSPCs) behave much like somatic HSPCs exhibiting robust clonal proliferation and multilineage hematopoietic capacity^{39-41, 43-47}. Thus, iPS hold great promise since they are amenable to large-scale production and can overcome the challenge of finding immune-compatible donors. Nonetheless, the utilization of iPS-HSPCs in transplantation is limited by the relative scarcity of finding them in the bone marrow following transplantation due to a number of inherent reasons⁴⁷⁻⁵². We postulated that the low engraftment of iPS-HSPCs could partially be due to a deficiency in migration⁵³ and thus in the expression of properly glycosylated selectin ligands. As illustrated in **Figure 5A (left panel)**, iPS-HSPCs lack the sLe^x epitope as indicated by the absence of HECA-452 staining. Moreover, the absence of sLe^x was consistent with the absence of E-selectin binding activity as shown by Western-blot analysis ($n = 3$) (**Figure 5A, right panel**). Inadequate fucosylation of selectin ligands can result in poor HSPCs homing and engraftment¹¹. However, this defect can be circumvented by

exogenous fucosylation of the ligands. To test whether the iPS-HSPCs express adequate amounts of $\alpha(1,3)$ -fucosyltransferase compared to CB-HSPCs, we performed q-PCR analysis to quantify the relative expression of FT-VII, which is widely expressed on hematopoietic cells including CD34⁺ cells from bone marrow and appears to be the dominant FT responsible for producing leukocyte selectin ligand activity ⁵⁴⁻⁵⁶. Our data demonstrated that iPS-HSPCs express a significantly lower amount of FT-VII (50 ± 5 -fold; data are mean $\pm$ SD, $n = 3$, p -value = 0.007) than HSPCs from CB. Given this stark difference in fucosyltransferase expression, we decided to determine if *ex vivo* treatment of the iPS-HSPCs with rhFT-VI (yeast system) would be sufficient to create E-selectin ligands. Following treatment with rhFTVI, iPS-HSPCs gained HECA-452 reactivity indicating sLe^x structures were created (**Figure 5B**, *left panel*). The ability of the fucosylated ligands to bind E-selectin was confirmed by Western-blot analysis (**Figure 5B**, *upper right panel*). Furthermore, immunoprecipitation of the known E-selectin ligands on HSPCs ^{27, 28, 31} shows that both CD43 and CD44 were decorated with HECA-452 antigenic determinants following treatment (**Figure 5B**, *lower right panel*). To determine whether the treatment affected the clonogenic activity of iPS-HSPCs, clonogenicity assays were performed. As evident in **Figure 5C**, the treatment did not significantly influence changes in the clonogenic ability of the iPS-HSPCs colonies compared to control treated cells ($n = 3$, p -value=0.91).

Figure 5: iPS-HSPCs gain E-selectin binding determinants following treatment with rhFTVI. (A) Human iPS cells (*left panel*) were differentiated toward HSPCs (see **Figure S3D** showing 30% CD34⁺ cells were generated following differentiation of iPS cells to HSPCs) and then the cells were sorted for CD34⁺ cells and double stained for CD34 surface antigen and HECA-452 antigenic determinant. The lack of sLe^x structures and E-selectin binding on iPS-HSPCs was confirmed by Western blot analysis. Unlike CB-HSPCs, human iPS-HSPCs do not bind to E-selectin (*right panel*). This is a representative experiment of *n* = 3 independent experiments. The gates were set based on the isotype controls after compensation. (B) Flow cytometric analysis of iPS-HSPCs treated with rhFTVI shows that the cells were appropriately fucosylated and gained HECA-452 reactivity on their surface (*left panel*). Western blot analysis revealed the binding of E-selectin following rhFTVI treatment to iPS-HSPCs whole cell lysates (*upper right panel*) and immunoprecipitations of CD43 and CD44 from iPS-HSPC lysates (*lower right panel*). (C) CFU assay was performed on iPS-HSPCs either treated with rhFTVI (+) or with buffer alone (-). On day 21 of culture, total colonies were enumerated for erythroid burst-forming units (BFU-Es), granulocyte macrophage colony-forming units (CFU-GMs), and granulocyte-erythroid-megakaryocyte macrophage colony-forming units (CFU-GEMMs). Results show that treatment with rhFTVI (+) did not affect the multipotent clonal behavior of iPS-HSPCs.

