Expression and Purification of Glycosyltransferases in *Pichia Pastoris*: Towards Improving the Migration of Stem Cells by Enhancing Surface Expression of Sialyl Lewis X

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Recruitment of circulating cells towards target sites is primarily dependent on E-selectin receptor/ligand adhesive interactions. Glycosyltransferase (GTs) are involved in the creation of E-selectin ligands. A sialofucosylated terminal tetrasaccharide like glycan structure known as sialyl Lewis x (sLe\(^x\)), is the most recognized ligand by selectins. This structure is found on the surface of cancer cells and leukocytes but is often absent on the surface of many adult stem cell populations. In order to synthesize sLe\(^x\), GTs must be endogenously expressed and remain active within the cells. Generally, these stem cells express terminal sialylated lactosamine structures on their glycoproteins which require the addition of alpha-(1,3)-fucose to be converted into an E-selectin ligand. There are a number of fucosyltransferases (FUTs) that are able to modify terminal lactosamine structures to create sLe\(^x\) such as FUT6. In this work we focused on expressing and purifying active recombinant FUTs as a tool to help create sLe\(^x\) structures on the surface of adult stem cells in order to enhance their migration.
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Chapter 1: Introduction and Background

1.1 Cellular migration

Cell migration is an important process involved in a variety of physiological and pathological functions such as attracting immune cells to inflammatory sites, migration and engraftment of therapeutic stem cells to their target tissue, and metastasis of cancer cells. The mechanism of delivery of cells to these sites has been well described in the context of inflammation and/or injury (1-3). The is a sophisticated process that is controlled by a number of adhesion molecules including the selectins, chemokines and integrins which all function in a coordinated stepwise manner.

The multistep paradigm of cellular migration is illustrated in Fig. 1.1. Briefly, it begins with tethering and rolling of flowing cells onto the endothelial cells within the vasculature which is mainly mediated by the selectins and their ligands. It causes the tethered cell to roll along the endothelium at a slower speed. Next, chemokine binding to its receptor on the flowing cells leads to integrin activation on the cell in flow. This activation leads to conformational changes in the integrin by inside-out signal transduction events resulting in high-affinity binding of the integrin to their cellular adhesion molecules (CAMs) on the endothelium. This results in firm adhesion and arrest of the cell that was in flow onto the endothelial cells. Ultimately the last step follows where the cell transmigrates to reach the extravascular space. Although each step in this process is important and dependent on the previous step, the interaction of selectins with their ligands are the gatekeepers of the multistep paradigm (4, 5).
Figure 1.1: The multistep paradigm of cell migration that take place between the cells in flow and the endothelial cells lining the blood vessels of an organ or tissue (such as the bone marrow). Step 1: Selectin-dependent tethering and rolling has effect on the cells velocity. Step 2: Integrin activation which controlled by the expression of chemokines that activate integrin conformational changes that result in high affinity binding to its counter receptor on endothelium cells. This leads to Step 3: Firm adhesion and arrest mediated by the integrins; and Step 4: the transmigration of the cell in flow into the intended site. Figure adapted from (4).

1.2 Selectins

Selectins are type-I transmembrane C-type (Ca\textsuperscript{2+}-dependent) lectins that bind to carbohydrate ligands in a calcium-dependent manner. The family includes E-selectin, L-selectin and P-selectin, which all have similar structures (6-8) consisting of an amino-terminal lectin-like domain, an epidermal growth factor (EGF)-like domain, a variable number of consensus repeats (CRs; also, known as complement regulatory proteins or “sushi” domains), a single membrane spanning domain, and a carboxyterminal cytoplasmic domain (Fig. 1.2). The binding of selectins with their ligands mainly depends on the lectin domain. All selectins have affinity towards Sialyl Lewis (sLe\textsuperscript{a})
carbohydrate structures (9). The fucose and sialic acid of this 4-sugar structure provide the negative charge for binding to positively charged amino acids that are found in all selectins. In addition to the sLe\(^x\), P- and L-selectin also require sulfation of nearby tyrosines or sugars respectively.

**L-selectin** is expressed on leukocytes and has a role in migration of naïve lymphocytes to secondary lymph organs via interactions with peripheral node addressins (PNAd) such as CD34, GlyCAM-1, MadCAM-1, and endomucin expressed on high endothelial venules (HEV) (10). Besides, sLe\(^x\), L-selectin also requires sulfation of the 6-hydroxyl group of N-acetyl-glucosamine.

**P-selectin** is expressed inside secretary granules of endothelial cells and platelets, which when activated fuse to a membrane and release the P-selectin onto platelets or inflamed endothelial cells. Meanwhile, it found at low level on bone marrow endothelium, thymus endothelium and skin post capillary venules. Activation is mediated by histamine or cytokines such as IL-1 or TNF-\(\alpha\) and helps to recruit leukocytes to inflammatory sites as well as having a role in promoting blood clotting and wound healing on platelets. The major P-selectin ligand is P-selectin glycoprotein ligand 1 (PSGL-1).

**E-selectin** is a glycosylated glycoprotein expressed on endothelial cells upon activation by inflammatory stimuli. It is also expressed on hematopoietic bone marrow endothelial cells and skin microvessels. It becomes maximally expressed on the endothelium 3-4 hours after activation by IL-1 and TNF-\(\alpha\). E-selectin is the main player in the recruitment of hematopoietic stem/progenitor cells (HSPCs) to the bone marrow (reviewed by Sackstein (11)).
Figure 1.2: The selectin family of adhesion molecules share a common structure composed of five different domains: lectin binding domain, (epidermal growth factor) EGF domain, consensus repeat, transmembrane region and short cytoplasmic tail.

1.3 Selectin ligands

The selectins bind to specified terminal carbohydrate determinants (Fig. 1.3) that are composed of tetrasaccharide sialyl Lewis $x$ (sLe$^x$; or also to its isomer sLe$^a$). sLe$^x$ is a sialofucosylated sugar comprised of a sialic acid linked to galactose in an $\alpha(2,3)$ bond and a fucose linked to a N-acetylglucosamine in an $\alpha(1,3)$ bond. Both fucosylation and sialylation are essential for binding to selectins (12). These determinants could be displayed on either a protein scaffold (i.e., a glycoprotein) or a lipid scaffold (i.e., a glycolipid) and are identified by anti-sLe$^{x/a}$ antibodies such as CSLEX-1, KM93, and HECA-452 (13). Sometimes recognition of ligands by selectins requires additional modifications, such as sulfation, to increase the binding affinity of P-selectin and L-selectin, but this is not needed for E-selectin binding.
E-selectin is constitutively expressed on the endothelial cells lining the blood vessels in the bone marrow. Consequently it plays a dominant role in HSPC homing to the bone marrow (BM). It has also recently been implicated in controlling the quiescence, self-renewal, and chemoresistance of HSPCs (14).

1.3.1 E-Selectin Ligands

Many types of cells express E-selectin ligands including HSPCs, monocytes, eosinophils, neutrophils, memory and activated T-cells, natural killer cells and many types of cancer cells (15). A number of studies have described the role of E-selectin ligands in recruitment of immune cells to inflammatory sites in vivo using ligand or selectin deficient mice. Hidalgo et al. (16) reported that 3 glycoproteins, PSGL-1, ESL-1 (E-selectin Ligand 1), and CD44, were considered active E-selectin ligands on murine neutrophils and implicated that PSGL-1 and ESL-1 were the most prominent ligands involved in neutrophil recruitment to inflammation. I have summarized some of these studies in Table 1.1.

**E-selectin ligands:**

**ESL-1**

E-selectin-ligand-1 (ESL-1) is the major ligand of E-selectin that was first described in 1993 (Levinovitz et al, 1993) and cloned in 1995 by Steegmaier et al. While Hidalgo group (17) characterized the first mice fully deficient in ESL-1 and demonstrated its role in E-selectin binding, rolling, and recruitment of neutrophils to inflamed tissues. ESL-1 acts as an E-selectin ligand on mouse HSPCs (18) but not on human HSPCs (19).
**P-selectin glycoprotein ligand-1 (PSGL-1)** is a type-I transmembrane protein, which has the capability of binding to all three selectins. It is a homodimeric structure with a molecular weight of 240 kDa (dimer) (two 120 kDa monomers that are linked by disulfide bonds) and is found on most leukocytes, CD34+HSPCs and on platelets.

**CD44** is a type-I transmembrane glycoprotein, which is expressed on leukocytes and HSPCs. There is an E-selectin binding glycoform of CD44 known as, hematopoietic cell E- and/or L-selectin ligand (HCELL) that is expressed on the surface of HSPCs and many cancer cells. There is also strong evidence that HCELL functions as E-selectin ligand on activated human T-cells (15) and is likely involved in the recruitment to inflammatory sites (20, 21).

**CD43** is expressed on most hematopoietic cells (22, 23) and has been described as an E-selectin ligand on CLA+ T cells. In addition a recent report (24) illustrated CD43 as an E-selectin ligand on Th17 cells *in-vitro* and *in-vivo*. A recent report from our lab however showed that CD43 is not a major E-selectin ligand on activated human T cells that are primarily of the Th2 phenotype (15).

