UNIVERSITÀ DEL PIEMONTE ORIENTALE

Department of Health Science
School of Medicine

Master's degree in Medical Biotechnologies

Juvenile Bone Marrow Transplantation to Correct Hemophilia A Bleeding Phenotype

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Summary

HSC transplantation has become a promising therapeutic option for treating a variety of diseases. However, establishing a potential cell source that is ethical and capable of reconstituting hematological compartment is necessary. Previous studies have shown that adult bone marrow (ABM) cells can engraft in hemophilia A (HA) mice following total body irradiation (TBI). Less adverse pre-conditioning is needed, especially in diseased individuals. Our previous studies have shown fetal liver (FL) cells can engraft in newborn HA mice without any pre-conditioning, however this approach would be difficult to translate in clinics.

In this thesis we considered juvenile bone marrow (JBM) cells a suitable alternative to FL, and we evaluated their ability to engraft and achieve long-term engraftment without any pre-conditioning. To this purpose, we set up several transplantation experiments, comparing JBM cells with ABM cells transplantation in busulfan-treated HA mice or in absence of preconditioning. Finally, we evaluated the possibility to use a mobilizing agent used in clinic (G-CSF) to improve/maximize the engraftment of JBM cells. Our results can pave the way to additional studies aiming at achieving efficient and long-term engraftment levels capable of mediating bleeding phenotype of HA in absence of preconditioning regimens.

1) Introduction

1.1 Hemophilia A

Hemophilia A (HA) is an X-linked recessive disease caused by mutations in the blood clotting factor VIII (FVIII) gene present in the long arm region of the X chromosome. Because of the reduction/absence of FVIII activity, hemophilia A results in internal and external bleeding episodes that can occur both spontaneously or following trauma. Frequent bleeding episodes can cause a multitude of complications, most notably, anaemia, hemarthrosis, and joint bleeding (Castaman & Matino, 2019).

In normal conditions when a tissue is injured platelets are activated at the site of injury and the contact activation pathway (Figure 1.1) of coagulation is triggered. This specific pathway is dependent on the interaction between Factor VIII and Factor IX, leading to the activation of Factor X, required to convert prothrombin to thrombin (Hulfin et al., 1976). Finally, thrombin converts fibrinogen into fibrin which is important for the stabilization of the blood clot formed following platelets activation.

The severity of HA is classified according to the residual levels of FVIII activity in the blood. Coagulation assays are used as the preliminary tools for diagnosing HA based on severity: with levels of FVIII lower than 1% HA is classified as severe, for levels from 1% to 5% is classified as moderate, whilst mild HA is classified for FVIII activity ranging from 5% to 30% (Ali Shah et al., 2022).

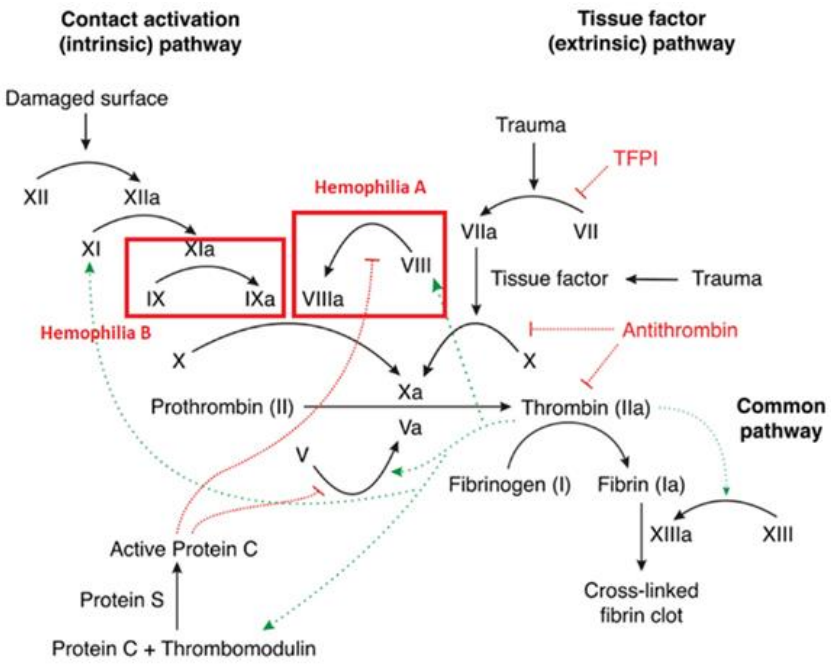

Fig. 1.1. Scheme of coagulation cascade. **Contact Activation:** Initiated by contact with a damaged surface, it involves the sequential activation of factors XII, XI, IX, and VIII, ultimately leading to the activation of factor X. **Tissue Factor Pathway:** Triggered by trauma, it starts with the activation of factor VII by tissue factor (TF), which then activates factor X. This detailed illustration provides an overview of the complex interactions and regulatory mechanisms within the blood coagulation process (Hoffman et al., 2018).

1.2 Hemophilia: Current Treatment Options

HA patients are treated with regular infusions of plasma-derived FVIII (pdFVIII) or recombinant FVIII (rFVIII). (Noone et al., 2019).

The traditional methodology for the treatment of HA, the so called “replacement therapy”, started in the 1960’s with the infusion of whole blood plasma from healthy donor in the attempt to correct the bleeding phenotype of hemophilic recipients (Franchini, 2013).

With advancements in cryo-precipitation, plasma-derived FVIII (pdFVIII) concentrates became readily available, but without any viral inactivation procedure, thus transferring blood-borne related infections to hemophilic recipients. It was only following 1985 with the implementation of procedures for viral inactivation that the risk of blood-borne infections was greatly reduced (Franchini, 2013).

The major drawback of replacement therapy is the development of anti-FVIII antibodies that neutralize the infused factor (inhibitors) in approximately 30% of

severe HA patients. These neutralizing antibodies are frequently polyclonal immunoglobulin G (IgG) isotype 4 (IgG4) and 1 (IgG1) (Prezotti et al., 2022).

With advancements in research, a safer replacement therapy option was created. SHL (Standard Half-Life) recombinant products, SHL rFVIII, are laboratory-made recombinant proteins with same capabilities and function of pdFVIII (McCall et al., 2019). These products did not pose any risk of bloodborne diseases transmission, and with no reliance on the availability of donor plasma.

SHL rFVIII, consist of three different generations, each with its own post-translational modifications that can influence their biosynthesis, half-life/clearance, and immunogenicity (Prezotti et al., 2022).

Continuous injections with SHL rFVIII are required to reduce severe HA bleeding episodes. However, the main issue with SHL rFVIII injections is their short half-life thus compelling HA patients to frequent injections, approximately three times a week intravenously, to maintain FVIII levels >1% in the peripheral blood. Furthermore, SHL rFVIII are not effective against joint bleeding, a key and clear disadvantage for using SHL rFVIII to correct the bleeding phenotype of HA (Ar et al., 2019).

The average half-life of SHL rFVIII ranges from 10 to 14 hours, even less in paediatric patients, hence advancements in SHL rFVIII replacement therapy led to the development of extended half-life recombinant FVIII (EHL rFVIII) in the attempt to create a more user-friendly treatment approach which can reduce the frequence of weekly injections (Ar et al., 2019) .

To extend the half-life of SHL rFVIII, fusion with polyethylene glycol (PEG) is showing promising results, since PEG-conjugated products are cleared at a much slower rate by the kidneys due to its larger molecular weight (Solms et al., 2020). Indeed, PEGylated rFVIII shows half-life 1.5-1.8 times longer than SHL rFVIII.

Other EHL rFVIII products have been evaluated, among which in 2016 rFVIIIFc (Efmoroctocog alpha, Eloctate®) received the approval for the treatment of patients with HA, both for prophylaxis and on demand. This drug showed a 1.5-fold increase in half-life compared with full-length rFVIII: the binding to Fc-domain of immunoglobulin G creates a recombinant fusion of B-domain deleted

recombinant FVIII and the dimeric constant region of Ig1 shows very favourable pharmacokinetics since it exploits the recycling mechanism by the neonatal Fc receptor (FcRn), thus increasing the rFVIIIFc half-life (Giraud et al., 2021).

Despite this half-life extension, these products did not reach an optimal value that can greatly reduce weekly injections. To further reduce the need for continuous injections of rFVIII, more research was needed to identify the causality behind the rapid clearance of rFVIII (Lambert et al., 2018).

The von Willebrand factor (VWF) plays an important role in the half-life potential of rFVIII. In fact, VWF is responsible for stabilizing FVIII, while also playing a role in the clearance of FVIII by endocytosis. This intricate balance between FVIII and VWF binding is important to achieve stable blood haemostasis (Swystun et al., 2017).

As of 2021, a recently developed rFVIII showed extended half-life of rFVIII by three to four times by combining two techniques, one that focuses on increasing the half-life of rFVIII, and another that prevents the binding between VWF and rFVIII: rFVIIIFc-VWF-XTEN, named BIVV001, efanesoctocog alfa (Ozelo et al., 2022)

BIVV001 consists of a rFVIIIFc, VWF D'D3 domain, and two XTEN hydrophilic molecules. XTEN increases the molecular weight of rFVIII, slowing down clearance by the kidney and VWF D'D3 domain stabilizes FVIII in circulation while avoiding the interaction with plasma VWF which is not only stabilizing but also limiting FVIII half-life in the bloodstream (Chhabra et al., 2020).

Despite the increased blood lifespan of EHL rFVIII, these molecules showed the same risk of inhibitors development, i.e. ~30%, in HA patients.

