Human Umbilical Cord Mesenchymal Stem Cell-Based Gene Therapy for Hemophilia B using scAAV-DJ/8-LP1-hFIXco Transduction

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Research Article

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Abstract

Background

Hemophilia B is an X-linked bleeding disorder caused by a mutation in the gene responsible for encoding coagulation factor IX (FIX). Gene therapy offers promising potential for curing this disease. However, the current method of relatively high dosage of virus injection carries inherent risks. The purpose of this study was to introduce a novel scAAV-DJ/8-LP1-hFIXco vector transduced human umbilical cord blood derived mesenchymal stem cells (HUCMSCs) as an alternative cell-based gene therapy to conventional gene therapy for Hemophilia B.

Methods

The human FIX activation system was employed for detection. The RNA and protein expression levels were evaluated using PCR and western blot techniques. In animal studies, clotting time was utilized as a parameter for bleeding assessment. The immunohistochemical analysis was used to assess the distribution of HUCMSCs. The safety of this cell-based gene therapy was evaluated using hematoxylin-eosin staining.

Results

Our findings demonstrate that transduction of HUCMSCs with the scAAV-DJ/8-LP1-hFIXco vector results in consistent and sustainable secretion of human FIX both in vitro and in vivo. The secretion level is comparable to that observed following intravenous injection with a high dose of the viral vector. After a 5-month observation period, no tumor-related tissues were observed in any of the mice studied.

Conclusions

we have successfully developed a novel cell-based gene therapy for the potential and safer treatment option for Hemophilia B.

Background

Hemophilia B is an X-linked bleeding disorder resulting from a mutation in the gene responsible for encoding coagulation factor IX (FIX). Gene therapy utilizing an adenovirus-associated virus (AAV) vector has been shown significant potential for long-term treatment of hemophilia B with just one injection of the vector. In November 2022, the U.S. Food and Drug Administration (FDA) approved the first gene therapy for the treatment of adult patients with hemophilia B, using AAV5 as the vector to deliver the FIX variant padua [1]. However, the efficient transduction of AAV is hindered by the requirement to convert its single-stranded (ss) genome into double-stranded (ds) forms that can be transcribed in target cells.

AAV-DJ is a chimera of adenovirus-associated virus types 2, 8, and 9, while AAV-DJ/8 is a mutant of AAV-DJ in heparin binding domain. AAV-DJ/8 differs from its closest natural relative (AAV-2) by 60 capsid

amino acids. In culture, recombinant AAV-DJ/8 vectors outperformed eight standard AAV serotypes, and in livers, they greatly surpassed AAV-2[2].

With low immunogenicity in humans[3,4], high transduction efficiency in liver cells[5,6], and high factor IX (FIX) production, we have developed a novel self-complementary adeno-associated virus vector DJ/8 (scAAV-DJ/8) that expresses human codon-optimized factor IX (scAAV-DJ/8-LP1-hFIXco). This vector shows promise as a potential candidate for gene therapy for hemophilia B, which mainly exists in target cells as episomes in the form of circular genomes or concatemers [7].

Numerous studies have consistently shown that recombinant adeno-associated virus (rAAV) exhibits a favorable safety profile with minimal toxicity in various cells and tissues[8–10]. Nevertheless, the intravenous injection of high doses of vector (approximately 10^{11}-10^{12} vector genomes (vg) per kilogram) necessary for a therapeutic response may pose potential risks of liver toxicity and immune reactions. Furthermore, in animal models, high vector doses are observed to be associated with non-specific biodistribution of the vector[11,12]. Moreover, the production of clinical-grade vectors at a dosage of 10^{14} vg per patient can be economically burdensome [13]. A recent case report revealed that a patient with advanced Duchenne's muscular dystrophy (DMD) treated with high-dose rAAV9 (1×10^{14} vg per kilogram) gene therapy died from an innate immune reaction caused acute respiratory distress syndrome (ARDS)[14]. Although AAV-mediated gene therapy for hemophilia B has been proved to be safe, its exorbitant cost[15] may have limited its application.