**CD34** has been found to contain sulfated and fucosylated glycans, sulfation of CD34 has been shown to be required for L-selectin recognition (25). A recent study showed CD34 in hematopiotic stem cells act as active E-selectin ligand (unpublished).
Table 1.1: E-selectin ligands expressed on mammalian cells.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Expression</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESL-1</td>
<td>Hematopoietic progenitors cells.</td>
<td>(17, 26)</td>
</tr>
<tr>
<td>CD34</td>
<td>Human Hematopoietic Stem/Progenitor Cells</td>
<td>(25, 27, 28)</td>
</tr>
</tbody>
</table>
| PSGL-1  | -Platelets (29)  
          -Hematopoietic cells  
          -Some Cancer cells  
          -CD34+ HSPC.  
          -All peripheral leukocytes (30) (31) | (32, 33) |
| CD44    | -Leukocytes, erythrocytes and brain. | (20, 21) |
| CD43    | Hematopoietic cells. B-precursor ALL cell lines (34) | (22-24) |

1.4 The role of glycosyltransferases (GTs) in cell migration

Glycosyltransferases (GTs) act in an assembly line fashion to build post-translational carbohydrate modifications on proteins. GTs are located in the endoplasmic reticulum (ER) and Golgi apparatus of cells. Two main types of glycans exists. N-glycans are found on asparagine residues within a consensus sequence of serine/threonine-X amino acid-asparagine where a N-acetylglucosamine (GlcNAc) residue is linked to the carboxy amide group of the asparagine. O-glycans, on the other hand, occur on serine/threonine residues where a N-acetylgalactosamine (GalNAc) is added to the hydroxyl group of Serine/Threonine residue.
Carbohydrates are involved in many cellular processes, for example, topogenesis, signaling, cell-cell interaction, cell migration, cell growth, differentiation and apoptosis. To understand the role of GTs in cell migration we focus on their contribution to selectin ligands formation through the creation of only sLe\(^x\) and sLe\(^a\) glycan motifs. The knowledge gained about the expression and regulation of GTs plays a critical role in the formation of E-selectin ligands sLe\(^x\)\(^a\) and will significantly help to manipulate the migration of human cells in circulation. Indeed, understanding the specificity of GTs which are required to create E-selectin ligands permitted researchers to manipulate and induce ligand formation on cells \(ex \, vivo\) to help direct migration of therapeutic cells (such as HSPCs) to specific tissues (such as the bone marrow) that express the corresponding selectin counter-receptors (5, 35). Specific GTs involved in selectin ligand formation will be discussed in the following section.

### 1.4.1 Glycosyltransferases (GTs)

The glycosyltransferases catalyze the transfer of sugar residues from nucleotide-sugars to specific acceptor (carbohydrates or glycan chains) according to the following general equation:

\[
\text{Nucleotide-sugar} + \text{Acceptor} \rightarrow \text{Sugar-Acceptor} + \text{Nucleotide}
\]

GTs are type II transmembrane glycoproteins consisting of a short amino-terminal cytoplasmic tail, transmembrane region, an extended stem region and a large carboxy-terminal catalytic domain (36) which is oriented to the lumen of the ER or Golgi apparatus. There are many factors that affect the GTs activity such as, availability of sugar nucleotides, the location of the GTs in the ER/Golgi stacks in relation to the
substrates that it can recognize, and even the posttranslational modification of GTs (37). Most GTs are themselves glycosylated and this is a critical requirement for their activity (38, 39). The display of glycan structures on selectin ligands requires the expression and activity of various GTs, including the action of α1,3- or α1,4-fucosyltransferases (FUT), α2,3-sialyltransferases (ST), β1,4-galactosyltransferases (GalT), and β1,6-N-acetylglucosaminyltransferases (GlcNAcT) (4). Basically, each enzyme catalyzes only one of several sugar nucleotide substrates (including UDP-Galactose (Gal), UDP-Glucose (Glc), UDP-N-acetylgalactosamine (GalNAc), UDP-N-acetylglucosamine (GlcNAc), GDP-fucose (Fuc), GDP-mannose (Man), UDP-xylose (Xyl), or CMP-sialic acid (SA)). In addition, the acceptor for each GT is quite specific with few exceptions and is only capable of forming one particular glycosidic bond (i.e., either an α or β anomer).

Figure 1.3: GTs involved in the formation of sLeα and sLeβ structures on selectin ligands. The expression of the GTs responsible for capping the galactose (Gal) of the type 1 or type 2 lactosamines [Galβ1,4GlcNAc or Galβ1,3GlcNAc] with sialic acid (NeuAc)—sialyltransferases (ST)—and the terminal N-acetylglucosamine (GlcNAc) of the lactosamine with fucose (Fuc)—fucosyltransferases (FUT)—to create sLeβ are often correlated with cells that migrate or home to tissues where selectins are expressed.
In O-glycan synthesis, it starts by the action of GalNAcT enzymes that transfer GalNAc residue to a serine or the threonine on the polypeptide. Then, by the addition of Gal in a linkage β1-3 by β1-3GalT enzyme to form core 1 structures (Galβ1-3GalNAc). After that core 2 branching is formed by the addition of GlcNAc from C2GlcNAcT enzyme action in β1-6 linkage. These branches are then extended by the addition of GlcNAc and Gal alternately to form polylactosamine side chains. In this step, the terminal cap or sialylated Lewis epitope is the most important to determine if the molecule will play a role in cell adhesion selectin ligand activity (40); this is determined by two enzymes α1,3- or α1,4-fucosyltransferases (FUTs) and α2,3-sialyltransferases (STs). This pathway finally ends up with the formation of the O-linked core two based tetrasaccharide sialyl Lewis\( ^{x/a} \), which is composed of sialic acid, Gal, GlcNAc and Fuc. Generally, from STs family ST3Gal-III create sLe\(^a\) by acting on type 1 Lactosamine while ST3Gal-IV and ST3Gal-VI give sLe\(^x\) that mainly act on type 2 Lactosamines. On the other hand, α1,3 fucosylation has imperative role for E-selectin ligand creation. This is outlined above in Fig. 1.3. Such work will be discussed in the following section.

1.5 Fucosyltransferases (FUTs)

The fucosyltransferase family share the same structural characteristics (Fig. 1.4) including a 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 (41). This family
consists of 13 enzymes that have been identified in the human genome and classified either according to the type of linkage, based on the site of fucose addition, into α1,2, α1,3/4, α1,6, and O-FUTs or according to sequence analysis and their similarities (42, 43). FUTs are differentially expressed throughout development and contribute to many biological functions in normal and disease (malignant transformation) settings. The complexity of regulation and expression in FUTs genes are dependent on many factors in a tissue-specific and stage-specific manner (44, 45).

All FUTs enzymes bind GDP-fucose that imply they have the same consensus sequence in donor substrate binding (Lys300) (41). FUTs add fucose on sialylated precursors (42), so they catalyzed the final step in glycoconjugate synthesis resulting in sLe\(^{x/a}\) expression. They transfer the fucose residue from GDP-fucose (donor substrate) to GlcNAc in Gal-GlcNAc-sequences (acceptor substrate) in α1,3/4 linkage (46) to form sLe\(^{x/a}\) that could bind to counterpart selectins (Fig. 1.3).
In mammalian cells, there are six α1,3/4 FUTs, FUT3–7 and FUT9 (or Fuc-TIII–VII and Fuc-TIX), all of which have α1,3 activity, but FUT3 and FUT5 also have α1,4 activity (43, 45). There is about 85% sequence identity shared between FUT3, 5, and 6. FUT10 and FUT11 were found in the human genome by comparison with fucosyltransferase sequences in the Drosophila melanogaster genome (47) but this needs further studies to determine their function and the acceptor specificity. In addition, FUT4 and FUT7 are the only human FUTs enzymes expressed in leukocytes to create functional selectin ligands. FUT6 (48) is expressed in epithelial cells, liver, kidney and gastrointestinal tissues but not detected in brain or leukocytes. FUT9 is expressed in stomach, spleen, blood cells and brain (49). While FUT1 and FUT2 have α1,2 activity FUT1 and FUT2 and their primary function is to synthesize H blood group antigens and related structures (50). The only enzyme that has α1,6 activity is FUT8 (51).

1.5.1 Fucose

Fucose is a deoxyhexose, monosaccharide sugar that is expressed on many N- and O-linked glycans on mammalian cells. Fucose lacks a hydroxyl group on the carbon at the 6-position (C-6), it has the L-configuration and is frequently found as a terminal modification on glycan structures. GDP-fucose has two pathways for synthesis, the GDP-mannose-dependent de novo pathway and the free fucose-dependent salvage pathway (42). FUTs use GDP-fucose as donor substrate and as a result it plays a significant role in fucosylated glycans and its deficiency leads to such abnormal phenomena or disease as leukocytes adhesion deficiency type II (LADII) syndrome.
1.5.2 Fucosyltransferase 6 (FUT6)

Selectin ligands must be α1,3-fucosylated to form the terminal glycan sLe\(^x\) determinant. FUT6 like other glycosyltransferases are resident membrane within the Golgi apparatus. FUT6 encodes the major α1,3fucosyltransferase activity in human plasma and putatively released from the liver and kidney and FUT6 was found to be the only FUT expressed in Hep-G2, human hepatocyte/liver carcinoma, cells (Borsing, 1999) (52). FUT6 is extensively expressed in epithelial cells and gatroentisinal tissue specifically, stomach, jejunum, and colon and also abundantly in brain, lung and cervix uteri. For acceptor substrate specificity, FUT6 has a preference for N-Acetyllactosamine (Galβ1→4GlcNAc) and also good specificity towards 3′-Sialyl-N-acetyllactosamine, (NeuAcα2→3Galβ1→4GlcNAc) (48, 52, 53). Another classification of fucosyltransferases is made according to whether they are sensitive to inhibition by N-ethylmaleimide (NEM) (a cysteine-modifying reagent); FUT6 is sensitive to NEM which results in more than 98% FUT6 inhibition (54). FUT6 has five N-linked glycosylation sites, five cysteines within the catalytic domain which are either free or involved in disulfide-linkages. FUT6 can synthesize three type 2 Lewis antigens, Le\(^x\), Le\(^y\) and sLe\(^x\), and exhibits the strongest α1,3 FUTs activity among the five α1,3FUTs.