Replacement therapy options has always been the standard treatment option for HA. However, non-replacement therapy options have recently been developed, particularly with advancements in monoclonal antibody engineering, and the need to create therapeutic alternatives for inhibitors positive HA patients (Marchesini et al., 2021). Hence, the development of bispecific antibodies that can activate factors in the coagulation pathways have slowly become a viable treatment option for HA.

Emicizumab (Hemlibra®) is the first licensed monoclonal treatment option: it is a humanized antibody with sites that can bind to activated factor IX (FIXa) and factor X (FX), thus mimicking FVIII co-binding capabilities and triggering the coagulation pathway (Gelbenegger et al., 2020). The advantages Emicizumab plays over traditional replacement therapy options are the administration (subcutaneous), and the reduced injections, since it requires an injection once every 1,2,4 weeks according to the dosage, thus eliminating the need for frequent injections as is the case with replacement therapy.

Additionally, emicizumab has lower immunogenicity as compared with pdFVIII and rFVIII: in clinical studies (HAVEN 1–5, HOHOEMI, and STASEY) out of 668 HA patients, approximately 0.1% showed neutralizing anti-emicizumab antibodies (Schmitt et al., 2021).

A newly developed monoclonal antibody, which is currently in clinical trials as of 2023, is Marstacimab, and is showing promising results for treatment of severe HA. Marstacimab targets and inhibits tissue factor pathway inhibitor, or TFPI, thus inactivating its function and, allowing coagulation cascade to proceed with the tissue factor pathway (Mahlangu et al., 2023). Marstacimab showed promising results in clinical trials. Fig 1.2 shows the mode of action for bispecific antibodies, that can activate coagulation pathway.

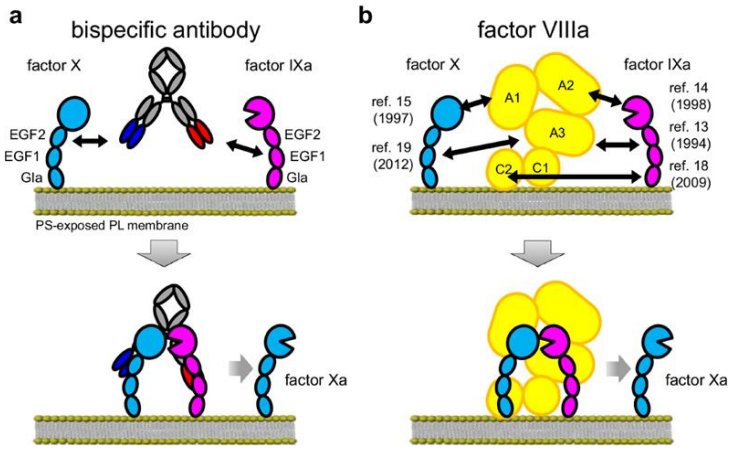

Fig 1.2 illustrates mechanism of action of a bispecific antibody, activating factors in the coagulation cascade.

However, some adverse reactions have been reported, such as haemorrhages, hepatic disorders, hypertension, and allergic reactions in the injection site for patients suffering from hemophilia (Gualtierotti et al., 2022).

Although rapid biotechnological advancements are being achieved in both replacement and non-replacement therapy for HA, the preliminary disadvantage of using such therapeutic options is that they are not long-term treatment, and repeated/continuous injections are still required (Luo et al., 2022).

1.3 Hemophilia: A Promising Candidate for Cell/Gene Therapy

Since HA is a monogenic disease, it the perfect candidate for long-term treatment options that can combine novel strategies in the realm of gene and cell therapy (Castaman et al., 2022).

Over the past two decades, significant research has been done to possibly treat HA by using gene therapy. Initial promising results have been achieved by the intravenous administration of adeno-associated viral vector (AAV) directed at the liver for severe cases of hemophilia B (Manno et al., 2006) Following infusion, factor 9 (FIX) expression ranged between 1 and 6 IU/dL, which resulted in the massive reduction of bleeding without the need for continued prophylaxis injections (Nathwani et al., 2011). The patients treated with the AAV, showed stable FIX expression even after eight years of monitoring (Nathwani et al., 2014).

With such promising results, a gene therapy treatment approach for the treatment of HA started to be envisaged. However, the size of the FVIII gene has hindered its use in AAV. This problem was solved with the removal of the FVIII B-Domain, which is not required for the coagulation activity, thus reducing the FVIII expression cassette and allowing proper insertion within the AAV platform (Nathwani, 2019).

Roctavian is the first breakthrough gene therapy treatment option designed for hemophilia A. Roctavian carries the correct copy of the BDD-FVIII gene into liver cells via a one-time infusion process. Upon in vivo administration, liver cells receiving the correct copy of the FVIII begin producing the coagulation factor. Roctavian is currently in phase 3 clinical trials, done on patients with no FVIII inhibitor presence, and with no pre-existing immunity against the AAV5 serotype (Ozelo et al., 2022). Roctavian has been recently approved by the european

medicines agency (EMA) and U.S. food and drug administration (FDA) for the use of adults with severe HA, leaving out the possibility for using such a treatment option on young HA patients.

Hence, gene therapy is still limited by AAV pre-existing immunity (>50% of the population), a field for which research is still ongoing (Rajavel et al., 2018; Ronzitti et al., 2020).

Hence, a gene therapy treatment option that might not work on the majority of the population and that is not clinically approved for use in juvenile patients (Machin et al., 2018) cannot be considered a long-term effective treatment option for HA patients. These disadvantages leave the therapeutic market for HA wide open and, additionally, the need for creating a sustainable long-term treatment option that works on both juvenile and adult patients is a crucial necessity.

1.4 Cell Therapy: A Sustainable Long-Term Treatment Approach

Recent research has revealed the possible therapeutic potential of cell therapy, offering a potential long-term correction for a multitude of monogenic diseases, including HA. From cancer to monogenic diseases, the potential of harnessing stem cells to correct different (possibly any) diseases has gained the interest of the scientific community (Ramaswamy et al., 2018). Stem cells hold the blueprint for regeneration by holding the potential to rejuvenate any damaged tissue and organ due to their strong regenerative capabilities (Ajmal et al., 2023).

The preliminary goal of a cell-based long term therapeutic approach is the establishment of a viable source of stem cells that can be easily acquired, show potential engraftment capabilities, possibly without the use of adverse pre-conditioning regimes, and are able to reconstitute the desired cell compartment in patients; in the case of HA, the stem cell approach should use cells able to engraft for long term and capable of producing enough FVIII levels to reduce the bleeding phenotype in patients, potentially achieving long-term correction of the bleeding phenotype already at a young age.

In cell and gene therapy approaches, the long-term goal is the autologous transplantation of stem cells: these cells should be isolated from HA patients, ex-vivo corrected with a correct copy of the FVIII gene, and finally transplanted back into the HA patients. This therapeutic combination between cell and gene therapy

can pave the way towards the creation of a potential therapeutic option for HA patients.

Among the stem cells that can be used, hematopoietic stem cells (HSC) are the most easily obtainable and the best characterized. The experimental methodology of autologous ex-vivo gene corrected HSC CD34+ transplantation has been established before. The idea behind this type of treatment focuses on the isolation of CD34+ cells from the patient and, following gene correcting the cells using a viral vector carrying the correct copy of the gene of interest, the corrected cells are transplanted back into the patient.

However, the major limitation behind this type of therapeutic option is the establishment of a viable engraftment levels of corrected cells that can translate into a high enough expression of the wildtype protein, to treat or even potentially cure the disease (Hasan et al., 2024a).

To pave the way for the treatment of HA using transplantation, an idea about the engraftment levels required to provide sufficient FVIII levels that can correct the bleeding phenotype is still a crucial requirement. Additionally, long-term engraftment needs to be established. Furthermore, studies are needed to understand HSC engraftment capabilities between different donors at different intervals of time and therefore with HSC at different developmental stage, such as at pre-natal, juvenile, and adult.

1.5 Bone Marrow Cells/HSC As Cell Therapy Approach For Hemophilia A

In the past, liver was considered the primary site of FVIII production due to the fact that orthotopic liver transplantation showed the ability to correct HA (Storb et al.,1972.). For this reason, hepatocytes were considered the main FVIII-producing cells. However, more recent studies showed that endothelial cells, mainly liver sinusoidal endothelial cells (LSEC), are the main source of FVIII in the body (Benten et al., 2005; Follenzi et al., 2008; KUMARAN et al., 2005; Pan et al., 2016; Shahani et al., 2014) and that, in a lesser extent, FVIII is produced by HSC (Follenzi et al., 2008; Shahani et al., 2014). Indeed, these cell therapy studies showed that LSEC and bone marrow (BM) cells are able to produce and secrete FVIII upon cell transplantation and engraftment. However, the availability of LSEC is limited and would require invasive procedures, especially for patients with

HA, for their transplant, while bone marrow cells offer greater availability, their transplant is minimally or non-invasive and BM transplantation is a well-established procedure, used in clinics for over 50 years.

The first study using BM transplantation as a cell therapy approach to treat HA was published in 2012 by Follenzi et al. in which they transplanted total bone marrow cells from healthy donor wildtype (WT) mice to treat the bleeding phenotype of recipient HA mice. This is the first study that aimed at understanding the potential of bone marrow transplantation treatment, focusing on adult WT murine total bone marrow cells, their engraftment capabilities and their therapeutic potential in restoring FVIII levels in the blood.

