Lentiviral-Engineered Mesenchymal Stem Cells for Hemophilia B Gene Therapy

Lentiviral-Engineered Mesenchymal Stem Cells for Hemophilia B Gene Therapy

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I. ABSTRACT

Hemophilia B patients may have frequent, spontaneous and life-threatening bleeds that are currently managed by an invasive and expensive treatment. Mesenchymal stem cells (MSCs) are increasingly being applied to clinically therapeutic strategies and lentiviral gene vectors have been shown to be safe and efficient tools for modifying stem cells for long-term expression of high levels of transgenes. In this study, MSCs were engineered with a lentivirus to express sustained and therapeutic levels of human FIX protein *in vitro* and in mice. The modified MSCs secreted human FIX protein at levels exceeding 4 μg/10^6 MSCs/24 h with high FIX coagulant activity of greater than 2.5 mIU/10^6 MSCs/24 h for 6 week *in vitro.* Functional FIX transgene was continually expressed by these cells when they were induced to differentiate into adipocyte, osteoblast and chondrocyte lineages *in vitro*. However, the modified MSCs transplanted via tail vein into NOD-SCID-γ mice expressed low levels of FIX *in vivo*. The transplantation procedure had an increased risk of death that was more pronounced in mice that received cell doses exceeding 2 million cells. Organ examinations suggested the deaths resulted from entrapment of MSCs in pulmonary capillaries. Modified MSCs encapsulated in alginate-PLL microcapsules and transplanted into the peritoneal cavity of both NOD-SCID-γ and hemophilia B mice at 9 million cells/mouse resulted in therapeutic expression around 100 ng of human FIX/mL of plasma only for a few days *in vivo* as human FIX expression quickly decreased to basal values by the end of the first week. Cultured *ex vivo*, human FIX expression by retrieved capsules indicated an innate immune response to the encapsulated cells prevented sustained expression of FIX. These investigations demonstrate that lentivirally modified MSCs have the potential to express therapeutic human FIX for sustained periods *in vitro*, even after their differentiation. However, they also highlight the challenges to overcome to optimize cell engraftment and survival following transplantation, and to minimize the immune responses associated with the xenogeneic translational models used.

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III. Chapter 1: Literature Review

A. Hemophilia

Hemophilia A and B, are X-linked genetic disorders that result of a deficiency in the plasma of clotting factors VIII and IX respectively. Together with congenital factor XI deficiency (hemophila C) and von Willibrands disease, these genetic disorders account for 95-97% of inherited blood coagulation disorders [1]. Hemophilia B is caused by insertions, deletions, missense, nonsense and splice-site errors within the Factor IX (FIX) gene, and is passed down via the X chromosome. This inheritance pattern dictates that affected males will beget unaffected males and carrier females, and that carrier females will have a 50% chance of giving birth to an affected male or a carrier female [Fig. 1A]. Sporadic cases of Hemophilia B also result from *de novo* mutations and account for 30% of cases in mild and moderate forms and over 40% of severe forms [2]. Sporadic mutations in the factor IX gene occur twice more frequently in males than females [3].

Hemophilia B occurs in every 1 in 25 000-30 000 male births, and is most common in its severe form, where patients have less than 1% of normal circulating levels of factor IX (FIX). Moderate and mild hemophilia B patients have between 1-4% and 5-25% the levels of normal circulating FIX, respectively. It is estimated that around the world as many as 70% of all hemophiliacs are underdiagnosed and untreated, mostly within developing countries [4]. In these cases, patients with severe hemophilia rarely live beyond childhood due to spontaneous or accidental bleeding episodes and the absence of diagnostic and treatment tools. The two main

approaches for the genetic diagnosis of hemophilia B are: linkage analysis, which uses single nucleotide polymorphisms and microsatellite variable tandem repeats to track familial hemophilia; and direct mutation detection, which uses polymerase chain reaction (PCR) and various screening methods or DNA sequencing [1]. The latter method is becoming increasingly popular in countries with access to more advanced molecular techniques because it has a higher rate of success and can detect both familial and sporadic FIX mutations.

The primary symptom of severe hemophilia is recurrent bleeding into muscles and joints from a young age, leading to arthritis and joint pain in later years [Fig. 1B] [5, 6]. Patients with limited or no access to factor IX replacement therapy will likely develop disabling arthritis as a result of progressive joint damage at an earlier age [5]. Bleeding episodes can be spontaneous and sometimes fatal when they occur in a critical area, such as the intracranial space. Moderate hemophiliacs are at risk for less frequent sporadic bleeds, and mild hemophiliacs are not in danger of the sporadic life-threatening bleeds common to the severe phenotype, and generally aren't at serious risk unless confronted with a major hemostatic challenge such as major elective surgery or trauma [7]. Many mild hemophiliacs are not even diagnosed until the later stages of life due to a lack of obvious symptoms [4].

Some hemophiliacs develop factor IX-neutralizing antibodies (inhibitors) against therapeutic FIX given as treatment. Reports on the number of hemophilia B patients that develop inhibitors varies, but are generally around 3%, which makes it about a ten-fold less likely complication than it is for patients with hemophilia A [8-

10]. The development of inhibitors has been linked to FIX mutation type, with the risk of FIX inhibitor development being higher in patients with large FIX gene deletions, or severe phenotype [11, 12]. Hemophilia B patients with inhibitors are more likely to suffer from the symptoms of hemophilia B because treatment becomes much more difficult.

B. Current Treatment for Hemophilia

The first documented form of treatment of hemophilia in the mid-1800s was transfusion from an unaffected donor [9]. Over a century later, prothrombin complex concentrates, plasma products containing a concentrated mixture of prothrombin and factors VII, IX, and X were used for several years [9]. The drawback with prothrombin complex concentrates was that its use was commonly associated with thrombotic events, particularly in surgical applications [9]. The Cohn-Oncley plasma fractionation process was first developed in the mid-1940s as a method to extract therapeutic proteins from pooled human plasmas [13]. In 1993 it was demonstrated that a highly purified human FIX concentrate derived using the Cohn-Oncley fractionation process demonstrated reduced thrombogenic complications than prothrombin complex concentrates, and thus a better therapeutic product [14]. It was widely publicized that in the 1980s the majority of hemophiliacs in North America who received plasma-derived products were exposed to a variety of viral pathogens, as many developed symptoms of infections with human immunedeficiency virus (HIV), Hepatitis B virus and/or Hepatitis C viruses (HBV,HCV) [9]. In the 1990s there was also a fear of blood-borne

transmission of prion diseases including Bovine Spongiform Encephalopathy and Creutzfeldt-Jakob disease. Despite increases in purification of the extraction of donor FIX from plasma followed by pathogen inactivation strategies, there was call to create a safer therapeutic FIX. To fulfill this demand, the USA Food and Drug Administration in 1998 approved the first commercial recombinant FIX (rFIX), BeneFIX®, manufactured by Genetics Institute [15]. BeneFIX® is produced in Chinese Hamster Ovary cells, and though at first produced in high concentrations, less than 1% of the protein was active due to inadequate carboxylation in the Gla domain [16]. Two enzymes are now used to induce post-translational modifications and the purification of rFIX from conditioned cell media is completed in seven steps [17]. BeneFIX® is still the only recombinant FIX product on the market today, although there are a wide variety of plasma-derived FIX (pdFIX) products available [Fig. 1C] [17]. In the United States, pdFIX accounts for only 20-25% of users despite the fact that there are some documented benefits to the use of pathogen-inactivated pdFIX products [17]. For example the *in vivo* recovery of rFIX is about 25 to 30% lower than the recovery from pdFIX infusion, especially in patients younger than 15 years of age [18, 19]. A number of new rFIX products are currently in development that can be produced at higher concentrations more easily, are biosimilar and thus less expensive, or are more active and would require less frequent doses [17].

Current North American treatment for hemophilia B is based on the World Federation of Hemophilia guidelines. The first choice of treatment for children involves the initiation of prophylaxis following either the first major haemarthrosis

event or when the child is two years old [20]. The treatment consists of a twice-weekly intravenous injection of concentrated FIX (recombinant or plasma-derived) at a dose of 30-40 international units (IU) per kilogram of body weight, where 1 IU equals the amount of FIX normally found in 1mL of plasma (approximately 5 μg) [5, 20]. Based on a regimen initiated in Sweden called the Malmo protocol, prophylactic treatment aims to minimize spontaneous bleeding episodes, and post-operative bleeding [5]. Doses vary according to the severity of symptoms, but generally aim to maintain a level above 1% of normal FIX level at the troughs. With the half-life of current therapeutic FIX products ranging only from 16.4-20.8 hours, frequent injections are often necessary [21].

Figure 1. *Inheritance pattern, symptoms and current treatment of hemophilia B.* **A)** Inheritance pattern of hemophilia B. Carrier females have a 50% chance of producing an affected male or carrier female. Affected males will produce either carrier females or unaffected males. **B)** Bleeding into the joints can cause hemarthrosis and early-onset arthritis in uncontrolled sever Hemophilia B [6]. **C)** Current treatment consists of regular injections of either recombinant (BeneFIX®) or plasma-derived (eg. MonoFIX®) FIX protein.

Due to the high cost of treatment, less than 50% of patients with severe hemophilia B in the US are estimated to be on prophylactic regimens [21]. The expense of treatment is due to the high cost of rFIX and pdFIX proteins. One study in 2001 stated that the average treatment for a patient with severe hemophilia in the United States cost $139,000 per year [4]. Patients with inhibitors require higher and more frequent doses of FIX, adding significantly to treatment costs. There is no evidence supporting a greater risk of inhibitor development for those receiving rFIX rather than pdFIX [22], though this risk has been suggested in Hemophilia A

patients. The treatment of patients with inhibitors is challenging as it is much more difficult to achieve haemostasis in these patients. Patients with low levels of inhibitors can be treated with more frequent doses of FIX. Hemophiliacs with high levels of inhibitors can be treated with the Immune Tolerance Induction (ITI) regimen with the goal of inducing antigen-specific tolerance to FIX. This can involve the regular infusion of FIX at high doses or the use of immunoregulatory drugs [23]. The rate of success of ITI therapies in Hemophilia B patients is much lower than in patients with Hemophilia A at 13-31% [23]. Alternatively, treatment of patients with high levels of FIX inhibitors that cannot be successfully treated with the ITI regimen can be treated with doses of recombinant activated Factor VII or activated prothrombin complex concentrate (aPCC) [9].

C. FIX Structure and Activation

The complete nucleotide FIX gene sequence was first reported in 1985 [24]. The FIX gene is located on the long arm of the X chromosome at Xq27.1 containing 8 exons and 7 introns, with a total length of about 34kB [Fig. 2A] [25]. Synthesis of FIX takes place in the liver by hepatocytes and circulates in its inactive vitamin-K dependant protein form as the proenzyme of the serine protease FIXa [Fig. 2E] [25]. The FIX transcript is 2803 bases in length, and is comprised of a short 5' untranslated region (29 b), open reading frame plus stop codon (1383 b), and a 3' untranslated region (1390 b) [Fig. 2B] [26]. The FIX zymogen enters circulation following the removal of the pre- and pro-sequences that are included in the precursor protein that direct FIX for secretion and provide a binding domain for

vitamin K-dependant carboxylase [Fig. 2C]. Some additional post-translational modifications during biosynthesis include the γ-carboxylation of the first 12 Glu residues; partial hydroxylation of Asp-64; sulfation of Tyr-155; phosphorylation of Ser-158; and glycosylation at Asn-157, Asn-159, Ser-53, Ser-61, Thr-159, Thr-169, and Thr-172 [Fig. 2E] [24]. The unactivated form of FIX contains an N-terminal and Gla domain (residues 1-40), short hydrophobic stack, two epidermal growth factor (EGF)-like domains that are connected by linker residues, an activation peptide, and a C-terminal protease domain [Fig. 2C&E] [25]. The binding of calcium within the Gla [25, 27], EGF1 [28] and protease domains of FIX imparts structural properties to FIX that confer biologic function [29, 30].

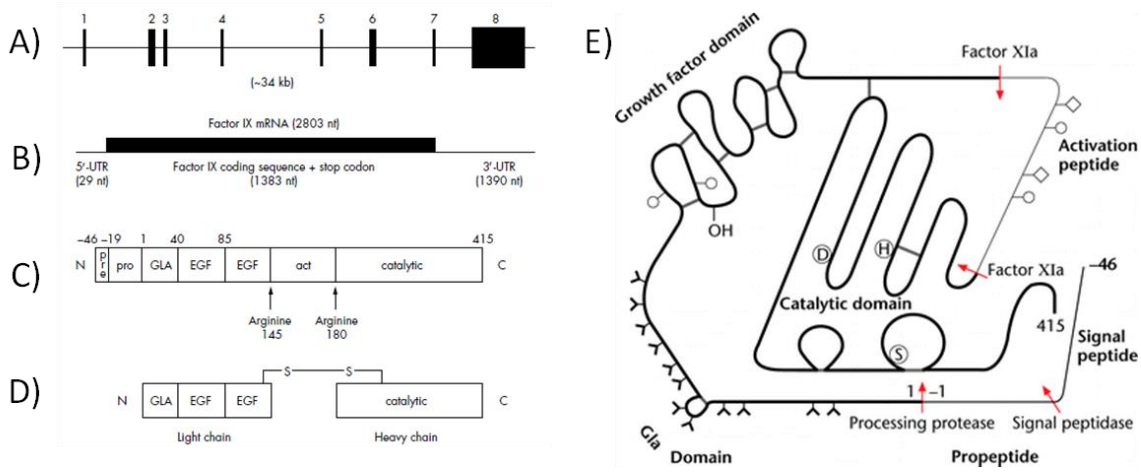

Figure 2. *Structure of human Factor IX protein.* **A)** Genomic organization of the human factor IX gene. **B)** Factor IX mRNA showing the relative size and location of the open reading frame. **C)** The newly synthesized factor IX protein molecule comprising a pre- and pro-sequence (27 and 19 amino acids, respectively) and a mature peptide of 415 amino acids (total length, 461 amino acids). **D)** Activated factor IX comprising an N-terminal light chain and a C-terminal heavy chain held together by a disulphide bridge between cysteine resides 132 and 279. **E)** An alternate diagram of the hFIX protein illustrating various active sites. GLA, "Gla" domain, in which 12 glutamic acid residues undergo post-translational γ-carboxylation by a vitamin K dependent carboxylase; EGF, epidermal growth factor-like domain; act, activation peptide released after proteolytic activation at arginine 145 and arginine 180; catalytic, the serine protease domain. A-D) Adapted from Bowen *et al* [26] E) Adapted from Berkner [31].

Activation of FIX results from the proteolytic cleavages at both the α-cleavage site (Arg145–Ala146) and the β-cleavage site (Arg180–Val181) [32] [Fig. 2C]. Both the TF/FVIIa/Ca^{2+} complex (extrinsic pathway) and FXIa/Ca^{2+} (intrinsic pathway) covert FIX to FIXa, its activated form. The newly formed FIXa is illustrated in Figure 2D and consists of an N-terminal light chain (residues 1–145) containing the Gla, EGF1, and EGF2 domains, and the C-terminal heavy chain (residues 181–415) containing the serine protease domain with catalytic triad of residues Ser-c195 (365), His-c57 (221), and Asp-c102 (269) [26]. The two chains are held together by a single disulfide bond between cysteine residues 132 and 279 [Fig. 2D].

D. Overview of the Role of FIX in Coagulation

Coagulation is an important component of hemostasis and impedes blood loss from damaged vessels. The process has been highly conserved throughout mammalian evolution and can be traced back over 400 million years to ancestral serine protease cascades [33]. Current understanding states that there are three main interconnected stages of the coagulation pathway in humans: the tissue factor-initiated pathway (extrinsic), which is the most important part of coagulation cascade and is designed to generate a thrombin burst; the contact-initiated pathway (intrinsic) which plays a more minor role to initiate clots and is not well understood, but is responsible for the clots formed in association to biomaterials; the final common pathway responsible for thrombin activation. An overview of the pathways is illustrated in Figure 3.

1. The Contact Activation (Intrinsic) Pathway

The contact-activation pathway is initiated by the activation of the blood zymogen Factor XII via surface contact interaction with phospholipids or bacteria [34]. This leads to the assembly of an activation complex involving FXIIa and the allosteric proteins: prekallikrein (PK), high-molecular-weight kininogen (HMWK), and FXI [34]. This assembly activates prekallikrein to kallikrein, which in turn activates FXII to FXIIa. Factor XIIa then activates FXI to FXIa and establishes a reciprocal activation cascade by hydrolyzing more prekallikrein to kallikrein [Fig. 3]. Kallikrein then acts on HMWK to cause the release of bradykinin, a potent vasodilator. Factor XIa activates FIX into the serine protease FIXa in a reaction requiring Ca^{2+} binding to the Gla residues in the amino-terminal region of FIX [Fig. 3]. The activation of FVIII is initiated by small amounts of thrombin. Conversely, high concentrations of thrombin will cleave FVIIIa, resulting in its inactivation and control of the coagulation cascade. The activation of FXa requires Ca^{2+} and the assembly of the tenase complex where FVIIIa acts as a receptor for FIXa and FX in conjunction with exposed phospholipids on the surface of activated platelets. Factor X is activated via the cleavage of an internal arg-ile (R-I) bond by FIXa. The point of activation of FX to FXa is the site at which the intrinsic and extrinsic coagulation cascades converge into the final common pathway.

2. The Tissue Factor (Extrinsic) Pathway

In the tissue factor pathway the coagulation process is initiated when tissue factor (TF) becomes available on cell surfaces as a result of vascular injury in the extravascular space and where it binds to small amounts of activated Factor VII (FVIIa) circulating in the blood [35]. The Factor VIIa-TF complex then activates both FIX and FX [Fig. 3]. Additional amounts of TF/FVIIa are generated following TF/FVII activation via a feedback loop of FIXa, FXa and TF/FVIIa [Fig. 3]. This augments the initiation phase of coagulation. Small amounts of thrombin are generated by the FXa that is formed, and this in turn activates FV, FVIII, and more FXI on the platelet surface through a feedback mechanism [36, 37] [Fig. 3]. Larger amounts of FX are then activated to form FXa via the assembly of FIXa and FVIIIa on the platelet surface and the formation of the intrinsic tenase complex as described for the contact activation pathway [38] [Fig. 3]. Some FXa initially generated by the TF/FVIIa complex later combines with TF pathway inhibitor (TFPI) produced by endothelial cells to inhibit TF/FVIIa activity [39] [Fig. 3].

This pathway illustrates how a deficiency or dysfunction of FIX or FVIII would compromise the activation of FX, and thereby result in decreased availability of fibrin deposition and clot formation in the succeeding final common pathway [Fig. 3]. From this perspective it is evident why hemophilia A and B exhibit similar symptoms.

3. The Final Common Pathway

In the final common pathway FXa forms a prothrombinase complex with FVa, and Ca^{2+} on phospholipid surfaces [Fig. 3]. This complex rapidly converts prothrombin into thrombin [37]. The generation of a small amount of extrinsically generated thrombin is enough to initiate the coagulation mechanism and the expansion of thrombin generation via the intrinsic mechanism.