DISCUSSION

Use of an α -1,3-FT enzyme preparation and enzymatic conditions that are specifically designed for treating live cells have been reported in many studies ^{13, 34, 36, 57}. Creating efficient active GTs is not a trivial task. Eukaryotic expression systems are preferred over bacterial systems in the production of GTs. The rhFTVI was expressed and purified in several eukaryotic systems including CHO cells ¹⁰, insect cells ⁷ and yeast expression systems ⁹. Although all these systems produced functional rhFTVI, none of these studies characterized the purified enzyme on living cells, limiting the tested enzymes on cells to those produced commercially ¹¹⁻¹⁴. In this study, we used *Pichia Pastoris* yeast and the *Bombyx mori* silkworm-baculovirus expression systems to express and purify rhFTVI. Both expression systems are capable of expressing highly active proteins compared to the currently available commercial enzymes, displaying posttranslational modifications that are more similar to higher eukaryotes.

rhFTVI was detected intracellularly in *Pichia pastoris* yeast system while it was secreted in *Bombyx mori* silkworm system in presence of the silkworm 30k6G signal peptide. However, rhFTVI enzyme from silkworm hemolymph was not easily recovered from the total protein by IMAC purification. Therefore, technically, it is better to choose an alternative expression method in order to preserve biological activity and enhance recovery. Gene fusion technology has been widely used to improve protein stabilization and solubilization situation. Currently, our results show that a stable active rhFTVI enzyme was recovered by using BmApoLpIII as a fusion partner.

The rhFTVI enzymes expressed in *Pichia pastoris* yeast and *Bombyx mori* silkworm were detected at various molecular weights following separation of eluted proteins. Mass spectrometry analysis confirmed that the rhFTVI enzyme from the *Pichia pastoris* yeast system were specifically found in all of the detected bands in the Western-blot.

Additionally, all the purified enzymes at these different molecular weights showed nearly similar catalytic activity. Strikingly, these activities were found to be much higher than commercially available sources.

For such applications, the most crucial factors to consider are that the purified GTs are active and relatively pure.

A recent study ⁵⁸ describes the creation of a GTs vector library and demonstrates the expression and secretion a number of these GTs in mammalian and insect cells. However it is important to note that this method does not always result in optimum levels of active enzyme and, in fact, many of these enzymes exhibited partial proteolysis following secretion ⁵⁸. Moreover, each specific GT enzyme may have a unique set of folding constraints within the eukaryotic secretory pathway that cannot be generalized to all enzymes equally and therefore could require significant optimization to consider characteristics such as: type of tag used, tag placement, promoter/plasmid used, etc. Additionally, the expression of the same enzyme family was different in mammalian and insect cells, demonstrating the contribution of host-specific factors and highlighting the limitation in this strategy.

To characterize the activity of the purified rhFTVI enzymes, we used K562 cells as a model, a leukemic cell line lacking sLe^x epitopes and unable to bind E-selectin ^{59, 60}, and showed that the enzymes were able to successfully fucosylate and create sLe^x structures on live cells which equated to E-selectin binding. Indeed, an analysis of GTs in K562 cells revealed that although STs that support the synthesis of sLe^x, ST3Gal-III and – IV, were expressed, little to no expression of FTs that support sLe^x synthesis were found. This suggested that α -2,3 sialylation was intact in these cells but α 1,3 fucosylation was not. This is consistent with previous work indicating that K562 cells bind E-selectin when transfected with FTVII cDNA ⁶¹. Interestingly, the main E-selectin ligand that formed following rhFTVI treatment of K562 cells was CD43 although small amounts of CD44 and PSGL-1 also served as E-selectin ligands^{59, 60}. This is likely due to the fact that CD43 is highly expressed in these cells ^{62, 63}.