The transplantation of $2x10^6$ to $10x10^6$ total bone marrow cells from WT adult mice into irradiated adult C57BL/6 HA mice resulted in high (≥80%) long-term (≥16 weeks) engraftment levels of donor-derived cells (Follenzi et al., 2012) (Fig 1.3A). Following donor cells engraftment, FVIII activity was detectable in plasma of recipient adult HA mice with FVIII levels directly correlating with the number of transplanted cells (Fig. 1.3 B).

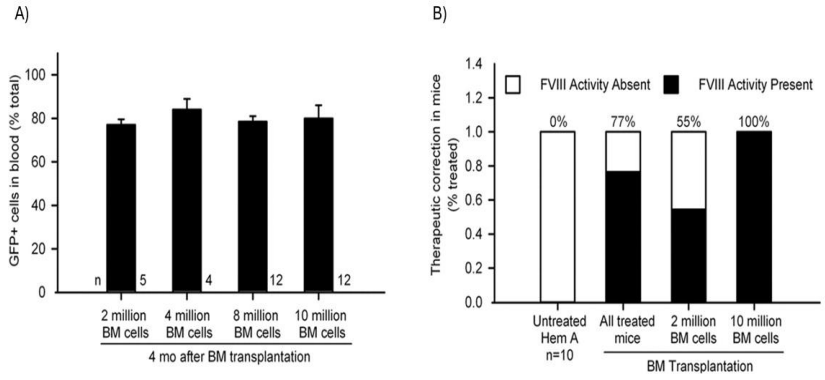

Fig 1.3(a) illustrates % of engrafted adult total bone marrow cells in HA adult recipients depending on n. of transplanted cells, **Fig 1.3(b)** illustrates % of FVIII present after successful engraftment (Follenzi et al., 2012)

Thus, this transplantation experiment demonstrated that FVIII is produced by hematopoietic cells and confirmed the possibility of using bone marrow transplantation to correct the bleeding phenotype of HA. However, the use of adverse pre-conditioning regimens, like in the case of this study total body irradiation (TBI), is detrimental especially in particular pathological conditions. TBI is used to efficiently transplant donor cells into the bone marrow since

irradiation reduces the host bone marrow volume, creating space, or “room”, for efficient engraftment of donor cells (Sabloff et al., 2021). Additional and less damaging pre-conditioning regimens use drugs such as busulfan (BU) in place of radiations. BU is a myeloablative alkylating agents that plays a huge role as a conditioning agent in HSC transplantation. Conditioning with BU helps in generating ‘space’ in the recipient’s bone marrow and supress immune reactions against donor transplanted cells (Garcia-Perez et al., 2021). BU has a cytotoxic effect against host hematopoietic stem cells and even progenitor cells by preventing DNA replication, through the process of DNA crosslinking, slowly leading the recipient cells to undergo apoptosis. This leads to the depletion of recipient non-cycling stem cells, allowing high levels of donor-derived long-term HSC to efficiently engraft within their specialized niche in the bone marrow. In summary, BU creates space by eliminating existing cells and preparing the bone marrow for successful HSC engraftment (Mehdizadeh et al., 2021).

However, the use of an adverse pre-conditioning regimen, such as TBI or BU, can cause a plethora of complications for HA patients (Pearlman et al., 2021). In this sense, a breakthrough concerning HSC transplantation would be the possibility of achieving long-term engraftment without the use of any adverse pre-conditioning agent. In a study performed in our laboratory, it has been compared the engraftment ability of fetal liver (FL) cells and adult BM (ABM) cells (Merlin et al., 2023). FL cells isolated at embryonic day 13.5 (FLE 13.5) showed greater engraftment ability in comparison to ABM cells in BU pre-conditioned recipient mice.

When transplantation was performed without the use of any pre-conditioning agent, FLE 13.5 showed long term engraftment in neonatal mice whilst total ABM cells did not show any engraftment ability. This means that phenotypic and molecular differences exist between fetal HSC and adult HSC creating a major difference between their engraftment capabilities, one of which might depend on their different cycling state. Fig 1.4 shows the engraftment capabilities of the two groups, fetal liver and adult total bone marrow with (left) and without (right) BU pre-conditioning.

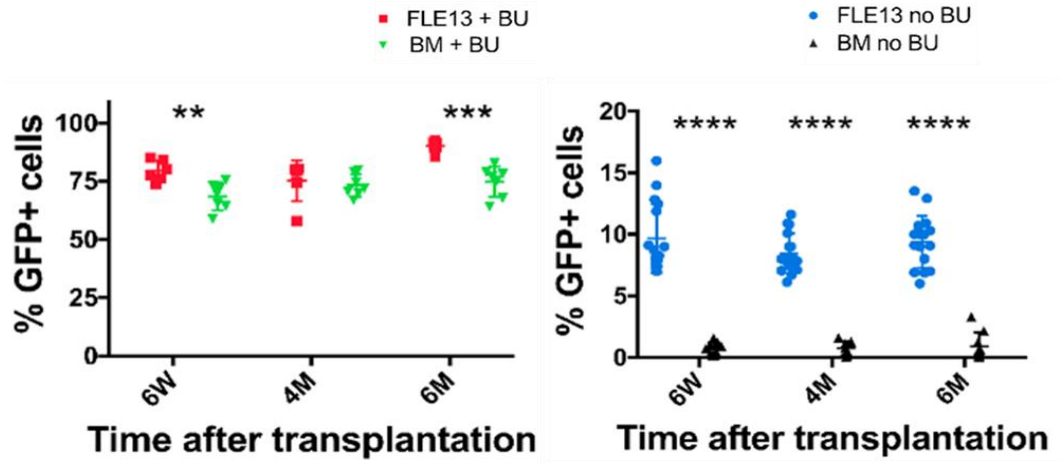

Fig 1.4 Left % of engrafted adult total bone marrow cells and FLE13 in neonatal recipients pre-conditioned with BU. **Right** % of engrafted adult total bone marrow cells and FLE13 in neonatal without any pre-conditioning (Merlin et al., 2023).

1.6 HSC Homing, Migration, and Cycling

In the mouse embryo, long-term HSC with high repopulating ability first start appearing in the aorta-gonad-mesonephros region during the ninth day of gestation (E9). On E10.5, HSC start migrating to the fetal liver, whereby they begin to proliferate substantially. On E13, fetal liver cells start migrating to the bone marrow (BM). Hence, HSC on E13 might be programmed to migrate to the bone marrow (Ganuza et al., 2022).

HSC homing and engraftment play a crucial role for the successful engraftment and the long-term reconstituting capabilities of HSC following transplantation (Hasan et al., 2024). Homing refers to the HSC capabilities to migrate from their site of origin to the bone marrow where they establish long-term residence (Caocci et al., 2017).

HSC actively cross the endothelial barrier between blood vessels and the bone marrow, where they pass through the endothelial vasculature with the help of chemokines and cytokines, and finally settle in specialized microenvironments within the bone marrow, which provide the necessary support to ensure long-term proliferation and survival of HSC (Mukaida et al., 2017). This process from blood circulating HSC to specialized microenvironments within the bone marrow is referred to as homing and it typically occurs in a timeframe ranging from a few hours to a couple of days at maximum (Hasan et al., 2024a)

There are several chemokines, cytokines, and adhesion molecules that are responsible for HSC homing. The most crucial cytokine described to be involved in homing is CXCL12, also known as stromal cell-derived factor-1 (SDF-1), secreted mainly by bone marrow stromal cells and endothelial cells. HSC express the SDF-1 receptor CXCR4, which binds to the cytokine CXCL12 aiding in the homing process to the bone marrow (Teicher & Fricker, 2010). An additional molecule that showed to have a role in the homing process is the sialyl Lewis-X (sLe-x), also known as cluster of differentiation 15s (CD15s) or stage-specific embryonic antigen 1 (SSEA-1), an essential chemokine that allows binding to endothelial cells for the facilitation of migration to the specialized niches in the bone marrow (McFerrin et al., 2014).

Another interesting aspect of HSC migration and homing is the difference in the expression patterns between long-term HSC (LT-HSC) and short-term HSC (ST-HSC). Indeed, CXCR4 receptor is expressed at higher level in LT-HSC as compared to ST-HSC, while the sLe-x expression is higher in ST-HSC compared to LT-HSC (Al-Amoodi et al., 2022). However, HSC engraftment ability is not limited to CXCR4 and sLE-x, therefore this area is still an active subject of study.

Fetal hematopoietic stem cells exhibit differences in surface markers, proliferative state, and gene expression. One of these differences in gene expression has to do with a specific cellular pathway, Lin28b–let-7–Hmga2 axis. Lin28b and Hmga2 act as antagonists for let-7 miRNA, which confers lower self-renewal potential and plasticity to HSC, thus shifting their status to a quiescent phase (Copley et al., 2013). The majority of adult HSC are quiescent, meaning they are not in an active cycling state, whilst the majority of fetal HSC are in an active cycling state, due to a reduced expression of let-7 miRNA compared to adult HSC. Lin28b is a protein encoded by the lin28b gene, and its primarily role is to facilitate the pluripotency of stem cells by binding to and sequestering miRNA let-7 (Tsialikas & Romer-Seibert, 2015). Hmga2 is responsible for promoting self-renewal of HSC and it is more expressed in fetal HSC as compared to adult HSC, promoting self-renewal of fetal HSC (Kubota et al., 2021). Fig 1.5 shows the gene expression levels of Lin28b, let7, and Hmga2 during murine lifetime.