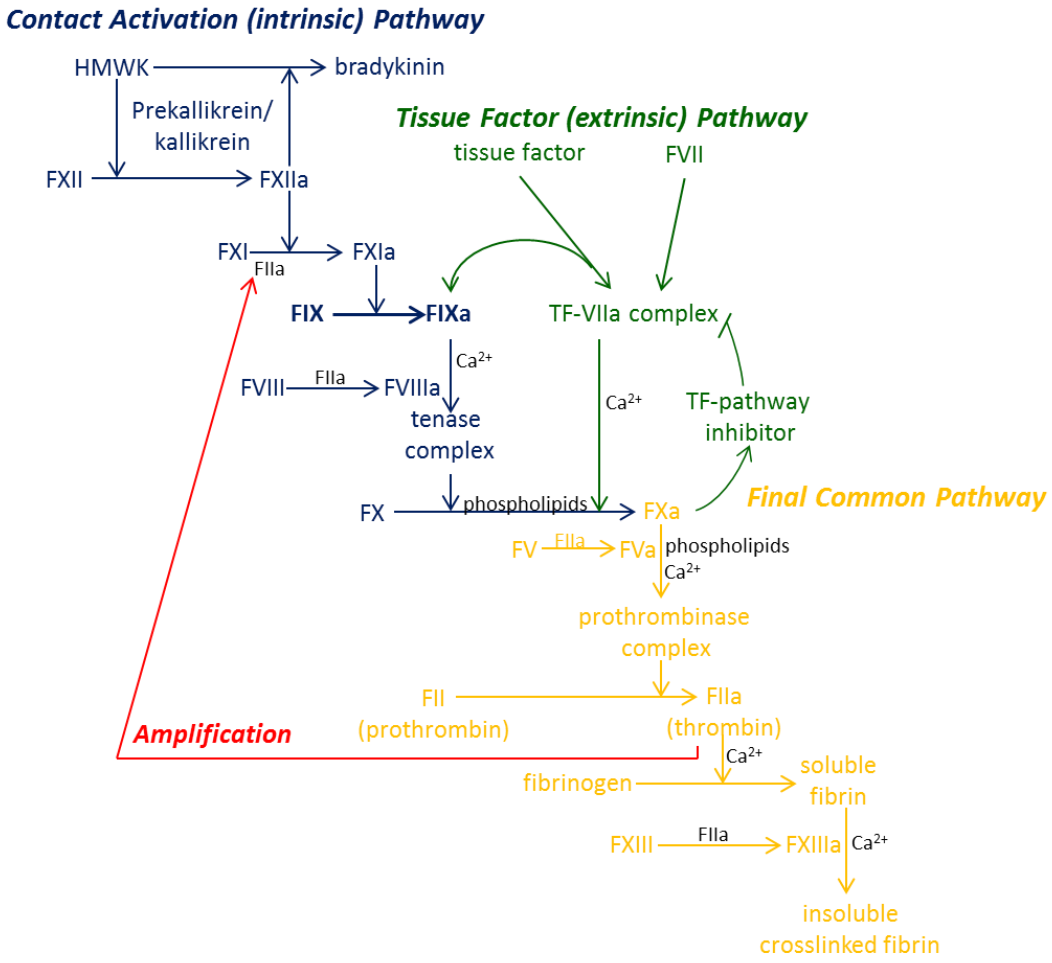

Figure 3. *The Role of Factor IX in the Coagulation Cascade.* The coagulation cascade is activated via one of two main pathways; the contact activation (intrinsic) pathway [blue], or the tissue factor (extrinsic) pathway [green]. These pathways converge to form the final common pathway [gold] which activates thrombin (FII) and forms insoluble crosslinked fibrin which is the basis of coagulation. The role of FIX is bolded.

E. Gene Therapy

Gene therapy can be generally described as the 'introduction of genetic material and/or cells in the body to treat or prevent disease' (American Society of Gene and Cell Therapy). There are many gene therapy protocols with a variety of applications for conditions including cancer, cardiovascular, monogeneic, infectious and neurological diseases. Approaches to gene therapy generally fall under two main categories; *in vivo* and *ex vivo* [Fig. 4]. *In vivo* gene therapy requires the direct administration of the gene to the patient, resulting in direct *in situ* gene modification [Fig. 4A]. This could use a variety of vector delivery methods including viral, liposomal and cationic polymers. The risks and challenges associated with this approach can be significant and include: evasion of immune system; cell-targeting; successful integration and expression in host cells; minimization of toxicology or infectivity of vector and sustained and adequate expression levels. *Ex vivo* gene therapy involves the isolation of target cells from the patient or source, *in vitro* modification of the patient's cells, re-administration of modified cells to the patient, and successful cell engraftment [Fig. 4B]. This is safer but more labour-intensive than *in vivo* therapies. With *ex vivo* approaches delivery can be more precisely targeted, and exposure of foreign DNA to the immune system can be minimized. To date, over 1500 gene therapies have gone to clinical trial [40]. This is significant progress from a field only initiated in 1973 when Rogers *et al* first attempted to use a virus to deliver genes to cells *in vitro* [41].

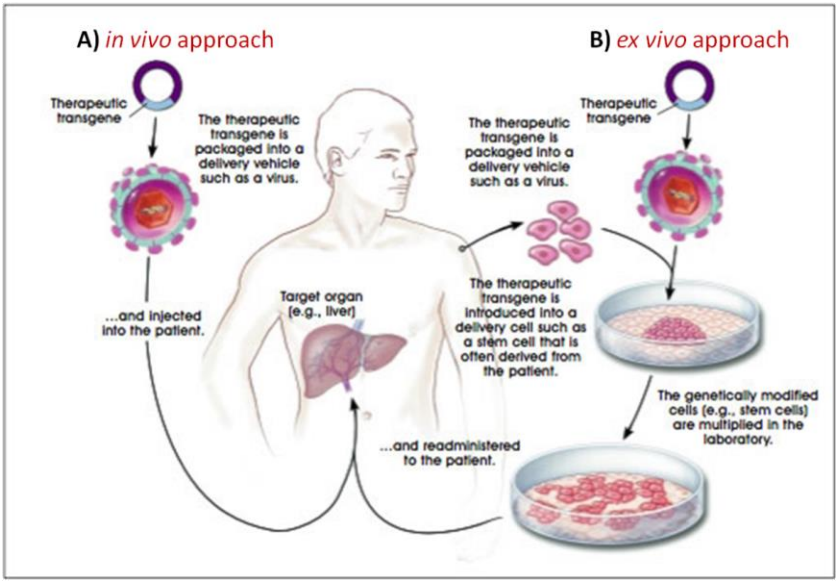

Figure 4. *Gene Therapy.* **A)** The *in vivo* approach utilises the administration of the vector directly to the patient and *in situ* cell modification. **B)** The *ex vivo* approach isolates cells and modifies them *in vitro,* prior to (re)administration to the patient.

1. Hemophilia B as a Model for Gene Therapy

Hemophilia is a prime disease model for gene therapy, with research to date placing emphasis on the use of viral vectors. Replacing the lost function of the FIX gene is the aim for hemophilia B treatment, and gene therapy is ideally suited to accomplish this for the following reasons:

A. Low levels of therapeutic protein result in great improvements in the quality of life of the patients. Factor IX has a plasma half-life of 18–24 hours, and a production rate matching that of only 1% of normal levels should be sufficient to convert severe hemophilia B to its moderate form [42].

B. A single gene source of a relatively small protein simplifies the task of delivery. A FIX gene has been engineered that expresses well and is small and easy to carry. Relatively few post-translational modifications are needed for FIX, and

these can be carried out by many mammalian cell types [15]. Additionally, the levels of FIX protein within the blood can be easily monitored by the activated partial thromboplastin time (APTT) test.

C. Factor IX protein can be delivered from a variety of cell types and make its way to the circulatory system. It is therefore not necessary to target or restrict production of FIX to a specific locale within the body. Factor IX production also does not need to be tightly regulated, and can be continually expressed at a constant level [43].

D. Small and large animal models exist for testing experimental hemophilia B therapies. In 1997 and 1998 three separate groups developed a FIX knockout murine model [44-46]. A hemophilia B dog with a missense mutation resulting in a complete lack of circulating FIX was identified in 1989 [47] and hemophilia B rhesus macaques are readily accessible to researchers [48].

E. Clotting factors expressed as high as 150% of normal levels have been shown to exhibit no negative effects in small animal studies, so there is little risk of complication due to FIX over-expression [49].

A successful gene therapy requires a gene delivery method that is efficient, safe, non-immunogenic, and long-lasting. For a therapy to enter the market it must have efficacy, ease of administration, or economical improvements over the existing therapy. Hemophilia research has explored a number of approaches over the last fifteen years hoping to achieve this goal. Therapies utilizing viruses have proven most successful to date because they are the most efficient gene delivery vehicles at

hand. Although plasmid DNA approaches are safer, they are less efficient and often transient.

In vivo viral approaches, though popular because of their efficiency, still raise many safety concerns including adverse immunological reactions, cytotoxicity, germ-line transmission and oncogenesis. *Ex vivo* viral approaches use controlled cell modification outside of the body. This can maximize the efficiency of a viral vector while increasing the level of safety. This also allows for cell-line assessment prior to implantation. Researchers have primarily used replication incompetent retrovirus, lentivirus and adeno-associated viruses for their stable and permanent integration into the host genome.

Hemophilia is one of many monogeneic disorders for which an ideal treatment would consist of a single application of a life-long therapeutic agent. *Ex vivo* viral approaches for hemophilia B apply modified FIX-expressing cells to the patient's body. The cells are either terminally differentiated, immortalized or a form of stem/progenitor cells. Endothelial and endothelial progenitor cells were initially a logical choice due to their natural role in coagulation. Fibroblasts and myoblasts are an easily obtained progenitor cell-type used commonly in experimentation and are easily manipulated *in vitro*. More recent advances in stem cell culture technology have made progenitor and stem cells a more feasible choice. The inherent capacities of stem cells to self-renew and give rise to identically modified progeny make it possible to expand populations *in vitro* for therapeutic applications while maintaining their multi-potent ability to inhabit a biological niche. However, the

usefulness of more primitive stem cells has been precluded by a high rate of tumorigenesis [50]. Mesenchymal stem cells were the first off-the-shelf stem cell to gain regulatory approval; they have the advantage of being immune privileged, have the ability to self-renew and differentiate into other cell lineages in host cell populations, have the ability to respond to chemotactic gradients which predisposes them to home to bone marrow and injury sites, and possess a relative ease with which they can be isolated and expanded *in vitro* [51, 52]. Overall, a number of cell types have been explored as potential vehicles for the treatment of hemophilia in both small animal and clinical trials.

F. Mesenchymal Stem Cells (MSCs)

1. Discovery, Nomenclature and Origin

Mesenchymal stem cells were first identified by Friedenstein *et al* as a subpopulation of bone marrow cells in the late 60's and early 70s [53-55]. Investigation into their role in tissue regeneration increased following the proposal by Caplan *et al* in the early 90s to define MSCs as a stem cell capable of differentiation into a variety of cell types [56, 57]. Multilineage capabilities were eventually demonstrated *in vitro* in the late 90s by Pittenger *et al,* and they were quickly promoted as an ideal cell type for regenerative medicine, tissue engineering and therapeutic applications [58]. The clear demonstration of MSCs undergoing differentiation and self-renewal *in vivo* remains to be exhibited, though clinical applications of MSCs are still on the rise. The most common applications are diabetes, bone and cartilage disease, autoimmune diseases, cardiac repair,

gastrointestinal disease and cancer cell therapy. Clinical trials.gov indicates 265 ongoing studies using mesenchymal stem cells worldwide as of October 2012, and most trials are in phase I, phase II or a mixture of I/II.

There is some confusion surrounding the nomenclature of MSCs. Whether the MSC moniker stands for mesenchymal stem cell, mesenchymal stromal cell, or marrow stromal cells, and whether the terms are synonymous is not clear. MSCs are generally agreed to be a heterogenous mix of cell types due to the fact that the term "MSC" has been applied to all kinds of expanded stromal cell populations. However, a cell population does not need to be pure to be clinically useful and this sometimes makes them safer to use [59]. New discoveries highlight the importance of strict definitions for these cell types as an increasing number of reports have identified distinctions between MSCs originating from different organs and tissues, and the method of isolation or *in vitro* cell expansion [59]. A number of groups have gone so far as to propose the existence of different MSC sub-populations with varying levels of "stemness" such as unrestricted somatic stem cells [60], embryonic-like stem cells [61] and very small embryonic–like cells [62].

Mesenchymal stem cells (MSCs) were first thought to originate from cells of the mesodermal tissue and differentiate into a variety of tissues *in vitro*. However, new evidence indicates they may be neuroectodermal in origin [63]. A cell surface marker exclusive to MSCs has yet to be identified, however the International Society for Cell Therapy (ISCT), have defined criteria for identifying human MSCs. True MSCs: must adhere to plastic when maintained in standard culture conditions;

≥95% of the population must express the cell surface antigens CD105, CD73 and CD90, by flow cytometry and lack expression (≤2% positive) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II; be able to differentiate to osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions [64]. Standards relating to the isolation, definition and expansion of MSCs are continually being updated as more information is discerned.

2. Sources of MSCs and their Differentiation Potential

MSCs can develop *in vitro* into many cell types including bone [65], cartilage [65], adipose [66], tendon [67], ligament [67], muscle [68], stroma [67], and some non-mesodermal lineages such as neurons [69] and Schwann cells [70] [Fig. 5A]. MSCs can be isolated and expanded from a growing variety of sources including bone marrow [62], umbilical cord blood [71, 72], peripheral blood [73], muscle [74], vasculature [75], skin [76], and adipose tissue [77]. Though the proportion of stem cells in the bone marrow is quite low (estimated to be between 0.001% and 0.01% of total nucleated cells) [78], isolation from human bone marrow is fairly simple and bone-marrow-derived stem cells can be expanded *in vitro* to allow for implantation and testing in humans. This is the most common source of MSCs used for therapeutic research today, although, the derivation of cells from umbilical cord blood and adipose tissue is becoming more prevalent, and avoids the invasiveness required of bone marrow procedures.

3. Immunomodulatory Effects of MSCs

MSCs interact with the immune system in unique ways [Fig. 5B]. This has been demonstrated *in vitro*, and *in vivo,* in both humans and animal models [79-82]. Generally, MSCs are said to have immunosuppressive properties or to be immune privileged [83]. Initial studies focused on the interaction of MSCs with T lymphocytes. MSCs were seen to inhibit T cell proliferation by up to 60-98% *in vitro* [84] and also inhibited the proliferation of mouse memory T cells [85]. While in the presence of MSCs, T cells were observed to remain in a quiescent state with a reduction in IFN-γ production [86].

Aggarwal *et al.* co-cultured MSCs with subpopulations of immune cells and found an altered cytokine secretion profile of dendritic cells (DCs), naive and effector T cells (T helper 1 [T_H1] and [T_H2]), and natural killer (NK) cells to induce a more anti-inflammatory or tolerant phenotype. Induced regulatory T cell production was also noted [52].

In 2005 several studies reported the effects of human MSCs on dendritic cells (DCs). It was shown that MSCs could suppress the differentiation of monocytes and CD34+ cells into DCs, and mature DCs that had differentiated in the presence of MSCs were impaired in their function [87-89].

Reports that MSCs inhibit B-cell proliferation first surfaced in 2005 [86], while further investigation by Corcione *et al* confirmed in 2006 that soluble elements played a role in the inhibition of B cell proliferation through an arrest at the G1/G0 phase in the cell cycle [90].

MSCs suppress the proliferation of natural killer (NK) cells after stimulation with IL-2 or IL-15 [89, 91]. Sotiropoulou *et al* demonstrated *in vitro* that at low NK/MSC cell ratios, MSCs altered the phenotype of NK cells and suppressed proliferation, cytokine secretion, and cytotoxicity against human leukocyte antigen (HLA) class I-expressing targets [91]. However, MSCs are not completely protected from the action of NK cells as they are still susceptible to lysis by autologous and allogeneic IL2-activated NK cells [92, 93]. Spaggiari *et al* presumes that although MSCs express normal levels of MHC class I (protection from NK cells), they display ligands that are recognized by activating NK receptors that trigger NK allo-reactivity [93].

A number of *in vitro* studies have investigated the immunomodulatory effects of MSCs in allogeneic transplants. Notable positive effects on T lymphocyte production, dendritic cell differentiation, B cell and NK cell proliferation have elucidated the immune privileged qualities of MSCs. Our current understanding demonstrates that MSCs are a worthwhile avenue of research for *ex vivo* gene therapies, and continuing studies to further the characterization of human immune responses to allogeneic stem cells will only aid in this progress.

4. Homing and Engraftment of MSCs

MSCs have an inherently migratory nature, as they exist in a constant state of unrest entering and exiting the bone marrow to complete a surveillance of peripheral circulation and responding to chemotactic gradients [94]. The ability of systemically implanted MSCs to preferentially target or non-specifically localize to

sites and tissues within the body make MSCs ideal gene delivery vehicles. The molecular signals responsible for the trafficking and homing of MSCs are still being investigated, but some specific chemokine ligand-receptor relationships have been identified and tested with implications for therapeutic use.

MSCs derived from bone marrow that are systemically infused have been demonstrated to non-specifically home to tissues including bone marrow (BM), lung, liver, kidney, and spleen. They have also been noted to preferentially target tumors, ischemic and inflamed tissues and sites of injury due to genetic defect [95], myeloablative therapy [96], irradiation [97] and myocardial infarction [98]. The most notable chemokine recognised by CXCR4 receptors and responsible for MSC homing both within and away from the bone marrow is stromal-derived factor 1α (SDF-1α/CXCL12) [67, 99]. Son *et al* demonstrated that MSCs express CXCR4 among other receptors implicated in homing, and are strongly attracted to SDF-1α for up to passage 15-18 *in vitro* [67]. Another investigation utilised pancreatic islets to discover that a minority of MSCs express a restricted set of chemokine receptors: CCR1, CCR7, CXCR4, CXCR6, and CX3CR1 [100]. Accordingly, MSCs showed appreciable chemotactic migration to the chemokines CXCL12, CX3CL1, CXCL6, CCL3, and CCL19 [100]. The methods of isolation and culture expansion have been reported to influence the homing efficiency of MSCs *in vivo* [101].

Experimentation with the over-expression of signals that promote MSC engraftment would be advantageous as the reported rate of engraftment of systemically implanted MSCs is very low [102]. As demonstrated by other groups,

the expression of CXCR4 regulates the homing mechanism in MSCs and results in a higher percentage of cells taking up residence in the bone marrow and at sites of injury [67, 99]. Over-expression is a likely method to increase the homing and engraftment of implanted therapeutic MSCs and has been increasingly explored with respect to myocardial repair [103-105].

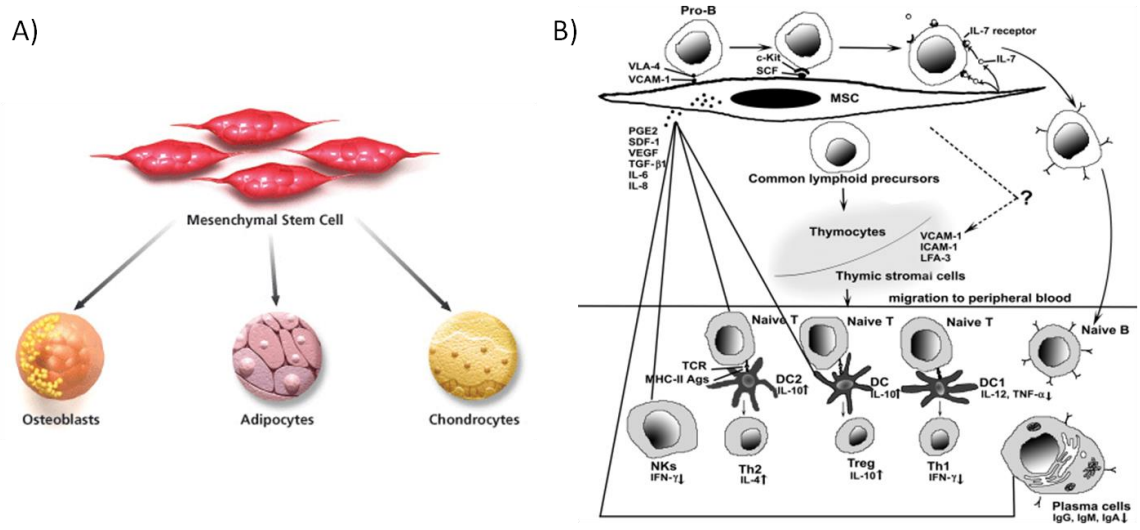

Figure 5. *Differentiation and Immunomodulation of Mesenchymal Stem Cells.* **A)** The three main differentiation lineages of MSCs; osteoblasts, adipocytes, chondrocytes. **B)** Illustration of the identified MSC relationships with immune cells [78]. Of note, altered T cell and NK cell secretion profiles and inhibited proliferation combined with increased T-reg production contribute to immunotolerance induction.

G. Applications of MSCs to *ex vivo* Therapies

As MSCs have only become a major research focus in the past two decades, defining criteria and isolation protocols of a true mesenchymal stem cell can vary, and it is important to take this into consideration when interpreting study results. As our knowledge base of MSC behaviour expands, so too do the variety of *in vivo* studies utilising MSCs for therapeutic end. To date, no tumors have been found in humans recipient of MSCs [59]. Some investigation into this topic has been

completed and it was found that transient and donor-dependent aneuploid MSCs did not give rise to tumors [106]. A second report stated that MSCs were capable of forming sarcomas [107], and another report indicating that MSCs could spontaneously transform was retracted [108]. As previously mentioned, MSCs have been approved for use in many clinical trials and therefore over 2000 people have likely been treated with MSCs [59] which would present MSCs as generally safe, however not enough time has elapsed to highlight possible rare adverse events or late treatment complications so we must keep this possibility in mind. MSCs have been used in therapies designed to treat myocardial defects, kidney injury, connective tissue injury, and acute cerebral injury. For the majority of these therapies cells are applied via local or systemic injection, and some use of scaffolds has being explored. A review of hemophilia therapies that have utilized MSCs for the delivery of therapeutic factors follows in a later section.