In this study, we introduced the human umbilical cord mesenchymal stem cells (HUCMSCs) for cell based gene therapy in hemophilia B. HUCMSCs are stromal cells that possess the capacity for self-renewal and demonstrate differentiation into multiple cell lineages[16,17]. Furthermore, they exhibit low immunogenicity and have immunomodulatory effects which make them suitable for targeted cell-based therapies[18]. The safety of the commercial source of HUCMSCs infusion has been demonstrated in patients, and its clinical application for the prevention and treatment of graft versus host disease (GVHD) or stimulation of hematopoiesis recovery after hematopoietic stem cell transplantation is already under way[19]. In this study, instead of directly injecting a high dose of vector into the tail vein of the mouse, we have discovered that HUCMSCs transduced by a novel ScAAV-DJ/8-LP1-hFIXco vector (DJ/8-hFIX) could be employed in a specific cell-based gene therapy approach for hemophilia B. This alternative method may help mitigate the potential risks associated with high-dose vector injection, enhance the therapeutic applications of HUCMSCs, and usher in a new era of cell-based gene therapy.

Materials and methods

Materials

The refined human FIX gene sequence was designed by our team. Then the gene sequence was sent to GeneChem Biotechnology Company (Shanghai, China) to integrate into ScAAV vector. The AAV-DJ/8

Helper Free Bicistronic expression system (IRES-GFP) kit and QuickTiterTM AAV Quantitation kit were purchased from Cell Biolabs (San Diego, USA). The human embryonic kidney 293T cell line (HEK293T), Chinese hamster ovary cell line (CHO), normal human liver cell line (HL7702), and human amnion cell line (FL) were acquired from ATCC and cryopreserved in the Hematology-Oncology Laboratory at the Division of Hematology-Oncology, Children's Hospital, Zhejiang University School of Medicine. Treatment grade HUCMSCs were purchased from Qilu Cell Therapy Engineering Technology Co. Ltd (Shandong, China). Mouse anti-human FIX monoclonal antibody was purchased from Abcam (#ab17196; Billerica, MA). Mouse anti-human CD105 antibody was purchased from proteintech (10862-1-AP; PTG, USA). Basal medium for HUCMSCs supplemented with 10% fetal bovine serum (FBS, Gibco), mesenchymal stem cell growth factor, and 1% penicillin/streptomycin (Invitrogen) was purchased from Viraltherapy Technologies (Wuhan, China). HyQ RPMI1640 (improved type) cell growth medium containing 15% FBS for HEK293T, CHO, HL7702, and FL cell lines was purchased from Hyclone (Logan, USA). The animal anesthetic tribromoethanol was purchased from Nanjing AIBI Bio-Technology. co. Ltd (Nanjing, China).

Methods

The construction of scAAV-LP1-hFIXco vector

The LP1 promoter was constructed with consecutive segments of the human apolipo-protein(HAPO) hepatic control region (HCR) and the human alpha-1-antitrypsin (hAAT) gene promoter including the 5' untranslated region, which was cloned upstream of a modified SV40 small t antigen intron (SV40). A codon-optimized human FIX (hFIXco) was designed by codons, which were most frequently found in highly expressed eukaryotic genes[20], synthesized by oligonucleotides, and subsequently assembled by ligation, PCR amplified, and sequenced prior to cloning into the ScAAV backbone to create scAAV-LP1-hFIXco vector (Fig. 1a). The synthesis, identification of scAAV-LP1-hFIXco was performed by GeneChem Biotechnology Company (Shanghai, China).

Construction and Amplification and the titer detection of DJ/8-hFIX

The HEK293T cell line was used for DJ/8-hFIX packaging and amplification. Three vectors including scAAV-LP1-hFIXco, pAAV-DJ/8 and pHelper for packaging was prepared at 1:1:1 ratio (F1b). The amplification process of DJ/8-hFIX was carried out in accordance with the instructions provided by the AAV-DJ/8 Helper Free Bicistronic Expression System (IRES-GFP) kit. The titer of DJ/8-hFIX was determined using the Quick Titer TM AAV Quantification kit as per the provided instructions.