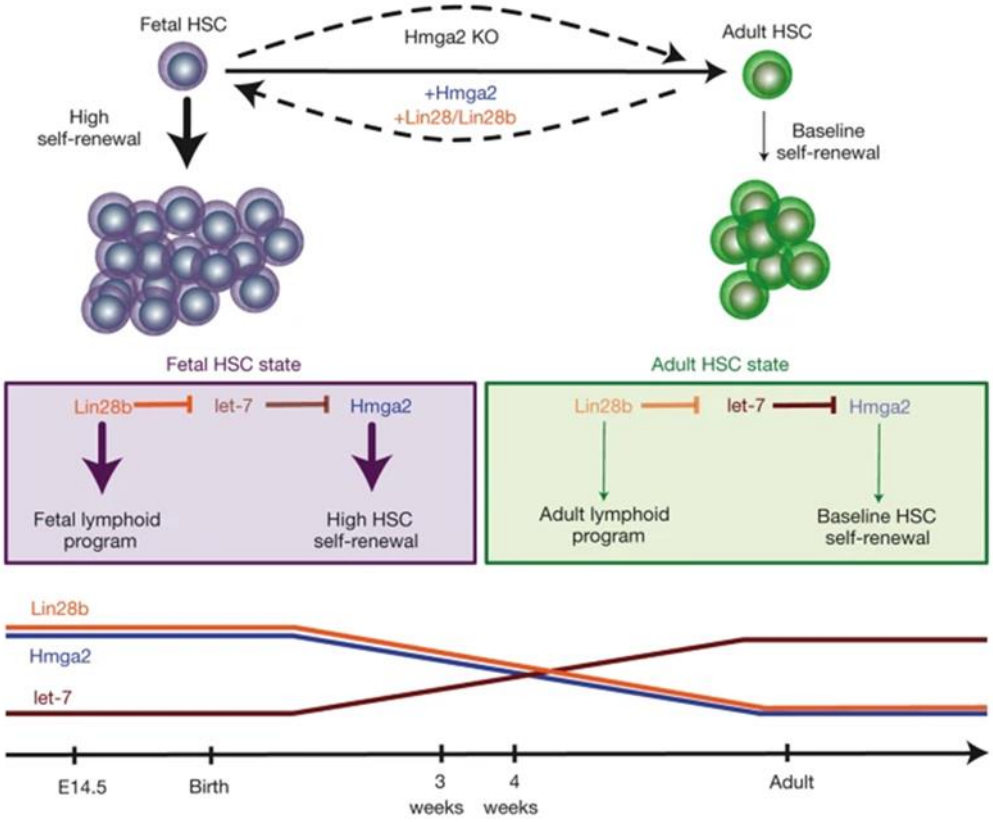

Fig. 1.5. Schematic differences between Hmga2, Lin28b, and let-7 miRNA in terms of expression from E14.5 till adult, the expression of these three markers gives rise to pluripotency of HSC (Bowie et al., 2006)

Differences in these molecules' expression could give a plausible explanation for the higher engraftment ability showed by FL cells compared to ABM cells in neonatal mice without any pre-conditioning observed in our previous study (Merlin et al., 2023).

It would be also interesting to know if HSC become quiescent immediately after gestation. In a study performed by Bowie et al., HSCs cycling activity was assessed to determine the cycling status of HSC at specific time points during murine lifetime. To further investigate the pace and timing of the transition of HSC into a largely quiescent population, they additionally analysed the cycling status of competitive repopulating units (CRU) in lineage negative (Lin–) BM cell suspensions from FLE 14.5, 3 weeks, 4 weeks, and 10 weeks. Following cell fractionation into G0 and G1/S/G2/M subsets they confirmed that the CRUs

detected in the 3-wk BM were grouped to the G1/S/G2/M fraction. Surprisingly, 98% of the CRUs in the 4-wk BM were found in the G0 fraction, Fig 1.6 (Bowie et al., 2006). Thus, HSC lose their proliferative ability at 4 weeks in a murine animal model.

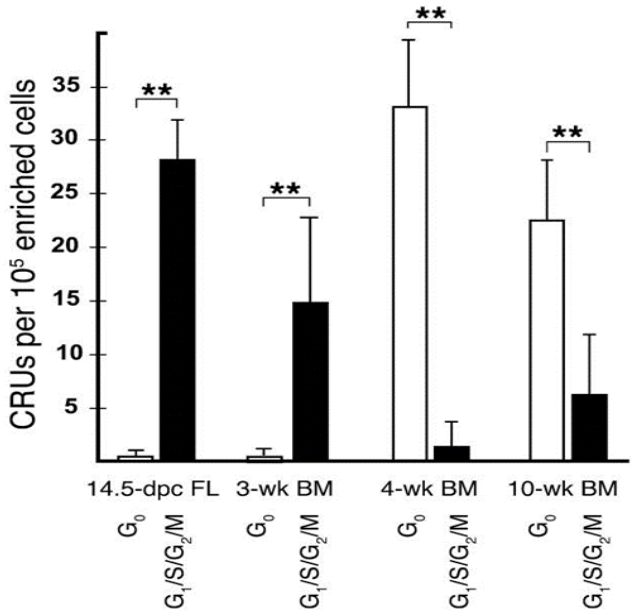

Fig 1.6 CRU of cycling cells present at different murine age time-points. Murine HSC continue in their active state until week 3, reaching a quiescent G0 phase at 4 weeks (Bowie et al., 2006)

Thus, because the active cycling phase of fetal HSC could be the explanation for and reflect their better engraftment capacity, they represent a valuable source for HSC-based cell therapy approaches.

However, there are huge concerns related to the use of human fetal liver cells: the difficulty of the right timing for their isolation and the ethical issues. This means that an alternative source of suitable HSC is needed, one that has the engraftment capabilities of fetal HSC and are easier to acquire, with much less or nonethical concern, and according to the abovementioned studies juvenile HSC could represent this cell source.

In murine models, juvenile is considered to be at a timepoint of age that ranges from 1.5 weeks to 6 weeks (Cumbermack et al., 2011). Based on the literature explained above, at approximate 2-week time-point levels of let-7-miRNA, which aids in adult HSC quiescence, is not as high as in adult HSC. Furthermore, at a 3-

week time-point, murine HSC are still highly proliferating, and their cell cycle state is still considered as active until the 4-week time-point. Whether juvenile bone marrow possess an engraftment capacity comparable to fetal HSC that can be used as a potential source of cells that can engraft efficiently, without the use of any adverse pre-conditioning agent, thus possibly representing frontier for the application of a regenerative cell-based approach for treating the HA bleeding phenotype, still represent a scientific question with major implications on regenerative medicine. Moreover, an additional way to help the homing of transplanted HSC could be represented by the use of clinically approved mobilizing agents.

1.7 Mobilizing Agents and Their Role in engraftment of transplanted HSC

The bone marrow provides a specific niche where HSC reside and this microenvironment contains various different cell types, chemokines, and signalling molecules which are involved in HSC retention within these specialized niches.

Osteoblasts play a crucial role in regulating HSC retention within the bone marrow by producing factors like CXCL12/SDF-1 which bind to CXCR4 receptors on HSC, “anchoring” them within the bone marrow (Ratajczak et al., 2013).

Additionally, HSC interact with the perivascular niche within the bone marrow, specifically with stromal or mesenchymal cells, which aid in retaining HSC within the bone marrow. (Pinho & Frenette, 2019)

Mobilizing agents act on these retention molecules through different mechanisms depending on which agent is used, allowing HSC to” detach” from their specialized microenvironments in the bone marrow and move to the peripheral blood (mobilization).

G-CSF, granulocyte colony stimulating factor, is a mobilizing agent used in clinic which helps mobilize HSC from their niche within the bone marrow and aid in their migration to the peripheral blood. Although the G-CSF mechanism of action is not completely understood, there are different proposed mechanisms to explain how G-CSF works. The first relies on osteoblast remodelling, whereby G-CSF induces changes in osteoblast conformation leading to a decrease in osteoblast produced

CXCL12 (Hopman & DiPersio, 2014), a major retention marker for HSC within the bone marrow niche.

G-CSF mobilization disrupts the retention signalling pathway between CXCL12 and CXCR4 via a protease dependant mechanism, promoting the release of proteases specific for CXCL12 and CXCR4, thus leading to the cleavage of these retention receptors and mobilizing HSC to the peripheral blood from their specialized niches in the bone marrow (Hopman & DiPersio, 2014).

Additionally, more evidence linking the effect of G-CSF to the retention markers CXCL12 and CXCR4 have been presented. In fact, mice knockout for CXCR4 did not show any mobilizing effect when treated with G-CSF, implicating the importance of CXCR4-CXCL12 receptor pathway in HSC mobilization following G-CSF administration, thus making it a major contender for HSC retention within the bone marrow. Fig 1.9 shows the methodology behind G-CSF in cleaving retention markers of HSCs'.

2) Materials and Methods

Animals

Animal studies were approved by the Animal Care and Use Committee of the Università del Piemonte Orientale "A. Avogadro" (Novara, Italy) and the by the Italian Ministry of Health with the authorization no. 758/2021-PR, and the Ethical Review Board of the Universidad Pablo de Olavide (Seville, Spain) according to the European Union regulations. In vivo experiments were performed on recipient newborn, Juvenile and adult HA mice in a C57BL/6 background (C57BL/6-HA) and Ly5.1 Mouse/CD45.1 (B6.SJL-Ptprca Pepcb/BoyCrl, cat #:494). Donor juvenile bone marrow (JBM) cells and adult bone marrow (ABM) cells were isolated from green fluorescent protein-positive (GFP+) mice (C57BL/6-Tg(ACTbEGFP)1Osb/J, strain #:003291) or Ly5.1/CD45.1 mice (B6.SJL-Ptprca Pepcb/BoyCrl ,cat #:494) in C57BL/6 background (Charles River Laboratory). We crossed GFP+ mice with C57BL/6 hemophilia A mice (Bl6HA) to obtain HA mice expressing GFP (Bl6HA-GFP+). For G-CSF experiment, recipient mice were treated with daily subcutaneous injection of G-CSF (Accofil, Filgrastim; 250ug/kg) in saline for 4 days (G-CSF 4x) or for 2days (G-CSF 2x) before transplantation. For busulfan (BU) experiment, adult mice (7-8 weeks old) received Busulfan

(BUSigma Aldrich; 25 mg/kg/injection) resuspended dissolved in acetone (25 mg/ml), diluted in corn peanuts oil and delivered by intraperitoneal injection 72 h, 48 h and 24 h before transplantation (total dosage = 75 mg/kg/mouse).