Previous work using rhFTVI to augment sLe^x expression on cells that exhibit either low to no binding to E-selectin such as CD34⁺HSPCs ⁶⁴ hMSCs ³⁶ or neural stem cells ³⁴, show significantly improved binding of cells to E-selectin reaching upwards of 90-95% of cells binding. This is consistent with our findings where we show that following treatment of K562 cells or MSCs with rhFTVI from either recombinant enzyme (*Pichia*

pastoris or *Bombyx mori*), all cells expressed sLe^x positive epitopes that bound E-selectin. Notably, cell viability was unaffected following treatment with our rhFTVI enzymes and the K562 cells retained sLe^x expression for 24-h, which in agreement with previous work ³⁶. In contrast, the cells treated with the commercial enzymes resulted in more than 50% cell death (data not shown) and did not show any increase in sLe^x expression (**Figure 2B**). This is likely due to the presence of higher levels of glycerol, imidazole and manganese, agents that promote cytotoxicity^{36, 65, 66} in the commercial enzyme storage buffer. Interestingly, a recent study using MSCs ¹³ illustrated that the sLe^x expression following fucosylation treatment was retained for over two days if stored at 4°C, while *ex vivo* fucosylation of CB CD34⁺ cells was stable for 2-h at room temp ¹⁴. Collectively, our results support the applicability for enzymes produced from these eukaryotic systems, and specifically the novel silkworm system in the treatment of cells for therapeutic endeavors whilst preserving cell viability.

For HSPCs to navigate towards their niches, they should follow a cascade of events that involve critical interactions between several adhesion molecules on the surface of HSPCs with their counterreceptors on the opposing bone marrow endothelium ¹. The first step is regulated by the interaction of E- and P- selectins expressed on bone marrow endothelium, with their glycoconjugate ligands expressed on the circulating HSPCs ⁶⁷⁻⁶⁹. We demonstrate here that the iPS-HSPCs lack the appropriate antigenic determinant for HECA-452 binding, thus leading to defective binding to E-selectin, a key adhesion molecule important for directing the migration of stem cells to the bone marrow which in agreement with ⁵³. Our data shows that this lack of HECA-452 is due to inadequate expression of the appropriate FTs, specifically FT-VII^{14, 32-36}. Fucosylation using rhFTVI was sufficient to decorate iPS-HSPCs with HECA-452 antigenic determinants leading to substantial increases in E-selectin binding activity. In addition to the enhancing binding capacity to E-selectin, the fucosylation of HSPCs showed higher binding capacity with P-selectin as well ^{11, 70}. This *ex vivo* approach is likely more effective than using the FT mRNA-mediated glycoengineering protocol since the stem cells' phenotypic characteristics and viability are better preserved ⁵³. Recently, reports have shown that ectopic expression of master hematopoietic transcription factors in iPS cells leads to the derivation of engraftable

HSPCs with some potential for migration when injected intrafemorally ^{71, 72}. In these studies, the cells were injected into one femur and engraftment was observed in both femurs, suggesting that stem cells from the initially injected femur migrated to the contralateral femur that wasn't injected. Through the development of improved protocols to derive HSPCs and such technologies as those outlined here, these sources of HSPCs could be that much closer to clinical use ⁷³.

Overall, we outline an advanced approach for purifying high quality and quantity of active GT enzymes from the yeast expression system using a simple one step purification scheme. In addition, we describe, for the first time, the use of the *Bombyx mori* silkworm system to produce highly active rhFTVI fused to BmApoLpIII that can be use as alternative source to insect, yeast and mammalian systems. The *Bombyx mori* silkworm system is one of the most promising systems for production of recombinant active eukaryotic proteins offering several benefits including: optimal folding and post-translational modifications resulting in proper biological activity, an extremely high yield with no limit to the size of the protein (several milligrams in one standard-scale experiment), great amenability to scaling-up, and a short time period for production of the protein. Using this system, researchers will be able to express secretory, membrane or nuclear proteins for research or industrial applications. We recently demonstrated that active β -1,4-galactosyltransferase 1 (β 4GalT1) could be effectively expressed and purified from the *Bombyx mori* silkworm system ²⁰ and now we have added the efficiency of this expression system to generate active rhFTVI. Using these expression platforms for the production these and other recombinant GTs paves the way to the development of many robust applications beyond the *ex vivo* creation of glycan structures on live cells. For example, these enzymes could be used to search of inhibitors, which could be used in cancer since many cancers upregulate the expression of sLe^x specific GTs. In colon cancer, for example, elevated sLe^x expression is linked to up-regulation of STs and FTs ⁷⁴. In addition, basal breast cancers typically show higher sLe^x expression and more E-selectin bound cells than luminal cancers indicative of higher metastatic potential ⁷⁵.