Human bone marrow MSCs were intrasplenically implanted for hemophilia A gene therapy using a retroviral vector expressing FVIII, and resulted in therapeutic expression for only 6 days [109]. The same group later transduced human bone marrow mesenchymal cells with a lentiviral vector expressing canine FVIII and s.c.-implanted them in a collagen scaffold into immunodeficient mice [110]. This resulted in the engraftment and long-term survival of the cells. FVIII expression was not monitored *in vivo* but GFP expression was noted for the 2 month duration of the study, indicating the capability of modified MSCs to express a transgene over an extended period *in vivo*.

Human MSCs derived from cord blood, umbilical cord and adipose tissue were modified with the erythropoietin (EPO) gene [111] and one million BM MSCs were injected into the intraperitoneal cavity (IP) of NOD/SCID/γC (NSG) mice. Levels of EPO peaked at 20 days post-injection and declined to near-normal levels after 60 days. This study demonstrates the potential to modify MSCs derived from a variety of sources to supply therapeutic protein. A recent study has developed a three-dimensional scaffold system to support the engraftment and differentiation of FIX-expressing MSCs [112]. Expression of FIX was noted for over 12 weeks.

As demonstrated by these studies, the potential of a low number of MSCs to persist *in vivo* expressing therapeutic proteins for sustained durations is a real and likely possibility. Admittedly in its infancy, this approach is backed with sound preliminary evidence. Further characteristics of MSCs that support the attractiveness of their use as a successful gene therapy tool for hemophilia applications have been demonstrated, and details will follow.

H. The Viral Modification of Cells for *ex vivo* Therapies

Gene therapy aims to introduce gene material to the body, and viruses are vehicles that have evolved over millions of years to accomplish just that. A wide variety of viruses have been investigated to introduce transgenes and modify populations of cells as a method of disease treatment or prevention. These viruses include the non-integrating; adenovirus, herpes-simplex virus, baculovirus and sendai-virus, and the integrating; adeno-associated, retro- and lenti- viruses. Each virus offers different characteristics that must be selected carefully to match the

desired application such as the targeted cell type, efficiency, safety, and level and duration of expression.

1. Lentiviral Vectors

Lentiviruses first demonstrated as effective gene delivery vehicles in 1996 and have become a popular choice for gene transfer in *ex vivo* approaches [113, 114]. They are an ideal selection for the modification of MSCs for hemophilia therapy for a number of reasons. Lentiviruses integrate transgenes into host somatic cell DNA, resulting in stable protein expression that is transferred to daughter cells. Human immunodeficiency virus type 1 (HIV-1) is well characterized and commonly used to develop lentiviral vectors. Viral particles are generally produced using trans-complementation in packaging cells co-transfected with both the plasmid containing transgene and viral packaging genes [Fig. 6B]. Several generations of lentiviral vectors have been developed to include different promoters, pseudotypes and other safety and enhancing elements that improve the vectors' ability to express transgene in target cells.

Lentivirus is a prime candidate for gene therapy for a number of reasons. Firstly, the ability to integrate of up to 10kb DNA into the host chromosome without transferring viral genes is an advantage over a number of other viral vectors [115]. The ability to transduce both dividing and non-dividing cells with high efficiency is of great practical importance. Long-lasting expression even after the differentiation of stem cells [116, 117] and the resistance to transcriptional silencing [118] are great benefits for therapies requiring long-term expression of transgenes. The lack

of interference from pre-existing viral immunity in host [119] and low immunogenicity versus encapsidated vectors [120] are also advantageous for *ex vivo* therapies where the immune response is a great challenge. A lack of precedence associating tumor formation with lentiviral trials [121] exists, and unlike γ-retroviral vectors, lentiviral vectors exhibit no preference for transcriptional start sites, areas close to DNAase1 hypersensitivity sites, or CpG islands [122], adding to the safety of lentiviral trials. In addition, the tropism of lentiviral cells can be modified to target cells to specific cell and tissue types [123]. Pseudotyping for the G protein of the vesicular stomatitis virus (VSV-G) is the most common because it recognises a phospholipid ubiquitous to mammalian cells [121], stabilizes the vector from shear force during centrifugation [124] and provides direct cell entry into an endocytic path [125]. Though there are many benefits to lentiviral vectors over other gene delivery vehicles, there still remains the risk of insertional mutagenesis resulting from the insertion of a viral vector into, or near, genes that confer a survival advantage to target cells [121]. That being said, there is a lack of precedence associating HIV vectors with tumor formation [121]. For these reasons the lentivirus is currently a viral gene vector with strong potential in gene therapy, and would be the ideal choice for hemophilia B *ex vivo* approaches.

2. Lentiviral Modification of Stem Cells and MSCs

A study comparing high doses of lenti- and retroviral transduction has demonstrated the low genotoxicity of lentiviral integration into hematopoetic stem

cells (HSCs) [126]. A lentiviral vector expressing FIX from HSC in irradiated non-obese diabetic – severe combined immunodeficient (NOD-SCID) mice was demonstrated to express FIX *in vivo* for over 8 weeks [127]. This highlights the safety and ability of integrated lentiviral constructs to express transgenes for extended periods of time *in vivo*. The efficiency of lentiviral, retroviral and adeno-associated viral-based infection of cultured, bovine-derived aortic endothelial cells were compared in a 2007 study [128]. Transduction efficiency was demonstrated to be 20-30% higher in lentivirally transduced cells when compared to retrovirus and as much as 50% higher when compared to adenovirus.

Purified murine MSC have been modified using lentivirus to over-express a number of paracrine and growth factors and applied *in vivo* to investigate the effects on cardiomyocyte repair following myocardial infarction [129, 130]. Similarly, lentivirally-modified rat and porcine MSC have also been applied to ischemic and myocardial infarction allogeneic investigations [131-134]. In addition to therapeutic gene expression, lentiviruses have also been applied to human MSC to introduce small hairpin RNA (shRNA) [135], RNAi [136], ZFNs [137], and as a method for tracking cell biodistribution [138].

An *ex vivo* gene therapy for hemophilia A using endothelial progenitor cells modified with a lentivirus to express FVIII implanted cells intravenously (i.v.) or sub-cutaneously (s.c.) on a matrigel scaffold was investigated [139]. Therapeutic levels of FVIII were noted for up to 27 weeks and no anti-FVIII inhibitors were detected. Similarly, long-lasting expression of FIX from lentivirally modified

progenitor cells was demonstrated in transduced bone marrow cells of hemophilia B mice with a dual expressing erythroid-specific lentiviral vector encoding hFIX and methylguanine methyltransferase (MGMT) mutant P140K [140]. Transplantation in syngeneic murine recipients following non-myeloablative conditioning and *in vivo* MGMT selection greatly increased the number of hFIX expressing cells. Factor IX protein expression reached therapeutic levels, and high levels were sustained in secondary and tertiary engrafted mice for up to 18 months after the initial primary bone marrow engraftment. These studies denote the applicability of lentiviruses to hemophilia *ex vivo* gene therapies.

Current research supports an emphasis in applying lentiviral vectors for long-term expression in *ex vivo* therapies. Specifically, lentiviral-driven expression from stem and progenitor cell types has showed significant success compared to other viral vectors. This supports the use of a lentiviral vector to express FIX in MSCs for an *ex vivo* approach to gene therapy of hemophilia B.

I. Overview of the Most Prominent *ex vivo* Approaches to Hemophilia

The development of *ex vivo* approaches to hemophilia therapy that transplant modified and expanded cells as sources of coagulation factors has been explored with a variety of cell types. Endothelial cells, myoblasts and fibroblasts, megakaryocyte, hematopoietic stem cells and finally MSCs are the most prominent cell types that have been utilized. High levels of sustained expression followed by

persistence or engraftment and the absence of formation of inhibiting antibodies are required for the success of these approaches.

1. Endothelial Cells for *ex vivo* Hemophilia Therapy

Vascular endothelial cells and their progenitors were an initial obvious choice for hemophilia gene therapy due to their natural function to modulate clot formation and location lining the surface of blood vessels [141]. They also express von Willibrand Factor (VWF), which has been demonstrated to prolong the circulation time of FVIII when bound in complex [142, 143] and protect FVIII from antibody recognition. Endothelial progenitor cells are present only in low numbers and thus must be expanded *in vitro*, and this offers some technical challenges. Blood outgrowth endothelial cells (BOECs) have been utilized in therapies for hemophilia A beginning in 2002 [144]. Stable FVIII human BOEC transfectants were administered to immune-deficient mice and an increase in circulating FVIII levels was observed in a dose-dependent manner and maintained for the entire 156-day study. Up to 4.8% of cells in their bone marrow and 3.6% of their cells in their spleen were found to consist of the transfected donor cells, representing greater than 100-fold expansion of cells *in vivo*. Similar studies that followed have shown no significant improvements to this approach but have highlighted challenges in promoting the engraftment and *in vivo* survival and proliferation of autologous endothelial cells [50]. A recent FVIII-BOEC study utilized a Matrigel® scaffold for the subcutaneous implantation of modified BOECS, and very low levels of FVIII were maintained for 20 weeks [139]. Mice that did not receive immunosuppressive

treatment or pre-emptive tolerizing doses of FVIII developed inhibitors to FVIII. Endothelial progenitors have demonstrated some capabilities in the long-term expression for hemophilia A therapies, but recent studies demonstrate challenges in immune tolerance and engraftment that need to be addressed.

2. Myoblasts and Fibroblasts for *ex vivo* Hemophilia Therapy

A number of cell-based treatments for hemophilia have explored the use of myoblasts as they are abundant and easy to culture. Two early landmark studies utilized mouse myoblasts transduced with a retrovirus to express human [145] and canine [146] FIX. In both cases cells expressed high levels of FIX *in vitro* (>1μg/10^6 cells/24 h). Cells were injected into multiple sites of posterior limb muscle groups and recipients were given immunosuppressive treatment. Expression peaked at 2-3 weeks and then dropped by 4 weeks. Similar studies demonstrated sub-therapeutic but long-term expression, highlighting the challenge of the immune response to allogeneic cells [147]. A novel approach of cell encapsulation was pioneered by Hortelano *et al* in response to challenges with the immune system, where FIX-transfected fibroblasts were encapsulated with cross-linked alginate and implanted to the intraperitoneal cavity [148, 149]. Minimal *in vivo* expression of FIX was detected in the first few days but disappeared shortly after. Anti-FIX antibodies were detected following the disappearance of FIX expression for the 200 day study period, suggesting that low levels of FIX continued to be secreted. The development of optimized vectors resulted in minimal increases of FIX levels *in vivo*, but again were not sustained beyond week 2 following the detection of anti-FIX antibodies

[150]. A similar investigation with FIX-deficient nude mice was then completed using nearly identical treatment conditions, however levels peaked at day 4 *in vivo* and had disappeared by day 5 [151]. Antibodies to human FIX were detected by day 10 though inhibitors were detected only in one mouse. An increase in dose during the same study resulted in therapeutic expression on day 77 and phenotypic improvements to clot formation, however this resulted in tumour formation in 6 of 7 mice, indicating escape of some fibroblast cells from capsules. A follow-up study utilised monoclonal anti-CD4 antibodies to decrease the CD4+ T cell response [152]. This approach was successful for 6 weeks, however a full immune response subsequently developed accompanied by reduced FIX levels. A change in cell type to fetal G8 murine myoblasts modified with a retrovirus and encapsulated in alginate-PLL resulted in the *in vivo* FIX expression of about 6% of normal levels until day 60 in hemophilia B mice [153]. A similar study was attempted with human primary myoblasts however the low levels of FIX that were initially detected were no longer present after 2 weeks [154]. Overall this approach has demonstrated progress in the survival of cells capable of expressing therapeutic protein, however triggering of the immune response remains a significant challenge.

A clinical trial has investigated the application of FVIII-modified autologous skin fibroblasts for the treatment of 6 patients with severe hemophilia A [155]. Unfortunately, the patients had only minimal or undetectable levels of FVIII following the implantatation of 1-4x10^8 cells in the abdominal wall even though no anti-FVIII inhibitors were detected in response to this therapy. Myoblasts and

fibroblasts were one of the first cell types explored for hemophilia therapies, however some limitations in terms of the continued expression and elicitation of immune response have hampered progress in this cell-based approach to hemophilia therapy.

3. MSCs for *ex vivo* Hemophilia Therapy

One of the first investigations to utilize MSCs for hemophilia evaluated the efficacy of donor MSCs modified with a retrovirus to induce immune tolerance to FVIII in mice [156]. Whole body irradiated-Hemophilia A mice received 1-2x10^6 of FVIII-transduced BM cells and were placed on an immunization schedule 16 weeks later. Expression of FVIII was not detected from implanted cells, however half the mice had a reduced antibody titer and there was a lack of response of CD4 T cells to the FVIII protein. A number of studies have described the challenges in modifying MSCs and their low receptivity for foreign DNA, which is common of primary cells [157]. The first *in vitro* study to address this problem modified human and murine bone marrow stromal cells with a variety of retroviral vectors to find maximum expression of 200ng/10^6cells/24h in human MSCs and 900ng/10^6cells/24h in murine MSCs [158]. A following study modified human bone marrow stromal cells with a FVIII retroviral vector and intrasplenically injected cells into immunodeficient mice [159]. The cells had demonstrated high levels of FVIII expression *in vitro* for a month, however *in vivo* only very low levels were detected on day 1 that dropped to negligible levels by day 8. The percentage of cells that had engrafted was about 0.3% and since no inhibitory antibodies were detected the

declining expression was presumed to be a result of promoter inactivation. To improve on these results the group modified the long terminal repeat (LTR) of the vector to address promotor inactivation [109]. Initial improvements in expression were noted but were again silenced by day 6 and no differences in engraftment were noted. An eight year lapse in MSC-based hemophilia therapies was ended following the investigation of retrovirally modified murine MSCs used in conjunction with a scaffold for the delivery of FIX in 2011 [112]. The three-dimensional scaffold consisted of a polylactic-co-glycolic acid (PLGA) and hydroxyapatite composite about 200μm in diameter. Scaffolds seeded with cells were implanted and hFIX was detected for 12 weeks *in vivo* at levels below the therapeutic threshold. Promoter silencing was again suggested as the explanation for declining expression levels. Recent studies have explored the use of lentivirally-modified paternal ovine MSCs transplanted into the intraperitoneal cavity of two pediatric Hemophilia A sheep [160]. The treatment resolved all existent hemarthroses and ceased spontaneous bleeds. There was evidence of engraftment particularly at affected joints; however the development of inhibitors resulted in a decreased effectiveness of the therapy. Complete bone marrow transplants have also been identified as therapeutic for hemophilia A patients, and an investigation into the responsible cell populations has been completed [161]. Transplants into full-body irradiated mice indicated that hepatocytes and endothelial cells did not contribute to therapeutic benefit, however fractions of Kupffer cells (liver macrophage and mononuclear cells) which arise from healthy BM-derived MSCs were found to protect Hemophilia A mice from

bleeding challenges. Overall MAC-based therapies for hemophilia indicate the potential to express high levels of coagulation factors and the potential for long-term expression, however they continue to be challenged by vector inactivation and the development of inhibitors. Adaptations to therapies are needed to address these challenges in order to develop effective hemophilia therapies.

4. Megakaryocytes for *ex vivo* Hemophilia Therapy

A few groups have explored the potential of megakaryocytes for *ex vivo* hemophilia therapies. Megakaryocytes are derived from the bone marrow, are responsible for producing platelets and naturally synthesize VWF, which would be advantageous for the protection of FVIII [162, 163]. The production of platelets that could deliver therapeutic proteins to sites of vascular damage would also be beneficial. An early megakaryocyte therapy for hemophilia used a promoter specific to human promegakaryocytic cell lines to express FVIII [164]. The same group also transduced peripheral blood cells with a FVIII retrovirus and induced megakaryocyte lineage via exposure to cytokines [165]. Platelet agonists induced the release of FVIII and VWF and demonstrated a 3-fold increase in FVIII activity compared to controls. An *in vivo* investigation transplanted human modified megakaryocytes via the tail vein to sub-lethally irradiated immunodeficient mice and later treated with growth factors to induce platelet activation [165]. After 2 weeks donor platelets consisted of 35% of the total platelets in circulation and exhibited co-localized FVIII and VWF. Mononuclear cells isolated from the bone marrow were transduced with a Lenti-FVIII vector and induced to form

megakaryocytes. Between 6 to $10x10^6$ cells were transplanted to lethally irradiated littermates and four weeks following transplantation FVIII activity was undetectable in the plasma [166]. However, FVIII activity in platelet lysates was similar to that of heterozygous mice and no antibodies to FVIII were detected for duration of the 11 month study. A secondary transplant was conducted with similar results. These studies show that though the modification of megakaryocytes to co-express transgenic FVIII and endogenous VWF is technically difficult and inefficient in itself, the overall concept may be an improvement to cell-based hemophilia therapies as it reduces the FVIII dose required for therapeutic benefit by delivering it directly to its site of need. Therapies for Hemophilia B have not yet been explored using this approach but may evolve down the line.

5. Hematopoietic Stem Cells for *ex vivo* Hemophilia Therapy

Hematopoietic stem cells (HSC) have also been explored for applications in hemophilia therapy for their role in hemostasis and immune-tolerance. Most investigations have focused on bone marrow transfers aiming to tolerize FVIII-null mouse models with FVIII-inhibitors and bring sustained therapeutic expression of FVIII. Following the first bone marrow transplants for hemophilia therapy in 1998, exploration with bone marrow cells transduced with a murine stem cell virus (MSCV)-based onco-retroviral FVIII-expressing vector were transplanted into mice pre-treated with either lethal or sublethal radiation, or a busulfan treatment [156, 167]. Therapeutic levels of FVIII were detected in mouse plasma for over 6 months [167]. Further adaptations of this approach have involved the use of a platelet-

specific promoter in a FVIII-lentiviral vector that demonstrated a lack of antibodies and sustained FVIII-expression in even secondary recipients of bone marrow transplants [166]. Later studies bred transgenic mice expressing FVIII from platelets into FVIII-null mice and completed BM transplants with these cells into immunized FVIII-null mice that had received lethal and sub-lethal doses of irradiation [168]. The FVIII-specific immunity did not appear to affect the engraftment of transplanted cells as 85% of the treated mice survived the tail clip assay. Additionally they found that even transplants of low proportions of cells improved hemostasis [168]. Isolated HSCs modified with a porcine FVIII transgene transplanted into myeloablated FVIII-null mice with anti-human FVIII inhibitors also demonstrated high level-pFVIII expression that resulted in steadily declining antibody titers [169]. However, there was no correlation between pre-transplantation antibody titers and post-transplantation FVIII activity or donor engraftment to confirm the effects were specifically related to the transplanted cells [169]. A later investigation utilizing this approach transplanting the same pFVIII-expressing HSCs into both naïve and immunized mice using a non-myeloablative pre-treatment showed that treated mice were non-responsive to rFVIII challenges [170]. The most recent modification of HSCs for hemophilia therapy utilised a lentiviral vector containing an immunoglobulin heavy chain enhancer-promoter that directed protein synthesis to B-lineage cells [171]. Stem Cell Antigen-1^+ ($Sca\text{-}1^+$) HSCs modified with this vector were transplanted into hemophilia A mice and resulted in FVIII circulation for the 6-month duration of study. A subsequent challenge with rFVIII indicated that immune

hyporesponsiveness had been induced. Researchers were additionally successful at conducting BM and B cell transplants from the spleen of treated mice to secondary recipients resulting in therapeutic expression of FVIII over the short term. Hemophilia therapies using HSCs have demonstrated some success in terms of sustained therapeutic expression, secondary transfer and tolerance induction. However the challenge of engraftment is still significant as myeloablative treatment is an invasive an undesirable approach for hemophiliacs.

IV. CHAPTER 2: PROJECT OVERVIEW

A. Context, Challenges and Scope of Project

Hemophilia is a debilitating disease whose current treatment is expensive, invasive and in some cases ineffective. While hemophilia is an ideal model for gene therapy, past gene therapy treatment approaches have encountered hurdles resulting in a limited ability to provide a source of long-lasting FIX expression. In recent years lentiviral vectors have demonstrated a number of benefits for their use in stem cell modification and cell-based gene therapies [110, 116, 117, 172]. Mesenchymal stem cells (MSCs) have demonstrated novel characteristics such as immunomodulation and multi-lineage differentiation capacity that make them an ideal cell type for application in cell therapies and tissue engineering [58, 83]. These two biological entities can be used in conjunction to develop a more effective and less invasive gene therapy treatment for hemophilia B. Additionally, immune-isolation of therapeutic cells using alginate microcapsules has proven effective at delivering therapeutic levels of transgene in mouse models [149-151, 153].