Cell Isolation and Transplantation

Adult (6 weeks old) and Juvenile (2-3 weeks old) BM cells were isolated by flushing tibias and femurs of 6-week-old or 2-3 week-old CD45.1 HA/WT or CD45.2 HA/WT donor mice (depending on the experiment), red blood cell were removed by lysis with suitable buffer (red blood lysis buffer, RBLB: 155 mM NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA), then washed and resuspended in D-PBS 1% FBS. For transplantation, $5x10^6$ cells were resuspended in 50 µL of D-PBS 1% FBS and injected in ~30 seconds in the superficial temporal vein or facial vein of newborn mice or resuspended in 300µL D-PBS and injected into the tail vein or retro-orbital sinus (Yardeni et al., 2011) of adult and juvenile recipient mice.

Flow cytometry

The engraftment of transplanted mice was evaluated and quantified as the percentage of CD45.1+ or CD45.2+ donor cells present in the peripheral blood (PB) of recipient mice and assessed every 4 weeks for up to 29 weeks. The assessment of engraftment was done by collecting PB using the retroorbital puncture procedure. To assess the engraftment levels, PB was treated with RBLB (described above), and neutralized using FACS buffer (PBS1X, FBS 2%, and 2 mM EDTA), before staining leucocytes with the appropriate antibodies mix. Antibodies used for flow cytometry are: CD45.1 FITC-conjugated antibodies (Miltenyi Biotech; 1:100), CD45.2PE-conjugated antibodies (Miltenyi Biotech; 1:100). Further antibodies used to evaluate the percentage of donor-derived leucocytes are: CD19 APC-conjugated, CD3 pecy7-conjugated, and CD11b PE-conjugated antibodies. Splenocytes and BM cells were obtained at the last time-point after mechanical disaggregation and analysed for engraftment of donor-derived cells. Samples were acquired on the Attune NxT Acoustic Focusing Cytometer (Thermofisher Scientific) and analysis was performed by FlowJo (Tree Star Inc.).

Statistical analysis

The statistical analyses were performed with GraphPad Prism 10.0 (GraphPad Software). All data are expressed as an average ± standard deviation. A 2-way analysis of variance was performed to compare the engraftment percentages of donor cells between the groups and to resolve the overall effects between the different mice groups over time. Statistical significance was assumed for $P < 0.05$.

3) Objectives

To assess the engraftment ability of juvenile bone marrow cells, several experiments were performed. The first experiment aimed at evaluating their engraftment abilities by comparing juvenile bone marrow cells and adult bone marrow cells, using myeloablative conditioning (busulfan, BU).

This experiment was perfomed as a control experiment aimed at understanding whether juvenile bone marrow cells do indeed possess higher engraftment capabilities as compared to their adult bone marrow counterparts, using the same dosage of BU and the same recipient mouse model.

The next experiment aims to recreate the fetal liver experiment but with the use of juvenile bone marrow cells into neonatal recipients, without the use of any pre-conditioning, to assess the engraftment ability of juvenile bone marrow cells as compared to fetal liver and adult total bone marrow.

The final experiments aimed at evaluating juvenile bone marrow cells engraftment capabilities in recipient mice treated with a mobilizing agent, G-CSF, to increase their engraftment potential.

4) Results:

4.1 Comparing Juvenile and Adult Bone Marrow Engraftment using Busulfan:

Juvenile total bone marrow cells, (JBM), and adult total bone marrow cells, (ABM), were transplanted in adult recipients pre-treated with busulfan (BU), to assess the engraftment capabilities between the two groups to evaluate whether juvenile HSC possess higher engraftment ability compared to adult HSC following treatment with myeloablative agents. For this purpose, we proceeded by setting up 2 experimental groups of mice. The first experimental group consisted of donor

JBM cells isolated from CD45.1 mice into BU-treated adult HA recipient mice (CD45.2). The second group consisted of donor ABM isolated from CD45.1 mice into BU-treated adult HA recipient mice (CD45.2). The experimental design is represented in Fig 3.1.

The engraftment potential of JBM was compared to engraftment potential of ABM in BU pre-conditioned HA adult mice over the course of 16 weeks. Gating strategy used for detecting/quantifying donor and recipient cells is shown in Fig. 3.2. As shown in Fig. 3.3, JBM cells demonstrated higher engraftment capacity as compared to ABM during all the experiment up to 16 weeks. At 4 weeks JBM cells engrafted better than ABM cells (JBM=79.3%±10.96, ABM=47.38%±12.09; $p< 0.05$) At 8 weeks the number of donor-derived cells increased in both groups, with JBM cells still showing higher engraftment ability (JBM=87.90%±5.1, ABM=59.89%±17.51; $p<0.05$). At 12 weeks, the engraftment of both groups stabilized, with JBM displaying a higher engraftment compared to ABM (JBM=85%±6.09, ABM=54%±18.20; $p=0.05$), and at 16 weeks, the last time-point, JBM group showed an increased percentage of donor-derived cells compared to ABM group (JBM=91.63%±5.05, ABM=54.3%±16.3 $p<0.05$). According to these results, JBM possess better engraftment ability compared to ABM in BU-treated adult HA recipient mice.

4.2 Assessing Engraftment Capabilities of Juvenile Bone Marrow cells in Neonatal Mice:

To determine if JBM cells possess the ability displayed by FL cells to engraft in neonatal mice without any pre-conditioning, we transplanted JBM cells in newborn HA and WT mice.

JBM cells were able to engraft in untreated HA and WT neonatal mice and donor-derived cells were found in recipient mice up to 14 weeks after transplantation (Fig. 3.4). However, during the follow-up engrafted cells reduced in number and did not achieve long-term engraftment, eventually virtually disappearing at 16 weeks post-transplantation. WT neonatal recipients showed higher engraftment ability and at 8 weeks post transplantation donor-derived cells in WT recipient were almost double than in HA recipients (WT=8.16%±0.88 vs HA=5.31%±3.39; p=ns). At 10 weeks post-transplantation, we observed a reduction in engrafted cells in both groups of recipients (WT=6.1%±0.84 vs HA=4.68%±1.86; p=ns). The engraftment

continued to decline at 14 weeks for both WT (5.6%±1.29) and HA (3.9±2.00) recipient mice (p=ns), and eventually at 16 weeks, the long-term engraftment time-point, the engrafted donor cells were completely lost in both groups (Fig. 3.4).

4.3 Use of G-CSF to Achieve Sustainable Long-term Engraftment:

We evaluated the possibility to use a mobilizing agent such as G-CSF to create space within the niche in the bone marrow with the aim of increasing the engraftment capabilities of transplanted cells and reaching the long-term engraftment (16 weeks) while avoiding myeloablative conditioning. For this purpose, we investigated the dosage required to achieve this aim. In a first attempt recipient mice were treated with two doses of G-CSF (G-CSF 2x) before transplantation and JBM HA cells were transplanted in juvenile and adult CD45.1 recipients (Fig 3.5).

JBM cells were able to engraft in both juvenile and adult recipients (juvenile = 0.7%±0.42 vs adult = 0.61%±0.40; p=ns) 4 weeks after transplantation. At 8 weeks engraftment percentage increased in both groups (juvenile = 1.12%±0.9 vs adult = 1.33%±0.7; p=ns) 12 week after transplantation we observed a slight decrease in engraftment percentage in both groups (juvenile = 0.88%±0.78 vs adult = 1.17%±1.17; p=ns). Noteworthy, long-term engraftment was achieved with both juvenile and recipient mice showing ~1% of donor-derived cells 16 weeks after transplantation (juvenile = 0.96%±0.70 vs adult = 1.2%±1.17; p=ns) (Fig. 3.6).

We then evaluated the possibility to increase the dosage of G-CSF (from 2 to 4 injections, G-CSF 4x) to improve the engraftment of transplanted cells (Fig. 3.7).

Following G-CSF 4x, JBM cells showed higher engraftment in adult recipients compared to juvenile recipients (adult = 3.7%±1.27 vs juvenile = 1.8%±0.3; p=ns) 4 weeks after transplantation. Additionally, comparing these results with G-CSF 2x transplantation experiment we noticed that both groups showed an increase in engraftment of transplanted cells. 8-weeks after transplantation the engraftment of donor-derived cells increased, with JBM cells showing better engraftment in adult recipients compared to juvenile recipients (adult = 6.2%±1.31 vs juvenile = 3.5%±0.54; $p<0.05$) as observed for the 4 weeks' time-point. As observed for the

G-CSF 2x experiment, both groups showed a slight decrease in their engraftment percentage 12 weeks after transplantation, with adult recipients still showing higher engraftment compared to juvenile (adult = 4.9%±1.27 vs juvenile = 2.4%±0.74; $p<0.05$). Contrary to what observed for with G-CSF 2x treatment, G-CSF 4x further increased engraftment percentage at 16 weeks(adult = 5.7%±1.74 vs juvenile =2.2%±0.51; $p<0.05$) followed by a decrease in engraftment 22 weeks after transplantation in both groups, with adult recipients still showing higher engraftment compared to juvenile (adult = 3.3%±2.11 vs juvenile = 2.5%±1.11; p=ns). The engraftment observed was stable since, even though with a slight decrease, 29 weeks after transplantation both groups were still showing the presence of donor-derived cells (adults = 2.7%±1.58 vs juvenile = 2.3%±0.98; p=ns), demonstrating that JBM cells possess the ability of long-term engraftment in both adult and juvenile recipients (Fig. 3.8).