Cell culture

The HUCMSCs, HEK293T, CHO, FL and HL7702 cells were cultured in a humidified incubator at 37°C with a 5% CO2 atmosphere. The cells were seeded in T75 flasks and used for experiment when they reached 80–95% of confluence.

The activity assay of hFIXco

The activity assay of hFIXco was determined using a CS-5100 automatic blood coagulation analyzer (Sysmex America Inc., Mundelein, USA).

The transduction of host cells by DJ/8-hFIX vector

HUCMSCs, CHO, HL-7702, and FL cells were co-incubated with DJ/8-hFIX at a ratio of 1:1000. Cell supernatants were collected at 24, 48, and 72 hours, and hFIXco activity was assessed using an automatic coagulation analyzer. The DJ/8-hFIX transduced HL-7702 cells (tr-HL7702) served as the positive control, while the DJ/8-hFIX transduced CHO (tr-CHO) and FL cell lines(tr-FL) were employed as negative controls.

RNA assay

Total mRNA was extracted using the Trizol method. Amplification of the hFIXco gene was performed using RT-PCR in the transduced cells. The forward and reverse primers for hFIXco were 5'-TACAACTCTGCAAGCTGGA-3' and 5'-GTTCTTGCTCAAAGCCAA-3', respectively. The size of the product was 243bp. GAPDH served as the housekeeping gene. The forward and reverse primers for GAPDH amplification were 5'-TTCACCACCATGGAGAAGGC-3' and 5'-GGCATGGACTGTGGTCATGA-3', respectively. The size of the product was 192bp.

Western blot analysis

Pre-treated with the 0.2mL M-PER protein extraction buffer, the DJ/8-hFIX transduced HUCMSCs (tr-HUCMSCs, 10^7) and its supernatants (from 24 hours to 5 months) were mixed with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. the DJ/8-hFIX untransduced HUCMSCs (untr-HUCMSCs) and its supernatant served as negative control. A positive control was established using pure human FIX protein (8ng/lane). Subsequently, the samples underwent resolution on an SDS-Tris-glycine acrylamide gel and were transferred onto a nitrocellulose membrane. Immunoblotting was conducted using the anti-hFIX monoclonal antibody (mouse anti-human), followed by incubation with the Goat anti-mouse horseradish peroxidase (HRP)-conjugated antibody and SuperSignal chemiluminescence substrate, respectively. The bands were visualized by exposing the x-ray film.

Animal procedures

NOD-SCID gamma mice (NSG), (NOD.Cg-Prkd^{cscid} IL2rgtm^{1Wjl}/SzJ) mice and *F9*-Knock out (F9-KO) mice(strain name: C57BL/6JSmoc-*F9*^{em1Smoc}) were obtained from the Shanghai Model Organisms Center, Inc (Shanghai, China) and kept in specific pathogen-free conditions. All experimental procedures involving animals followed institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC), Zhejiang Center of Laboratory Animals(ZJCLA). The mice used in the study were all male, 4 weeks old, and weighed approximately 20 gram(g). For the experimental groups, NSG mice and *F9*-Knock out mice were injected with tr-HUCMSCs (1×10^3/g) via tail vein. Another group of mice received untr-HUCMSCs (1×10^3/g) and 0.9% Normal Saline (0.9% NS) as a negative control and blank, respectively. The DJ/8-hFIX (1×10^{11}vg/kg) tail vein injection group served as the positive control. Each

NSG and F9-KO group consisted of four animals. Blood samples were collected at designated time points post-injection from the retroorbital venous plexus of NSG mice and the tail veins of F9-KO mice. Citrated plasma was centrifuged at 5000 g for 5 minutes, and the supernatant was stored at -80℃ for further assays.

Human FIX activity assessment in F9-KO mice

Tribromoethanol was used to anesthetize the F9-KO mice.To obtain blood spots, we transected the tail of each experimental mouse at a length of 1 cm using a scalpel. Subsequently, blood spots were imaged using a SONY 5600W camera, captured 30 seconds post-incision. At the end of the 30-second period, we gently touched the wound site on the tail with filter paper at regular intervals until the blood spots disappeared. The clotting time was then recorded.

