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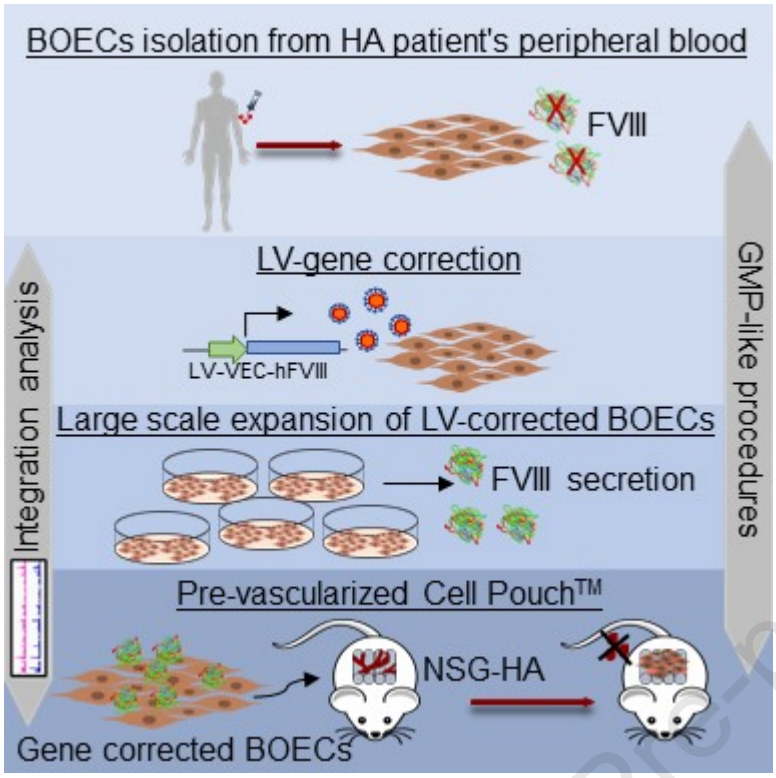

Efficient and safe correction of hemophilia A by lentiviral vector-transduced BOECs in an implantable device

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Short title: **BOECs within a medical device to cure hemophilia A**

Abstract

Hemophilia A (HA) is a rare bleeding disorder caused by deficiency/dysfunction of the FVIII protein. As current therapies based on frequent FVIII infusions are not a definitive cure, long-term expression of FVIII in endothelial cells through lentiviral vector (LV)-mediated gene transfer holds the promise of a one-time treatment. Thus, here we sought to determine whether LV-corrected blood outgrowth endothelial cells (BOECs) implanted through a prevascularized medical device (Cell Pouch™) would rescue the bleeding phenotype of HA mice. To this end, BOECs from HA patients and healthy donors were isolated, expanded and transduced with an LV carrying FVIII driven by an endothelial-specific promoter employing GMP-like procedures. FVIII-corrected HA-BOECs were either directly transplanted into the peritoneal cavity or injected into a Cell Pouch™ implanted subcutaneously in NSG-HA mice. In both cases, FVIII secretion sufficient to improve the mouse bleeding phenotype. Indeed, FVIII-corrected HA-BOECs reached a relatively short-term clinically relevant engraftment being detected up to 16 weeks after transplantation, and their genomic integration profile did not show enrichment for oncogenes, confirming the process safety. Overall, this is the first pre-clinical study showing the safety and feasibility of transplantation of GMP-like produced LV-corrected BOECs within an implantable device for the long-term treatment of HA.

Introduction

Hemophilia A (HA) is an X-linked disorder caused by mutations in the *F8* gene.[1,2] These mutations result in deficiency or reduced activity of the coagulation factor VIII (FVIII), leading to a lifelong bleeding tendency, whose clinical severity is proportional to FVIII reduction.[1] Although the current standard of care is to infuse intravenously HA patients with clotting factor concentrates, the short half-life of FVIII requires frequent and multiple infusions, with a negative impact on the patient's quality of life (QoL). New generation of standard rFVIII was obtained by refinements of the recombinant protein through the optimization of relevant post-translational modifications like glycosylation, that improves the stability of the mature FVIII protein,[3] or the introduction of a covalent link between the FVIII heavy and light chains, preserving FVIII from premature degradation and conferring a higher binding affinity to VWF with a reduction of the needed injection dose.[4–6]

Other new bioengineered molecules were developed with higher extended plasma half-life (EPHL) [7] and improved pharmacokinetics by the fusion of rFVIII with the Fc portion of immunoglobulin[8] or by the conjugation with polyethylene glycol (PEGylation).[9]

However, several issues are still to be solved as the recurrent intravenous (iv) route of administration and the inhibitor development, common in 20-40% of patients with the severe form,[10] worsen the clinical outcome, making the treatment ineffective.[11,12] Therefore, a new clinical approach emerged more recently, i.e. emicizumab, to overcome the difficulties of iv delivery and to improve and prolong the effectiveness of the therapy in all patients, regardless of the inhibitor presence or absence.[13,14] However, bleeding events can still occur after trauma requiring the use of additional hemostatic agents, according to the patients' inhibitor status.[15,16]

This has led researchers to explore innovative cell and gene therapy strategies that may ensure continuous endogenous FVIII expression with only a one-time treatment. Another good reason for choosing gene therapy over traditional approaches is that HA is a monogenic disease i.e., entirely ascribable to the lack of one protein, FVIII and that a small increase in FVIII plasma levels is enough to ameliorate the bleeding phenotype of HA patients.

Given the growing number of cell and gene therapy approaches being developed, it is becoming increasingly important to identify the most suitable cell target. Even though *F8* mRNA is expressed in different human and mouse organs, such as liver, spleen, lymph nodes, kidney,[17–20] and in hematopoietic cells,[21,22] transplantation studies in hemophilic animal models have shown FVIII expression to be mainly localized in liver sinusoidal endothelial cells (LSECs),[23–25] making these cells attractive targets for HA gene therapy. This is also supported by the fact that endothelial cells (ECs) secrete FVIII and can act as tolerogenic cells.[26,27]

Over the years, several gene therapy approaches for HA have been attempted using adeno-associated virus (AAV) vectors to induce FVIII expression in the desired cell type. Despite the encouraging preliminary results obtained in few ongoing clinical trials testing the efficacy of AAV-mediated hepatocyte-targeted FVIII expression in HA patients,[28–30] some medical issues still need to be addressed, such as the use of these vectors in patients with pre-existing immunity to AAV or with FVIII inhibitors. As AAV vectors do not actively integrate into the host cell genome, they are lost upon cell division during liver growth or in case of liver disease, thus potentially limiting their use in pediatric patients and questioning their life-long maintenance. Therefore, lentiviral vectors (LVs) could represent a viable approach able to overcome some AAV limitations. Moreover, several studies have demonstrated, by the use of endothelial specific promoters, specific expression of human FVIII in LSECs.[23–25,31] Recently, we have shown that induced pluripotent stem cells (iPSCs) derived from $CD34^+$ HA cells can be differentiated into ECs and genetically corrected by LV to express the FVIII transgene, deleted of the B domain (BDD), driven by the endothelial specific vascular endothelial cadherin (VEC) promoter. After transplanting these cells into the liver of monocrotaline-conditioned NOD-*scid* $IL2Rg^{null}$ HA (NSG-HA) mice, we were able to correct the bleeding phenotype of these mice and maintain a stable FVIII activity over time.[32] Moreover, BDD-FVIII-transduced ECs encapsulated in microcarrier beads have been shown to survive for a prolonged time in the peritoneal cavity of NSG-HA mice secreting therapeutic level of FVIII.[32]