In summary, this project aims to make use of a number of experimental successes recently achieved in the fields of viral vector, stem cell and *ex vivo* technologies. The aim is to use mesenchymal stem cells (MSC) engineered with a lentivirus to deliver therapeutic levels of FIX protein to hemophilia B mice for a sustained period of time.

B. Objectives

PART A: Develop a FIX-Expressing human cord blood-derived MSC cell line using a lentivirus

- Develop a reproducible method of cell-line generation
- Assay Expression of Functional human FIX *in vitro*
- Investigate ability of CB MSC to differentiate following modification, and ability to continue to express functional FIX transgene *in vitro*

PART B: Develop an optimal CB MSC transplantation treatment regimen *(in vivo)*

- Develop a reproducible method for modified CB MSC transplantation
- Determine ideal mouse model for CB MSC transplantation
- Assess Expression of FIX *in vivo*
- Assess for dose effect *in vivo*

PART C: Complete *in vivo* trials of encapsulated modified CB MSCs (with Bahareh Sayyar)

- Encapsulate CB MSC with alginate-PLL using previously optimized protocols
- Assess Expression of FIX *in vivo*

C. Hypothesis

A lentiviral vector engineered to enclose a human FIX gene will enable the development of a FIX-expressing cell line that expresses sustained high-levels of FIX from cord blood-derived MSCs. The transplantation of these cells into mice via tail vein injection or IP transplantation of capsules will result in therapeutic expression of FIX *in vivo*.

V. CHAPTER 3: Part A – Development of FIX-Expressing MSCs

A. Part A: Methods

1. Generation of a FIX-Expressing Lentiviral DNA Vector

A lentiviral DNA vector containing the human FIX gene (PLVX-FIXi) was generated using traditional restriction enzyme digest and ligation techniques [Fig. 6A]. This vector was then used to generate viral particles and transduce cells for gene therapy.

a) Source and structure of lentiviral vector and FIX transgene

The human FIX gene utilized to generate the lentiviral vector is 1682 bp in length, and contains fragments of intron 1 from the human FIX gene (FIXi), specifically the first 135 bp and the last 293 bp [150]. The gene was obtained from a vector constructed of the pBluescript II SK+/- phagemid vector containing the FIXi gene of interest (pBSK-FIXi). The inclusion of DNA from intron 1 of the human FIX gene has been demonstrated to enhance the activity of the FIX protein [173].

The PLVX-Puro 4th generation vector from Clontech Laboratories (Mountain View, CA) [Fig. 6B] is replication incompetent and designed to infect cells only once. It is an ideal vector for the *ex vivo* modification of MSCs. The PLVX-Puro vector has many features that make it attractive for the purpose of sustained expression of FIX from MSCs. These follow:

A. The vector contains only 25% of the genes from the original wild type HIV-1 virus.

B. The trans-acting elements of the vector are separated onto 5 helper plasmids, thus lowering the chance of replication competent virus (RCL) generation to five chance recombinant events.
C. The vector contains a wild-type LTR, thus increasing transcription while making it Tat dependent.
D. Expression of the transgene is driven by the CMV promoter which has demonstrated sustained expression in other tested lentiviral vectors [174].
E. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) that facilitates effective transcription termination at the 3' LTR is included. WPRE both elevates the level of functional viral genomic RNA in packaging cells, and the level of mature transgene mRNA in transduced target cells [175, 176]. Thus both titer and transgene expression are increased.
F. The inclusion of the central polypurine tract/central termination sequence element (cPPT/CTS) in the PLVX vector creates a central DNA flap that increases nuclear importation of the viral genome during target cell infection. This results in improved vector integration and greater transduction efficiency [177] and this has been demonstrated in hematopoetic cells [178].
G. Enhanced gene expression and viral titers are provided by the Rev-responsive element (RRE). This allows the transport of unspliced RNA out of the nucleus [179].

H. Fusion of the polymerase (pol) gene responsible for the coding of transcriptase, integrase and protease to the viral protein R (vpr) gene which regulates the import of the HIV- preintegration complex ensures the transport of reverse transcriptase (RT) and integrase into the recombinant viral particle.

Various companies have recently introduced lentiviral vectors to the market with features designed for specific applications. For the above mentioned reasons this vector was chosen in addition to the ease with which a transgene can be introduced by conventional cloning, and simple protocol for generation of viral particles.

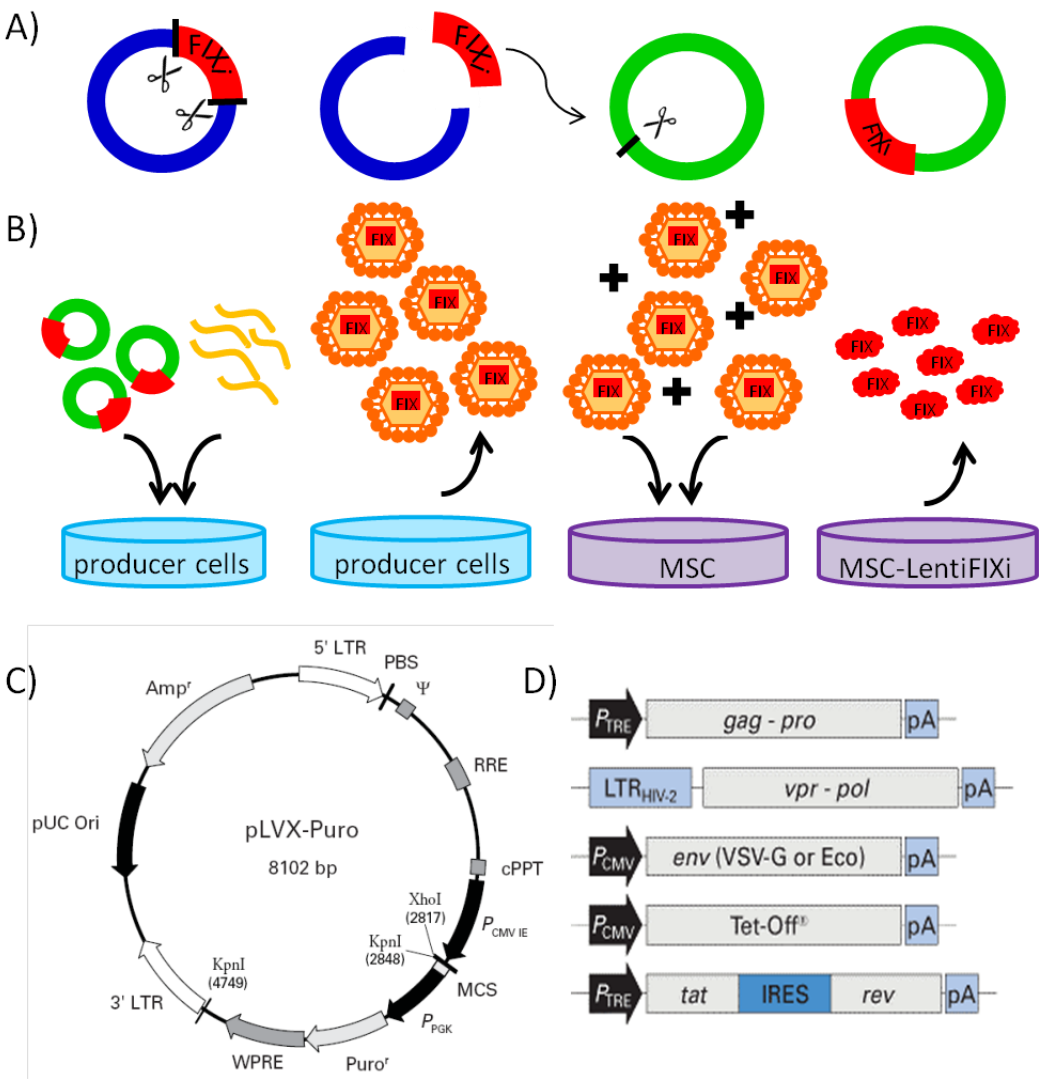

Figure 6. *Generation of PLVX-FIXi lentiviral particles and PLVX vector structure.* **A)** Restriction enzyme digest to extract FIXi **B)** Co-transfection of PLVX-FIXi and helper viral plasmids to generate viable PLVX-FIXi Lenti particles. **C)** DNA vector map with prokaryotic *AMP*^{R} and Eukaryotic *PURO*^{R} selection factors. Multi-cloning site (MCS) contains many restriction enzyme digest sequences including *xhoI* and *xbaI* **D)** Structure of trans-acting packaging elements for generation of replication incompetent lentiviral particles.

b) Lentiviral vector and FIX DNA replication and purification

Large quantities of pBSK-FIXi plasmid DNA for excision of FIXi transgene were expanded in *E. Coli* bacteria and extracted using traditional DNA purification methods. An inoculation of 1 mL of Luria-Bertani (LB) media containing 1 µg of ampicillin from frozen pBSK-FIXi bacterial stock was cultured in a 37° C horizontal

shaker at 225 rpm overnight. The following morning this 1 mL culture was used to inoculate 400 mL of LB media containing 400 µg of ampicillin selection antibiotics and identically cultured for 16 h. The pBSK-FIXi DNA was purified from bacteria using Qiagen's QIAprep® Spin Maxiprep Kit (Toronto, ON) according to manufacturer's instructions. Briefly, bacteria was pelleted, resuspended and lysed and DNA extracted using successive washes, filtering and elution followed by precipitation.

Lyophilized PLVX-Puro vector (Clontech Laboratories, Mountain View, CA) was reconstituted in double deionized water (ddH_20), to be used in the engineering of a PLVX-FIXi vector. PLVX-Puro stock DNA was transformed into One Shot® Stbl3™ Chemically Competent *E. coli* cells (Invitrogen, Burlington, ON) for replication. Briefly, a vial of 50 µL of competent cells was thawed on ice for 20 min and 100 pg of PLVX-Puro vector DNA was added and mixed gently. This was incubated on ice for 30 min. Cells were then heat-shocked in a 42° C water bath for 45 s and returned to an ice bed for 2 min. One millilitre of LB media was added and cells were incubated for 1 h at 37° C on a horizontal shaker at 225 rpm. The bacterial suspension was then used to streak an LB/agar plate containing 100 µg/mL ampicillin. This plate was then incubated in an inverted position overnight in a 37°C incubator. Single bacterial colonies were isolated and inoculated into 5 mL of LB media in a 15 mL polyethylene tube and cultured overnight in a 37° 225 rpm horizontal shaker. A fraction of this culture was then used to inoculate larger volumes of LB while the remaining volume was subjected to DNA extraction using

the Qiagen's QIAprep® Spin Miniprep Kit. The identity of vector DNA in these samples was confirmed following digestion with restriction enzymes and subsequent assessment on an agarose gel.

c) Restriction enzyme digestion and generation of DNA fragments for vector construction

Excision of the FIXi gene from the pBSK-FIXi DNA vector was completed using Fast Digest® XhoI and XbaI restriction enzymes (Fermentas, Burlington, ON). Briefly, purified pBSK-FIXi DNA was gently combined with restriction enzyme(s), water and Fast Digest® 10X buffer and incubated for 15 min at 37° C. To confirm digestion of the plasmid, samples were combined with 6X DNA loading dye (Fermentas, Burlington, ON) and run on a 1% agarose gel alongside GeneRuler™ 10,000 bp DNA ladder (Fermentas, Burlington, ON). Agarose gel was prepared by adding 1g of agarose powder to 100 mL of 1% TAE electrophoresis buffer (Tris, acetate, EDTA) at pH 8.0. This mixture was gently heated and mixed to dissolve the agarose powder. After a few minutes of cooling the gel was poured into a BioRad® gel box containing a comb, and cooled at room temperature for 15 min, or until gel had completely solidified. The gel was then submerged in a bath of TAE buffer, followed by the addition of samples to individual wells. The gel was run at 15 V for 14 h and stained in an ethidium bromide bath for 10 min. Following a de-stain period of 10 min in ddH_20, gels were visualized using ultraviolet light (UV). Digestion fragments were visually assessed for correct size in comparison to the DNA ladder, 1861 bp (~2 Kb) for FIXi gene and 2895 bp (~3 Kb) for pBSK backbone.

The PLVX-Puro vector DNA was similarly linearized for ligation of FIXi transgene ligation via digestion of DNA plasmid with XbaI and XhoI Fast Digest restriction enzymes and run on an agarose gel to confirm digestion and linear size of 8095 bp (~8 Kb) [Fig. 6C].

Following confirmation of digestion and generation of DNA fragments of interest, DNA was extracted from the agarose gel to be used in the ligation of the PLVX-FIXi DNA vector. DNA bands containing the FIXi product (~2 kB) or PLVX linearized vector (~5.5 kB) were excised with a scalpel and eluted from agarose gel using the protocol and components inclusive to the Qiagen QIAquick® Gel Extraction Kit. These DNA fragments were then stored at -70° until required for ligation.

d) Ligation of lentiviral vector and FIX transgene DNA

DNA fragments were eluted from agarose using methods previously described, and used in a ligation reaction to form a PLVX-Puro FIXi-containing DNA vector. The reaction consisted of an overnight incubation at room temperature of the FIXI and PLVX linear DNA strands digested with XbaI and XhoI at a ratio of 3:1 with 1 μL of Fermentas T4 DNA ligase enzyme and ddH20 to a final volume of 20 μL.

e) Verification of PLVX-FIXi sequence

Ligation reactions were then cultured for 1 hr and plated overnight on LB/agar containing 100 μg/mL ampicillin. Resistant clones were then inoculated into 5 mL LB with ampicillin selection and cultured overnight. Qiagen's QIAprep® Spin Miniprep kit was then used to purify the DNA from 4 mL of the overnight

culture according to manufacturer's protocol. The remaining 1 mL of culture was stored at -70°C for later use. Restriction enzyme digests with XbaI and NdeI were run on a 1% agarose gel to confirm the identity of the newly formed plasmid. Plasmids indicating ligation of PLVX-FIXi DNA were used for large-scale preparations.

DNA samples were sent to the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University for sequencing using primers 5' GCGAGATCTTAGCAGATT-GTGAACATGATC 3'and 5 'CAACCATGGACCTTGGAAATCCATCTTTCA 3' designed to flank the FIXi region.

f) Verification of PLVX-FIXi functionality

The PLVX-FIXi plasmid was tested for expression of FIX when transfected into G8 murine foetal myoblast cells using Invitrogen's Lipofectamine™ 2000 transfection agent. One day prior to transfection, cells were seeded on a 10 cm plate to reach 90-95% confluency within 24 h. PLVX-FIXi vector DNA (24 µg) was diluted in 1.5 mL of Opti-MEM® I Reduced Serum Medium and mixed. A volume of 60 µL of Lipofectamine™ 2000 was also added to 1.5 mL of Opti-MEM® I Reduced Serum Medium and incubated for 5 min at room temperature. The diluted DNA and transfection reagent were then combined, mixed and incubated at room temperature for 20 min. After incubation, the solution complex was added to cell plates and medium, and rocked gently back and forth. After 6 h the media was

changed and cells passaged normally. Cell media supernatant samples were measured by hFIX ELISA to determine FIX levels.

g) Large-scale DNA replication and purification

Cultures containing correctly ligated PLVX-FIXi DNA plasmids were then cultured on a large scale for DNA purification. A 500 µL volume of LB media was inoculated with the frozen sample of PLVX-FIXi and cultured at 37°C for 2 h on a horizontal shaker at 225 rpm. This culture was then streaked on an LB/agar plate containing ampicillin and incubated in an inverted position at 37°C overnight. Individual colonies were then cultured in 3mL of LB containing ampicillin at 37° C for 2 h on a horizontal shaker at 225 rpm. A portion of this culture was then used to inoculate 400 mL of LB media containing ampicillin selection antibiotics and identically cultured overnight. Qiagen's QIAprep® Spin Maxiprep Kit was used to purify DNA from the bacterial culture and stored at -70°. Prior to use in any experiment, each Maxi preparation was confirmed for PLVX-FIXi identity via restriction enzyme digest and assessment on a 1% agarose gel.

h) Measuring DNA concentrations

The optical densities of all DNA preparations at 600 nm were measured to ascertain the concentration of DNA in ddH_20. The concentration of suspended DNA in ddH_20 provides necessary information to set up digestion, ligation and transformation reactions. The Beckman Coulter DU 530 Spectrophotometer was used set to an optical density of 600 nm (OD600) under the settings for double ratio and concentration of nucleic acid, and 50X dilution and 50X concentrations. A 2 µL

DNA sample and 98 µL ddH20 were added to the cuvette and mixed well with pipette tip. Triplicate readings were taken and averaged to determine DNA concentrations.

2. Development of Lenti-FIXI Viral Particles

Following the generation of the PLVX-FIXi DNA plasmid, the Clontech Lenti-X™ HT Packaging System kit (Clontech Laboratories, Mountain View, CA) was used to generate viable replication-incompetent lentiviral particles that can be used to transduce cells to express the human FIX transgene.

a) Co-Transfection of all plasmids to generate lentiviral particles

Lentivirus particles were generated using the Clontech Lenti-X™ HT Packaging System kit and Lenti-X 293T cells. The simultaneous transfection of PLVX-FIXi plasmid DNA and Lenti-X HTX packaging DNA was completed with Xfect™ transfection reagent. Briefly, PLVX-FIXi and Lenti-X HTX helper DNA, and Xfect transfection polymer were added to identical volumes of reaction buffer and mixed gently. Polymer and DNA suspensions were combined, vortexed and incubated for 10 min at room temperature to allow for the formation of DNA-nanopolymer complexes. DNA-nanopolymer complexes were then added to Lenti-X 293T cells that had been plated the night before to achieve a confluency of 50%. Complexes were incubated with the cells for about 8 hours followed by a change in media. Media samples containing live virus were collected 48 h after this media change. Medium samples containing lentivirus were immediately filtered through a low-

protein binding 0.45 μm polyethersulfone (PES) filter, aliquot to 2 mL vials and stored in the -70° freezer for titration or later use.

b) Viral collection and titration

Lentiviral titer was determined using antibiotic selection. HT-1080 cells were plated in one 6-well polystyrene plastic tissue culture plate at 2 x 10^5 cells/well in 2 mL of Dulbecco's Modified Eagle Medium. Complete cell medium was prepared with a concentration of 12 μg/mL of polybrene and aliquot to 1.5 mL tubes. Virus stock was thawed at room temperature and added to the first media aliquot so that 5 serial ten-fold dilutions of virus in media were generated. Dilutions were mixed well and 1 mL of each dilution was added to the existing 2 mL of medium on the HT1080 cells in each well. One well was left containing no viral dilution as a control, and the final concentration of polybrene was diluted to 4 ug/mL in 3 mL. The 6-well plate was then centrifuged for 90 min at 32° at a speed of 1500 g to increase levels of viral transduction. After a 20 h infection period the supernatant was removed, cells washed with 2 mL PBS and medium replaced. Antibiotic selection began at this time to determine viral titer. Optimal puromycin selection level previously determined (2.5 μg/mL) was maintained in each well for 14 d. Media and selection antibiotic were replaced and cells washed with PBS every two days. After 14 d the number of visible colonies in the well with the lowest viral dilution was visually counted, and viral titer determined.

c) Determination of optimal puromycin concentration for selection of transduced cells

Optimal puromycin antibiotic concentration for selection of resistant cells containing the PLVX-FIXi vector was determined using HT-1080 cells suggested for viral titering. Cells were seeded to 2x6-well plate (200 000/well) and after 12 h varying concentrations of Puromycin antibiotic were added to cell media. Media was changed every two days and after 10 d cell survival was analyzed. The lowest concentration of puromycin that resulted in a clear well with no attached cells was determined to be optimal for selection of resistant lentivirally transduced cells.

3. Development of a stably-expressing CB MSC FIX-Expressing cell line

a) Source and culturing of human cord blood-derived mesenchymal stem cells

Cord blood-derived mesenchymal stem cells (MSCs) were derived from human umbilical cord blood with full consent of donors. MSCs were isolated by the Janowska-Wieczorek lab in Edmonton, AB according to previously published protocols [67]. Cells arrived between passages 1-5 and were cultivated in Iscove's Modified Dulbecco's medium (IMDM) (Life Technologies™, Burlington, ON) supplemented with 10% fetal bovine serum in standard tissue culture-treated polystyrene dishes. Upon reaching 70% confluency cells were washed with PBS, detached using TrypLE™ (Life Technologies™, Burlington, ON) and replated in fresh medium. Cell medium was replaced every 2-3 days and two population doublings were considered to represent 1 cell passage. Cells in suspension were counted using the Beckman Coulter Z1 Coulter® particle counter.

b) Generation of stably-expressing FIX cell lines from lentiviral transductions

Stably-expressing FIX cell lines were generated via transduction of FIX-expressing lentivirus using methods described. Cord blood (CB) MSC cells were seeded onto a 6-well or 10 cm plates and transduced with LentiFIXi at a multiplicity of infection (MOI) of ~10 in appropriate cell medium containing a 4 μg/mL concentration of polybrene. Cell plates were incubated overnight at 37°C in an incubator with 5% CO_2 and medium was changed after 24 h. Selection with puromycin at 2 μg/mL began 48 h following media replacement. Selection with puromycin continued for 14 days, leaving a completely transduced cell pool.