Immunohistochemical analysis and hematoxylin-eosin staining

After the 7-day and 5-month post-transplantation periods, tissues including lung, liver, brain, bone marrow, and spleen were collected from various groups following carbon dioxide-induced euthanasia. The samples were fixed in 10% formalin and then embedded in paraffin wax. The paraffin sections were then deparaffinized, followed by antigen retrieval via microwave heating (at 500W, 5min x 2) in a pH 6.0 citrate buffer. The endogenous enzymes in the sections were blocked using 3% H_2O_2 for 20 minutes. Then, the sections were incubated overnight at 4°C with the primary antibody (anti-CD105 antibody, the specific membrane antigen of HUCMSCs), followed by HRP-conjugated goat anti-rabbit immunoglobulin G and then immersed in Diaminobenzidine chromogen substrate for 10 minutes. After being washed with distilled water and stained with Mayer hematoxylin, the sections were dehydrated and mounted with a coverslip. For the negative control, PBS (phosphate buffer saline) was substituted for the primary antibody. Tissues from 0.9% NS treated NSG mice incubated with anti-CD105 antibody were also employed as a negative control. Immunohistochemical analysis, Hematoxylin-eosin staining was conducted by Wuhan Pinuofei Biological Technology Co., Ltd. Software used for image processing is 3DHISTECH's Slide Converter. The 40x magnification of immunohistochemical photos was taken and used in this article.

Statistical Analysis

The statistical analysis was conducted using SPSS-13.0 software from Chicago, USA. The numerical data was presented as mean ± standard deviation. The paired Student's t-test was utilized to assess significant differences between various treatments. A p-value below 0.05 was considered to be statistically significant.

Results

Using the AAV-DJ/8 Helper Free Bicistronic expression system (IRES-GFP) kit, DJ/8-hIX was effectively constructed and amplified in HEK293T cells with a vector ratio of 1:1:1 (scAAV-LP1-hFIXco:pAAV-DJ/8:pHelper). The high titer of DJ/8-hFIX was achieved using the PEG concentration method. The titration of the DJ/8-hFIX was performed according to the instructions of the QuickTiterTM AAV Quantitation Kit.

HUCMSCs, CHO, HL-7702, and FL cells were co-incubated with DJ/8-hFIX at a ratio of 1:1000. We also tested ratios of 1:10000 and 1:100000, but the activity assay of hFIXco secreted by HUCMSCs and HL7702 remained almost the same at all three ratios. The optimal mineral ratio was found to be 1:1000. The DJ/8-hFIX demonstrated efficient transduction of HUCMSCs and HL7702. However, low transduction efficiency was observed in CHO and FL cell lines. After 24 hours of transduction, the activity assay revealed that the hFIXco secreted by the tr-HUCMSCs was 113% ±4.92% compared to that of HL7702 (95.56%±1.29%). In contrast, the tr-CHO and tr-FL only exhibited a detection rate of active hFIXco at 2.52 ± 0.08% and 3.66 ± 0.12%, respectively (Fig. 2a).

To assess the authentic biological function of hFIXco secreted by tr-HUCMSCs, a serum sample was obtained from a hemophilia B patient, displaying a FIX activity of 1.7% and an APTT of 62.4 seconds (within the normal range of 23–38 seconds). Employing the supernatant of tr-HL7702 as the positive control, the tr-HUCMSCs supernatant was combined with the hemophilia B patient's serum at varying ratios: 1:8, 1:4, 1:2, 1:1, and 2:1. Consequently, the APTT was reduced to 50.6, 48.5, 48.4, 46.2, and 35.1 seconds, whereas the FIX activity increased to 40.4%, 44.3%, 47.2%, 49.3%, and 62% respectively. When compared to tr-HL7702, which exhibited APTT values of 51.7, 47.9, 45.9, 43.7, and 34.5 seconds and FIX activities of 41.6%, 43.7%, 45.4%, 53.5%, and 64% respectively, the hFIXco secreted by tr-HUCMSCs displayed coagulation capabilities that were equivalent to those of HL7702. Both were capable of elevating the patient's FIX activity to approximately 40% (as shown in Fig. 2b).