Several studies have focused on defining different cell sources and matrices to transplant FVIII-expressing ECs.[33–35] A readily available EC source is represented by patient-derived blood outgrowth endothelial cells (BOECs).[36] BOECs are isolated from adult peripheral blood[37] and can be fully differentiated into mature ECs. They promote neovascularization *in vivo* when transplanted into immunodeficient mice[38] or when cultured on three-dimensional biodegradable vascular scaffolds.[39–41] In addition, they can be considered a valuable source of cells to understand EC biology and model disease and can be used in regenerative medicine due to their ability to promote neovascularization, thus representing an optimal candidate for HA cell and gene therapy. Indeed, BOECs transplanted in NSG-HA mice after gene modification for FVIII expression were able to partially rescue the hemorrhagic phenotype of these mice.[36,42] Moreover, autologous transplantation of FVIII-expressing BOEC cell-sheet allowed long-term phenotypic correction and survival of transplanted cells.[35] Noteworthy, BOECs can promote neovascularization *in vivo* in combination with synthetic or natural materials.[38]

A combination of LV-corrected BOECs with a medical device is classified by the European Union as a combined gene therapy medicinal product (GTMP).[43] The mandatory non-clinical study scheme prior to the first administration of a cell-based GTMP to human subjects includes the comprehensive characterization of the transduced cells and the evaluation of the medical device contribution.[44] Moreover, the proof-of-concept pharmacodynamics along with the molecular mechanism of action must be identified in preclinical models *in vivo* and/or *in vitro*. These studies are deemed essential to determine the GMP cell dose to be used in clinical trials.[45]

Here, we show extensive characterization of LV-transduced BOECs isolated from healthy donors or HA patients for FVIII production *in vivo*. These cells were transplanted in a small scalable, implantable, and prevascularized medical device, namely Cell Pouch™ (Sernova Corp.), previously developed for diabetes treatment.[46]

Our findings, showing that Cell Pouch-transplanted LV-corrected HA-BOECs are capable of correcting the bleeding phenotype of HA mice, open new avenues for the treatment of HA in humans.

Results

Characterization of BOECs isolated from HA patients or healthy subjects and LV-mediated FVIII gene transfer

Upon isolation and expansion in culture medium, both BOECs from healthy donors and HA patients showed the classical endothelial cobblestone-like morphology (Figure 1A). Of note, despite being all isolated from severe HA patients, HA BOECs gave rise to many colonies (Figure S1A) and there was not a significant difference in the number of isolated colonies between healthy donors and HA patients (Figure S1B). For transgene expression, isolated cells were transduced with a lentiviral vector (LV) carrying the BDD form of FVIII driven by the vascular endothelial cadherin promoter (LV-VEC.hBDD-FVIII) or with an LV carrying the green fluorescent protein under the control of the same promoter (LV-VEC.GFP), both at an MOI of 20. FACS analysis showed 98±1% GFP^+ cells after transduction (Figure S1C), indicating excellent transduction efficiency. The number of integrated LV copies/cell was ~6 and ~3 for LV-VEC.GFP and LV-VEC.hBDD-FVIII-transduced cells, respectively (Figure S2A). Thus, this protocol ensures a very high transduction efficiency while maintaining a safe number of integrated LV copies/cell.[47,48]

We next assessed the endothelial phenotype and functionality of transduced *vs* non-transduced healthy or HA BOECs. As shown in Figure 1B, all cells expressed the classical endothelial markers (e.g., *PECAM1*, *KDR, TEK, CDH5*, and V*WF*) as well as other genes specific to blood endothelial cells (BECs)[49,50] (Figure 1C). The endothelial phenotype of healthy and HA BOECs was further verified at the protein level (Figure 1D and E, respectively), while the hematopoietic phenotype was ruled out upon CD34 and CD45 staining, which resulted negative (Figure 1D and E).

The endothelial functionality of the transduced cells was confirmed by their ability to form tubule networks upon Matrigel cell culture (Figure 1F). *F8* mRNA expression was measured in transduced BOECs by RT-PCR (Figure 1G), while FVIII protein expression levels were detected by flow cytometry (Figure H and I for healthy and HA BOECs, respectively) and immunofluorescence (IF)

(Figure 1J). Interestingly in healthy BOECs we detected low levels of FVIII (Figure 1H and 1J). This is in accordance with previous works where FVIII in healthy BOECs was barely observed. [42,51,52] When cell supernatants were subjected to activated partial thromboplastin time (aPTT) assay, we noticed a consistent shortening in LV-VEC.hBDD-FVIII-transduced BOECs (69± 3.5 sec for transduced healthy BOECs, 66± 4 sec for transduced HA BOECs) compared to non-transduced cells (80± 2.8 sec for healthy BOECs, 84± 2.8 sec for HA BOECs) (Figure S2B), in good agreement with the amount of secreted FVIII (35.9± 2.3 ng/ml for LV-VEC.hBDD-FVIII healthy BOECs, 4.5± 1.3 ng/ml for non-transduced healthy BOECs; 54± 7.5 ng/ml for LV-VEC.hBDD-FVIII HA BOECs, 0.15± 2.5 ng/ml for non-transduced HA BOECs) (Figure S2C).

To further evaluate the safety of LV transduction, healthy BOECs were transduced with different MOIs (MOI 10, 20, 50, 100), and HIV-1 p24 expression on cell supernatant was assessed. As shown in Figure S2C, all LV-transduced BOEC supernatants were negative for HIV-1 p24 at any of the MOIs tested 10 days after transduction, suggesting the reliability of our protocol. HIV-1 p24 on supernatant of HA BOECs transduced with an MOI of 20 showed comparable results (Figure S2D).

Secretion of the FVIII gene product by LV-transduced HA or healthy BOECs in NSG-HA mice

Since healthy and HA BOECs were both able to secrete FVIII *in vitro*, we evaluated their ability to survive and secrete FVIII in NSG-HA mice following intraperitoneal (i.p.) injection in association with Cytodex® 3 microcarrier beads. Following injection, FVIII-transduced GFP^+ healthy BOECs were able to partially restore FVIII activity, which reached a peak of approximately 10% at 4 weeks post injection (pi) and persisted above 5% for up to 10 weeks pi (Figure 2A). As expected, LV-VEC.GFP BOEC controls showed only basal FVIII secretion, which only lasted for 4 weeks. Importantly, mice receiving FVIII-transduced HA BOECs revealed sustained therapeutic FVIII activity (up to 10%) for up to 13 weeks pi, which persisted at a level < 5% throughout the 18 following weeks (Figure 2A). Blood loss assays, run between 7 and 10 weeks p.i. of FVIII-transduced GFP^+ HA BOECs, demonstrated partial hemostasis restoration (Figure 2B) accompanied by detectable amounts of plasmatic FVIII antigen (12.4±3.9 ng/ml) (Figure 2C). At week 13 p.i., three mice in each

experimental group were killed, and beads were recovered from the abdominal cavity. IF staining was performed on the recovered beads using an anti-GFP antibody that would detect the transplanted cells, which were previously transduced with both LV-VEC.GFP and LV-VEC.FVIII. Therefore, the presence of GFP+ cells confirm that they are still associated with the beads and that they maintained an endothelial phenotype, as shown by the co-staining with CD31 (Figure 2D).

Large scale expansion of FVIII-transduced cells

With the aim to translate this approach into the clinic, we developed a protocol that would allow us to obtain a large amount of transduced HA BOECs for our *in vivo* experiments. LV-VEC.hBDD-FVIII-transduced HA BOECs from four patients were large-scale expanded to reach 10^8 cells, frozen and sent to the partners in accordance with GMP-like procedures. Upon arrival, cells were re-cultured by simulating a centralized cell production process with long-term cryopreservation. After large-scale expansion and cryopreservation, upon thawing and reseeding, all cells showed normal cobblestone-like morphology (Figure 3A). Even though their size was slightly enlarged, no significant changes in their doubling time, cell density, and length of time required for expansion were noticed (Figure 3B). In addition to maintaining expression of the classical endothelial markers (CD31, KDR, Tie-2, VEC), expanded BOECs became $CD34^+$, a transmembrane phosphoglycoprotein involved in cell adhesion,[53] while they retained the classical $CD45^-$ phenotype (Figure 3C).