4.4 Engrafted cells Migration and Differentiation:

Since 4X dosage of G-CSF resulted in long-term engraftment, we evaluate the presence and repopulating ability of hematopoietic compartment of donor-derived cells in the bone marrow (BM), spleen (SP), and peripheral blood (PB). Fig 3.9 (A) shows the engraftment levels at the last time-point. Thus, we evaluated the presence and percentage of donor-derived progenitor cells, T Cells, B Cells and myeloid cells in the peripheral blood, spleen, and bone marrow of recipient mice (Fig. 3.9 A and B)

Flow cytometry analysis showed that most donor-derived cells were found in the spleen (Fig 3.9 A) and most donor-derived progenitors were of B Cell origin (Fig 3.9 C). The percentages of donor-derived progenitors, in spleen and bone marrow correlate well with percentages observed in normal mice (Kraal & Janse, 1986; Pang et al., 2017) however, donor-derived cells in peripheral blood are showing a higher repopulation of B Cells, in normal mice T Cells are the most abundant in peripheral blood (Hayakawa et al., 1983), indicating a difference in this organ.

Figures:

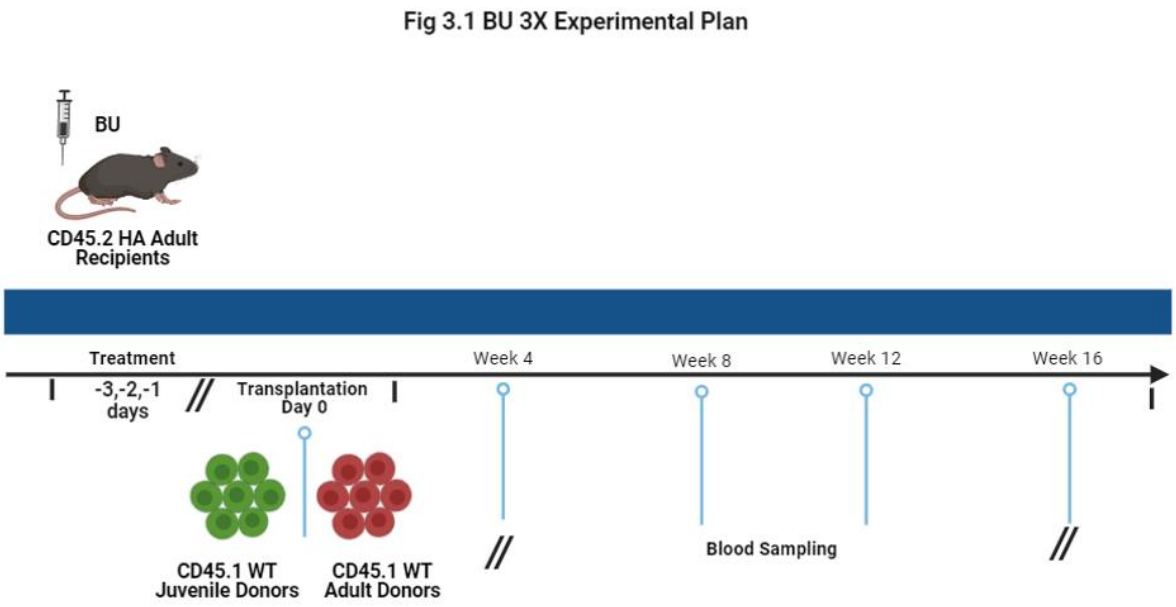

Fig 3.1. Schematic representation of the experimental plan of BU3x experiment, CD45.1 WT Juvenile bone marrow and CD45.1 WT adults bone marrow were transplanted into adult HA CD45.2 recipients.

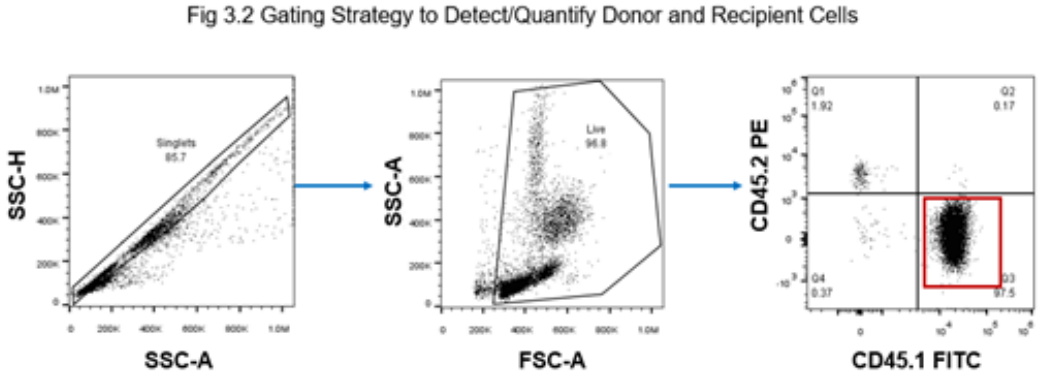

Fig 3.2. Gating strategy used to detect and quantify donor and recipient cells by flow cytometry analysis.

Fig 3.3 BU3X Transplantation Engraftment

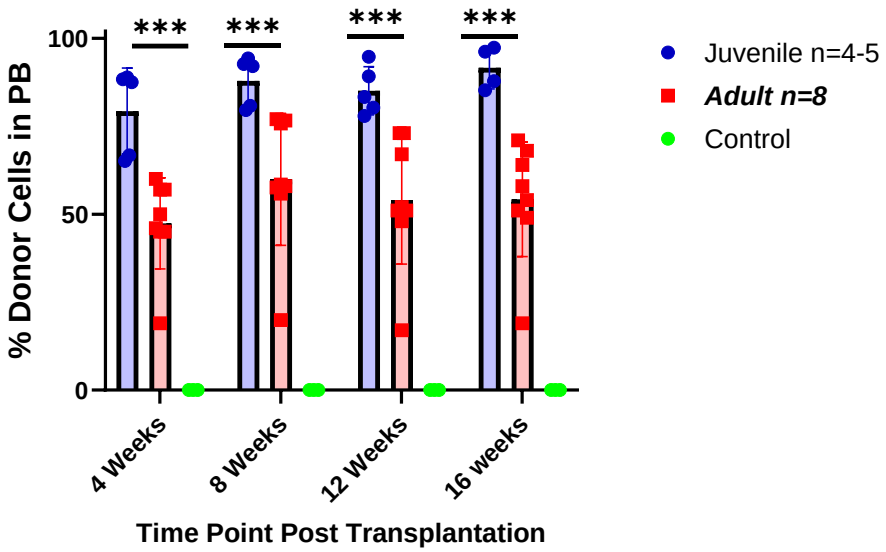

Fig 3.3. Engraftment efficiency of donor-derived cells in adult recipients **Juvenile BM cells (blue circles);Adult BM cells (red squares); Control (green circles)**

Fig 3.4 Engraftment Levels in Neonatal Mice (No- Pre-Conditioning)

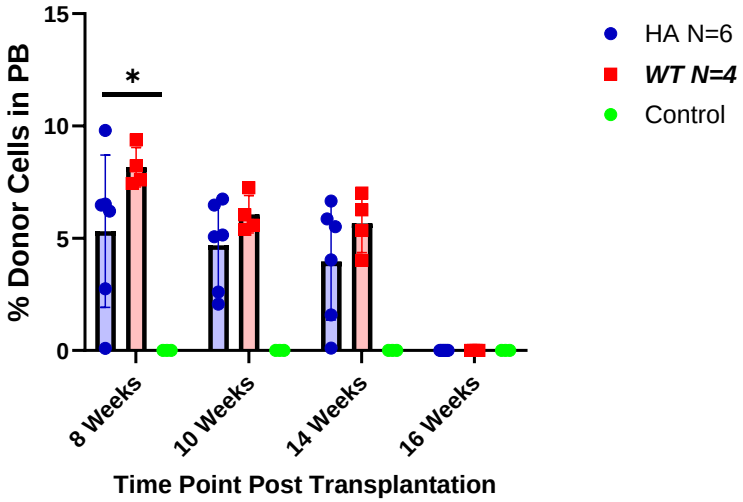

Fig 3.4. Donor-derived cells in peripheral blood (PB) of neonatal mice. Data points represent the mean percentage of donor cells in PB, with error bars indicating the standard deviation The three groups being compared are newborn HA mice transplanted with juvenile bone marrow HA cells (blue circles) or JBM wt cells (red squares), and a Control group (PBS; green circles). Recipients mice showed presence of donor cells across all time points (8, 10 and 14 weeks after transplantation). All donor cells were lost at long-term time point (16 weeks)