FUT6 was first purified from human liver which involved many successive purification methods including chromatographies on CM-Sephadex, Phenyl Sepharose, GDP-hexanolamine- Sepharose, and HPLC and specific antibodies directed to FUT6 (53) (55). FUT6 is expressed in many expression systems beginning with the prokaryotic system up till higher order eukaryotic systems such as yeast, insect, and mammalian cells. In more
recent years, purified FUT6 has been used to create sLex structures on therapeutic cells such as mesenchymal stem cells (35, 56) and HSPCs (57, 58) to promote their migration to target organs. Furthermore some studies have focused on FUT6’s expression level in disease (59) in order to target its activity.

1.5.2.1 FUT6 gene and protein

The human FUT6 gene is located on chromosome 19p13.3 and it has six exons. It encodes for a 359 amino acids including N-terminal region that is composed of the cytoplasmic sequence, signal-anchor for type II membrane sequence while the C-terminal region consist of luminal sequence that contains catalytic domain (composed of 325 aa), the third part between the membrane-spanning region and catalytic domain is a region called stem region as in (Fig. 1.4). FUT6 exceeds the size of FUT3 by 15 amino acids. To determine which portion is required for activity, there were a lot of truncation studies of FUTs that demonstrated the N-terminal region is not required for activity, so it can be deleted without any effect on enzyme activity while any change in C-terminal region results in the production of an inactive enzyme (60).

1.5.2.2 Effect of glycosylation on FUT6 enzyme activity

There were many studies that have exhibited the impact of glycosylation on FUT activity. FUTs harbors N-linked glycosylation sites (Asn) (61); FUT6 has three sites for glycosylation. Although Asn 105 was not glycosylated when expressed in COS cells, the other two sites were glycosylated and these sites are conserved for other FUTs such as FUT3 and FUT5 (61). Indeed glycosylation inhibitors and site directed mutagenesis at
these Asn sites resulted in lower activity and reduction in molecular weight (39) demonstrating that N-glycosylation is critical for optimal activity of FUT.

1.5.2.3 Recombinant FUT6

It is possible to use genetic approach to introduce GTs genes into cells to form the glycoconjugates on the cell surface for E-selectin ligands formation. The transfection of GTs genes into cells could be either transient or stable. More recently, studies have focused on using these enzymes ex vivo to treat cells in a more transient, simple and efficient manner (4, 35, 62-64).

Along with the advancements in recombinant DNA technologies to express and produce target protein with large yield, using GTs to manipulate glycans became much more practical. In order to produce soluble form of FUT6 that has been used extensively in the past years (53) by replacing cytoplasmic tail and transmembrane region with a cleavable signal sequence, this resulted in efficient transport of membrane bound GTs into the extracellular space as an active soluble enzyme. The enzyme activity and the primary structure could be significantly affected by removal and addition of amino acids from either the N- or C-termini, as in some glycosyltransferases the C-terminus has been found to be sensitive to any changes in primary structure. Several mammalian Golgi glycosyltransferases, β-1,4-galactosyltransferase, α-2,6-sialyltransferase and α-2,3-sialyltransferase, have been shown to misfold in the yeast ER when expressed as full length proteins (65). Release of FUT6 upon cleavage was accompanied by a reduction in molecular mass of the enzyme and it has been speculated that FUT6 occurs in two native
MW forms 47 kDa and 43 kDa, as well as a 43 kDa secreted form. These differences in MW could also be related to different posttranslational modifications. In addition, the position of tag that is used in the purification step is crucial, since correct position of the fusion purification tag with respect to the catalytic domain of FUT6 is important to promote the proper folding of FUT6. Another major concern in GTs activity during purification is the requirement of divalent cations for their catalytic activity (66). The different expression systems that could be used to express recombinant GTs will be discussed in the following section.

1.6 Recombinant FUTs protein in many expression systems

FUTs enzymes can exist naturally in the human body in a soluble form. FUT6 has been found naturally in body fluids such as amniotic fluid, milk, serum, and semen (Mollicone et al, 1990). There are different expression systems that could be used to express functional GTs including bacterial cells, mammalian cell lines (67), baculovirus-infected insect cells (68, 69), yeast cells (70), and even silk worm (71). The choice of the system to use depends on many factors such as genetic stability, optimal folding and posttranslational modifications that could affect the function of the recombinant protein and also biosafety. In addition, the yield produced with the ability to scale it up because secreted proteins concentration will become more with increase cell density, and the time requirement for protein production. Over the past decade, scientists started to produce recombinant GTs, especially fucosyltransferases, in different expression systems. The majority of the α3-FUTs activity in plasma is due to α3-FUT6 which is encoded by the FUT6 gene (Brinkman et al., 1996). They obtained very similar acceptor specificity profile
with cloned α3-FUT6 expressed in a mammalian system in CHO cells (53), COS cells (48), or insect cells (72).

Although relatively practical and simple with large potential yields, bacterial expression systems do not result in enzymatically active GTs likely due to the absence of glycosylation machinery required for enzymatic activity i.e. N-glycosylation (36, 38, 39, 61). The yeast, *Pichia Pastoris*, expression system has been used to express many glycosyltransferases involved in the biosynthesis of N- and O-linked oligosaccharides (70, 73). This is summarized in Table 1.2. In summary, the choice between expression systems depends on many factors, the nature and use of the recombinant protein, and the related production costs. Yeast and silkworm expression systems combine the ease, simplicity and cost effectiveness of bacterial systems to the high quality post-translationally modified protein of mammalian systems.

**Table 1.2:** The expression of recombinant GTs in different expression systems.

<table>
<thead>
<tr>
<th>Glycosyltransferases</th>
<th>Expression system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1,4-Galactosyltransferase</td>
<td><em>Pichia pastoris</em></td>
<td>(73, 74)</td>
</tr>
<tr>
<td></td>
<td>Insect cells</td>
<td></td>
</tr>
<tr>
<td>α-2,6-Sialytranseferase</td>
<td><em>Pichia pastoris</em></td>
<td>(73, 74)</td>
</tr>
<tr>
<td>α-1,3-Fucosyltransferase3</td>
<td>Baby Hamester Kidney cells IBHK-21B)</td>
<td>(75) (76)</td>
</tr>
<tr>
<td></td>
<td><em>Pichia pastoris</em></td>
<td>(77)</td>
</tr>
</tbody>
</table>
|                              | CHO cells                              | (Auge, 2000)
<p>|                              |                                        | (Auge, 2000) |</p>
<table>
<thead>
<tr>
<th>Enzyme Type</th>
<th>System</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-1,3-Fucosyltransferase5</td>
<td>Insect and Mammalian systems</td>
<td>(78)</td>
</tr>
<tr>
<td>α-1,3-Fucosyltransferase6</td>
<td><em>Pichia pastoris</em> Insect cells</td>
<td>(79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Borsig, 1998)</td>
</tr>
<tr>
<td>α-1,3-Fucosyltransferase7</td>
<td>Insect cells Yeast cells</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Smithers., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Salo., 2004)</td>
</tr>
<tr>
<td>α-1,3-Fucosyltransferase9</td>
<td>Insect cells Hela Cells</td>
<td>(74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Seelhorst K, 2013)</td>
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<tr>
<td></td>
<td></td>
<td>(Brito et al., 2007)</td>
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<tr>
<td></td>
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<td>(Toivonen et al, 2002)</td>
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<tr>
<td></td>
<td></td>
<td>(Brito et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Stack et al., 2010)</td>
</tr>
<tr>
<td>α-2,3 sialyltransferase</td>
<td>Yeast cells</td>
<td>(74)</td>
</tr>
<tr>
<td>ST3GalIII</td>
<td></td>
<td>(Mattila., 1996)</td>
</tr>
<tr>
<td>α-2,6 sialyltransferase</td>
<td>Yeast cells</td>
<td>(74)</td>
</tr>
<tr>
<td>α-2,6 sialyltransferase</td>
<td>Yeast cells</td>
<td>Malissard (1998)</td>
</tr>
</tbody>
</table>
1.6.1 Yeast expression system

Two yeast species have mainly been used, i.e. *Saccharomyces cerevisiae* and *Pichia pastoris* (80) to express mammalian proteins. Both of them have been used to produce glycosyltransferases on a large scale (74, 81-83). With *S. cerevisiae* cells, GTs were expressed and immobilized at the cell surface through the function of yeast cell wall protein Pir, and can then be used as an enzyme source Pir anchor proteins to immobilize the target proteins at the yeast cell surface. Although many advantages exist for the use of *S. cerevisiae* to express GTs, a major limitation due to the synthesis of polymannans chains (50 to 100 mannose residues) could impair protein activity as well as non-human glycosylation patterns could be potentially immunoreactive (84). *Pichia pastoris* tends to be a more favorable candidate to express GTs (85) as it has the ability to overcome the hyperglycosylation of recombinant proteins, the possibility to secrete soluble forms of proteins and has been used to produce proteins for many therapeutic purposes (70, 86) (35, 87-89). Many glycosyltransferases have been expressed successfully in this yeast system and are summarized in Table1.2.