Functionally, FVIII-transduced cells preserved their tubulogenesis activity (Figure 3D) and led to partial restoration of FVIII activity once transferred into NSG-HA mice (Figure 3E), similar to the kinetics of non-expanded BOECs. Thus, LV-VEC.hBDD-FVIII HA BOECs maintain their ability to secrete FVIII at therapeutic levels even after large-scale expansion.

Tissue matrix development and safety of LV-VEC.hBDD-FVIII-transduced BOECs within a Cell Pouch™ implanted in NSG-HA mice

The Cell Pouch™ is a medical implantation device specifically designed to enable the development of a vascularized tissue matrix environment that ensures long-term survival and function of transplanted therapeutic cells. Thus, we first evaluated safety and survival of transduced HA BOECs

within the Cell Pouch™ implanted in NSG-HA mice. For this purpose, 4-week implanted Cell Pouches™ were transplanted with one of three doses (2×, 5× or 10×10^6) of LV-VEC.hBDD-FVIII HA BOECs isolated from two separate HA donors. The Cell Pouches™ transplanted with BOECs were explanted at 4, 8, or 12 weeks, and a gross pathological assessment was performed. HA BOECs were safe across doses and time points with no visible tumors observed (n = 60 total; HA1 n = 37; HA2, n = 23) (data not shown).

Overall, the tissue matrix developed within the Cell Pouch™ internal chamber and transplant area was viable among all groups according to time, dose, and cell lot, with no apparent signs of inflammation, hemorrhage, fibrosis, or necrosis (Figure 4A and Table S1). The center of the transplanted chamber area showed mild to moderate collagen deposition without any difference due to donor lot, time, or dose. Within the area of pre-vascularization, there was comparatively an increase in established collagen, indicating that the Cell Pouch™ promoted, over time, the development of a natural scaffold to provide strength and structure to the environment irrespective of the transplant. Regarding tissue vascularization, there was moderate neovascularization of the central, transplanted tissue of the Cell Pouch™ that was present in both donor lots, as well as the controls, along with evidence of established vessel growth, indicating that the tissue development within this area included new blood vessel formation (Figure 4B). Established vessels within the central, transplanted zone appeared to be more prominent and donor-dependent at the latest time points (Figure 4A).

Transduced HA BOECs improve the bleeding phenotype and cell survival in mice after transplantation into the vascularized Cell Pouch™

The therapeutic efficacy of LV-VEC.hBDD-FVIII HA BOECs transplanted into the Cell Pouch™ was evaluated by performing a tail bleeding assay on NSG-HA mice four months after the cell transfer. Remarkably, we noticed a significantly improved presence of clotting as judged by a reduction in blood volume recovered in animals transplanted with 20×10^6 LV-VEC.hBDD-FVIII HA BOECs compared to non-transplanted mice (Figure 5A). Notably, this was not significantly different when compared to NSG mice, confirming that correction of the missing coagulation factor had been

achieved in the transplanted HA mice. Relatively long-term cell survival (4 months post-transplant) was confirmed by co-staining with anti-HLA-ABC and anti-vWF antibodies as well as by the formation of blood vessels within the transplanted area (Figure 5B, 5C and Table S2).

Overall, these data indicate that corrected HA BOECs are able to engraft and persist for prolonged periods of time within the tissue matrix supported by the Cell Pouch™ and secret enough FVIII to correct the hemophilia phenotype of the implanted NSG-HA mice.

Complex composition of BOEC clonal populations

Sonication Linker Mediated PCR was performed on 53 samples of genomic DNA extracted from LV-transduced BOECs derived from 3 healthy donors (D45, D2, and D3) and 3 HA patients (pHA1, pA, and pC), collected at different expansion passages or procedure time points. By grouping the samples according to the BOEC source (i.e., healthy donors or HA patients) and the type of vector used (i.e., LV-VEC.hBDD-FVIII or LV-VEC.GFP), we obtained 4 main groups: HA.FVIII; HA.GFP; Healthy.FVIII; Healthy.GFP. Overall, we retrieved 142,349 integration sites (IS) (HA.FVIII: 28,069; HA.GFP: 106,554; Healthy.FVIII: 5,864; Healthy.GFP: 1,862.) (Table S3). We compared the distribution of IS of the 4 groups along the whole human genome and with respect to gene transcription start site (TSS). The profile of LV integrations was similar for all the groups and confirmed the marked tendency of the LV to integrate within gene bodies, without bias for promoter regions (Figure 6A-B), in line with previously published results.[54–57] Following enrichment analysis of genomic position and gene annotations, none of the ontological gene classes showed cancer or tumor suppressor gene enrichment (Table S4).

Common Insertion Site (CIS) analysis showed few highly targeted genes in all datasets (e.g., *NPLOC4*, *PACS1*, and *MROH1*) (Table S5). The quantification of IS Abundance showed only a few clones with abundance > 10% in LV-VEC.hBDD-FVIII-transduced BOECs from pA and pHA1 (Figure 7). Only 2 IS were retrieved from D45 cells transduced at an MOI of 30, thus resulting in both to be 50% abundant. One clone with an IS in the GNL3 gene, which may interact with p53 and may be involved in tumorigenesis, and with abundance > 25% was also observed in pHA1 BOEC,

but only at a single time point (P11-UK). To address the clonality of transduced BOECs, we analyzed the diversity of the clonal population through Shannon diversity index. The highest Shannon diversity index, between 9 and 11, was observed in BOECs from pHA1 and pA, transduced with the VEC.GFP vector. All the other BOECs showed a Shannon diversity index between 4 and 8, which remained constant throughout the various cell passages. A lower diversity index directly correlated with a lower number of IS, in particular for the HA Beads and Cell Pouch™ samples at different time points (Figure 8A). To better understand if, especially in the Cell Pouch™ samples, the clonal diversity was reduced, we compared the H Index between samples grouped by type (Expansion, HA Beads, Cell Pouch™ and LV used (VEC-FVIII, VEC-GFP) (Figure 8B). IS analysis revealed a high level polyclonality of LV-transduced BOECs, with no significant difference between the FVIII- and GFP-transduced samples. The clonal composition heterogeneity of FVIII-transduced samples remained constant over time *in vitro* and *in vivo*. Finally, Cell Pouch™ samples had a significant lower H index when compared to BOECs in expansion.

DISCUSSION

Although the current therapy for HA involves the administration of plasma-derived or recombinant FVIII, there is to date no definitive cure for this inherited bleeding disorder. While several ongoing phase 1-3 clinical trials assessing the feasibility and safety of AAV-mediated hepatocyte-directed HA gene therapy have been able to achieve therapeutic FVIII plasma levels,[58–61] further experiments are in progress to assess the long-term stability of transgene expression. In this regard, the fact that AAV vectors do not actively integrate into the host cell genome and, thus, can be lost upon cell division during liver growth or liver disease questions their life-long maintenance besides limiting their potential use in pediatric patients.