NOMENCLATURE

E-Ig: Recombinant E-selectin-Ig chimera; HSPCs: Hematopoietic Stem/Progenitor Cells; mAbs: Monoclonal antibodies; PSGL-1: P-selectin glycoprotein ligand 1; sLe^{x/a}: Sialyl Lewis X/A; GDP: guanosine diphosphate; LacNAc: N-Acetylactosamine; GTs: glycosyltransferases; rhFTVI: recombinant human fucosyltransferase VI; STs: sialyltransferases; BEVS: baculovirus expression vector systems; IMAC: immobilized metal affinity chromatography; MSCs: Mesenchymal stromal cells; HSPCs: hematopoietic stem/progenitor cells; iPSCs: induced pluripotent stem cells; CB: cord blood.

CONFLICT OF INTEREST

The authors declare the following competing financial interest(s): One or more US or international patent applications submitted by the authors relate to this work.

AUTHOR CONTRIBUTIONS

A.S.A. designed, performed and analyzed experiments and wrote the manuscript. K.S., A.J.A., R.Z. and J.M.L. contributed equally to this study. A.S.A. and K.S. designed and performed enzyme expression and purification from the yeast expression system while J.M.L., R.Z. and T.K. characterized the enzyme expression and purification from the silkworm expression system. M.T. helped in discussing and analyzing the enzyme purification from the yeast expression systems. A.J.A. designed, conducted and analyzed work related to the iPS-HSPCs experiments. M.L. and J.C.I.B. supported A.J.A in the culture and derivation of HSPCs from iPS cells. K.S., A.J.A., J.M.L., and T.K. all contributed to the writing of the manuscript. J.S.M. conceived, designed and analyzed this study and wrote the manuscript.

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ACCESSION IDs

The UniProt accession ID for the human fucosyltransferase 6 is P51993. The UniProt accession ID for the *Bombyx mori* Apolipophorin-III is Q17180.

Table I: MS analysis for fractionated bands of purified rhFTVI in yeast system, potential rhFTVI identification and the corresponding peptide sequence identified. Unmatched peptide sequences are summarized in **Table S1**.

	Molecular Weight Range	Protein Probability	Unique Peptide Count	Peptide Sequence	Coverage of peptide sequence, %
rhFTVI produced from <i>Pichia pastoris</i> yeast expression system	~70-kDa (i.e. 60-70kDa)	100%	13	FLPPDAFIHV DDFQSPK	5.2
	~50-kDa (i.e. 45-55kDa)	100%	14	SHKPLPQGT MMETLSR	4.9
	~38-kDa (i.e. 35-45kDa)	100%	8	VYPQADAVI VHHR	4.0

Supporting Information. The Supporting Information is available free of charge and includes:

- Supporting methods: SDS-PAGE and immunoblotting, mass spectrometry, deglycosylation assay, flow cytometry and cell sorting, RT-PCR analysis, Stamper-Woodruff assay and details related to differentiation and analysis of hematopoietic stem cells from induced pluripotent stem cells.
- Supporting figures and tables: outlining details related to results from mass spectrometry analysis, RT-PCR primers, controls to determine enzyme specific activity, cell viability analysis and the maintenance and differentiation capacity of human iPS cells.

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Domain Structures of Human FTVI

Pichia pastoris vector construction rhFTVI (N-tag)

Bombyx mori vector construction rhFTVI (N-tag)

Biochemistry

W.B.: human FTVI

(B)

(B) Biochemistry

(B) Biochemistry

(D)

(D)

(E)

(B)