The cultivation of yeast cells requires its own growth media (84, 90) either in shaker flasks or fed-batch cultivation with fermentation (73). The construct design should focus on the selection of promotors (induced or constitutive) and whether the target protein will be expressed intracellularly or extracellularly (i.e. released into the supernatant/media).

There are two phases for methylotrophic *P. pastoris*. It begins with the growth phase to produce high growth rates (>100 g/l on glycerol media) and then production of target protein at low growth rates by induction with methanol as carbon source at an optimum
temperature. The selection of adequate yeast promoters, secretion signals and selectable markers have been reviewed (73). In summary, the yeast expression system is cost-effective, easily transformed and simple to cultivate with safe nutrients and growth media (90). It is considered a suitable host for the study of enzymes relevant to the glycosylation pathway and can be used to express glycosyltransferases from a variety of eukaryotic organisms but it is necessary to evaluate each protein independently since each protein has its own requirements for folding, glycosylation and maturation-associated cleavage. Extensive studies are important for comparative evolution and some novel protocols - PichiaPinkTM- could be followed (91).

1.7 Medical target and application of recombinant FUTs

Most intravenous therapeutic adult stem cells have limited engraftment efficiency to their target tissue due to lack of key homing molecules. In such cases, these therapeutic cells require powerful methods to improve homing. Glycan-Engineering methods could be used to create glycan structures such as sLe\(^x\) and sLe\(^a\) on the cell surface in order to guide the delivery of cells to their target tissues where specific selectins are expressed. GTs can be introduced genetically or by using recombinant GTs ex vivo. Furthermore, since the sLe\(^x\) determinant synthesized by the GTs plays an extensive role in cancer metastasis, direct suppression of enzyme activity would be valuable for cancer treatment.
1.7.1 FUTs application in clinical therapeutic

As discussed previously, from 13 human FUTs genes, about eight of them have $\alpha$-1,3-fucosyltransferases activity, which has a strong potential to form sLe$^x$ structures as the last step in the pathway (42, 92). So, targeting these enzymes as a potential candidate could help. Firstly, if they intended to use in a direct injection of therapeutic cells to target tissue sites then they will have a role in sLe$^x$ formation and enhance binding to E-selectins. Secondly, inhibiting FUTs in many types of cancers such as prostate cancer cells, and gastric cancer cells, could help inhibit metastasis since it relies on selectin-selectin ligand interactions (93-96). Glycosyltransferases should be endogenously expressed and active to create sLe$^x$ structure. The understanding of these GTs and which are required to synthesize a particular structure allows researchers to do many studies for cell manipulation and enzyme ex vivo treatment to modify and express the selectin ligands structure (62, 64).

*Enhancing Fucosylation*

Selectin ligands should be $\alpha$-1,3-fucosylated to form the terminal sLe$^x$ epitope and usually stem cells lack expression of these enzymes. Extensive studies using HSPCs (from human cord blood) has demonstrated improved migration and engraftment of these cells following ex vivo treatment with either FUT6 or FUT7 (57, 58, 97-99). The activity of FUT6 and FUT7 could be different since FUT7-treated HSPCs showed much greater binding to P-selectin than the FUT6-treated cells (98). FUT6 treated mesenchymal stem
cells enhanced their trafficking to bone (35) whereas FUT6 treated neural stem cells lead to improved migration to spinal cord and brain in multiple sclerosis models (89). In kidney injury, engineering the glycan of CD44 on MSCs through FUT6 transfection enhanced renotropism and the regenerating ability of MSCs in ischemic kidney injury (56). While (100) showed both the transfection of MSCs with FUT6 or ex vivo treatment had similar results in increased in cell surface E-selectin ligand.
**Aim of study:**

The general aim for this research is to identify a new source for production of functional fucosyltransferase 6 (FUT6) enzyme using an eukaryotic expression system. Since FUT6 has a critical role in catalyzing the final step in glycoconjucate synthesis for cell adhesion activity, it will be promising to utilize this enzyme in creating relevant selectin-binding glycan determinants on the cell surface Sialyl Lewis x (sLe\(^5\)). Following are the specific aims of this research:

**SPECIFIC AIM (I):**

To express, purify and characterize human FUT6 using the *Pichia pastoris* yeast expression system.

**SPECIFIC AIM (II):**

To treat the model cells *ex vivo* using the recombinant FUT6 generated in Specific Aim 1 for glycan engineering of cell surface glycoproteins and to investigate the effect treatment on selectin binding.
Chapter 2: Material and Methods

2.1 Construction FUT6 recombinant vector and transformation to Pichia Pastoris

Briefly, a pPink-αHC vector was used (Invitrogen) to integrate the human FUT6 cDNA encoding amino acid 35-359 of the FUT6 protein sequence that omits the cytoplasmic and transmembrane regions of full length human FUT6, and encompassed the entire catalytic domain of the enzyme. The vector was propagated in E. coli strain TOP 10F (Invitrogen). The recovered DNA was linearized with restriction enzyme and then the digested DNA were used to transform Pichia Pastoris strains according to the manufacturer’s instructions (Invitrogen). Stable transformants were selected on minimal medium agar plates (MD plates) for further processing.

2.1.1 Construction of recombinant vector and transform to E. coli cells

A) The cDNA encoding soluble form of human FUT6 were generated by PCR with FUT6 primers and the FUT6 contained six histidine (His-tag) at N-terminus and must have a phosphorylated 5’ blunt end (adding an Mly I site) and a 3’ overhang after the stop codon that is compatible to the restriction enzyme used to linearize pPinkα-HC (Kpn I). FUT6 lack any internal restriction site for Mly I and the restriction enzyme used.

B) pPink-αHC vector was used from (Invitrogen) to subclone the human FUT6 open reading frame (ORF) downstream of the α-mating factor pre-sequence. The PichiaPink vectors contain the ampicillin resistance gene to allow selection of the
plasmid using ampicillin. About 0.5 μg/μl vector linearized by double digestion with 10 units/μL Stu I restriction enzyme (created a blunt end) and 10 units/μL Kpn I restriction enzyme in the multiple cloning site downstream of the Stu I site that does not cut within FUT6. It was incubated for 2 hours overnight at 37°C then added Calf Intestinal Alkaline Phosphatase (CIAP) (1 unit/μL) to dephosphorylate the vector and then we checked the proper digestion by a gel.

C) Ligation of vector with human FUT6 cDNA

We set up a ligation reaction in a 0.5 mL micro-centrifuge tube by gently mixing 2μL of 5X ligase buffer, 0.5 μL of T4 DNA ligase 1 μL of 20 ng/μL pPinkα- HC and 1μL of 20 ng/μL of FUT6 gene and then centrifuged briefly, and incubated the mix at 25°C for 1–2 hours, and/or at 16°C overnight.

![Figure 2.1: pPink-αHC Plasmid map and integration of human FUT6 sequence to the plasmid with specific digestion with assigned restriction enzymes, GOI: is human FUT6. Picture from Invirogen.](image)

D) Transformation to E. coli: This step was to analyze the transformants for the presence and proper orientation of FUT6 gene. pPinkα-HC contains Ampicillin resistance gene,
following the pichiapink protocol for transformation step by electroporation with 0.1 cm cuvette, and plating the E.coli cells in LB agar contain ampicillin including, one plate for cells only and one for vector only as a control. To identify the correct clone, we picked up 6-8 colonies per plate and analyze positive colonies by PCR and sequencing. We purified the colony and make a glycerol stock for long term storage. The plasmids were isolated from E. coli by using PureLink Quick Plasmid Miniprep Kit to transform them to pichia strain.

2.1.2 Transformation to Pichia Pastoris strain

Pichia PinkPlasmid DNA was purified and linearized before transformation and selection in PichiaPink strains from (invitrogen). First, we prepared PichiaPink Strain 1 by placing it in YPD media and then we used 5–10 μg of plasmid per transfection that linearize within the TRP2 region of PichiaPink vectors each transformation by electroporation. The wild-type ade2 knockout Pichia strain was used. The ade2 knockout renders the PichiaPink strain an adenine auxotroph, which requires an external adenine source for growth and the pichiaPink vector had this ade2 gene. These cells are unable to grow on minimal medium or adenine dropout medium unless it adequate transform with recombinant vector. We included the "vector only" and “cells only” controls to evaluate the experiment. The positive transformants were identified by direct PCR screening. The transformants were plated into yeast agar plate minimal media called synthetic dropout that lack only one nutrient as Adenin and another nutritional agar YPD contained 1% yeast extract, 2% bactopeptone and 2% Dextrose. Then incubated for 2-3 days at 30°C.
2.2 FUT6 Expression in *Pichia Pastoris*

**Growing and expression in Shake Flask Cultures**

By using BMGY and BMMY (buffered complex glycerol or methanol medium), for expression of FUT6. These media are buffered with phosphate buffer and contain yeast extract and peptone to stabilize secreted proteins and prevent or decrease proteolysis of secreted proteins. All expression is done at 30°C, in a shaking incubator at 200 rpm. Buffered Glycerol-complex Medium and Buffered Methanol-complex Medium (1 liter) composed of 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0 1.34% YNB, 0.0004% biotin and 1% glycerol or 0.5% methanol.