A promising alternative approach is represented by a combination of cell and gene therapy, which would however require the identification of a suitable cell type able to effectively secrete FVIII while meeting all the necessary conditions for successful cell transplantation. In this regard, it is widely

acknowledged that the liver is the main organ producing FVIII, where LSECs appear to be the main source of FVIII[62–64] and can play a tolerogenic role. In addition, because of the important role of the interaction between FVIII and vWF in the stability and activity of FVIII, LSECs may represent the most suitable target for cell and gene therapy-based strategies aimed to correct the HA phenotype.[65] Unfortunately**,** LSEC are not easy to obtain and maintain *in vitro*, therefore, in this study we explored the feasibility of using gene-corrected autologous BOECs, more manageable and previously shown to be able to secrete FVIII *in vivo*.[66] Here we show that BOECs isolated from both healthy and HA donors can be efficiently cultured, transduced, expanded, and used to correct the bleeding phenotype of HA mice. In this regard, it is important to point out that the large number of corrected cells we obtained allowed us to reach more quickly FVIII therapeutic concentrations, thus reducing the risk of cell senescence.

The current protocols for BOEC isolation are based on the culture of mononuclear cells (MNCs) from peripheral or umbilical cord blood on collagen-coated cell culture vessels in endothelial specific medium.[67] The fact that MNCs can be isolated directly through density gradient centrifugation of blood makes these cells a safe cell source for hemophilic patients. Normally, BOECs colonies arise after 2-4 weeks of culture, and the colonies are very rare since their number in the normal peripheral blood is quite low.[67] However, here we show that, under GMP-compliant conditions and using a chemically defined medium, it is possible to isolate BOECs from both healthy donors and HA patients with high efficiency and rapidly grow them to the desired amount to prevent the risk of cellular senescence once transplanted in mice.

In addition to being more easily obtainable, BOECs are fully differentiated ECs with a mature endothelial phenotype. Indeed, these cells originate from bone marrow-derived progenitors circulating in the blood or residing in the endothelium, which can be differentiated into BOECs *in vitro*.[68] Thus, the observation that the expanded pools of BOECs from healthy donors or HA patients retained the expression of endothelial markers and were able to form vessels indicates that our GMP-compliant conditions did not alter the endothelial phenotype and function of these cells, as previously

316 shown.[68] Moreover, the healthy BOECs showed low FVIII expression, as demonstrated by IF
317 staining and FACS analysis, in agreement with the low FVIII secretion found in the cell supernatant.
318 This is similar to what has been shown by previous studies in which healthy BOECs isolated from
319 both canine and human donors displayed low FVIII expression.[42,51,52] Indeed, FVIII expression
320 heterogeneity among different endothelial subpopulations has been reported, with the sinusoidal
321 endothelial cells shown as the main FVIII-secreting cells.[69]Another important aspect of this study is
322 that we efficiently transduced BOECs with an LV carrying a functional BDD form of FVIII driven
323 by the endothelial-specific promoter VEC. The efficiency and tissue specificity of FVIII transcription
324 under the control of this promoter has been previously demonstrated in gene therapy approaches
325 showing the restriction of FVIII expression in the desired cell type[70] and in cell therapy by secretion
326 of FVIII after genetic correction in target cells.[32] Here, we show that LV-corrected HA BOECs
327 transplanted in association with Cytodex® 3 microcarrier beads into the peritoneum of NSG-HA mice
328 rescues the hemophilic phenotype of these animals for up to 18 weeks, achieving 9% FVIII activity.
329 Importantly, we reached therapeutic levels of secreted FVIII through LV-VEC.hBDD-FVIII HA
330 BOECs injection into a prevascularized Cell Pouch™ device transplanted into a preclinical murine
331 model of severe HA. Notably, the correction of the bleeding phenotype by using LV-VEC.hBDD-
332 FVIII HA BOECs injected into the peritoneum lasted up to 13 weeks and then slowly decreased.
333 After 18 weeks FVIII activity was almost absent probably due to the death of BOECs.
334 Despite the encouraging results presented in this proof-of-concept study in a pre-clinical setting, there
335 still remain several important issues that need to be addressed before our approach can be brought
336 into the clinic. For instance, it will be imperative to characterize the cells within the Cell Pouch™ in
337 terms of cell markers, longevity, and proliferation/senescence status. It will also be important to assess
338 if we can increase the expression levels of FVIII using different EC-specific promoters, and if that
339 would translate into augmented FVIII secretion and functionality *ex-vivo*.
340 Overall, our findings indicate that cell transfer into a medical device is a suitable solution for cell
341 therapy as it confers a more physiological and protected environment where cells can proliferate at

an excellent rate and escape from the immune response of the transplanted organism, all the while allowing nutrient exchange and therapeutic protein secretion. Congruently, the safety and efficacy of the Cell Pouch™ for the transplantation of mouse pancreatic islets has been previously shown to provide insulin independence in diabetic animals in preclinical studies of type 1 diabetes mellitus.[46,71] Furthermore, a phase I/II clinical trial is ongoing for the treatment of T1DM patients whose result may support the potential application of this device to other diseases for cell therapy approaches, such as HA.[72]

The Cell Pouch™ is a biocompatible, safe, implantable device that forms an internal vascularized tissue matrix supporting the transplanted cells. When we analyzed the Cell Pouch™ injected with LV-VEC.hBDD-FVIII HA BOECs after 4 weeks from the cell transfer, we observed the presence of a viable vascularized tissue matrix supporting the cells, with no evidence of fibrosis-associated consequences, including inflammation and necrosis, or hemophilia-related hemorrhage episodes. Moreover, the bleeding assay demonstrated that LV-VEC.FVIII HA BOECs transplanted into the vascularized subcutaneous Cell Pouch™ were able to correct the clotting function of HA mice. FVIII secretion and activity measurement would support the data on hemophilic correction and strengthen our observations. These tests are planned for future studies.

As previously shown in a canine model of HA, BOECs transduced with an LV carrying the canine FVIII and implanted subcutaneously allowed secretion of therapeutic levels of FVIII up to 15 weeks in Matrigel scaffolds and up to a year after omental implantation.[66] Moreover, BOECs were shown to form tubule network *in vitro* when plated on Matrigel[73] or on the surface of synthetic vascular scaffolds[74] and to promote neovascularization *in vivo* when transplanted into immunodeficient mice,[38] suggesting that they can be directly involved in vessel formation.

In this context, our data attest the feasibility of a method to correct autologous cells based on a combined cell and gene therapy approach together with the use of a scaffold (i.e., Cell Pouch™) able to guarantee long-term cell survival and, in case of need, a re-injection of new therapeutic cells. In addition to the phenotypical and functional characterization of the transduced HA BOECs, our results

368 demonstrate the pharmacodynamics proof-of-concept in non-clinical models, which is mandatory
369 before any GTMP can be used in human clinical trial.[44,45] Thus, our next step will be to evaluate the
370 safety and toxicity of the GTMP *in vivo* based on these results so as to ensure patient safety and
371 promote product translation. Examples of required non-clinical studies are the evaluation of the
372 potential tumorigenicity and biodistribution of the transduced BOECs with or without the medical
373 device.[75] Our molecular analysis of the integration sites in BOECs shows that no enrichment for
374 oncogenes or expansion of clones with IS in CIS or biases toward gene classes related to cancer genes
375 occurred. IS analysis suggests a high level of polyclonality of LV-transduced BOECs, with no
376 statistical difference between the FVIII- and GFP-transduced samples. The heterogeneity of the clonal
377 composition of the FVIII-transduced samples remained constant over time (between different cell
378 passages) also when cells were coupled to the micro carrier beads. Furthermore, Cell Pouch™
379 samples had a statistically significant lower H index when compared to BOECs in expansion.
380 The process of BOEC engraftment within the subcutaneous space is novel and complex and further
381 studies will provide additional insight into the interactions between the developing tissue and the
382 transplanted cells, elucidating the role played in the kinetics of blood vessel formation and FVIII
383 secretion within the surrounding tissue.
384 In this study, we could not evaluate the immune response to the secreted factor because we used
385 implanted cells in immunodeficient hemophilic mice. Thus, in future studies it will be interesting to
386 evaluate antibody formation after transplantation of transduced BOECs encapsulated in the Cell
387 Pouch™ into immunocompetent mice. Finally, while several gene therapy clinical trials for HA are
388 ongoing, to our knowledge this is the first therapeutic approach that combines the GMP production
389 of autologous human BOECs with the use of a safe *ex-vivo* approach based on an implantable
390 prevascularized device.
391 In conclusion, our findings suggest that long-term encapsulation and survival of LV-corrected BOECs
392 by means of an implantable device may prove effective in ameliorating the HA patients' QoL. The
393 therapeutic dose of FVIII released by these autologous genetically modified cells would in fact