Fig 3.5 G-CSF 2X Experimental Plan

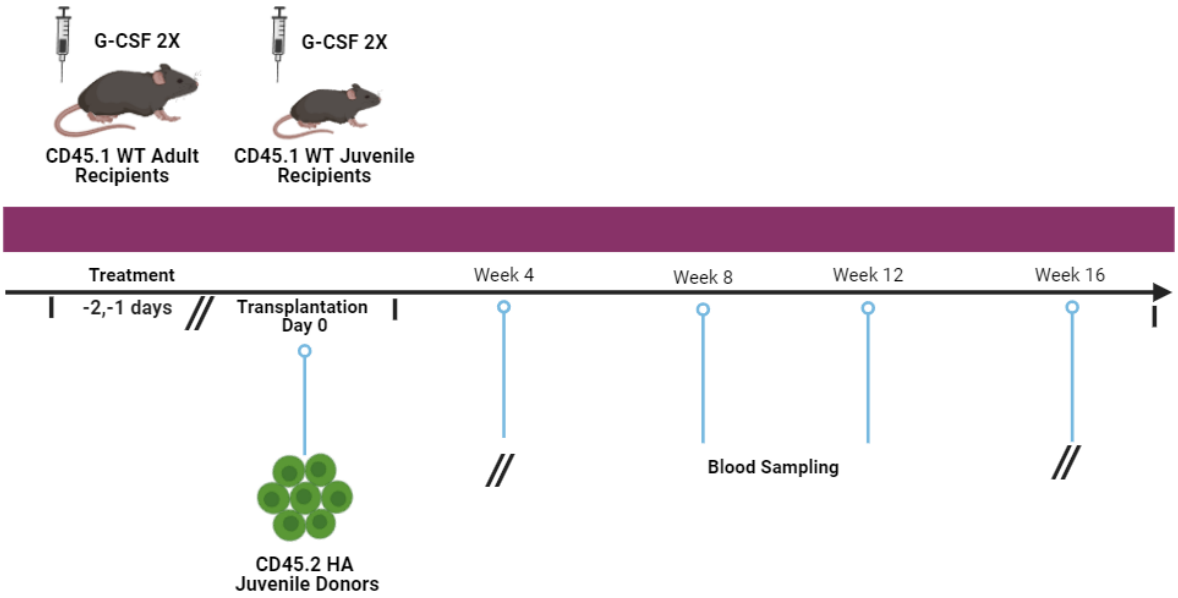

Fig 3.5. Experimental plan of G-CSF 2X experiment, CD45.2 HA Juvenile bone marrow were transplanted into CD45.1 WT adult and CD45.1 WT juvenile recipients, blood sampling was done until the 16-week time-point.

Fig 3.6 G-CSF 2X Engraftment

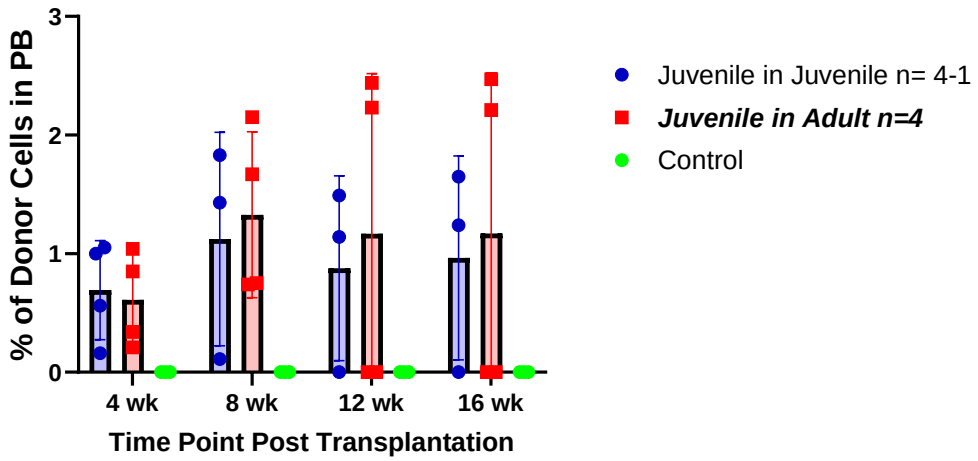

Fig 3.6 Engraftment levels following transplantation of juvenile bone marrow cells into juvenile (blue circle) and adult (red square) recipients using 2X doses of G-CSF. **Control** (green circles): No transplantation was performed.

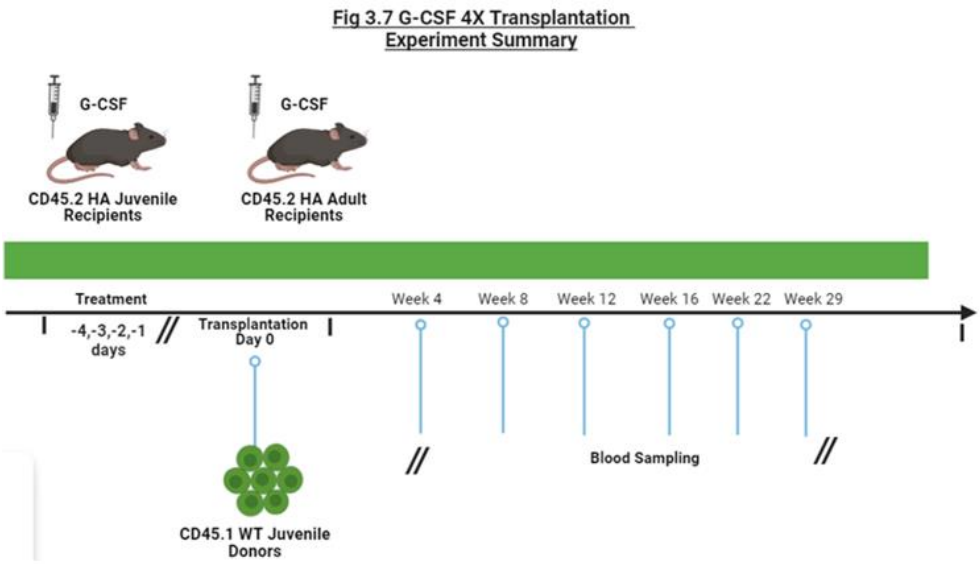

Fig 3.7. Experimental plan of G-CSF 4X experiment, CD45.2 HA Juvenile bone marrow were transplanted into CD45.1 WT adult and CD45.1 WT juvenile recipients.

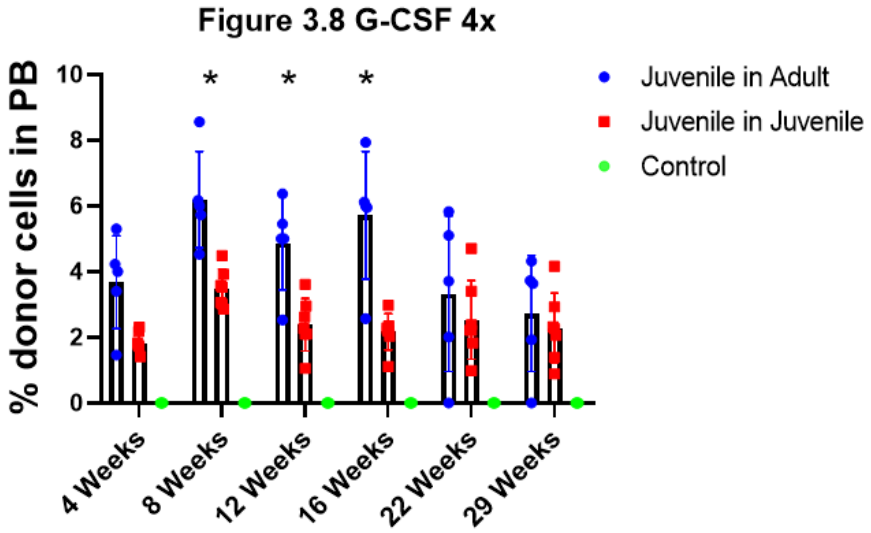

Fig 3.8 Engraftment levels following transplantation of juvenile bone marrow cells into juvenile (blue circle) and adult (red square) recipients using 4X doses of G-CSF. **Control** (green circles): No transplantation was performed.

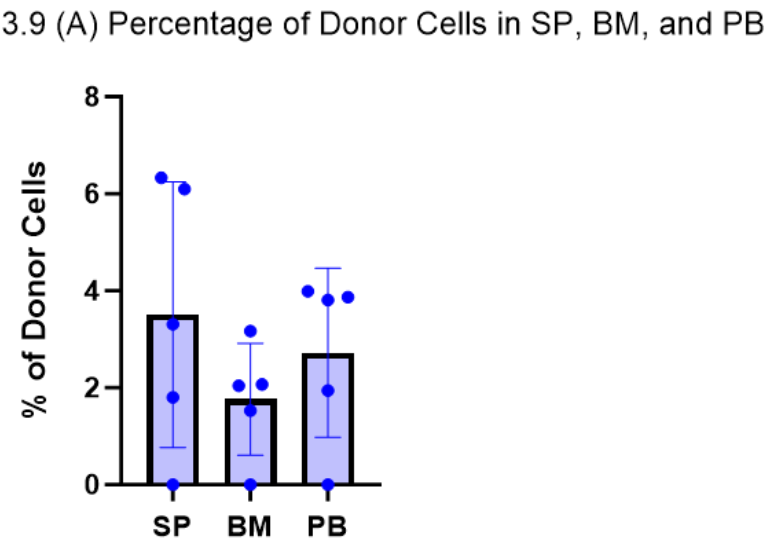

Fig 3.9(A). This figure displays the percentage of donor cells present in the spleen (SP), bone marrow (BM), and peripheral blood (PB) at last time point. The plot shows the median percentage of donor cells along with the interquartile range for each tissue type. In the SP, donor cells exhibit a higher median percentage compared to BM and PB.