Populations of CB MSC transduced with Lenti-FIXi viral particles and selected with puromycin were grown to 70% confluency in 10 cm dishes. Cells were washed, trypsinized and counted using the Beckman Coulter Z1 Coulter® particle counter. These cells were then equally divided amongst 4x10 cm plates and medium samples taken 24 h following cell plating. These samples were subsequently analyzed for FIX expression and FIX:C using a FIX ELISA and APTT respectively.

c) Quantification of hFIX expression via hFIX Enzyme-Linked ImmunoSorbent Assay (ELISA)

Quantitative measurement of hFIX protein expression was conducted using a sandwich-style FIX Enzyme-Linked ImmunoSorbent Assay (ELISA) kits from Affinity Biologicals™ (Ancaster, ON). Briefly, hFIX capture antibody was diluted in coating buffer, applied to wells in a 96-well plate, covered and left to sit overnight at 4°C. All wells were then washed with a 1/1000 dilution of PBS-Tween. A standard hFIX

reference was prepared with normal human plasma in half dilutions. These were applied to individual wells along with 100 µL samples of supernatant medium, covered and incubated at room temperature for 90 min. All wells were washed with PBS-Tween and hFIX detection antibody that had been diluted in substrate buffer was added to each well, covered and incubated at room temperature for 90 min. All wells were washed with PBS-Tween and 100 µL of freshly prepared o-phenylenediamine dichloride substrate was added to each well, covered, and incubated for 10 min at room temperature. Developing reactions were then stopped using 50 µL of 2.5 M H2SO4 and the optical density of the samples were taken at @ 490 nm using a plate reader. A standard curve was generated to calculate results.

d) Quantification of FIX activity via Activated Partial Thromboplastin Time (APTT)

Activated partial thromboplastin time (APTT) was used to quantify the functional FIX (FIX:C levels) of *in vitro* samples using human FIX-depleted plasma as the substrate plasma. Mouse plasma or media samples were diluted in Veronal buffer and combined with FIX-deficient plasma. These dilutions were aliquot to cuvettes containing a stainless steel ball for coagulation analysis. The addition of APTT reagent to the diluted samples followed by calcium chloride during agitation initiated the clot formation process and clotting times were quantified with the STart4® coagulation analyzer (Diagnostica Stago, Parsippany NJ). A standard curve was generated using increasing dilutions of pooled normal plasmas added to fixed concentrations of FIX-deficient human plasmas for the assessment of FIX:C values.

4. Assessment for Multi-Potential Differentiation Capability

Following modification with the Lenti-FIXi vector, CB MSCs were assessed for the ability to differentiate into the three primary lineages of MSCs: adipocytes, osteoblasts, and chondrocytes using differential mediums.

a) Differentiation and staining of MSCs to adipogenic, osteogenic and chondrogenic lineages

Adipogenesis: MSCs were seeded into a 12-well tissue culture coated plate at a density of 1 x 10^4 cells/cm^2. Cells were cultured for 4 hours until adherent in regular IMDM medium and the medium was then replaced with 1 mL Gibco's StemPro® Adipogenesis Differentiation Medium (Invitrogen, Burlington, ON). Media was changed every 2-3 days for 14 days and then cells were fixed and stained with Alizarin Red.

Osteogenesis: MSCs were seeded to 12-well plates at a density of 5 x 10^3 cells/cm^2 and cultured in IMDM for 4 hours until adherent in this medium. Medium was then replaced with StemPro® Osteogenesis Differentiation Medium and replaced every 2-3 days for 21 days and when the cells were fixed and stained with Oil Red O.

Chondrogenesis: Cells were pelleted via centrifuge for 5-10 min at 100 g. These cells were then resuspended in IMDM medium to a density of 1.6 x 10^7 cells/mL. These were seeded onto wells in 5 µL droplets (3 for each well) and incubated for 2 hours at 37°. Medium was then replaced with 1mL StemPro® Chondrogenesis Differentiation Medium and replaced every 2-3 days for 14 days. Cells were then fixed and stained with Alcian Blue.

b) Cell staining

Oil Red O Staining: Media was removed from adipo-differentiated MSCs and cells were washed 3 times with PBS. Cells were covered with 4% paraformaldehyde and allowed to fix at room temperature for 30 min. The pH of the Alizarin red solution was then verified to be between 4.1 and 4.3. Paraformaldehyde was discarded and cells washed twice with ddH_20. Well surfaces were covered with Alizarin Red for 3 min at room temperature and discarded. Cells were washed with ddH_20 4-6 times until wash ran clear and photos were taken with a digital camera through a light microscope at 100X magnification.

Alcian Blue Staining: Media was removed from chondro-differentiated cells and cells were gently rinsed with PBS 3 times using a micropipette. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min while the pH of the Alcian Blue was verified to be 2.5. Paraformaldehyde solution was discarded and cells rinsed twice with ddH_20. The well surface was covered with Alcian Blue and incubated at room temperature for 30 min. Wells were gently rinsed with ddH_20 4-6 times or until wash ran clear. Wells were then rinsed 3 times with 0.1N HCl. Water was then added photos taken using a light microscope at 40X magnification.

Alizarin Red Staining: Media was removed from osteo-differentiated cells and cells were gently rinsed with PBS 3 times using a micropipette. Cells were fixed with 4% paraformaldehyde at room temperature for 30 min while Oil Red O stain was diluted to working solution in a fume hood. This solution was filtered through Whatman® paper in a funnel. Paraformaldehyde solution was discarded and cells

rinsed twice with ddH_20. Isopropanol diluted to 60% was added to each well and incubated for 5 min at room temperature. Well surfaces were covered with Oil Red O solution and incubated at room temperature for 5 min. Stain was then discarded and cells rinsed 4-6 times with ddH_20 or until the wash ran clear. Cells were counterstained with hematoxylin for 1 min, followed by 4-6 repeated washes with ddH_20.

DAPI (4',6-diamidino-2-phenylindole) Staining: Cells were stained with DAPI to image for DNA. Cells were washed, harvested, centrifuged and resuspended in 100% cold ethanol for 15 min. Cells were then centrifuged, recovered in PBS and stained with DAPI at 3μM diluted in PBS containing 5% FBS for 15min.

c) Cell imaging

Cells stained with Oil Red O, Alcian blue and Alizarin Red and were visualized on a light microscope at 10x, 20x, and 40x resolutions. Photos were taken using a digital camera and processed using Microsoft software.

Cells stained with DAPI were visualized on a Ziess fluorescent microscope at 20x, 40x and 63x resolutions using either halogen or UV fluorescence. Photos were taken using a digital camera and processed using specialized software.

5. Statistics

Standard differentiation for triplicate or quadruplicate samples was indicated by error bars or stated values. A Student's 2-tailed T-test was used to

assess for the p-value of expression and activity of FIX levels in comparison to basal or control values. P-values below 0.05 were deemed significant.

B. Part A: Results

1. Engineering of a FIX-Expressing Lentiviral Vector and Generation of Stably-Expressing LentiFIXi-CB MSCs

The PLVX-FIXi lentiviral DNA plasmid was generated using traditional restriction enzyme digest and ligation reactions. Large-scale DNA preparations of the PLVX-FIXI plasmid were verified via restriction digest [Fig. 7A] and DNA sequencing. Additionally, significant levels of FIX protein were detected via hFIX ELISA in the medium of transfected hCB MSC and mG8 cell lines after 24 and 48 hours, confirming the integrity of the PLVX-FIXi lentiviral DNA plasmid [Fig. 7B]. The level of FIX protein expression from transfected cells should only be interpreted as a positive indication of the integrity of the vector, and not the amount of FIX capable of being expressed from the vector.

Cell lines expressing FIXi were generated from one round of transduction of hCB MSCs at a multiplicity of infection (MOI) of 10 with FIXi-expressing lentiviral particles generated from the Clontech Lenti-X™ HT packaging system. Following 12 days of antibiotic selection an initial test to confirm FIXi secretion and function were conducted using a hFIX ELISA and hFIX APTT (data not shown). All subsequent LentiFIXi-hCB MSC cell lines were generated identically with hCB MSC transduced at passages 4-6.

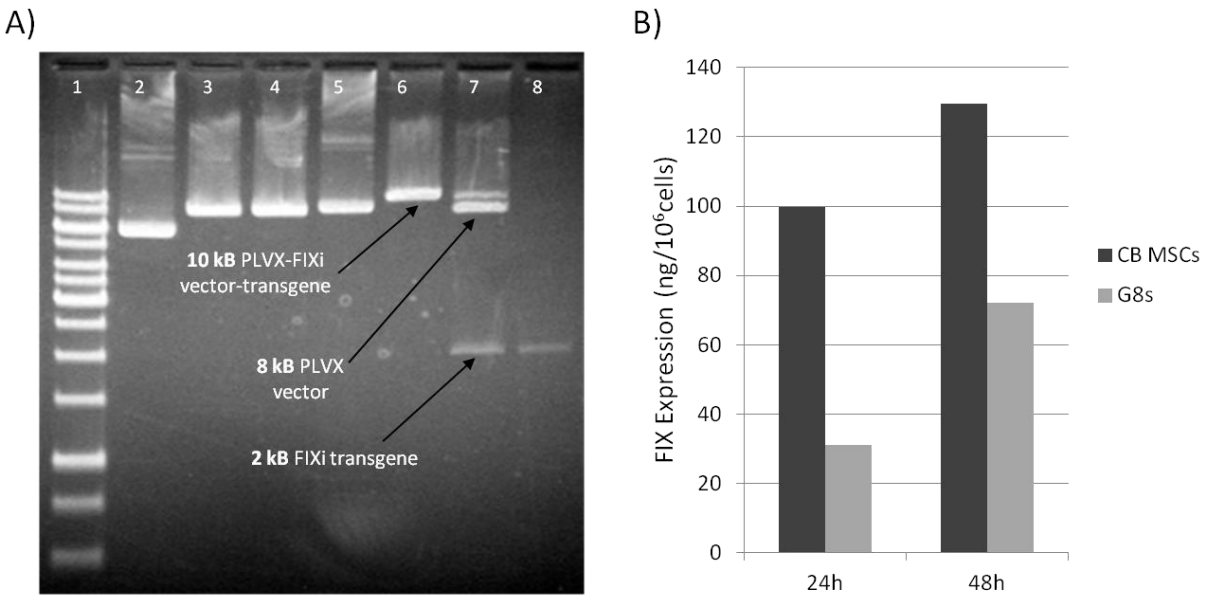

Figure 7. *Confirmation of PLVX-FIXi DNA Plasmid and FIX Expression*. **A)** Restriction enzyme digest of PLVX-FIXi plasmid to confirm ligation of transgene to vector. PLVX-Puro and FIXi insert controls were included. Lane 1: 1kB DNA ladder, lane 2: PLVX vector uncut, lane 3: PLVX-Puro XbaI digest, lane 4: PLVX-Puro XbaI and XhoI digest, lane 5: PLVX-FIXi uncut, lane 6: PLVX-FIXi XbaI digest, lane 7: PLVX-FIXi XbaI and XhoI digest, lane 8: FIXi XbaI and XhoI digest. **B)** FIX expression from *in vitro* samples via hFIX ELISA following transient Lipofectamine 2000™ transfection of G8 fibroblasts with PLVX-FIXi plasmid vector.

2. Long-Term Expression and Function of FIX Expressed from LentiFIXi-hCB MSC *in vitro*

LentiFIXi-CB MSCs were expanded *in vitro* and sampled for hFIX expression and function from 24 h quadruplicate samples over a 45-day period. The expression of hFIX protein from LentiFIXi-hCB MSCs was sustained at very high levels for the duration of the test period, averaging about 5000 ng/10^6cells/24 h [Fig. 8A]. The ageing of the cells did not appear to impact FIX expression, as cells on day 7 at passage 7 expressed 4517.6 ±194.2 ng/10^6cells/24 h of hFIX, and cells at passage 12 on day 45 maintained the expression of hFIX at a level of 5118.8 ±370.7

ng/10^6cells/24 h. LentiFIXi-hCB MSCs expressed three times the quantity of hFIX compared to the highest expressing hFIX cell line previously developed in the Hortelano lab, the RetroFIXi-G8 [153]. The levels of hFIX expression reported in Figure 8 were consistently reproduced in newly generated LentiFIXi-CB MSC cell lines.

The coagulant activity of the hFIX expressed from LentiFIXi-CB MSCs over the 45-day incubation period was measured using a hFIX APTT [Fig. 8B]. The FIX activity was sustained at a high level and averaged over 2500 mIU/10^6cells/24 h throughout the study period. As with FIX protein expression, the increasing passage of cells did not decrease the functional FIX secreted as the activity measured from cells on day 7 at passage 7 was 2156.1 ±155.0 mIU/10^6cells/24 h, and was similar on day 45 (passage 12) at 2930.1 ±263.2 mIU/10^6cells/24 h. The sustained high level of expression and function of hFIX expressed from LentiFIXi-CB MSCs make it a useful tool in the development of a gene therapy for hemophilia B, as expression and activity were significantly increased compared to the previous RetroFIXi-G8 cell line.

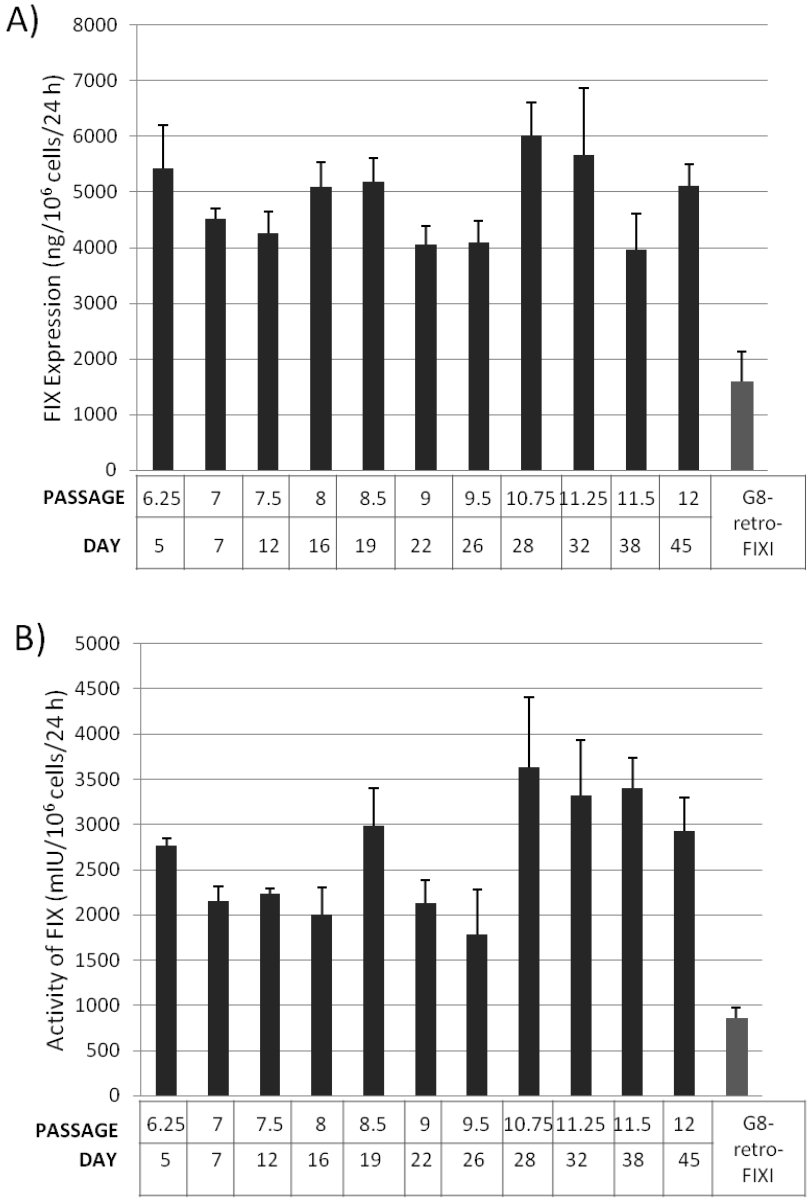

Figure 8. *Expression and functionality of hFIX from LentiFIXi-CBMSC* in vitro. **A)** Expression of FIX protein is sustained at high levels for over 45 days and up to passage 12 following transduction and selection of CB MSC-LentiFIXi cells *in vitro*. Values represent averages from quadruplicate samples. Error bars represent standard deviation. **B)** FIX coagulant activity of expressed from LentiFIXi-CBMSC cells as measured using FIX-APTT assay based on the time (s) necessary for 5 µL of media to added to FIX-depleted plasma to clot. Values are expressed from averages of quadruplicate samples in milliUnits of FIX in a 24 h period from 10^6 cells relative to passage and number of days in culture. A reference value of normal human plasma is indicated, where ~1000 mIU are present in 1mL. Error bars represent standard deviation.

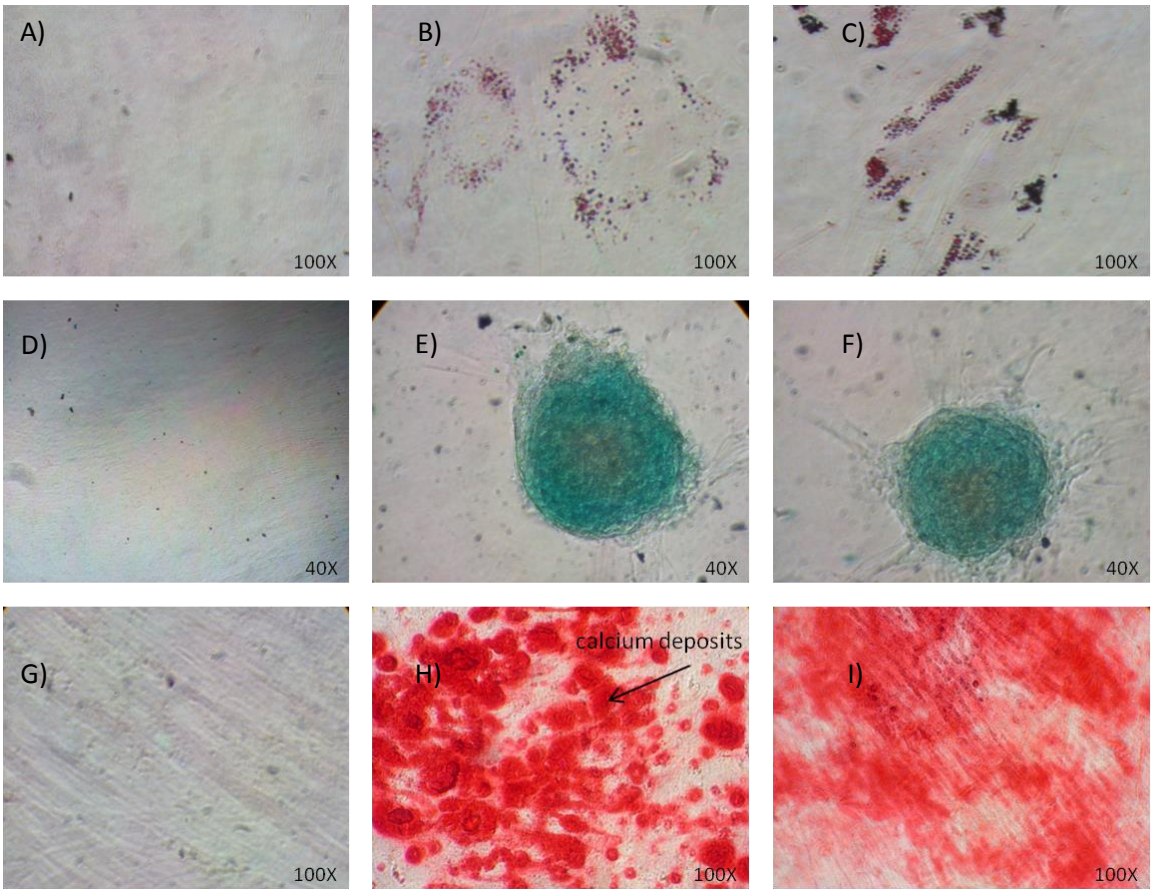

Figure 9. *Differentiation of CB MSC and LentiFIXi-CB MSC.* **A,D,G)** CB MSC at passage 6 cultured in control (IMDM) medium in monolayer (adipo and osteo) or pellets (chondro) according to differentiation protocol. Cells in **B,E,H)** are LentiFIXi-CB MSC at passage 6 and in differentiation medium, cells in **C,F,I)** are unmodified CB MSC at passage 6 in differentiation medium. **B,C)** Cells cultured in monolayer in adipocyte differentiation medium, red stain indicates lipid deposits in fat vesicles and adipogenic differentiation. **E,F)** Cells cultured in chondrocyte differentiation medium, blue stain indicates adic mucosubstances and chondrogenic differentiation. **H,I)** Cells cultured in osteoblast differentiation medium, red stain indicates calcium deposits and osteogenic differentiation.