394 prevent the need of frequent infusions of FVIII and significantly reduce the morbidity and the
395 frequency of the bleeding episodes in hemophiliacs.
396

Material and Methods

BOEC isolation form HA patients and healthy donors

Blood sampling from 4 adult severe HA patients, named pHA1, pA, pC, and pD, was performed at the hospital A.O.U Città della Salute e della Scienza, Turin, Italy. The blood was shipped at room temperature to Università del Piemonte Orientale (UPO), Novara, Italy. Blood sampling from adult severe HA patients was approved by the Ethics Committee "Comitato Etico Interaziendale A.O.U. Maggiore della Carità" (Protocol 810/CE, Study n. CE 125/17). Human BOECs were isolated as previously described,[67] with the introduction of an earlier cell passaging step seven days after initial isolation of the peripheral blood mononuclear cells to reduce expansion time and increase the final cell yield.[76] Isolated cells were cultured on CELLCOAT Collagen Type 1-coated tissue culture flasks (Greiner Bio-One) using MCDB 131 medium (Gibco®, Life Technologies) containing proprietary supplements. Primary cells from adult healthy donors (named D45, D2, D3) were isolated at Tissue Engineering and Regenerative Medicine, Würzburg, Germany, under informed consent according to ethical approval granted by the Institutional Ethics Committee of the University Hospital Würzburg (approval number 182/10). Cell viability and count were assessed using the Countess II FL Automated Cell Counter (Thermo Fisher Scientific).

Healthy and HA BOEC transduction

Healthy and HA BOECs were plated at a 10^4 cells/cm^2 density and after 6-8 h transduced with a lentiviral vector carrying the BDD form of FVIII under the control of the VE-cadherin promoter (LV-VEC.hBDD-FVIII) or with a lentiviral vector carrying the green fluorescent protein under the control of the same VE-cadherin promoter (LV-VEC.GFP), using a multiplicity of infection (MOI) of 20. After 14-16 h incubation, fresh medium was added to the cells and, 72 h later, half of the cells were harvested for subsequent analysis, while the other half was further cultured.

GMP-compliant (GMP-like) preclinical development of LV-VEC.hBDD-FVIII-transduced BOECs

BOECs were isolated and expanded using a GMP-compliant standardized approach between all partners, including a quality control strategy. The standardized expansion scheme defined within the project is based on the generation of Master Cell Banks (MCB) and a Working Cell Bank (WCB), which ensures not only a controllable defined expansion for each patient's BOECs but also an in-process quality control at defined crucial steps. After isolation and expansion, cells were transduced with LV lots produced with a GMP-compliant method (TFF, see Supplemental Material section). All freezing steps were performed using a cryopreservation solution based on compounds that are GMP-compliant free of toxic compounds (e.g., DMSO). The Cell Pouch™ was manufactured under GMP-compliant conditions. All steps were designed and conducted according to European GMP-regulations to ensure that the product would fully comply with the quality requirements of the European authorities. The main objectives were to provide sets of design and manufacturing protocols based on current European GMP regulations and to prepare an Investigational Medicinal Product Dossier (IMPD) for an Investigational Medicinal Product (IMP), composed of therapeutic cells and an implantable medical device (Cell Pouch™), a so-called combined Advanced Therapeutic Medicinal Product (combined ATMP).

Insertion site analysis

Integration sites (IS) were retrieved from genomic DNA of LV-transduced BOEC cells by Sonication Linker Mediated (SLiM)-PCR, an adaptation of a previously described method.[77,78] Genomic DNA (300 ng) was sheared using a Covaris E220 Ultrasonicator (Covaris Inc., Woburn, MA), generating fragments with a target size of 1000 bp. The fragmented DNA was split into 3 parts to generate technical replicates and, by using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA), subjected to end repair, 3' adenylation, and ligation to linker cassettes (Integrated DNA Technologies, Skokie, IL) containing a 8-nucleotide sequence barcode used for sample identification and a 12 random nucleotide sequence necessary for clonal abundance quantification. Ligation products were then subjected to 35 cycles of exponential PCR using primers

447 specific for the lentiviral vector LTR and the linker cassette. The amplification product was then re-
448 amplified with additional 10 PCR cycles using primers specific for the linker cassette and the LTR,
449 with the latter containing a second barcode in order to adopt a double barcode strategy for sample
450 identification. The final PCR products were quantified using a KAPA Library Quantification Kit
451 (Roche, Basil, Switzerland) and pooled in sequencing libraries with equimolar composition, avoiding
452 repeated barcode pairs. Primers incorporate the adapter sequences required for the Illumina paired
453 end sequencing technology (Illumina, San Diego, CA). Sequencing was performed on the Illumina
454 MiSeq and HiSeq. Sample processing and metadata were tracked within our laboratory information
455 management system.[79,80] Sequencing reads were processed by a dedicated bioinformatics pipeline
456 (VISPA2).[80] Briefly, paired sequence reads were filtered for raw reads quality, then cleaned by vector
457 genome, and the resulting cellular genomic sequence mapped on the human genome (version hg19),
458 and the nearest RefSeq gene assigned to each unambiguously mapped IS. Clonal abundance for each
459 IS was estimated using the R package sonicLength,[81] where the number of genomes with the same
460 integration site is calculated by counting the number of fragments with different sizes generated by
461 sonication belonging to each individual IS. Within each group, IS shared between different time
462 points of the same transduction were counted once. The relative abundance of each clone was then
463 calculated as the percentage of genomes with a specific integration site over the total genomes.
464 Common Insertion Sites (CIS) were identified through the Grubbs test for outliers.[82] Enrichment
465 analysis for ontological classes among the targeted genes by vector IS was performed by the Genomic
466 Regions Enrichment of Annotations Tool (G.R.E.A.T.).[83]

467 **Animal procedures**

468 Animal studies were approved by the Animal Care and Use Committee at UPO (Italian Health
469 Ministry Authorization n. 492/2016-PR, No. DBO64.5). NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (Jackson
470 stock No 005557) mice with hemophilic phenotype (NSG-HA) were previously generated and
471 maintained in our laboratory.[22] Eight-10 week old animals were used for cell transplantation studies.
472 Cell Pouch™ implantations were conducted under additional ethical guidelines and approval from

the Animal Care Committee at the University of British Columbia (Vancouver, British Columbia, Canada) in accordance with the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

BOEC transplantation

For cell transplantation with beads, $5x10^6$ FVIII-transduced healthy or HA BOECs were mixed with Cytodex®3 microcarrier beads (GE Healthcare Life Sciences) and intraperitoneally delivered in NSG-HA mice as previously described.[23] For Cell Pouch™ implantation, female NSG and NSG-HA animals were anesthetized and surgically implanted with a Cell Pouch™ in the subcutaneous space of the lower abdomen 4 weeks before cell transplantation, allowing incorporation with vascularized tissue and forming fully developed tissue chambers suitable for cell transplantation upon removal of a space holding plug. LV-VEC.hBDD-FVIII BOECs were cultured for 3 days post-thawing and finally transplanted into the Cell Pouch™. Mice received either a dose of viable BOECs ($2\text{-}20\times10^6$) or remained untreated. All animals received a prophylactic dose (2-4 IU) of recombinant human FVIII by tail vein injection prior to surgical procedures.