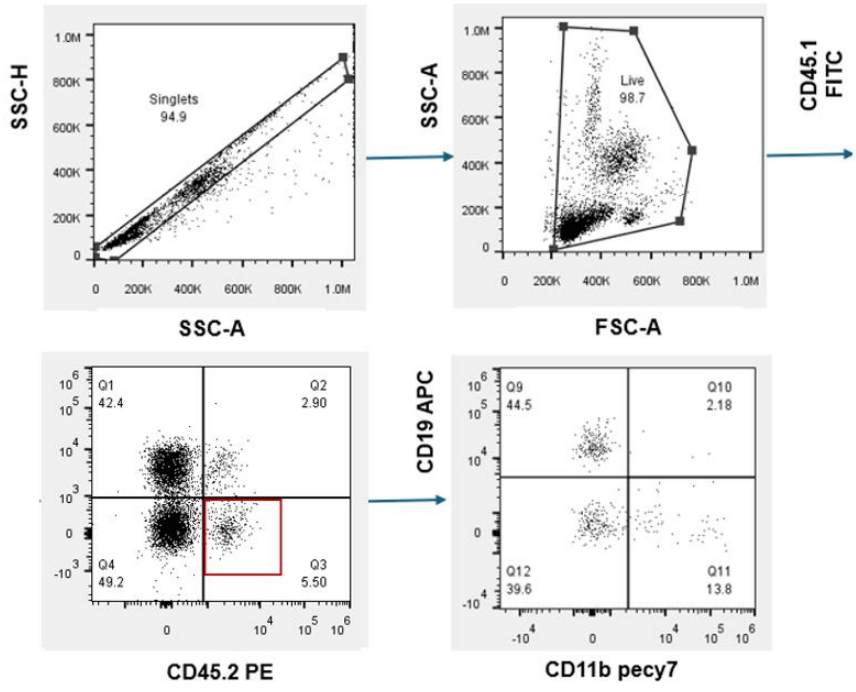

Fig 3.9(B). Flow cytometry gating strategy used to detect and quantify donor-derived progenitor cells. The gating strategy evaluated CD19+, CD11b+ and CD3+ cells in CD45.2+CD45.1- cells.

Fig 3.9 (C) Donor-Derived Progenitor Diffrentiation

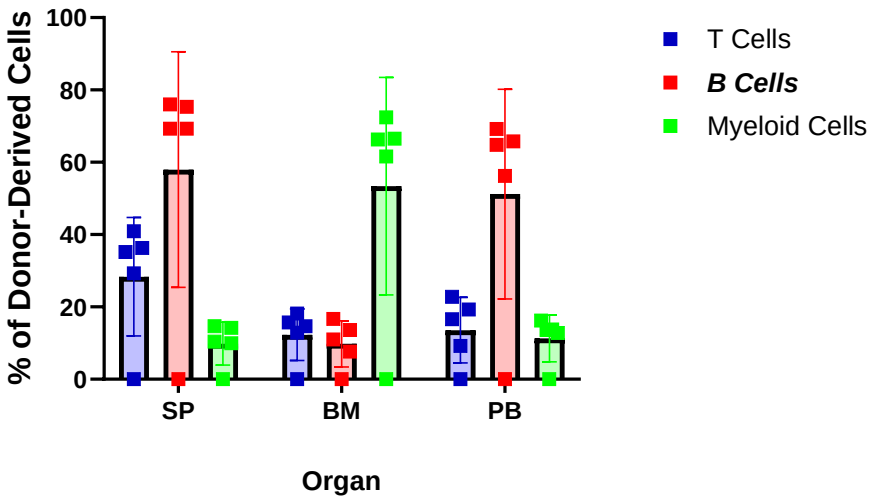

Fig 3.9(C) Differentiation of donor-derived progenitor cells into T cells, B cells, and myeloid cells across different organs: spleen (SP), bone marrow (BM), and peripheral blood (PB).

5) Discussion:

The development of a lifelong/long-term treatment for hemophilia A (HA) has involved the different studies and attempts over the last 2 decades. The current standard treatment for HA, i.e. replacement therapy, as well as the new/newly approved approaches such as emicizumab and gene therapy (ROCTAVIAN), despite been effective in the treatment of HA patients, they all presents issues related to inhibitors formation, pre-existing immunity to the treatment and/or safety-related doubts requiring deeper clinical investigations (Mancuso & Cannavò, 2015) (Brinza et al., 2024; Kitazawa et al., 2012; Mahlangu et al., 2018) (Aledort et al., 2019; Bowyer, Hickey, et al., 2023; Bowyer, Kitchen, et al., 2023)

Thus, new therapeutic approaches in form of cell and/or gene therapy able to ensure a stable long-term correction of the bleeding phenotype and while avoiding immune reaction against FVIII are extremely attractive, especially for the treatment of early age/neonate patients. In our studies, we demonstrated that following transplantation fetal liver (FL) cells from day 11 to 13 of gestation (E11-E13) showed long term (>1 year) engraftment potential in newborn HA mice and were able to correct the bleeding phenotype of recipient mice (Merlin et al., 2023). Noteworthy, FL cells, especially FLE13 cells, were able to engraft and produce therapeutic levels of FVIII even in absence of preconditioning, an ability not displayed by adult BM cells (Merlin et al., 2023).However, FL cells may not be suited for clinical uses due to various reasons, such as availability and immune responses, without forgetting ethical issues. Thus, we further explored the use of juvenile bone marrow (JBM) as alternative cell source to FL for efficient engraftment without preconditioning.

To evaluate their engraftment ability, we transplanted juvenile and adult bone marrow cells (ABM) into sub-lethally busulfan (BU)-conditioned adult HA recipient mice. Despite all showing long-term engraftment (>16 weeks), we observed higher engraftment of JBM-derived cells over time compared to ABM cells. These results are in line with our previous results on FL cells displaying a higher engraftment ability compared to ABM cells (Merlin et al., 2023), confirming that JBM cells can represent a suitable alternative source to FL cells.

To continue the comparison with FL cells, we evaluated the ability of JBM cells to engraft in newborn HA mice without any preconditioning by using the same experimental settings (Merlin et al., 2023). Contrary to what we observed for FL cells, JBM cells engrafted in the same way, but engraftment persisted only for <16 weeks showing their long-term repopulation defect which can be reverted by using mobilizing agents (Bowie et al., 2006) (Bowie et al., 2007).

Interestingly, with this experiment we compared the engraftment ability of both WT and HA JBM cells. WT and HA JBM showed engraftment in all recipient mice, with WT cells showing a trend of higher engraftment level compared to HA cells in short term. As far as we are concerned, there are no data in literature reporting a difference in engraftment ability between WT and HA without using precondition regimens, it would be interesting to further characterize the transplanted and engrafted cells and validate the results obtained.

To further investigate and better assess the potential for long-term engraftment of JBM cells, we exploited an indirect CXCL12 antagonist/mobilization agent, G-CSF, already used in clinic. JBM cells transplanted into juvenile and adult mice treated with 2 doses of G-CSF allowed us to achieve a lower engraftment (1-2%) engraftment that persisted for long term (>16 weeks). We hypothesised that this low engraftment could be due to a lower number of HSC mobilised into the peripheral blood thus resulting in a limited space made available for the engraftment of transplanted cells.

We then proceeded with 4 doses of G-CSF, the dosage used in clinics, trying to improve the engraftment of transplanted cells. Following transplantation in juvenile and adult mice receiving G-CSF 4x, JBM cells demonstrated long-term engraftment potential (≥5%), possibly attributed not only to the reversal of engraftment defect but also to the transient higher availability of space in the bone marrow niche due to the use of the mobilization agent (Bowie et al., 2006; Canarutto et al., 2023; Guderyon et al., 2020) Previous research has shown that it typically takes a minimum of 4-5 days of G-CSF treatment to reach the peak of HSC mobilisation and optimal collection of cells (Winkler et al., 2016).Therefore, we believe that we achieved higher long-term engraftment of JBM by administering G-CSF for 4 consecutive days. This approach aligns with previous

observations that the peak of HSC mobilisation occurs when most of these cells are in the peripheral blood and there is “sufficient room” for donor cells to engraft, along with the backflow of mobilised stem cells returning into the bone marrow niche (Canarutto et al., 2023). Moreover, transplantation of HA juvenile bone marrow cells into juvenile and adult wild type mice revealed higher engraftment in adult recipients, a trend that can possibly be due to the differential effects of G-CSF based on age, with adult mice exhibiting higher mobilization (Xing et al., 2006).

Previous transplantation studies using irradiated NOD/SCID mice have shown that the number and self-renewal activity of human lympho-myeloid stem cells within the CD34+CD38- population were similar in FL and cord blood (CB) and decrease during ontogeny in ABM. In addition, human CB HSC constitute promising fetal-like candidates for cell-based therapy purposes for efficient treatment of newborn HA individuals. More studies are necessary to determine the output of mobilization-based transplantation of human CB HSC starting from preclinical models, such as transplantation into immunodeficient newborn and adult HA mice. Further studies on different cell sources, in combination with the use of mobilization-based conditioning regimens in newborns will lay the foundation to delineate molecular mechanisms involved in transplanted HSC engraftment that should lead to new possibilities for bleeding phenotype correction in pediatric HA individuals. Moreover, we envisage the incorporation of gene therapy to these approaches, thus potentially further increasing FVIII production from transplanted cells along with more potent mobilization agents.

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