3. Differentiation of Modified Cells

Unmodified CB MSC and LentiFIXi-CB MSCs at passage 7 incubated in adipo-, chondro- and osteo- differentiation and control mediums for 22 days are illustrated in Figure 9. Positive staining for all three differentiation conditions was evident for modified and control cells and absent for cells cultured in control media. The Oil Red O stain indicates fat vesicles characteristic of adipocytes [Fig. 9B,C], Alizarin Red

identifies the presence of calcium deposits produced by oteoblasts [Fig 9. E,F] and Alcian Blue indicates acid mucosubstances characteristic of chondrocytes [Fig 9. H,I]. This differentiation test indicates that the modification of hCB MSCs with the PLVX-FIXi virus does not interfere with the ability of CB MSCs to differentiate into the defining mesenchymal lineages [Fig. 9].

4. Sustained Expression and Function of FIX Expressed from Differentiating LentiFIXi-CB MSCs

The sustained expression of hFIX by differentiating LentiFIXi-CB MSCs was confirmed using a hFIX ELISA and APTT. LentiFIXi-CB MSCs were cultured in adipocyte, chondrocyte and osteoblast differentiation conditions alongside controls and sampled every 2-3 days. The expression of hFIX was sustained throughout the test period by all three lineages indicating that transgene expression is not silenced during or immediately following the differentiation process [Table. 1]. The expression of hFIX by each lineage can be interpreted in reference to LentiFIXi-CB MSCs cultured under identical conditions in control medium. Initial cell numbers and replication rates for MSCs may differ throughout the differentiation period. For this reason, some disparities in cell number would be expected in the late stages of differentiation and control LentiFIXi-CB MSCs.

The level of hFIX protein expressed from LentiFIXi-CB MSC quantified by hFIX ELISA by cells in control and adipogenic media was maintained at a constant level for the 22 day investigation period following the initial 1 week cell growth period [Table 1]. The quantity of hFIX protein expressed by control cells was

maintained at about twice the level as that expressed by differentiating cells. This difference may be attributable to the lower cell numbers in the differentiating cell populations as replication slows during differentiation. Interestingly the level of hFIX activity for these populations indicates the same steady trend. However a greater amount of hFIX activity was secreted by cells differentiating in adipogenic than control media [Table 1]. Overall the continued expression of hFIX with a high level of activity is demonstrated in LentiFIXi-CB MSC differentiated into the adipogenic lineage.

The quantity of hFIX protein expressed from LentiFIXi-CB MSC in the osteoblast differentiation assay increased in both the control and differentiating populations over the 22-day test period and this is represented in both the protein quantification and functional assays [Table 1]. A closer trend in expression between control and differentiating cells is noted in the protein quantification assay rather than activity, which exhibited an even greater increase in activity for the differentiating cell population. In the last week of investigation, hFIX levels had more than tripled for both cell populations, and this can be at least partly attributed to cell proliferation that occurred in the first week of the investigation period.

Culture conditions for the quantification of hFIX protein secreted from control and chondrogenic differentiating LentiFIXi-CB MSC were slightly different from the adipo- and osteo- lineages; cells are cultured in a dense 3D environment. Both activity and protein quantification assays indicated a minimal and gradual drop in hFIX expression for control and chondro-differentiated cells, but overall

maintained significant levels of expression [Table 1]. The slight drop can be attributed to the reduced health of cells *in vitro* and/or the inability of secreted protein to escape the dense tissue environment and diffuse into cell culture media. Overall the level and function of hFIX secreted from both control and chondro-differentiated LentiFIXi-CB MSCs are maintained and follow the same trends.

For all three differentiation lineages, expression of functional and total hFIX protein by Lenti-FIXi CB MSCs is not impeded for the 22-day *in vitro* investigation period. Maintained expression of hFIX at levels similar to and even exceeding controls indicates that the differentiation process for LentiFIXI-CB MSCs does not impair the expression of total and functional FIX.

Day of Culture *in vitro* following transduction and selection		1	3	5	8	10	14	16	18	22	AVG
ADIPOGENESIS	**Adipogenesis FIX Expression ± STDEV (ng/mL)**	29.4 ±0.2	90.1 ±10.0	105.8 * ±14.9	125.0 * ±14.3	133.7 * ±22.8	93.6* ±34.1	119.0 * ±8.3	89.4* ±1.3	100.8 * ±3.5	98.5 ±34.1
	Undiff. Control FIX Expression ± STDEV (ng/mL)	39.7 ±6.0	158.8 ±1.4	175.2 ±12.4	304.9 ±6.0	275.3 ±5.6	217.2 ±10.9	225.3 ±14.3	224.0 ±9.0	241.4 ±8.1	206.9 ±75.2
	Adipogenesis FIX Activity ± STDEV (mIU/mL)	46.8* ±1.0	162.2 * ±11.0	157.9 ±18.4	156.1 * ±20.9	165.1 * ±45.9	145.5 * ±24.1	214.0 * ±7.1	196.9 * ±18.3	195.2 * ±30.6	160.0 ±53.7
	Undiff. Control FIX Activity ± STDEV (mIU/mL)	72.0 ±4.3	69.2 ±5.9	111.3 ±16.6	55.6 ±3.4	84.7 ±3.8	81.5 ±7.8	91.2 ±9.2	115.7 ±14.1	89.8 ±7.3	85.7 ±20.4
OSTEOGENESIS	**Osteogenesis FIX Expression ± STDEV (ng/mL)**	12.6 ±6.2	38.9 ± 6.3	81.4 ±10.3	138.2 ±23.3	86.1 ±18.8	154.2 ±7.9	201.3 ±0.9	182.9 * ±6.8	195.7 * ±7.0	121.3 ± 64.5
	Undiff. Control FIX Expression ± STDEV (ng/mL)	29.5 ±6.0	53.7 ±4.6	108.3 ±6.1	164.4 ±16.2	120.1 ±10.5	201.3 ±21.7	225.1 ±7.2	221.6 ±16.1	220.1 ±10.0	149.3 ±76.4
	Osteogenesis FIX Activity ± STDEV (mIU/mL)	34.5* ±0.81	46.4 ±4.0	45.4 ±9.8	64.6 ±1.5	87.9* ±1.7	83.7 ±15.7	91.3* ±6.1	84.3* ±11.4	101.6 * ±5.7	71.1 ±24.2
	Undiff. Control FIX Activity ± STDEV (mIU/mL)	22.2 ±0.84	17.3 ±7.8	29.9 ±16.8	47.3 ±2.31	37.3 ±2.63	72.8 ±6.7	76.5 ±6.4	46.4 ±4.1	59.8 ±12.3	45.4 ±20.7
CHONDROGENESIS	**Chondrogenesis FIX Expression ± STDEV (ng/mL)**	285.2* ±27.7	373.7 ±4.9	349.1 ±35.0	257.1 ±31.1	173.2* ±17.4	191.4 ±2.6	182.7* ±9.3	157.9* ±22.5	155.2* ±8.6	236.2 ±83.4
	Undiff. Control FIX Expression ± STDEV (ng/mL)	317.7 ±8.7	378.9 ±16.6	367.8 ±14.5	307.0 ±3.1	221.6 ±16.4	227.3 ±14.9	254.8 ±13.6	225.5 ±11.6	228.6 ±7.8	281.0 ±61.3
	Chondrogenesis FIX Activity ± STDEV (mIU/mL)	148.6 ±28.8	125.4 ±47.3	157.0 ±25.4	152.0 ±9.5	221.4 ±72.2	144.5 ±48.5	194.7 ±41.0	100.6 ±39.4	98.2 ±9.2	149.2 ±86.1
	Undiff. Control FIX Activity ± STDEV (mIU/mL)	161.4 ±10.3	124.3 ±22.2	107.7 ±12.8	99.4 ±28.3	109.4 ±10.3	75.9 ±6.9	85.2 ±18.5	76.0 ±11.1	84.3 ±4.0	102.6 ±35.8

Table 1. *Expression and functionality of FIX from differentiated LentiFIXi-CB MSC.* LentiFIXi-CB MSC at passage 7 were cultured in control (IMDM) and differentiation media in monolayer (adipogenesis and osteogenesis) and pellets (chondrogenesis) according to differentiation protocol. Media was changed every 3 days and samples were taken after 24 hours and assessed for FIX content using a hFIX ELISA and hFIX APTT. Expression and activity of FIX is sustained throughout differentiation to adipogenic, osteogenic and chondrogenic lineage. Errors indicate standard deviation. (*) indicates significant differences in FIX expression/activity (control and differentiating cells) to p=0.05.

C. Part A: Discussion

1. Engineering of a FIX-Expressing Lentiviral Vector and Generation of Stably-Expressing LentiFIXi-CB MSCs

It is evident that the use of a lentiviral vector for the modification of hCB MSCs was a viable approach for the induction of stable and high level hFIX expression by CB MSCs. There is a history of difficulty associated with the modification of primary cells due to their low receptivity of foreign DNA [167], however, a low MOI of 20 with the PLVX-FIXi vector was sufficient to modify the majority of cells to express the FIXi transgene. Previous *in vitro* investigations with the viral modification of MSCs have required very high MOIs that for the most part result in the transduction of less than 50% of cells. For example, MOIs in the range of 500-1000 were required for the successful modification of MSCs using the traditional adenovirus [180, 181]. Other viral vectors applied in MSC modification include the baculovirus which required a MOI of 200 [182], and the retrovirus with a MOI of 40-80 [183]. Previous work with lentiviruses and MSCs indicates that a MOI as high as 100 was often required for a 90% transduction [131]. There is evidence to suggest that CB-derived MSCs are more receptive to transduction than BM MSCs, however, this requires further investigation [167]. This efficient lentiviral vector is optimal for the *ex vivo* modification of MSCs because they are relatively slow growing compared to other commonly used cells, and thus benefit from the lentiviral ability to integrate non-dividing cells. This is also clinically relevant as

more efficient vectors result in a lower number of cells required from the donor, and shorter turnaround and expansions stages.

To our knowledge, levels of sustained transgene expression at the level of 4-6μg/10^6 cells/24 h have not previously been demonstrated for MSCs for a 6-week period. This is a significant finding as the use of MSCs in therapeutic research has greatly increased over the past ten years, with many leading to clinical trials [59]. There is a strong demand to express therapeutic transgenes from MSCs, or those that would contribute to the success of a therapy, and the high level of expression demonstrated in these findings is a promising step in that direction. With continued expression at this level, smaller amounts of cells would need to be applied, and feasible numbers for MSC-based hemophilia gene therapy are a possibility for clinical use. Assuming an expression rate of 5 μg of hFIX per million cells in a 24-hour period, just over 100 million LentiFIXi-CB MSC would be capable of secreting levels equivalent to 2% of normal in a 75 kg patient with hemophilia B. This does not take into account the additional levels of hFIX that would be secreted from cells arising from *in vivo* replication of transplanted MSCs.

In addition, the high level of expression of functional hFIX from modified human MSC *in vitro* may suit them for the production of rFIX. Current production methods of rFIX by Chinese hamster ovary (CHO) cells in a bioreactor require the cells to be supplemented with vitamin K and additionally modified to express enzymes to ensure cleavage of the propeptide and correct post-translational modification [15]. Additionally, expression levels of rFIX need to be controlled,

because high levels of expression cause a large proportion (>99%) of the produced rFIX to be inactive [16]. In this investigation, levels of activity in the range of 2-3 IU per million cells were attributed to the LentiFIXi-CB MSC without the addition of supplements or co-expression of enzymes. Another dispute concerned with current rFIX production methods is the lower *in vivo* recovery after infusion of rFIX into hemophilia B patients that has been associated with a deficiency of post-translational sulfation of tyrosine 155 and phosphorylation of serine 158 [184, 185]. Though no differences in the immunogenicity of rFIX and pdFIX have been demonstrated, they have been identified for rFVIII [186]. The described cell modification process for hCB MSCs can easily be adapted to express similar proteins and support the necessary post-translational and functional modifications required for protein activity. A similar approach regarding the production of rFVIII in human cells has already been reported [187].

2. Long-Term Expression and Function of FIX Expressed from LentiFIXi-hCB MSC *in vitro*

Both hFIX APTT and hFIX ELISA assays evaluating the long-term expression of undifferentiated Lenti FIXi-CB MSCs indicated continued stable expression of functional hFIX *in vitro* over the 6-week test period. Slight drops in expression around passage 9 and 9.5 were reflected in both assays, and increases were evident around passages of 10 and 11 [Table 1]. These variances within the generally stable trend of expression may be accounted for by small errors incurred from cell

counting, and would thus impact both the rates of hFIX expression and activity and become exaggerated when values are scaled up.

The relationship between the hFIX activity and protein quantities measured from LentiFIXi-CB MSCs is similar to those quantified in the retroFIXi-G8 cell line [Table 1]. For both cell lines, the activity of expressed FIX protein is equivalent to about twice the amount of activity that would be expected from the same quantity of FIX protein in pooled normal human plasma standards. This enhanced activity is consistent with previous studies demonstrating the effects of FIX transgenes including parts of intron 1 from the human FIX gene [173]. This is beneficial for a clinical therapy as less FIX needs to be delivered to reach the desired threshold of activity.

Human MSCs have a limited proliferative lifespan *in vitro*, and are known to become senescent at passages of 10 or higher [188], where 1 passage is indicative of 2 population doublings. Slowed replication was observed in both unmodified CB MSCs and CB MSC-LentiFIXi cells above passage 10, but this did not appear to affect the expression of hFIX as indicated by the sustained high levels of hFIX expression. In past investigations, hFIX expression has been confirmed from identically modified hCB MSC for up to 12 weeks *in vitro,* and cells demonstrated continued hFIX expression into sensecence. The passage where cells become senescent can vary depending on factors such as donor age, however, reasons are not fully understood at this time [189].

3. Sustained Expression and Activity of Lenti-FIXI CB MSC During Differentiation

It has previously been demonstrated that the modification of MSCs with a lentiviral vector expressing a transgene does not inhibit differentiation to the adipo-, osteo- or chondro- genic lineages [131, 138, 190]. However, few investigations have demonstrated the ability of a transgene to exhibit sustained expression throughout the MSC differentiation process. Studies with a retroviral vector have demonstrated the continued expression of green fluorescent protein (GFP) following MSC differentiation to the three lineages [191], and a similar investigation demonstrated continued expression of GFP from a lentiviral vector as MSCs differentiated to both the adipogenic and osteogenic lineages [131]. However, the continued expression of a *secreted* transgene throughout MSC differentiation has not been reported (to our knowledge) for a protein such as hFIXi for any of the three lineages. The continued expression of hFIX evidenced by the ELISA and APTT assays in differentiating LentiFIXi-CB MSCs in this investigation demonstrate sustained expression in all three lineages. These results have serious implications for a wide variety of therapeutic applications. For example, for therapies in which there is a desired cell lineage the differentiation process could be induced *in vitro* prior to cell transplantation. Additionally, therapeutic cells transplanted to niches in which a specific lineage is predisposed will continue to express therapeutic protein. This ensures that there are a variety of options that can be explored for optimal MSC transplantation, survival and engraftment. This capability can be applied to other

diseases in which therapeutic proteins would benefit from being expressed in any of bone, cartilage or fat tissues.

The general trends in hFIXi expression from control and differentiating cells are similarly represented in both the hFIX ELISA and hFIX APTT assays. The results of the hFIX APTT assay indicate a higher activity of hFIX from differentiating rather than control MSCs, however the opposite was true for the protein content as measured by the hFIX ELISA. Search of the current literature has not indicated a possible explanation for this observation. It is likely that this difference may be related to the growth phase of cells as they differentiate, but further investigation is required to draw a conclusion as this is outside the scope of this project.

D. Part A: Conclusions

Human CB MSCs can be efficiently modified with a non-replicating lentiviral vector at low MOI to induce the stable and high-level expression of functional hFIX protein for at least 6 weeks *in vitro*. The lentiviral modification does not impair the CB MSC ability to differentiate to the adipo-, osteo-, or chondro- lineages, and the viable cells continue to secrete hFIX throughout the differentiation process.

E. Part A: Future Research

The development of a LentiFIXi-CB MSC cell line that expresses sustained high levels of functional hFIX that are maintained throughout differentiation is a significant achievement. This is a valuable tool for the development of an *ex vivo* gene therapy for hemophilia. Future explorations should look into methods to

capitalize on these high levels of expression and prepare or adapt them for *in vivo* application. This approach could also be adapted for applications in a variety of conditions that could benefit from the sustained expression of transgenes.

More knowledge concerning the behaviour of MSCs *in vitro* would also be useful in the design of successful *ex vivo* therapies. Because MSCs are a recently identified cell type and have become very popular for applications in therapeutics and tissue engineering, there is a need to understand the differences in MSC characteristics derived from different tissue sources. Further experimentation of this lentiviral modification technique could be applied to MSCs derived from bone marrow, and adipose tissue.

Further investigation into the tendency of differentiating LentiFIXi-CB MSCs to secrete hFIX that exhibits higher coagulant activity/ng protein than non-differentiating LentiFIXi-CB MSC could be useful for the development of future hemophilia therapies, and other *ex vivo* therapies. Work in this vein would also contribute to the growing body of knowledge characterizing the behaviour of MSCs, and their usefulness in cell-based therapeutics. Finally, the application of these and similarly-modified cells *in vivo* is desired to evaluate their appropriateness for *ex vivo* therapies, and two approaches are explored in this thesis.

VI. Chapter 4: Part B – Attempts at Optimization of *in vivo* Delivery of FIX-MSCs

A. Part B: Methods

1. Hemophilia B and NOD-SCID-γ Mouse Models

All murine specimens were housed within the McMaster University Central Animal Facility and cared for under the university ethics and guidelines for ethical animal use. Male NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NOD/scid/γC or NSG) mice 3-5 weeks old were purchased from Jackson Laboratories (Bar Harbor, ME). All *in vivo* experimentation was completed between the ages of 6-12 weeks and followed the approved procedures outlined in the Animal Utilization Protocol 10-06-44 for the Hortelano lab. This mouse strain was selected for its lack of circulating mature T cells, B cells, deficiency of functional natural killer (NK) cells and deficiency in cytokine signalling. This NSγ mouse strain has been demonstrated to better engraft human hematopoietic stem cells and peripheral-blood mononuclear cells than any other published mouse strain [192, 193]. For this reason they were presumed a good model for translational testing of MSC implantation for therapeutic benefit.

A breeding colony of Hemophilia B$^{-/-}$ C57BL/6 mice was housed within the McMaster University Central Animal Facility. *In vivo* trials in hemophilia B mice were completed between the age of 6-12 weeks and in accordance to the procedures outlined in the Animal Utilization Protocol 10-06-44 for the Hortelano lab.

2. Injection of cells via tail vein

Cells to be implanted were washed, detached with TrypLE™®, counted and centrifuged at 700 g for 10 min at 4° C. Cells were gently resuspended in phosphate buffered saline (PBS), centrifuged as described and resuspended again in PBS to wash cells of any serum that may be lingering from cell media. Cells were pelleted one further time and gently and carefully resuspended in PBS at concentrations varying from 0.5-3 x 10^6 cells per 100 – 150 µL in 1 mL syringes and stored on ice. Care was taken to ensure that cells remained resuspended, and to reduce the presence of any cell clumps. Mice were anaesthetized using isofluorene gas and the injection area was sterilised with ethanol wipes. A 25G needle was used to inject suspended cells directly into the tail vein of anaesthetised mice with the aid of a heat lamp. Control mice received 100 µL of phosphate buffered saline (PBS). Mice were monitored for health and wellness in McMaster University Central Animal Facility. At the study conclusion mice were sacrificed by cervical dislocation and organs were necropsied and either flash frozen or preserved in 4% formalin. Slides were prepared by McMaster University CAF.