FVIII activity

aPTT assay was performed on plasma samples of transplanted mice to assess FVIII activity. Standard curves were generated by serial dilution of recombinant human BDD-FVIII (ReFacto) in hemophilic mouse plasma. Analyses were performed using a Coatron® M4 coagulometer (TECO Medical Instruments) and TEClot APTT-S kit reagents (TECO Medical Instruments).

Bleeding assay

A bleeding assay was performed on anesthetized mice. The distal portion of the tail was cut at a diameter of 2-2.5 mm. Tails were placed in a conical tube containing 14 ml of 37°C pre-warmed saline. Blood was collected for 10 min and, following centrifugation, resuspended in red blood lysis buffer (155 mM NH4Cl, 10 mM KHCO3, and 0.1 mM EDTA). The absorbance of the samples was measured at 575 nm. For cell transplantation experiments with Cell Pouch™, the tail bleeding assay

498 was performed by Sernova, as previously described,[84,85] at the end of the experimental period, 4
499 months post-transplantation. In brief, mice were anesthetized, and tail tips were placed in a guide,
500 ensuring the same diameter of 1-mm, and severed (~ a distal 10-mm segment) for each animal. The
501 tail was immediately immersed in pre-warmed saline at 37°C. Bleeding was carried out for a
502 maximum of 20 min, after which animals were euthanized as *per* approved animal use protocol
503 (AUP). Blood loss was evaluated by determining hemoglobin concentration by lysing collected red
504 blood cells (ACK Lysing Buffer, Gibco™), and the absorbance measured at 550 nm on a Synergy™
505 Mx (BioTeck) spectrophotometer. Results were analyzed by comparing the amount of blood loss
506 obtained from treated NSG-HA mice with control mice (untreated NSG-HA and NSG mice).

507 **Statistical analysis**

508 Data were expressed as means ± standard deviation (SD) or means ± standard error mean (SEM).
509 Statistical significance was analyzed using Student's t test with two-tailed distribution, assuming
510 equal standard deviation distribution, two-way analysis of variance (ANOVA) with Bonferroni post-
511 hoc test or Tukey's multiple comparison post hoc tests in GraphPad Prism 6 (GraphPad Software).
512 Statistical analyses involving IS were performed with the R software (r- project.org). Differences
513 were considered statistically significant when P values < 0.05.

514

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Authorship contributions

C.O., C.B., S.M., T.B., P.B., A.C., A.B.A., N.W., K.P. AC, FB, EU performed research and analyzed data. A.C., F.B. and E.M. performed integration analysis and analyzed data. A.B. and B.P. collected blood samples from hemophilic patients and performed analysis. K.P., D.M.M., and P.M.T. are/have been employees of Sernova Corp. which holds the patent US20190240375A1. A.F., J.B., A.S., D.M., P.T., M.Z. conceived the experiments generated funding, designed the research, and analyzed data. C.O., C.B., S.M. and A.F. wrote the manuscript that was revised by all authors.

Disclosure of Conflict of Interest

The authors declare that K.P., D.M.M., and P.M.T. are/have been employees of Sernova Corp.

Supplemental Information

Supplemental Information includes Supplemental experimental procedures, 2 figures, and 8 tables.

Keywords

Hemophilia A, Cell and Gene Therapy, Medical Device, BOEC, Lentiviral Vector, FVIII, Endothelial Cells.

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Figure legends

Figure 1. Healthy and HA BOEC isolation, LV transduction, and *in vitro* FVIII detection. (**A**) Light microscope pictures of cultured healthy and HA BOECs at passage 3. (**B**) Representative RT-PCR analysis for the expression of endothelial markers. HUVECs and fibroblasts were used as positive and negative control, respectively. (**C**) RT-PCR for endothelial markers specific for blood endothelial cells (BECs). iPSC-derived ECs and fibroblasts were used as positive and negative control, respectively. (**D**) Representative histograms of healthy non-transduced (black line) and LV-VEC.hBDD-FVIII transduced healthy BOECs (red line), showing endothelial marker expression and absence of hematopoietic markers. The filled-up histograms represent unstained BOECs. (**E**) Representative histograms of HA non-transduced (black line) and LV-VEC.hBDD-FVIII transduced HA BOECs (red line) showing endothelial marker expression and absence of hematopoietic markers. The filled-up histograms represent unstained BOECs. (**F**) Matrigel assay confirming tubule formation of transduced BOECs. (**G)** RT-PCR, using primers specific for the exogenous *F8* in non-transduced and LV-VEC.hBDD-FVIII BOECs. Unrelated transduced cells and fibroblast were used as positive and negative control respectively. (**H**) FVIII intracytoplasmic staining on non-transduced (black line) or transduced healthy BOECs (red line). The filled-up histogram represents unstained BOECs. (**I**) FVIII intracytoplasmic staining on non-transduced (Black line) or transduced HA BOECs (Red line). The filled-up histogram represents unstained BOECs. (**J**) FVIII detection by immunofluorescence: blue: DAPI, red: anti-FVIII. Data are expressed as mean ± SD and are representative of four independent experiments.

Figure 2. Intraperitoneal implantation of BOECs with Cytodex micro-carrier beads. (A) Kinetics of the percentage of FVIII activity measured by aPTT assay in the plasma of transplanted NSG-HA mice. BOECs used were transduced only with LV-VEC.GFP or with both LV-VEC.hBDD-

825 FVIII and LV-VEC.GFP. Data are expressed as mean ± SD and are representative of two independent
826 experiments using BOECs from two healthy donors (n = 7 mice), and four independent experiments
827 using HA BOECs from four patients (n = 23 mice). (**B**) Blood loss evaluation on NSG-HA mice
828 between week 7 and 10 (n = 4) after cell transplantation. (**C**) FVIII concentration in plasma of mice
829 transplanted with transduced or non-transduced BOECs at week 16. Data are expressed as mean ±
830 SD, ($P < 0.0001$ ***, $P < 0.001$ **) (**D**) Representative immunofluorescence on beads showing cells
831 co-expressing GFP and CD31.

832 **Figure 3. Large-scale expansion of HA patient derived BOECs.** (**A**) Light microscope pictures of
833 transduced HA BOECs pre- and post-expansion. (**B**) Cell size, cell density, culture time, and
834 population doubling level during pre- and post- large-scale expansion. (**C**) Endothelial marker
835 expression pre- and post- large-scale expansion expressed as stained cells *vs* cells with secondary
836 isotype controls. (**D**) Tubulogenic assay to assess the functionality of transduced HA BOECs after
837 pre- and post-large-scale expansion. (**E**) Kinetics of the percentage of FVIII activity measured by
838 aPTT assay in plasma of transplanted NSG-HA mice. Data are expressed as mean ± SD and are
839 representative of two independent experiments (n = 7).

840 **Figure 4. Pathological assessment after transplantation of LV-VEC.hBDD-FVIII HA BOECs**
841 **into the Cell Pouch™ device.** (**A**) Sernova Cell Pouches™ were removed at 4, 8, or 12 weeks and
842 stained by H&E and Masson's Trichrome for blinded histopathological analysis. Histology scores
843 and representative images at 12 weeks post- transplant with 10×10^6 LV-VEC.hBDD-FVIII BOECs
844 (animal groups n = 2-3). (B) Quantification of H&E and Masson's Trichrome for blinded
845 histopathological analysis.