The growing and expression required around 12 days. By using a single colony, inoculated 100 mL of BMGY medium in a 1L baffled flask. Then we grew the cells for 1–2 days at 30°C in a shaking incubator set 200 rpm. After the incubation we transferred the cells to a 1L of BMGY incubate another day with the same condition. Then in the following day we divided the cells into five/2L flasks of BMGY for two days in the same condition (30°C and 200 rpm). The approximate number of cells in a culture determined with a spectrophotometer by measuring the optical density (OD) at 600 nm. After that pellet the cells in a sterile centrifuge bottle at 3000×g for 15 minutes and decant the BMGY supernatant and resuspend the cell pellet in two/ 1 L of BMMY medium to induce expression. We returned the cells in BMMY medium to the 30°C shaking incubator for seven days and add 0.5% methanol daily which consider as inducer. In day 8 of induction we harvested the cells by centrifuging for 15 minutes at 1,500 × g.
2.3 FUT6 Purification

Intracellular FUT6 was purified by using immobilized metal affinity chromatography with His-Tag column. 2L of the culture were centrifuged for 15 min at 3000 g and 4°C and the pellets were resuspended in lysis buffer containing 100 mM KPi, 500 mM NaCl, 5 mM MnCl2 and 1 mM PMSF. PH=7.8 along with the EDTA free protease inhibitors. This and all the following procedures were carried on ice or at 4°C. After the lysis by cell disrupter machine with 40psi we added 10 mM MnCL2 and 2.5 mM imidizole to the 300mL lysate. The lysate was collected and centrifuged in 50 mL tubes for 2 h at 3000g. The supernatant was collect as it contained the crude extract and incubated with 2 mL of Ni-NTA agarose resin (Thermo Fisher Scientific) for 2 h at 4°C (patch procedure). After the incubation, the resin was collected by low speed centrifugation and loaded to the polypropylene columns 100/pk from (Pierce Thermo), equilibrated with the same binding buffer and collected the flow through. Then, 50 mL of washing buffer were added to the column this buffer contained 20 mM Tris-HCL. PH=7.8, 500 mM NaCl, 10% glycerol, 2 mM MnCl2 and 5 mM imidizole. After that the bound protein with resin were 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 imidizole that added to the column. The elution fraction was concentrated to 0.25 mL by using Amino Filtration tube 10kDa. All the fractions including crude extract, flow through, washing fraction and elution fraction were checked using 4-20% SDS-PAGE.


2.4 Determination of protein concentration

The protein concentration was determined by using bovine serum albumin as standard. We prepared 9 of defined albumin concentration, and then we run the same volume in SDS-PAGE gel with recombinant FUT6. After that we measured the intensity profile of the bands by Image J software and blotted the curve (intensities with concentration). We calculated FUT6 concentration by using the equation from the curve.

2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The proteins were separated on 4-20% SDS PAGE Criterion Tris Glycin Precast Protein Gels (Biorad). The samples (crude extract, washing fraction and elution fraction) mixed with 1x NuPAGE LDS Sample Buffer (Invitrogen) and 5% betamercaptoethanol as reducing agent then heated 10 min at 75°C. After that it loaded in the gel using 20 uL and for 45 minutes at 120V. Gels were stained with SimplyBlue Safe Stain (Invitrogen) for one hour and distained with water for another one hour.

2.6 Immunoblotting

A polyacrylamide gel was runnig using elution fraction, transferred by electroborted on PVDF membrane at 0.39A for 1:20h. The resulting membrane was blocked with Tris Buffer Saline Tween-20 (20mM Tris, 137mM NaCl, 0.1% Tween-20, PH 7.6) containing 5% non-fat milk 1-2h at room temperature and then incubated with anti-FUT6 antibody (1:1000; Abcam) 1h at room temperature, followed by incubation in secondary goat anti-
rabbit–horseradish peroxidase (1:20000) antibody. Detection was performed using standard chemilumiscence method by incubate the membranes with chemiluminescence reagent for 5 minutes. The same procedure was done with K562 lysate treated with FUT6 that then incubated with chimera E selectin-Ig.

2.7 Mass spectrometry for purified FUT6 protein

Mass spectrometry Sample Preparation (MS): Briefly, eluted fraction was separated using 4-20% SDS- PAGE gels and the protein bands stained visualized by Commasssee Stain. After that, we cut all bands that are in range of 70 kDa, 50 kDa, 37 kDa, 25 kDa and 15 kDa. The fractionated bands distained using distaining solution, the gel incubated with trypsin overnight at 37°C. The resulting peptides extracted using extraction buffer that contains 5% acetonitrile, 95% water, 0.1% formic acid. The peptides dried using speed vacuum until approximately 1ul sample volume. The peptides fractionated by nano-flow LC and analyzed using a LTQ Orbitrap Mass Spectrometer.

2.8 Deglycosylation assay

FUT6 was treated with 20mU/ml Peptide -N-Glycosidase F (PNGase-F, Biolabs). The reaction started by denaturation with 1X Glycoprotein Denaturing Buffer (0.5% SDS, 40 mM DTT) at 100°C for 10 minutes. After the addition of NP-40 and GlycoBuffer 2, two-fold dilutions of PNGase F were added and the reaction mix was incubated for 1 hour at 37°C. As a control, each treatment was performed under the same conditions with no added enzymes. Separation of reaction products were visualized by SDS-PAGE with comparing with untreated FUT6.
2.9 Fucosyltransferase assay

The specific activity of the purified FUT6 enzyme was determined by using the Glycosyltransferase Activity Kit (Promega), as per the manufacturer’s instructions. Briefly, 5 µL of recombinant FUT6 with serial dilution in six wells, 250µM GDP-Fucose (Sigma), 125µM of N-acetyl-D-lactosamine (Sigma) were mixed in 20 µL reaction buffer (25 mM HEPES, 5mM MnCl₂, pH 7.5 and HBSS) and one reaction well with no FUT6 used as a negative control. After that we incubated in a 96-well plate at room temperature for 1h. Then we added equal volume 25 uL of GDP detection reagent in each well to convert GDP that generated from the reaction to ATP and measured the ATP using luciferase/luciferin reaction. The light generated was determined using a luminometer. Luminescence correlated to GDP concentrations by using a GDP standard curve. All measurements were performed in duplicate.

2.10 FUT6 exofucosylation

K562 (human Erythroleukemia cell line) cells from (ATCC) were harvested, washed 2x with HBSS, and resuspended at density of 1x10⁶ cells/ml in FUT6 reaction buffer, containing 25mM HEPES pH 7.5 (Gibco Invitrogen), 0.1% human serum albumin (Sigma-Aldrich), 2.8mM GDP-fucose (Sigma), 5 mM MnCl₂ , and 150 mU/mL purified FUT6 enzyme in Hank’s Balanced Salt Solution (HBSS). Cells were incubated at 37°C for 30 minutes. We used buffer only controls excluding the FUT6 enzyme from the reaction as negative control. After the reaction, the cells were washed 2x with HBSS and used immediately for downstream experiments.
2.11 Flow Cytometry

Treated and un-treated (negative control) K562 cells were added in 96 wells plate and stained with Cutaneous Lymphocyte Antigen (PE-HECA-452) antibody to estimate the expression of sLe^x structure on the surface of K562 at a concentration of 1 μg/mL for 30 minutes at 4°C. After the incubation, we harvested the cells and suspended in FACS buffer continued 10 mM EDTA, 5% FBS and HBSS to wash them twice with 200 uL/well then analyzed for surface-marker expression using FACSCanto II platform and FlowJo software.

2.12 Immunoprecipitation of E-selectin ligands

The cell lysates from FUT6-K562 cells and untreated cells were precleared by incubating it with 30 μL Dynabeads Protein G for 2 hours at 4C with constant rotation to remove any non-specific binding between the Dynabeads and the lysates. Then the lysates immunoprecipated with incubated CD44mAb, CD43mAb and PSGL-1mAb separately with Protein G overnight at 4C. The supernatant was collected to verify the efficiency of IP while the complex lyasate-antibody-beads washed three times with lysis buffer. Then the complex resuspended in 2x LDS and 10% β-mecabtoethanol, followed by 10 minutes heating at 75C. The samples then were applied to western blot analysis by using E-Ig. (Refer to Western blot protocol above)

2.13 Stamper-woodruff assay

E-selectin was spotted on glass slides for 4 hours at 4°C and fixed with 3% glutaraldehyde followed by 0.2M lysine blocking then the slides were incubated in RPMI
1640, 5 mM CaCl2 and 2% FBS until the analysis. Treated and un-treated K562 with FUT6 were washed with HBSS, we cyto spin the cells on slides that coated with E-selectin for 30 minutes at 4°C and allowed to interact for 30 mins at 4°C with mild rotation (80 rpm). To exclude the possibility that the treated K562 are interacting non-specifically with E-selectin, EDTA reaction was used as a negative control for the binding.
Chapter 3: Results

3.1 Determining the transformation efficiency of FUT6-pPinkα-HC vector

*Pichia Pastoris* cells were plated on two different agars, one containing Yeast Extract Peptone Dextrose (YPD) and the other containing Dextrose-YNB medium without Adenine. Using the FUT6-pPinkα-HC vector constructed as described in Chapter 2, stable transformations were made. White and slightly pink colonies were observed on a selection plates upon transformation (*Fig. 3.1*). The pink colonies expressed low levels of the ADE2 gene product, while the white colonies expressed higher amounts of the ADE2 gene products (*Fig. 3.1*).

![Figure 3.1: Colony transformation of FUT6-pPinkα-HC vector into *Pichia Pastoris*. A: a modified synthetic agar dextrose-YNB agar without Adenin. B: Yeast Extract Peptone Dextrose (YPD) agar. Left, agar with parent *Pichia Pastoris* cells with integration of empty vector; right, agar with *Pichia Pastoris* cells with integration of FUT6-pPinkα-HC vector.](image)

White colonies were selected in order to more reliably ensure that the gene was integrated. It should be noted that the ADE2 gene on the plasmid enabled the *Pichia Pastoris* to grow on minimal medium lacking adenine whereas the parent *Pichia Pastoris*
cells would not grow on this minimal medium (Fig. 3.1A, left). 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 promotor AOX1 gene as explained in Chapter 2.