3. Collection and isolation of plasma samples

Blood samples (70 µL) were drawn from the orbital vein of anaesthetized mice using non-heparinized capillary tubes and transferred into 1.5 mL polyethylene tubes containing 8 µL of citrate and incubated on ice. Samples were then centrifuged at 13 000 x g for 12 min at 4° C to separate plasma from red blood

cell components. The top layer of plasma was aliquot and stored at -70° C until analysis of human FIX content could be completed via hFIX ELISA. hFIX ELISAs were conducted as previously described.

4. Size and Morphology Analysis

Cultured cells were washed, trypsinized and gently resuspended in PBS. Cells were counted and small aliquots of suspended cells in 100 μL volumes were added to glass microscope slides and covered with a sterile coverslip. Cells were analyzed under a Zeiss-Axiovert 100 microscope system equipped with AxioCam HR camera and Axiovision LE software.

5. Statistics

Standard differentiation for averaged samples was indicated by error bars. A Student's 2-tailed T-test was used to assess for the p-value of expression and activity of FIX levels in comparison to basal or control values.

B. Part B: Results

1. Expression of FIX in NSγ Mice Systemically Transplantated with Lenti-FIXI CB MSCs

The NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSγ) mouse strain lacking mature T cells, B cells, and functional NK cells with a deficiency in cytokine signalling was utilized for these *in vivo* studies [192, 193]. LentiFIXi-CB MSCs at passage 7 were suspended in PBS and injected via the tail vein into NSγ mice at varying doses. The resulting normalized expression of hFIX in the circulation of mice over the course of the 6 week investigation period treated with either a 2-3x10^6 (high), 1-2x10^6

(medium) or 0.5-1x10^6 (low) dose of LentiFIXi-CB MSCs is illustrated in Figure 10A. Expression levels of hFIX for mice treated with a high or low dose exhibited a trend of peak expression on days 3 and 7, and then a drop to lower levels for the remainder of the investigation period. Whereas, mice receiving the medium dose demonstrated an overall low level of hFIX expression throughout the investigation period with no distinctive peaks. At all time points, mice receiving the high dose demonstrated higher and more sustained levels of hFIX expression, compared to mice that received medium or low doses.

On average, no groups of mice that received systemically transplanted LentiFIXi-CB MSCs had hFIX levels that reached the therapeutic minimum of 50 ng/mL [Fig. 10A]. One mouse transplanted with the medium dose demonstrated hFIX expression at 52 ng/mL on day 3, but for following days registered levels similar to other mice in the same group. The high expression value was removed from the averaged group values on the basis that it was an outlier. All averaged and normalized values of hFIX expression *in vivo* registered below 10 ng/mL, and large errors represent variance between individual mice within the range of 0-15 ng/mL. For all *in vivo* trials a fraction of cells was maintained *in vitro* for the duration of the study in order to verify sustained levels of hFIX expression and integrity of LentiFIXi CB MSC.

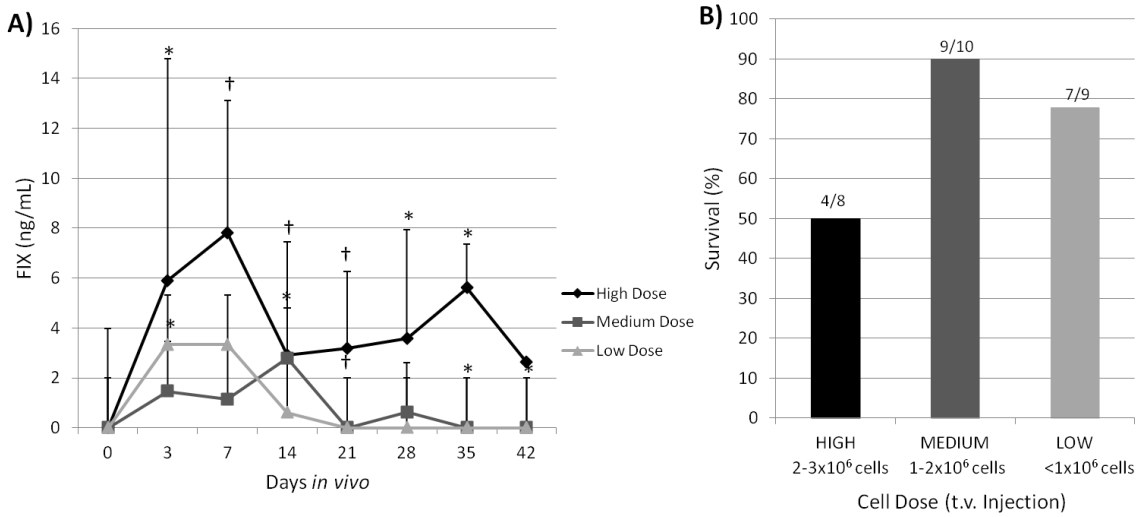

Figure 10. *Average normalized expression of hFIX* in vivo *and survival of mice recipient of LentiFIXi-CB MSC tail vein injection.* **A)** Expression of hFIX *in vivo* for mice that received either a 2-3x10^6 (high), 1-2x10^6 (medium) or 0.5-1x10^6 (low) dose of LentiFIXi-CB MSCs vial tail vein injection, quantified with an hFIX ELISA. For mice in the high and low dose range average expression peaked on days 3 and 7. Levels of expression significant from basal values are only apparent in mice recipient of the high cell dose. **B)** Survival rate in mice recipient of LentiFIXi-CB MSCs via tail vein injection in the same doses. Higher doses result in an increased likelihood of death immediately following transplantation procedure. *Indicates $p<0.05$, † indicates $p<0.1$ from day 0 values.

2. Survival of Mice Recipient of Tail Vein Cell Transplantation

The tail vein transplantation of LentiFIXi-CB MSCs caused immediate complications in some mice resulting in death. Mice would gasp for breath immediately following the injection and pass away within minutes. This phenomenon was observed to variable extents for all doses and as summarized in Figure 10B. Mice receiving the high dose were more likely to experience this effect as 50% of the eight mice recipients of the tail vein injection of LentiFIXi-CBMSCs did not survive the procedure. Lower doses decreased risk of this complication with only one of ten and two of nine mice recipient of the medium and low doses, respectively expiring.

3. Pathological Investigation of Mouse Response to Tail Vein Injection of CB MSC

A pathological investigation was performed to elucidate the cause of death in mice recipient of LentiFIXi-CB MSC tail vein injections. Repeat trails of CB MSC tail vein injections at passage 7 were completed to obtain samples for analysis by a veterinary pathologist to determine the cause of death [Fig. 11A,B]. Results indicate that human MSCs were substantially larger than comparable murine cells and murine lung capillaries and transplanted MSCs were the cause of distended and obliterated alveoli [Fig. 11A]. The presence of a granular material in the capillaries (the origin of which cannot be explained) was also described [Fig. 11B]. The cause of death was attributed to acute pulmonary failure with the alveoli unable to meet the need for gas exchange. This is consistent with observed behaviour of mice gasping for breath immediately prior to their death. This finding is also supported by some documented cases of systemically-implanted MSCs becoming lodged in the capillaries surrounding the lungs of recipient mice [194-197].

Samples from mice that survived the procedure and did not exhibit similar side-effects immediately following the injection were also analysed [Fig. 11C,D]. The Pathologist observed that septal capillaries and small arteries were often obliterated and that some small blood vessels had thrombi [Fig 11C]. The presence of human MSCs lodged in septal capillaries and surrounding interstitium was also observed [Fig. 11D]. The diagnosis was severe, acute multifocal vascular embolism and

thrombosis with the vascular changes attributed to the injection of cells that cause subsequent compromise of vascular flow.

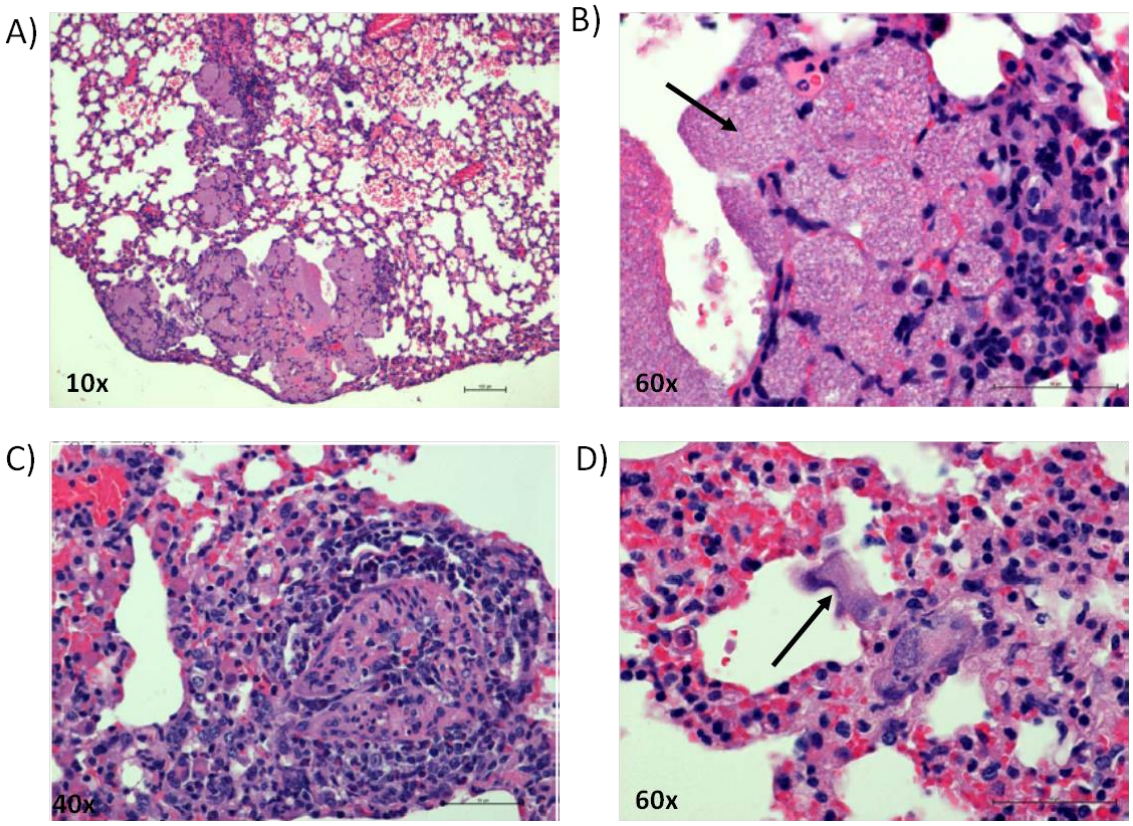

Figure 11. *Pathological Investigation of Lung sections following Tail Vein Injection of CB MSC.* CB MSC were injected via tail vein and organs retrieved following death or euthanization and preserved in 4% paraformaldehyde and sent to veterinary pathologist for sectioning and H&E staining. **A)** Lung section of mouse that did not survive transplant procedure, 10x. Large cells of human origin present in lung tissue exhibiting distended and obliterated alveoli. **B)** Lung section of mouse that did not survive transplant procedure, arrow indicates granular material present in capillaries. 60x. **C)** Lung section of mouse that survived transplant procedure, exhibits some obliterated septal capillaries and small arteries, and thrombosed small blood vessels 40x. **D)** Lung section of mouse that survived transplant procedure, 60x. Arrow indicates presence of human MSCs lodged in septal capillaries and surrounding interstitium.

4. LentiFIXi-CB MSC and CB MSC Cell Morphology and Size Characteristics

The morphology and size of LentiFIXi-CB MSCs was observed using a high power fluorescent microscope as a follow-up to the findings of the pathological investigation and previously published data indicating a tendency for systemically

transplanted human MSCs to become lodged in pulmonary capillaries. Adherent LentiFIXi-CB MSCs [Fig. 12A] and adherent un-modified CB MSCs at passage 7 [Fig. 12B] were photographed to assess for general size or morphology and no adverse characteristics were identified. Cells exhibited spindle-shaped morphology typical of MSCs [198]. Suspended LentiFIXi-CB MSCs [Fig. 12C] and un-modified CB MSCs at passage 7 [Fig.12D] were imaged and diameters measured using specialized software. The average diameter of LentiFIXi-CB MSCs ranged between 15-30μm, and a similar size range was observed for un-modified CB MSC. Some large cell aggregates were observed in suspension of both modified and unmodified cells with diameters in excess of 50 μm [Fig.12E,F].

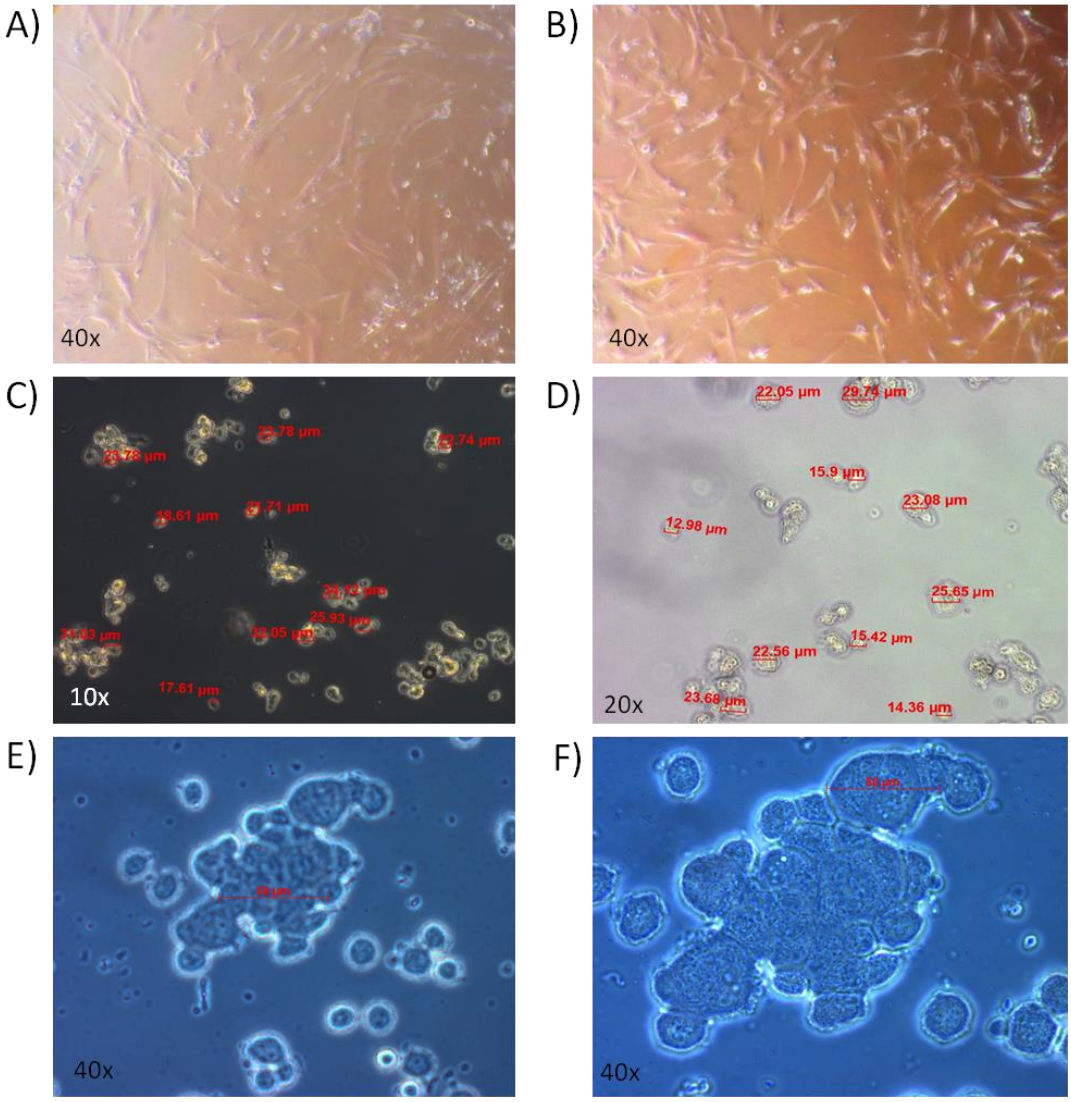

Figure 12. *Cell morphology and size characteristics.* Phase contrast electron microscopy of cells in suspension. **A,C,E)** LentiFIXi CB MSCs at passage 8 **B,D,E)** Unmodified CB MSCs, at passage 8. **A,B)** Adherent cells indicate no obvious differences in terms of morphology. **E,F)** Some large clumps of cells were detected in both cell populations with large diameters. Images and measurements taken with Zeiss-Axiovert 100 microscope system equipped with AxioCam HR camera using Axiovision LE software.

C. Part B: Discussion

1. Expression of FIX in NSγ Mice Systemically Transplanted with Lenti-FIXI CB MSCs

A large number of investigational therapies have applied human MSCs to both immunocompetent and immune deficient mouse models [199-201]. Though a

xenogeneic model is not ideal, it is currently the best available option for the first steps in the *in vivo* application of clinical therapies.

The low levels of circulating FIXi detected *in vivo* following the systemic transplantation of LentiFIXi-CB MSCs, despite high levels *in vitro* and prior to transplantation, indicate a significant shortcoming in cell engraftment and/or survival. FIX expression is not significant for the majority of the *in vivo* trials with the medium and low doses, and only mice that received high doses expressed hFIX that peaked in the first week. It can be said that the overall dose effect was notable in mice that received over 2 million cells; however at no point was therapeutic hFIX expression achieved.

There is sound reasoning behind the hypothesis that MSCs can be used to engraft and repopulate tissues *in vivo,* given their mesodermal origin and differentiation capabilities [102]. Additionally, many investigations are currently underway for the application of MSCs to this end for a variety of diseases and conditions [202, 203]. However, only minimal successes were achieved at this point, highlighting the major challenge of human MSC-based tissue repopulation in mice. A number of recent systemic transplantation therapies with MSCs in mice have reported <1% engraftment [194, 196, 204, 205]. Increasingly, there is evidence suggesting that the repair of damaged tissues by MSCs is a result of the secretion of bioactive molecules to help mediate repair and recruit immune and reparative cells, rather than direct cell replacement [59, 206]. The poor expression observed in the

current application appears to be comparable to the low engraftment reported in these similar studies.

For this investigation, large standard deviations at each time point represent inconsistencies within each dose, raising a concern for the clinical application of systemically administered modified MSCs. Reasons for inconsistency are not known, however this could be due to the lodging of MSCs in various capillaries, as has been previously demonstrated in systemically administrated hMSC studies in mice [194]. The low levels of expression observed in this investigation could be attributed to a few cells that were not initially cleared from the circulatory system and became lodged in the pulmonary capillary network. Inconsistent results from identical doses could also be explained by the very low levels of expression of FIX. Variance on the order of a few micrograms of FIX is not notable in terms of therapeutic value, however at this small scale represents a large variation.

Some reports have indicated that MSCs are capable of homing and adhering to the bone marrow, liver and spleen [65, 201, 207, 208]. However, some evidence indicates that the progressive subculture of MSCs can lead to changes in the phenotype that affect homing and has been associated with a decrease in the expression of adhesion factors, loss of chemokine receptors and chemotactic response [101]. A number of investigations have specifically highlighted the role of the SDF1-α/CXCR4 receptor in the homing process of MSCs [67, 204]. Based on this evidence, significant efforts were made to additionally engineer LentiFIXi-CB MSCs to over-express the CXCR4 receptor using a DNA plasmid and contribute to the

overall cell homing. However, recent investigations have highlighted the insignificant effects of this approach, and these efforts were therefore discontinued [204, 209]. The constantly evolving nature of health science research always presents a challenge to integrate and adapt to new knowledge, and this has been especially relevant in the past five years for MSC-based therapeutics.

2. Survival of Mice Recipient of Tail Vein Cell Transplantation, Pathological Investigation of Mouse Response to Tail Vein Injection and LentiFIXi-CB MSC Morphology and Size Characteristics

This study identified significant health risks associated with the transplantation of LentiFIXi-CB MSC via tail vein. Two studies have introduced greater doses and volumes of a variety of cell types using the same tail vein injection methods, with no reports of any undesirable health effects [210, 211]. Additionally, many investigations have reported the tail vein application of MSCs without mention of any adverse events [205, 212, 213]. Conversely, there have been a few reports that were previously mentioned that identify the tendency of MSCs to become lodged and form emboli in pulmonary capillaries following tail vein injection [194-197]. The lodging of human MSCs in the small mouse capillaries surrounding the lungs accounts for the gasping behaviour identified in some mice immediately following transplantation and leading to death. Cells block the flow of blood to the capillary bed and result in lack of oxygen transfer. Similar behaviour has been documented using intravital microscopy that demonstrates the tendency of transplanted MSC to become trapped in precapillaries [214]. The current study

found high rates of death in mice recipients of the high dose of LentiFIXi-CB MSCs. This is consistent with the proposed explanation that cells form emboli in capillaries, and that a greater number of transplanted cells results in an increased likelihood of cells becoming trapped in the capillary bed. Additional reasoning for this behaviour is that tail vein injection models are used to assess the rate of lung metastasis, indicating an underlying tendency for circulating cells to become caught in this location [215-217].