846 **Figure 5. Bleeding phenotype and cell survival of LV-VEC.hBDD-FVIII HA BOECs after**
847 **implantation in the Cell Pouch™ device.** (**A**) Bleeding assay on mice transplanted with 10×10^6 or
848 20×10^6 HA and LV-VEC.hBDD-FVIII BOECs, or left untreated (n = 3-6, mean ± SEM, $^{**}P < 0.05$,
849 ns: not significant). NSG mice were used as control for bleeding assay. (**B**) The transplanted Cell
850 Pouch™ devices were removed from the recipients NSG-HA mice, and immunofluorescence was

851 performed to detect cell survival within the mouse tissue by human cell staining (HLA-ABC) and
852 blood vessel formation through staining with cross-reacting human/mouse von Willebrand Factor
853 (vWF) antibody. The images shown are representative of two transplant groups (10×10^6 n = 5; 20×10^6
854 n = 12). (**C**) Quantification of HLA-ABC and blood vessel formation from blinded histopathological
855 assessment.

856 **Figure 6. Genome wide distribution of lentiviral vector IS.** (**A**) The pink track represents the
857 density distribution of genes (RefSeq annotation, hg19 genome). The green tracks are the density
858 distributions of all the IS retrieved in the HA transduced with LV-VEC.GFP and Healthy transduced
859 with LV-VEC.GFP groups. The blue tracks are the density distributions of all the IS retrieved in the
860 LV-VEC.hBDD-FVIII HA BOECs and LV-VEC.hBDD-FVIII Healthy BOECs groups. (**B)**
861 Distribution of IS of the 4 groups along the whole human genome and with respect to gene
862 transcription start site (TSS).

863 **Figure 7. Box plot representation of clonal abundance.** For each sample, the abundance values for
864 each clone are represented as dots. Clones over 10% are presented as dots labeled with the closest
865 gene symbol (RefSeq hg19).

866 **Figure 8. Clonal diversity comparison**. (**A**) Shannon diversity index for each transduced cell
867 population according to cell passage and time point. (**B**) H Index comparison between different
868 groups.

Figure 1

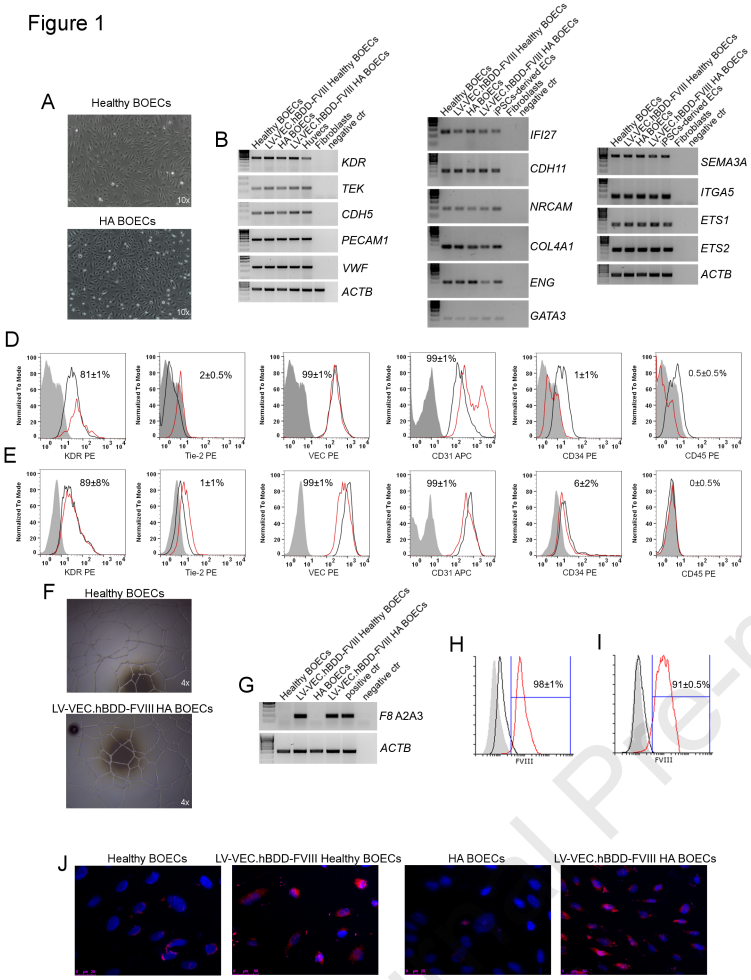
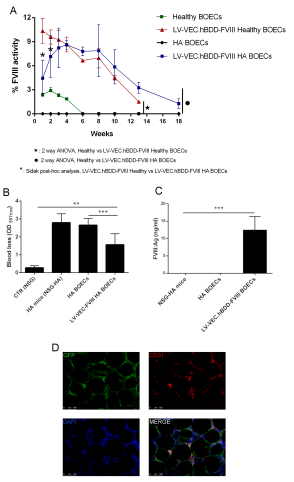


Figure 2



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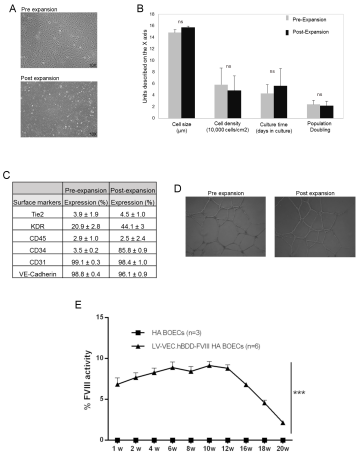
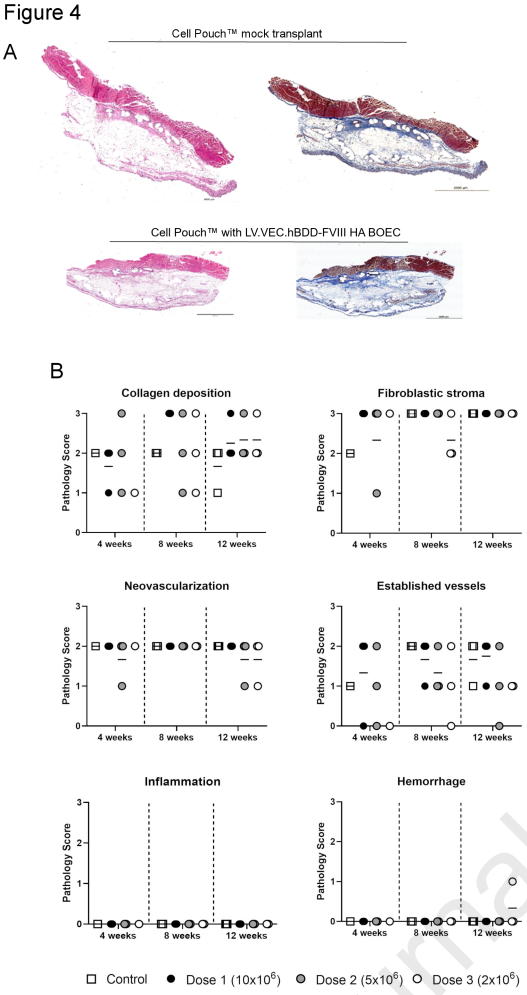


C

	Pre-expansion	Post-expansion
Surface markers	Expression (%)	Expression (%)
Tie2	3.9 ± 1.9	4.5 ± 1.9
KDR	20.8 ± 2.8	44.1 ± 3
CD45	2.9 ± 1.0	2.5 ± 2.4
CD34	3.5 ± 0.2	85.8 ± 0.9
CD31	99.1 ± 0.3	98.4 ± 1.0
VE-Cadherin	98.8 ± 0.4	96.1 ± 0.9