3.2 Expression and purification of FUT6

Recombinant Pichia pastoris strains designed as 6His-FUT6 were generated as described in Chapter 2. Small and large-scale cultures were grown and induced with 0.5% methanol. Lysates were then prepared according to Section 2.2 in Chapter 2. The expression levels of functional FUT6 enzyme in 0.5% methanol-induced recombinant Pichia pastoris cells were found to be highest on the seventh day following induction in BMMY media (data not shown). Cell lysates were then applied to 2 mL Ni-NTA agarose resin affinity column to trap histidine (His tag on FUT6). The columns were washed and the captured enzyme was then eluted using 250 mM imidazole and 400 mM imidazole consecutively. Prior to dialysis, the fractions were concentrated 30 fold. The eluted concentrated fractions were then run along with the crude extract, flow through and washing fractions on an SDS-PAGE gel as illustrated in Fig. 3.2. In addition a fraction from the media was also run in order to determine whether the enzyme was secreted or retained with-in the cells (data not shown).
Figure 3.2: Purification of FUT6 expressed in *P. pastoris*. Neat (A) and 60-fold concentrated (B) samples relating to the purification of histidine tagged FUT6 enzyme were run on a 4-20% polyacrylamide gel and stained with Coomassie blue. 1, protein ladder; 2, crude extract; 3, Flow through; 4, 5 mM imidazole washing fraction; 5, 250 mM imidazole elution fraction; 6, 400 mM imidazole elution fraction. The arrows refer to the potential molecular weight of the FUT6 enzyme.

Following the concentration of the eluate, the FUT6 enzyme appeared to be considerably purified following the elution using 250 mM imidazole (indicated by the arrows in Fig. 3.2 B). However, the sample contained some impurities and was not detected in the culture supernatant. The FUT6 enzyme was localized within the *Pichia pastoris* cells and was not secreted into the media (data not shown).

### 3.3 Recombinant FUT6 enzyme characteristics

In order to detect and identify recombinant FUT6 protein and to determine its molecular weight, we utilized two methods, one immunoblotting with anti-Fut6 and other was mass spectrometry (MS).
3.3.1 Determination of recombinant FUT6 molecular weight

To determine the molecular weight of FUT6 expressed by *Pichia Pastoris*, a Western blot was performed on the eluted protein and stained with FUT6 antibodies and anti-His antibodies as described in Section 2.6 of Chapter 2. Detection using anti-FUT6 antibody revealed a pattern of two major bands with molecular weights corresponding to 47 kDa and 43 kDa and two minor bands at 40 kDa and 37.5 kDa (Fig. 3.3). Both representing putative degradation products appeared as about 48 kDa protein and 37 kDa. Interestingly, a 70 kDa band was observed using the anti-His antibody (Fig. 3.3)

![Western blot analysis of purified FUT6 protein from *P. pastoris* cultures.](image)

Since the expected molecular weight of secreted FUT6 calculated from the amino acid sequence of the cloned enzyme is 38 kDa and in our case the FUT6 was expressed intracellularly the expected band should be around 50 kDa. We tried many FUT6
antibodies and the results were not consistent. Therefore we opted to identify the protein product using a mass spectrometry approach in order to confirm the purification of the FUT6 enzyme as well as confirm the results obtained using Western blotting.

3.3.2 FUT6 identification by Mass spectrometry

Purified FUT6 was run on an SDS-PAGE and the bands were prepared (Fig. 3.4) as described in Section 2.7 of Chapter 2. The raw data was converted to Mascot Generic Format files and a search using the online Mascot database was performed.

Figure 3.4: SDS-PAGE Coomassie stained gel of purified FUT6 protein prepared for Mass Spectrometry analysis. Coomassie stained bands indicated in the figure (70 kDa, 50 kDa, 37 kDa, 25 kDa and 15 kDa) were cut out, destained and incubated with trypsin overnight at 37 °C to prepare peptides for mass spectrometry analysis.
The MS analysis suggested that the FUT6 protein was found corresponding to molecular weights 75 kDa, 48 kDa and 37 kDa with 52%, 56% and 32% coverage respectively. FUT6 was not detectable at 25 kDa and 15 kDa bands. According to the Western blot in Fig. 3.3 these molecular weights indicate that the bands just below 50 kDa and at 37 kDa are likely FUT6 protein.

### 3.3.3 Determination of FUT6 protein concentration

Recombinant FUT6 protein concentration was calculated using bovine serum albumin standards. Since the FUT6 appears to correspond to different molecular weights, this required a reliable method for concentration estimation. SDS-PAGE was performed (Fig. 3.5) and the intensity profile was blotted against the defined concentration of albumin (Fig. 3.6).

**Figure 3.5: Determination of FUT6 concentration using BSA standards.** A range of known concentrations of BSA were used to determine the concentration of FUT6 in the purified eluate. The SDS-PAGE gel was stained with Coomassie in order to highlight the protein bands. Lane 1: protein ladder; Lane 2: 2 mg/mL BSA; Lane 3: 1.5 mg/mL; Lane 4: 1.0 mg/mL; lane 5: 0.750 mg/mL; Lane 6: 0.500 mg/mL; Lane 7: 0.250 mg/mL; Lane 8: 0.125 mg/mL; Lane 9: 0.025 mg/mL BSA. Lane 10 corresponds to 10 μL of the purified recombinant FUT6.
Recombinant FUT6 concentration was calculated using linear regression equation from BSA standard titration $Y = 262424x + 3473.9$. 75 kDa band concentrations were $\sim1.97$ mg/mL, 50 kDa band was $\sim1.1$ mg/mL and the 37 kDa band was $\sim0.8$ mg/mL. Total FUT6 concentration was found to be $\sim4$ mg/mL.

3.4 Impact of glycosylation on FUT6 activity

FUT6 has four potential $N$-glycosylation sites for which a shift of at least 2 kDa per site can be expected (REF). The FUT6 enzyme that we prepared is comprised of the luminal portion of the enzyme (refer to Fig. 1.4) and this portion should correspond to a molecular weight of about 36 kDa in its nonglycosylated form. In order to determine if the protein we purified is $N$-glycosylated, we compared treated recombinant FUT6 with PNGase F and Endo-H deglycosidase enzyme treated protein which removes $N$-linked glycans.

**Figure 3.6: BSA standard curve to determine concentration of FUT6.** BSA was titrated at 9 different concentrations 2 mg/mL to blank as shown in Fig. 3.5. The band intensities in Fig. 3.5 were recorded using Image J software and plotted against the concentration of BSA.
Figure 3.7: SDS-PAGE analysis for purified FUT6 treated with PNGase and Endo-H compared with untreated, protein bands stained by Comassie Stain.

As illustrated in Fig. 3.7, treatment of the FUT6 enzyme with PNGaseF caused a shift from ?kDa to 33 kDa and treatment with Endo-H caused a shift to 29 kDa. Or they were not sensitive to the treatments which need further analysis by other carbohydrate detection methods. The three pattern bands (75 kDa, 50 kDa and 37kDa) exhibited different form of N-linked glycosylation that could be fully or intermediate processed by *Pichia* cells and revealed that all three bands were *N*-glycosylated.

### 3.5 Determination of Fucosyltransferase 6 activity

The enzyme assay for FUT6 was conducted as described in Section 2.7 of Chapter 2. The enzymatic activity was assessed using a luciferase based assay that measures GDP released from the reaction and transferred to ATP (refer to the outline of the GT reactions outlined in Section 1.4.1 of Chapter 1). One unit (U) of enzyme activity corresponds to the transfer of 1 pmol of sugar (GDP-fucose) from the donor to the acceptor per min at 37°C (refer to Fig. 3.8).
To determine the specific activity of FUT6 enzyme, a GDP standard curve was prepared with concentration range (0-25 µM) in a total volume of 25 uL per reaction (Fig. 3.9). The GDP solutions were made from 10 mM GDP stock solution (provided with the assay kit) using buffer containing 25 mM HEPES, 5mM MnCl₂, pH 7.5 and HBSS. To 25μl of a GDP standard solution, 25 μl of the GDP detection reagent was added and the corresponding luminescence was measured (Table 3.1).

![Diagram](image)

**Figure 3.8:** General scheme of the principle used to determine the FUT6 activity.

![Graph](image)

**Figure 3.9:** GDP standard curve was prepared at the indicated GDP concentration range in 25 µl of GT reaction buffer.
Table 3.1: GDP titration using GDP-Glo Assay. RLU; Relative Luminescence Unit.

<table>
<thead>
<tr>
<th>GDP µM</th>
<th>25</th>
<th>12.5</th>
<th>6.3</th>
<th>3.1</th>
<th>1.56</th>
<th>0.78</th>
<th>0.39</th>
<th>0.2</th>
<th>0.05</th>
<th>0.02</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLU</td>
<td>39180</td>
<td>31158</td>
<td>18718</td>
<td>11512</td>
<td>7272</td>
<td>4276</td>
<td>2030</td>
<td>1174</td>
<td>680</td>
<td>624</td>
<td>490</td>
</tr>
</tbody>
</table>

We observed a linear relationship between the luminescent signal and the amount of GDP in the reaction buffer up to 25 µM GDP. In order to determine the activity of FUT6 in U/mL, we determined the amount of GDP generated of the reaction using a linear regression equation from GDP standard titration \( Y = 1673.7x + 2859.2 \). Recombinant FUT6 enzyme was titrated in 25µl in GT reaction buffer (25 mM HEPES, 5mM MnCl₂, pH 7.5 and HBSS) in a 96-well plate in the presence 40µM Ultra-Pure GDP-Fucose. After a 1 hour incubation at 23°C, GDP-Glo GT Assay was performed using 25µl of GDP detection reagent at room temperature as described in Section 2.7 of Chapter 2. Luminescence was recorded using a GloMax 96 Microplate Luminometer (Fig. 3.10).