The exact reasons why human MSC become trapped in the lungs are not known; however the leading explanation is physical, as human MSCs are too large to travel through small murine pulmonary capillaries [194, 196]. One investigation utilised the injection of microspheres of known size to determine that cells with diameters in the range of 10-15 μm were likely to become lodged in capillaries [194]. For the current investigation we recruited the analysis of an expert veterinary pathologist to assess for the cause of death in mice treated with a tail vein injection of LentiFIXi-CB MSCs. The opinion was that MSCs became lodged in the interstitial space of pulmonary capillaries, and the size of the transplanted cells was significantly larger than mouse vessels surrounding the lungs.

Further analysis of MSC was undertaken by determining the diameter of MSCs in suspension, and indeed many cells had diameters exceeding 15 μm. There is evidence for two morphological populations within MSCs, namely, self-renewing, small, round or spindle-shaped cells and slowly replicating, large, cuboidal or flattened cells [198, 218]. The dual population of MSCs may explain the presences of

larger MSCs. As the passage number of MSCs increases the proportion of the so-called slowly replicating larger cells also increases [218]. All *in vitro* and *in vivo* work was completed with CB MSC at passages 6 and 7, as a result of the limitation following modification with the lentivirus. An additional finding was that cells tended to clump and form aggregates in suspension. Though reasonable efforts were made to resuspend single cells, it is possible that some cell aggregates were also implanted and thus caused the significant blockage of vessels in recipient mice. To combat the challenge of physical blockage of capillaries, adapted transplantation methods have utilised vasodilators [194, 195] or the culture of MSCs in human instead of fetal bovine serum [194, 195, 219].

D. Part B: Conclusions

The systemic administration of LentiFIXi-CB MSCs resulted in low level and non- therapeutic expression of hFIX *in vivo*. Further investigation into the cause of the lack of FIX expression points to an absence of engraftment from tail vein injected cells. A further challenge identified with this approach was a health risk prominent in mice receiving cell doses greater than two million. This risk may be attributed to the large size of MSCs leading to a tendency to become trapped in pulmonary capillaries and form life-threatening emboli. The overall conclusion that was reached for this investigation was that the systemic administration of modified MSCs is inappropriate for the long-term expression of a therapeutic transgene for hemophilia B.

E. Part B: Future Research

As MSCs have only recently been exploited for therapeutic purposes, much knowledge regarding their characteristics and behaviour remain to be uncovered. Progress in defining these characteristics will aid in the development of more effective MSC-based *ex vivo* therapies for hemophilia.

Further investigation into the mechanisms at play causing the entrapment of transplanted MSCs in capillaries is required before an appropriate solution to systemic delivery will be forthcoming. More information concerning the basic size, morphology, and behaviour of transplanted MSCs would greatly facilitate the development of these methods. A number of investigations have tracked MSCs following transplantation, often using fluorescent markers with the aim of further understanding homing and engraftment behaviours [220-223]. Further development of a model that describes MSC behaviour *in vivo* would aid in the development of improved MSC administration techniques.

Some investigation into the use of alternative administration sites has been completed, with mediocre results. Intra-arterial injection (IA) results in the passive entrapment of implanted cells noted by microvascular occlusion, similar to the results witnessed in this investigation [224, 225]. Experimentation with cell injection directly into the bone marrow is an option that avoids the limitations of systemic clearance and passive entrapment, and situates them directly into the cell environment. One report has injected $3x10^7$ bone marrow cells into the bone marrow cavity via the knee joint of mice [226]. However, this procedure is

uncommon, highly invasive and difficult in mice and may not be ideally suited for clinical hemophilia applications. With these approaches the risk remains that implanted cells will not engraft efficiently and the use of a pre-conditioning agent (though not ideal for hemophiliacs) may be required to induce cell engraftment.

It is becoming clear that systemically transplanted MSCs do not efficiently engraft or repopulate tissues independently; therefore researchers are looking to identify methods that can increase the survival and delivery of transplanted MSCs for various therapeutic applications. Scaffolds provide transplanted cells with a predetermined niche and can be optimized for specific locations and tissues. Scaffolds using natural polymers such as chitosan, fibrin, alginate and collagen are increasingly being developed for MSC therapies for a variety of disorders [227-230]. These techniques contribute to the survival of cells and also reduce the invasiveness of the delivery method. Synthetic polymers such as polyurethane and poly(L-lactic acid)/poly(lactic-co-glycolic acid) have also been explored as MSC scaffolds and delivery, specifically for injury repair and tissue engineering applications [220, 231, 232]. Poly(N-isopropylacrylamide) (PNIPAAm) is one such injectable polymer that exists in liquid form at 4°C but becomes a hydrogel upon heating to 37°C and has been utilized in combination with MSCs in a few studies [226, 233-236]. A significant amount of exploration is currently underway for scaffolds that enhance transplanted MSC survival and proliferation *in vivo* and this knowledge may be applicable in hemophilia therapies. A recent investigation has already examined the effectiveness of a hydroxyapatite-PLGA scaffold for MSC delivery of FIX in mice

[112]. MSC-scaffolds are one option for hemophilia therapies that would address the challenges of *in vivo* survival and systemic transplantation identified in this investigation.

Insight on the nature of proliferation and identity of MSCs and the causes of changing cell size and morphology would also contribute to the design of more successful *ex vivo* MSC therapies. This type of knowledge is expected to develop as exploration with MSCs continues. With current understanding, systemic applications of MSCs are not considered an ideal approach for cell therapies, however further understanding of MSC behaviour may bring to light some procedural modifications that may make this a viable method of therapy for hemophilia.

VII. CHAPTER 5: PART C - *in vivo* Transplantation of Encapsulated FIX-MSCs

A. Part C: Methods

1. Encapsulation of Lenti-FIXI CB MSC in PLL-Alginate

Encapsulation of MSCs was developed by my colleague Bahareh Sayyar of this laboratory. Cells were washed 3 times by centrifugation at 1000gfor 10 min at 4° C and followed by resuspension in PBS. Following the final centrifugation cells were resuspended in a 1.56% solution of MVG Ultrapure alginate from FMC, BioPolymer (Philadelphia, PA, USA). The microencapsulation procedure was performed with an electrostatic encapsulator from Nisco Engineering Inc. (Zurich, Switzerland) using a protocol developed in the Hortelano lab, as previously described [237]. Briefly, the cell suspension was pumped through the electrostatic encapsulator (voltage: 7kV) at the flow rate of 0.9 ml/min and collected in a pool of 1.1% $CaCl_2$. Cell capsules were then washed in saline, cross-linked with poly-L-lysine, and coated in an additional layer of alginate. Capsules were cultured overnight in IMDM media supplemented with 10% FBS prior to *in vivo* transplantation.

2. Transplantation of Cell Entrapped in Microcapsules

Capsules were washed 3 times with PBS prior to intraperitoneal transplantation. Following the wash, capsules were collected in 5mL syringes containing minimal PBS and stored on ice until immediately prior to transplantation. Animals were anaesthetized with isofluourene and cleaned using

ethanol wipes in the general injection area along the midline. Capsules were administered to the peritoneal cavity using an 18G shielded IV catheter (BD, Franklin Lakes, NJ). Mice were warmed by hand for 5 min following the transplantation and monitored four times weekly for the duration of the *in vivo* experiment.

3. Collection and Isolation of Plasma Samples and Retrieval of Capsules

Blood samples were retrieved as described using heparinised capillary tubes from the orbital vein and plasma separated via centrifugation as previously described and stored at -70°. Capsules were surgically retrieved from the peritoneal cavity following cervical dislocation using a scoopula. Capsules were washed 3 times in PBS and then cultured in IMDM media supplemented with 10% FBS overnight prior to photographic analysis.

4. Statistics

Standard differentiation for averaged triplicate samples was indicated by error bars.

B. Part C: Results

1. Encapsulation of Lenti-FIXI CB MSCs

LentiFIXi-CB MSCs at passage 7 were encapsulated in alginate-PLL-alginate microcapsules using methods developed by the Hortelano lab [238]. Cells were previously assayed to express between 1-2 µg of hFIX per millilitre of encapsulated cells per 24 hours for the duration of a four week investigation period *in vitro* [238]. Viability of encapsulated LentiFIXi-CB MSCs *in vitro* remained high but dropped to

60% of the day 1 values for the same *in vitro* period. High sustained FIX expression and good viability indicate the suitability of microencapsulated LentiFIXi-CB MSCs for *in vivo* trials.

2. FIX Expression *in vivo* of Microencapsulated LentiFIXi-CB MSCs

In vivo trials of microencapsulated LentiFIXi-CB MSCs were conducted with both Hemophilia B and NSγ immune-deficient mice. Hemophilia B mice each received 3 mL of microencapsulated LentiFIXi-CB MSCs at passage 7 at a concentration of $3x10^6$ cells/mL. Microcapsules were transplanted into the peritoneal cavity of Hemophilia B mice, resulting in a total of $9x10^6$ cells per mouse. Plasma samples were assayed for concentration of FIX using a hFIX ELISA on days 0, 3 and 5. Expression of hFIX surpassed the therapeutic threshold at an average of 110.9 ± 50.6ng/mL on day 3, however this was not sustained, and had dropped to minimal subtherapeutic levels by day 5 at 22.5 ± 21.1 ng/mL [Fig 13A]. A xenogeneic immune response was suspected given the dramatic drop in hFIX expression and appearance of capsules following retrieval.

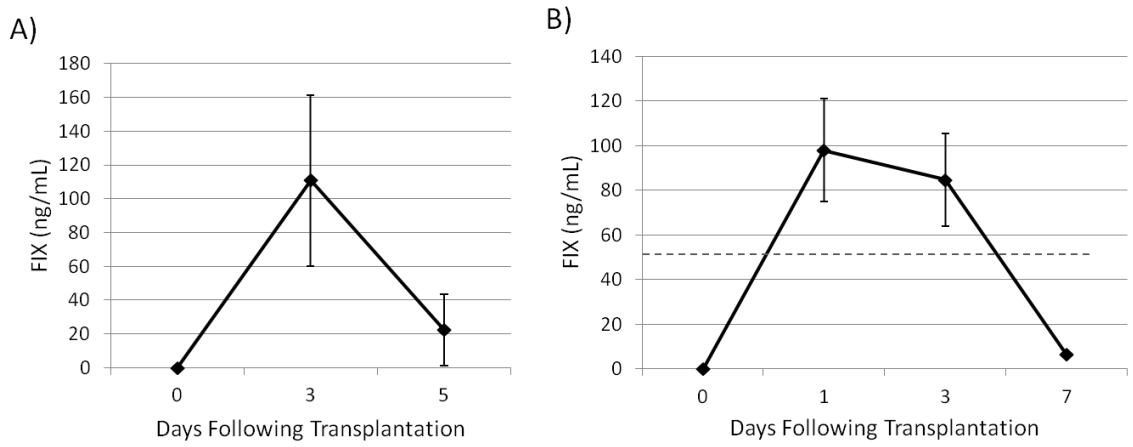

Figure 13. *Expression of FIX from transplanted CB MSC-LentiFIXi microcapsules* in vivo. Levels of circulating FIX were assayed by hFIX ELISA from plasma samples in mice recipient of alginate-PLL microencapsulated CB MSC LentiFIXi cells transplanted to the peritoneal cavity. **A)** Circulating levels of hFIX in Hemophilia B mice (n=3) that had received 3 mL of microcapsules containing $3x10^6$ cells/mL peaked at the therapeutic level of 110.9 ng/mL on day 3 and dropped to the sub-therapeutic level of 22.5 by day 5. **B)** Circulating levels of hFIX in NSγ mice (n=3) recipient of 4 mL of microcapsules containing $6x10^6$ cells/mL peaked at the therapeutic level of 98 $^+$/-23.1 ng/mL on day 1 and 84.7$^+$/-20.7 ng/mL on day 3 but dropped to the sub-therapeutic level of 6.4 $^+$/-0.17 ng/mL by day 7.

A second *in vivo* trial with immune-deficient NSγ mice was conducted under nearly identical conditions. Each mouse received 4 mL of LentiFIXi CB MSCs encapsulated at a density of $6x10^6$ cells/mL, for a total of $24x10^6$ cells per mouse. Plasma samples from these mice indicated that hFIX expression peaked at therapeutic levels of 98.0 ±23.1 ng/mL on day 1 and 84.7 ±24.7 ng/mL on day 3, but dropped to negligible levels of 6.4 ±0.2 ng/mL [Fig. 13B]. *In vivo* trials for both mice strains indicated a strong force opposing the long term expression of hFIX at levels similar to those observed *in vitro*.

C. Part C: Discussion

The alginate-PLL cell encapsulation procedure has been optimized for primary cells over the past 15 years in the Hortelano lab [149-151, 153]. Recent

adaptation of the procedure for LentiFIXi-CB MSCs was completed and high-level *in vitro* expression and good viability was demonstrated for a 4-week period [238]. Given the challenges associated with the tail vein transplantation of LentiFIXi-CB MSCs, encapsulation and intraperitoneal transplantation was explored as a method of FIX delivery. Results indicated a short peak in therapeutic-level expression of FIX in the first few days that dropped back to basal levels by day 7 and retrieved capsules indicated an immune response in the form of engulfed capsules. Previous work has demonstrated that an immune response is not mounted to identically-transplanted empty capsules. It is therefore reasonable to assume that the immune response was triggered by the presence of human LentiFIXi-CB MSC, The development of the immune response within the first 7 days of transplantation indicates the response is likely innate in nature, as previous experience has indicated that the adaptive response to secreted human FIX and other transgenes develops around the two-week mark [149-151, 239]. Even with the use of the immune-deficient NSγ mice optimized for stem cell transplantation, some innate immune mechanisms may still be triggered following the transplantation of xenogeneic cells. Overall an incompatibility of human cells in a murine model is the proposed source of failure for long-term FIX secretion. This finding requires further testing to confirm the response as a direct result of the innate immune response, and highlights the challenge of translational testing of hemophilia cell therapies.

D. Part C: Conclusions

This investigation demonstrated that intraperitoneally transplanted LentiFIXi-CB MSC in alginate-PLL capsules are capable of inducing short-term therapeutic expression in both hemophilia B and NSγ immune deficient mouse models. Levels of FIX expression were equivalent to about 2% of normal and lasted for a few days before returning to basal levels. This investigation also demonstrated the inability of the capsules to completely shield transplanted human cells from the immune response in both hemophilia B and NSγ immune deficient mouse models. The response was reasoned to be an innate response to the presence of xenogeneic cells but further testing is required to confirm this suspicion. This response highlighted the need for further optimization of a method to introduce human cells to mouse models for the therapeutic secretion of transgenes.

E. Part C: Future Research

Expression of FIX transgene from encapsulated LentiFIXi-CB MSC in optimized PLL-alginate microcapsules has been investigated *in vitro* with more results forthcoming [238]. These optimized CB MSC microcapsules show great promise for the support of MSCs *in vitro*, however require further investigation for their survival *in vivo* and long-term secretion of FIX.

The immune response has always presented a challenge for cell-based therapies, and the application of immune-privileged MSCs into immune-deficient mice has proven no exception. The significant drop in levels of hFIX in the circulation of both treated Hemophilia and NSγ immune-deficient mice around the

one week mark indicates either a decrease in cell viability, or the ability of cells to secrete viable hFIX protein. Capsules were therefore explanted for examination following the *in vivo* investigation period. In both cases it was found that a significant amount of host cells had engulfed the explanted capsules [Fig. 14C,D]. For the Hemophilia trial, capsules pictured on day 0 prior to implantation [Fig. 14A] differ greatly from those retrieved on day 5 [Fig. 14B]. Results are similar for capsules explanted from NSγ immune-deficient mice explanted after 4 weeks *in vivo* compared to day 0 [Fig. 14D]. In both cases it is suspected that the host immune response caused the engulfing of the capsules, and resulted in the impaired secretion of FIX into circulation. Further analysis into the characterisation of the immune response that is mounted to encapsulated cells is warranted in order to develop more effective therapies.

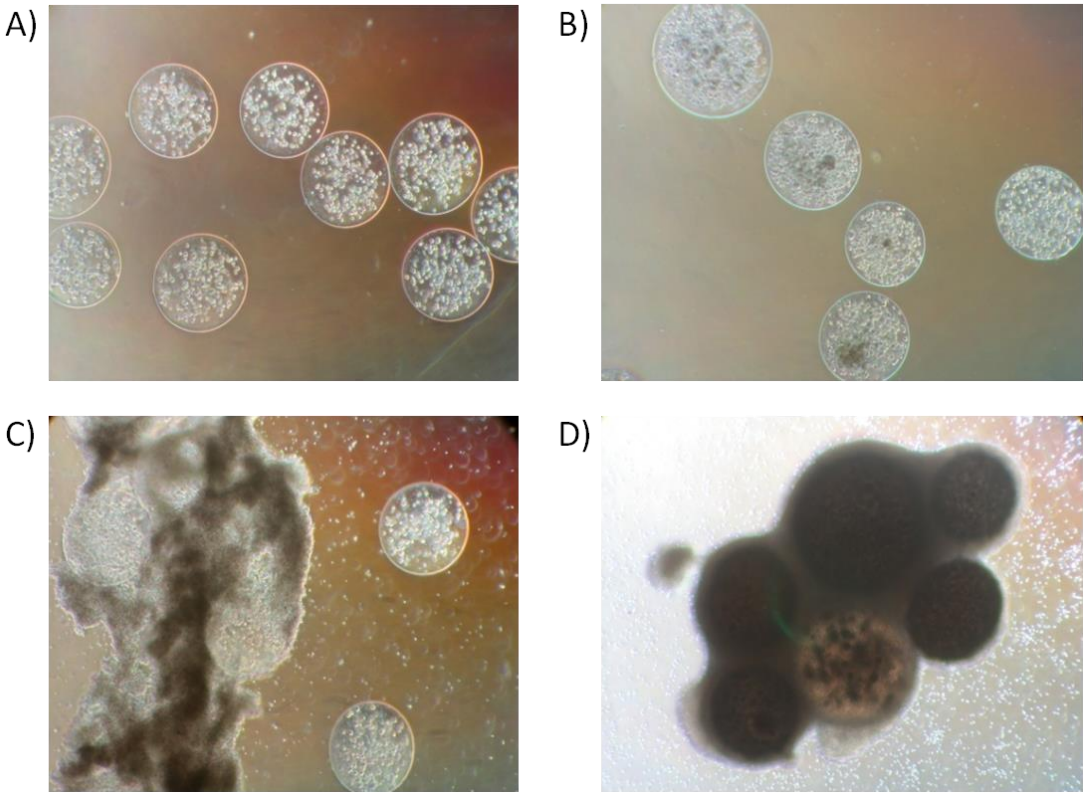

Figure 14. *Appearance of LentiFIXi-CB MSC microcapsules prior to and following* in vivo *treatment period*. Alginate-PLL microencapsulated LentiFIXi-CB MSC were photographed prior to implantation and following retrieval from the intraperitoneal cavity of treated mice. Capsules on day 0 prior to implantation in **A)** hemophilia B and **B)** immune-deficient NSγ mice. Capsules following explantation from peritoneal cavity on day 5 of treatment period in **C)** hemophilia B mice and day 28 of treatment period in **D)** immune-deficient NSγ mice.

Once the exact triggers for the immune response have been confirmed, alternate mediations to the capsules may be possible that can further protect the cells from interacting with the host immune system. One possible adjustment might be a decreased pore size of the alginate-PLL, as this is known to provide greater protection against immune rejection [240].

Another approach to demonstrate the ability of LentiFIXi-CB MSC to secrete FIX for longer periods *in vivo* is the use of immunosuppressive drugs. Clondronate is an experimental medicine that depletes macrophages which play a key role in the

innate immune response. Clondronate has been administered to mice to successfully induce macrophage dysfunction and interfere with the formation of an inflammatory innate immune response [241]. A similar approach may be applied to animal trials for encapsulated LentiFIXi-CB MSC, however this is not an ideal long-term solution for clinical practice.

An alternative solution to the xenogeneic immune response may be the development of an allogeneic cell line. Though efforts have been made in the Hortelano lab to develop methods to efficiently retrieve and culture murine MSCs, some challenges regarding low yield and short passage life *in vitro* exist. Further improvement of these protocols to overcome these inadequacies could render this a more feasible approach.

VIII. CHAPTER 6: REFERENCES

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