Figure 4



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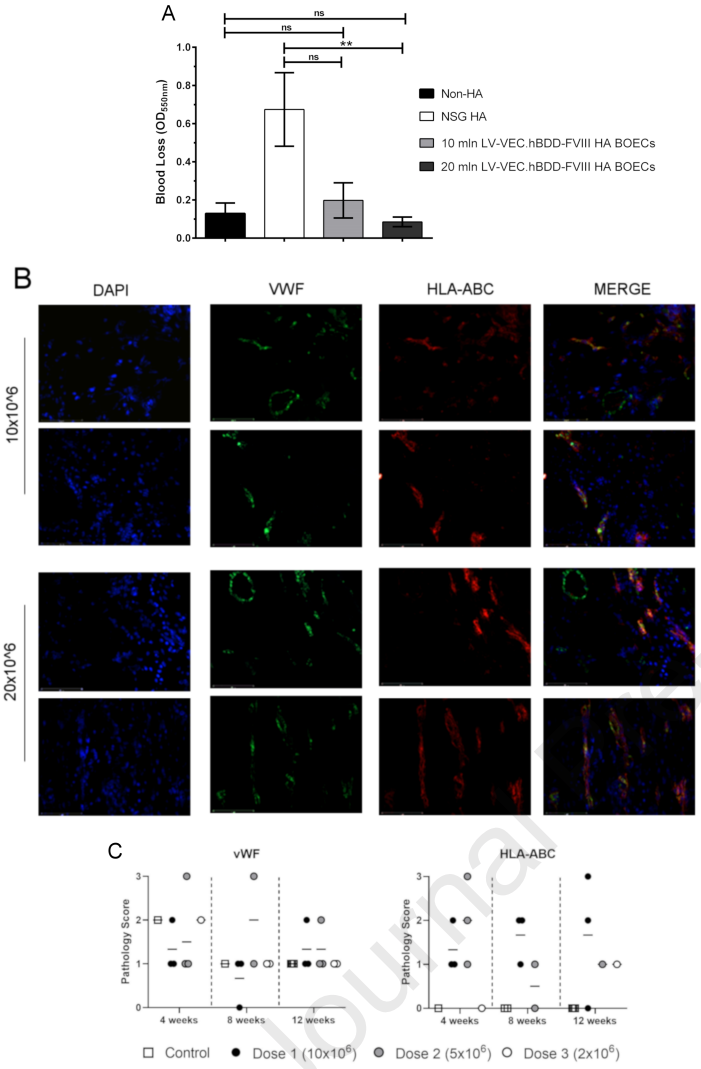
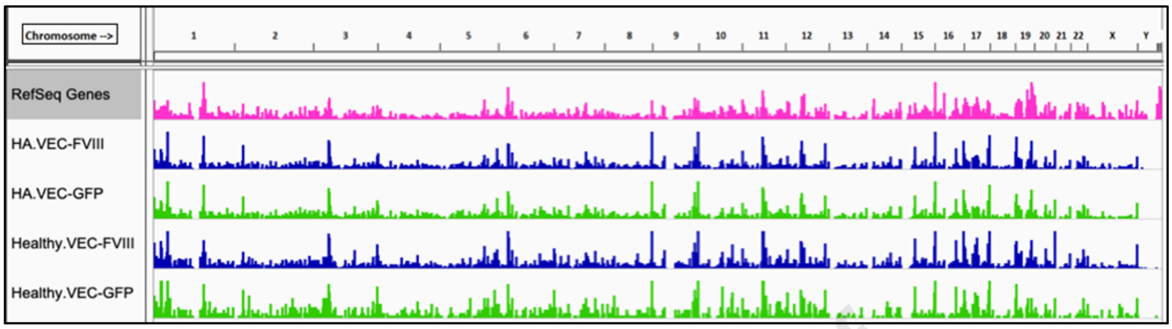


Figure 6

A



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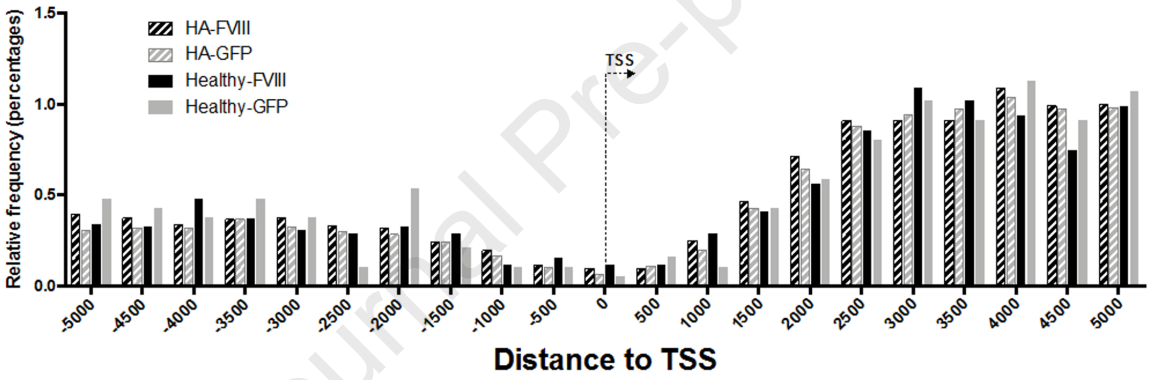


Figure 7

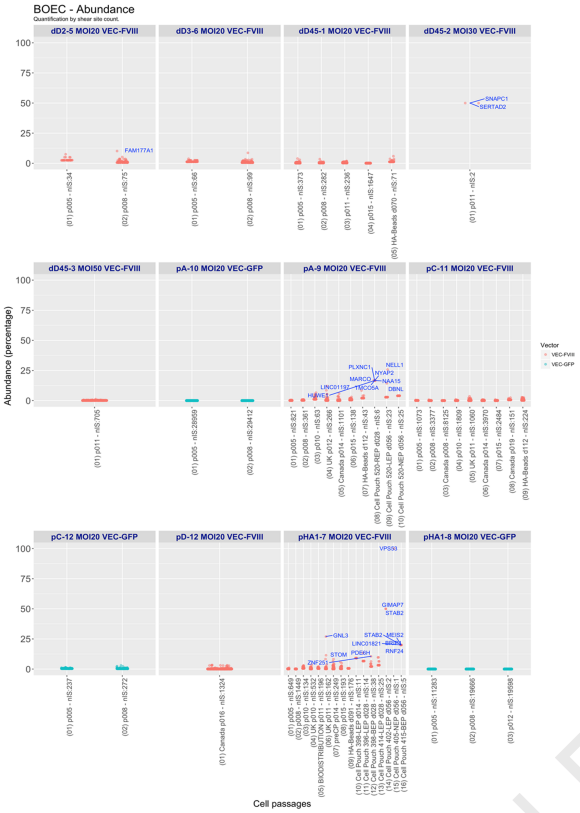
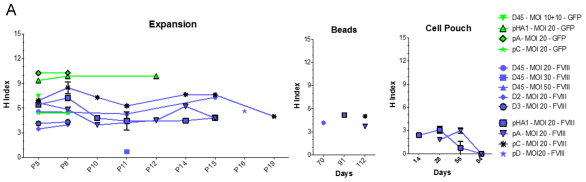


Figure 8

A



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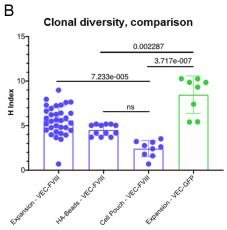

eTOC Synopsis

BOECs from HA patients and healthy donors were isolated, expanded, and LV-FVIII-transduced under GMP-like procedures. Amelioration of bleeding phenotype in a preclinical mouse model was reached by implantation of FVIII gene corrected hemophilic BOECs injected in a pre-vascularized Cell Pouch^{TM} reaching a long-term engraftment with a safe genomic integration profile.