Figure 3.10: Biochemical characterization of FUT6 using bioluminescent GDP Glo assay. A: the amount of GDP product in pmol with luminescence signal; B: FUT6 titrated in six serial dilutions with luminescence signal.
As shown in Fig. 3.9, we observed a linear relationship between the luminescent signal and the amount of FUT6. Specific activity of the FUT6 enzyme was calculated using the curve in Fig. 3.11 as pmol of GDP produced/min/ug of enzyme. The values are depicted in Table 3.2. The overall activity of FUT6 was ~13000 U/mL.

**Figure 3.11: Specific activity of FUT6.** Specific activity was calculated using the amount of GDP produced from a standard curve (Fig. 3.9) that was prepared on the same plate with a titrated amount of FUT6 enzyme.

**Table 3.2: FUT6 activity analysis in U/ml.**

<table>
<thead>
<tr>
<th>FUT6, µg</th>
<th>GDP pmol/min</th>
<th>U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.5</td>
<td>6.585397124</td>
<td>13170.79425</td>
</tr>
<tr>
<td>19.75</td>
<td>5.478891578</td>
<td>10957.78316</td>
</tr>
<tr>
<td>9.875</td>
<td>3.26668957</td>
<td>6533.37914</td>
</tr>
<tr>
<td>4.9375</td>
<td>1.734024915</td>
<td>3468.04983</td>
</tr>
<tr>
<td>2.46875</td>
<td>0.897649419</td>
<td>1795.298839</td>
</tr>
<tr>
<td>1.234375</td>
<td>0.241103792</td>
<td>482.207584</td>
</tr>
</tbody>
</table>
3.6 FUT6 exofucosylation

K562 cells were selected as model cells to test the ability of FUT6 enzyme to create Sialyl Lewis x (sLe\(^x\)) structures. K562 cells lack FUT6 gene (101). K562 cells were treated with FUT6 in a reaction buffer that contained GDP-fucose as donor for fucose, and MnCl\(_2\) as cofactor for the enzyme. Following treatment of the cells with the FUT6 enzyme, the cells were stained using antibodies (HECA452 clone) that recognize the sLe\(^x\) carbohydrate structure and analyzed by flow cytometry. Fig. 3.12 shows that following treatment, the HECA452 antibody recognizes K562 cells where prior to treatment with the FUT6 enzyme, they were not.

![Flow cytometric analysis of sLe\(^x\) expression.](image)

**Figure 3.12: Flow cytometric analysis of sLe\(^x\) expression.** K562 cells were treated with the appropriate concentration of purified FUT6 in HBSS, 0.1% human serum albumin, 0.5 mM GDP-Fucose, 5 mM MnCl\(_2\) and 25 mM HEPES pH=7.5 and incubated for 30 min at 37°C. Further cells were washed and stained with HECA452 antibody prior to analysis using the BD FACS Canto II.
K562 cells express low amount of sLe\textsuperscript{x} prior to treatment and ex-vivo fucosylation was sufficient to decorate K562 cells with sLe\textsuperscript{x} structures.

3.7 Optimization of Ex-vivo fucosylation treatment of K562 cells

Mn\textsuperscript{2+} was used in the enzymatic reactions as catalyst for high efficiency fucosyltransferase activity, but Mn\textsuperscript{2+} could induce prominent cell death (35). In order to minimize cell death, we determined the most effective concentration by titrating the concentrations in the range (0-5 mM) of MnCl\textsubscript{2}. In addition, we also determined the cytotoxicity resulting from exposure to stabilizer (glycerol) used to store the enzyme following purification. Results illustrated in Fig. 3.13 show that the enzyme is most active when either no glycerol is used as a stabilizer or with 50% glycerol as a stabilizer. In addition, in the absence of glycerol, the enzyme was sufficiently active at MnCl\textsubscript{2} concentrations corresponding to 3-4 mM.

![Figure 3.13](image)

**Figure 3.13:** Kinetics of sLe\textsuperscript{x} expression following fucosylation of K562. A: fucosylated K562 cells at 0 to mM MnCl\textsubscript{2}, B: Fucosylated K562 cells at 0 to 50% glycerol. The cells were stained for sLe\textsuperscript{x} using HECA452 antibody, and analyzed by flow cytometry. MFI: Mean fluorescence intensity.
Based on these results, we determined that the best conditions for treatment that maintained a high percentage of cell viability without affecting activity was to store the enzyme lyophilized without glycerol and use a MnCl₂ concentration from 3-4 mM.

3.8 Assessment of glycoprotein ligands created by FUT6 treatment

To determine the potential E-selectin ligands created by FUT6 treatment, we first stained K562 cells with antibodies directed against known ligands (19) (namely: CD44, CD43, and PSGL-1) and analyzed their expression using flow cytometry (Fig. 3.14).

Figure 3.14: K562 cells express PSGL-1, CD43 and CD44. K562 cells were stained for antibodies specific to PSGL-1, CD43, CD34 and CD44. Black, isotype control (mouse IgG and mouse IgG2a); Light Gray, antibodies specific for each ligand.
As shown in Fig. 3.14, K562 cells express PSGL-1, CD43 and CD44. Expression of these proteins does not equate to functional E-selectin ligand expression. This is determined by the decoration of sLe\(^x\) structures on these glycoproteins. Since K562 cells are not decorate by HECA452 reactive epitopes, the creation of E-selectin ligands on PSGL-1, CD43 and CD44 will need treatment with GTs.

3.9 Western blot analysis of E-selectin ligands created following FUT6 treatment of K562 cells

To determine the glycoproteins that act as E-selectin ligands following FUT6 treatment of K562 cells, we performed a western blot and probed the blot with a recombinant E-selectin-Ig chimera (E-Ig) to measure its ability to bind proteins from treated cell lysates. Lysates from FUT6 treated K562 was prepared and blotted onto two separate membranes and stained with E-selectin-Ig chimera (E-Ig) or HECA452 antibody that recognizes sLe\(^x\) (Fig. 3.15). Kg1a cell lysates were used as positive controls for these experiments as these cells carry high levels of functional E-selectin ligands (27).
FUT6 treatment was sufficient to induce sLe\(^x\) structures on proteins and the formation of E selectin glycoprotein ligands that appear at different molecular weights 120 kDa for CD43, 120-240 kDa for PSGL-1 and 100 kDa for CD44. This appears to indicate that these E-selectin ligands were created following treatment.

To analyze and more directly identify of E-selectin glycoproteins created by FUT6 treatment, we immuno-purified equal amounts of each ligand before and after treatment and assessed E-Ig binding activity by Western blot as described in Section 2.12 of Chapter 2 (Fig. 3.16).
Both CD44 and CD43 glycoproteins were decorated with α1,3 fucose after using α-1,3 linkage specific FUT6 treatment (Fig. 3.16). These data indicated that after fucosylation of K562 cells, E-selectin ligands were created as indicated by strong sLe^x expression and E-Ig binding. CD44 and CD43 may lack E-selectin binding in untreated K562 cells due to the absence of α-1,3-fucose at the terminal sialylated lactosamine unit.

**Figure 3.16:** CD44 and CD43 were immuno-purified from FUT6-K562 cells and Untreated K562 cells. The immuno-purified proteins were then prepared for Western blot and stained with either HECA452 or E-Ig as well as for each immuno-purified protein.
3.10 Functional E-selectin ligands were created on K562 cells following treatment with FUT6

To establish if the ligands created following FUT6 treatment on K562 cells were functional in flow based assays, the Stamper Woodruff assay was performed (27). Slides were coated with E-Ig and then both untreated K562 and treated FUT6-K562 cells were added onto slides and allowed to rotate for 30 min at 80 rpm. Following the incubated time, the numbers of rolling cells/mm² in seven distinct fields of view were counted (Fig. 3.17). As observed in Fig. 3.17, K562 cells treated with FUT6 bound E-Ig to a much greater degree than untreated cells or treated cells where EDTA was used to chelate the Ca²⁺ and show specificity, a requirement for mediating binding of selectins to their ligands.

Figure 3.17: Stamper Woodruff adhesion assay. K562 cells either treated with FUT6 (A) or left untreated (B) were viewed and counted with 20x magnification lens following the 30 min Stamper Woodruff assay. C: K562-FUT6 Treated No ESL, KG1a cells were used as a positive control (D). 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 (E) and K562-FUT6 treated cells (F).
Figure 3.18: FUT6-Treated K562 cells bind E selectin. The numbers of adherent cells on E-Ig coated slides from the Stamper Woodruff assay in Fig. 3.17 were quantified by counting cells under the 20x magnification lens. To determine the specificity of E selectin 20 mM EDTA was added to the buffers prior to the adhesion assay.

The results illustrate that K562 cells can roll on E-selectin in presence of Ca^{2+} after FUT6 treatment and this behavior was abrogated in the presence of 20Mm EDTA.
Chapter 4: Discussion

Ex vivo glycan engineering of glycoprotein ligands on stem/progenitor cells creates glycan structures that help to guide infused cells to endothelial beds that express E-selectin, thereby enabling efficient vascular delivery of these cells to sites where they are needed. Using an α-1,3-fucosyltransferase 6 (FUT6) enzyme preparation and enzymatic conditions that are specifically designed for treating live cells, we are able to synthesize E-selectin ligands on cells via the creation of sLeα determinants on glycoproteins and glycolipids. Creating efficient active GTs is not a trivial task. Eukaryotic expression systems are preferred over bacterial systems in the production of GTs. This work represents one of the methods that is used to express and purify FUT6 using the *Pichia pastoris* yeast expression system. These cells are able to carry out posttranslational modifications that are similar to higher eukaryotes. The first FUT6 expressed and purified in eukaryotic system was performed in CHO cells (53), then in insect cells (72) and then in the yeast expression system (74). Although all these systems produced functional FUT6, several of advantages and disadvantages exist.

Recombinant *Pichia.pastoris* strains were designed and the white clones in Dextrose-YNB medium were tested in shake flask culture. After 7 days of 0.5% methanol induction in BMMY medium, the supernatant and cell lysates were concentrated, and purified in a single step nickel column, then dialyzed against 20 mM Tris buffer pH 7.8 and tested for FUT6 activity. FUT6 was active in intracellular form but not in secreted form. The impaired secretion of functional FUT6 enzyme might have resulted due to many reasons including the construct design where the N-terminal site was not directly
after the alpha mating factor secretion signal or, as reported previously in \textit{S. cerevisiae} (102), the secretion was inhibited because N-glycosylation sites not present.

The concentrated purified FUT6 enzyme was analyzed on SDS-PAGE (Fig. 3.2). FUT6 was not totally pure since we detected many bands in the elution fraction at different molecular weights. The expected molecular weight based on the amino acids that include the luminal part of the enzyme (the catalytic domain and stem region) was around 38 kDa and the intracellular form was represented as homodimer (53). To further analyze our results we investigated each band by mass spectrometry. Out of the five proteins that were detected, three were identified as human FUT6 which corresponded to the 75 kDa, 50 kDa and 37 kDa bands. This molecular size of FUT6 by the SDS-PAGE seemed slightly different from its putative molecular size of 42 kDa, the small difference is correlated to the cell-type-specific glycosylation in \textit{Pichia} cells, and to the intracellular expression of FUT6 carries the secretion signal without a cleavage. The high homology between FUT3, FUT5 and FUT6 made it difficult to test the FUT6 enzyme by immunoblotting assay since commercial FUT6 antibodies recognized other FUTs?, but we address this problem by using different antibodies specific for FUT6 and target histidine tag. Collectively these results implied that FUT6 gave various molecular weights corresponding to 37 kDa, 50 kDa and 70 kDa (Fig. 3.3).

Since the molecular weight of FUT6 was not represented in one band, a reliable method to estimate its concentration and to address the glycosylation effect needed to be determined. Using an SDS-PAGE approach, the intensity of bands of known amounts of bovine serum albumin standards was used to determine the total FUT6 concentration in
the three bands (around 4 mg/mL). The fact that the fucosyltransfserses are themselves glycosylated, as FUT6 contained four N-linked sites (Asn), many reports exhibited the impact of glycosylation on fucosyltransferase activity. The variability in FUT6 molecular weight may be due to the difference in the glycosylation within *Pichia* cells which could be fully processed as in the 75 kDa band or an intermediate process as in 50 kDa and 37 kDa(103). The 37 kDa form of FUT6 is the deglycosylated form of FUT6 and when it was tested for activity, it was indeed impaired compared with the 50 kDa and 75 kDa which shown the full activity form. Thus, these results suggest the N-glycosylation of is FUT6 required for optimum functional activity. It has been reported in a study of the effect of glycosylation either by glycosylation inhibitors or site directed mutagenesis in Asn site and comparing the wild-type activity with the mutant. Peptide N glycosidase (PNGase) and Endo-H have been used in our study to remove N glycan from FUT6, we expected lower activity and reduction in molecular weight as with previous studies (53, 104). However we did not observe any detectable changes in the SDS-PAGE gel (*Fig. 3.7*) which needs further study.

The kinetic studies and the catalytic activity of FUTs depends on the presence of acceptor and substrates. We performed an *in vitro* activity assay using Glycosyltransferase Activity Kit (*Fig. 3.8-3.11*) in the presence of GDP-fucose as a donor and LacNAc as an acceptor. Specific activity of FUT6 was calculated as pmol GDP produced/min/μg of FUT6 and the activity was around 13 mU/mL.

We choose K562 erythroleukemic cell line model to enforce fucosylation *in vitro* and leading to the creation of sLe\(^\alpha\). As determined by flow cytometry, all K562 cells were
devoid of reactivity with HECA452 which recognizes sLe\(^x\)-like epitopes, indicating the absence of functional E-selectin ligands (Fig. 3.12). We analyzed the expression of E-selectin ligands on K562 cells by flow cytometry and found that CD43, CD44 and PSGL-1 were all expressed. Although they were expressed at the protein level, they did not bind E-selectin. It has been reported that K562 cells have very low α1-3-fucosyltransferase activity and likely this is the reason why these E-selectin ligands did not bind HECA452 (i.e. no sLe\(^x\) or E-Ig). Interestingly, K562 cells have been reported to express FUT4 however this was not sufficient to permit E-selectin binding (101). Next we used these negative E-selectin binding cells to test the ability of our purified FUT6 to steroselective add fucose to a membrane glycoproteins (CD43, CD44 and PSGL-1) on these cells. After enforced α-1,3-fucosylation, all K562 cells stained positive for HECA452 (sLe\(^x\) positive epitopes) (Fig. 3.13 and 3.14). FUT6 treatment was sufficient to decorate all K562 cells since the percentage of treated cells after staining with HECA-452 increased to 90% while it was 0% in untreated cells.

For cell surface glycan in vitro, it is critical that target cells remain viable and retain the native phenotype after manipulation. GTs are often stored in a buffer that includes 50% glycerol and 10 mM manganese to ensure optimal activity. Unfortunately such conditions are not optimal for the treatment of mammalian cells and have been (35) reported to induce cellular death starting at 8 hours after FUT6 treatment. Therefore, we optimized the enzymatic assay conditions to reduce both the amount of manganese and glycerol needed for the enzyme to work and ensure cell viability. Our results suggested that we could lower the manganese concentration to 3-5 mM and also remove glycerol from the
storage buffer (Fig. 3.15) in order to preserve optimal cell viability. These new conditions resulted in high-efficiency α-1,3-fucosylation of K562 cells with 100% cell viability.

To directly assess E-selectin binding activity, we examined the ability of fucosylated and untreated K562 cells to capture, tether and roll using a Stamper-Woodruf assay. Untreated K562 cells showed little or no interaction with E-selectin upon rotation, and this was consistent with their lack of E-selectin ligand expression. In contrast, ex vivo FUT6 treatment of K562 cells greatly enhanced in their ability to be captured by E-selectin during rotation (Fig. 3.16) and this was abrogated with presence of EDTA.

Overall, our results presented here demonstrate the dramatic increases of cell surface sLe\(^x\) observed by FACS, E-Ig reactivity by Western blot, and of capture/tethering and rolling collectively indicate ex vivo cell surface glycan modifications of glycoproteins can create functional E-selectin ligands on K562 cells.

After testing our hypothesis in K562 cells and we ending up with valid results, also in line with previous reported results with enhance selectin-mediated homing and engraftment in many cell types, including adult stem cells as umbilical cord hematopoietic cells (98), mesenchymal stem cells (35), neural stem cells (89), and other cells as regulatory T-cells (105). Thus, prompting us to analyze and hypothesize whether programming the expression of such molecules sLe\(^x\) by enforced fucosylation in induced-pluripotent stem cells derived hematopoietic stem cells (iPSCs-HSCs) could allow for more efficient migration to the bone marrow.
Chapter 5: Conclusion and Future Research

Toward our goal for enhancing homing and migration of therapeutic cells such as adult stem cells, we have made significant progress in purifying high quality and quantity FUT6 enzyme. The main objective of my research was fulfilled; using yeast expression system to produce active fucosyltransferases will be useful in order to scale up production. The favorability of our systems that was with single step purification compared to the previous reported studies, and also since the cost of our system is much lower than those for CHO cell mammalian expression system. Our results infer the choice of the signal sequence critically determines the fate of recombinant protein if it will be secreted in the medium or will be retained intracellular, but usually secreted form is more preferred. So, as a future improvement we will modify our plasmid construct to ascertain getting FUT6 in culture media. It has proved the activity of FUT6 on ex vivo treatment for glycan engineering on K562 cells by biochemical approaches and functional assays. The in vivo studies in our hypothesis will be compulsory to do subsequently.

Here we show that recombinant FUT6 will be a good candidate for medical and pharmacological studies. Our findings are important for informing future clinical applications using recombinant FUT6 which is ideal for glycoengineering strategies as it is simple, transient, and non-integrative.

Future work will focus on the expression and purification of other GTs such as sialyltransferases. Using both sialyltransferases and fucosyltransferases it could be envisioned that further improvement in the formation of sLex/structures may result. We hope to move this work to fundamental stem cell populations such as HSPCs derived
from iPSCs and CD34^{neg} HSPC populations, both of which are devoid of E-selectin ligands and are not able to migrate and engraft when tested *in vivo*. 
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