We used PCR to continuously monitor the RNA levels of hFIXco from the initial time point (24 hours) until the final time point (five months). The RNA expression levels in the tr-HUCMSCs were similar to those observed in the HL7702 cell line (Fig. 3a). The hFIXco RNA expression band displayed a notable difference compared to the non-transduced samples.

HFIXco protein in the tr-HUCMSCs and their supernatants was evaluated using western blot analysis. We observed that hFIXco protein in the supernatant secreted by the tr-HUCMSCs after 5 months of continuous culture was similar to that observed at 24 hours post-transplantation. (Fig. 3b).

hFIXco activity was detected in the supernatants of tr-HUCMSCs from the first day post-transplantation to 5 months of cultivation, with levels ranging from approximately 113%±4.92% to around 105%±1.13%, which was consistent with that of tr-HL7702, When compared to tr-CHO, a significant difference was observed (Fig. 4a).

In our study, we assessed the secretion of hFIX protein from tr-HUCMSCs in NSG mice over the course of 7 days to 5 months. The activity of hFIX protein remained stable and gradually decreased in both the tr-

HUCMSCs and DJ/8-hFIX injection groups. In the tr-HUCMSCs group, hFIXco activity decreased from 97.1 ± 2.3% at day 7 to 48.8 ± 4.5% at 5 months. Similarly, in the DJ/8-hFIX injection group, hFIXco activity decreased from 95.2 ± 2.2% to 40.8 ± 4.3%. However, almost no hFIXco activity was observed in the groups of mice injected with untr-HUCMSCs or 0.9% NS. These findings suggest that the hFIXco activities derived from tr-HUCMSCs exhibit a pattern of stability and gradual decline similar to that observed in the DJ/8-hFIX injection group (See Fig. 4b).

In the F9-KO study, the activity of hFIXco in sera was measured 60 days after injection using an activity assay. The tr-HUCMSCs group displayed an average hFIXco level of 67.4 ± 1.3%. This result was significantly higher than that of the untr-HUCMSCs group (0.3%±0.1%, $P < 0.05$) and the blank group (0.2%±0.1%, $P < 0.05$). Nevertheless, there was no significant difference in comparison to the DJ/8-hFIX injection group, which exhibited an average FIX level of 72.6%±1.7%.

To assess whether the hFIXco protein produced by exogenous transgenes has a physiological activity that improves clotting, the tails of F9-KO mice were surgically amputated at a length of 1 cm to measure their clotting times. At 90 days post-injection, the clotting times of mice in the DJ/8-hFIX injection group and the tr-HUCMSCs group were similar to those of wild-type mice (less than 240 seconds), and significantly shorter than those of mice in the untr-HUCMSCs group and the group injected with 0.9% NS. Mice from both the untr-HUCMSCs group and the group injected with 0.9% NS died due to severe bleeding. Mice that were directly injected with DJ/8-hFIX or tr-HUCMSCs survived despite experiencing minor bleeding for approximately 200 seconds. The results suggest that the injection of pure DJ/8-hFIX (1×10^{11} vg/kg) or transplantation of tr-HUCMSCs (1×10^{3} cells/g) can exert therapeutic effects as observed by the shortened bleeding time (Fig. 5).

To address concerns about long-term tumorigenicity, we conducted an experiment with NSG mice. Tissues from the brain, lungs, liver, and spleen of NSG mice that survived for 5 months post-transplantation were collected. Furthermore, animal survival was maintained even after 7 months post-transplantation. Hematoxylin-eosin staining of tissues revealed no significant differences among all groups. No tumor-related tissue was detected in either group after 5 months of experimentation (Fig. 6).

We analyzed the distribution of tr-HUCMSCs or untr-HUCMSCs in NSG mice using immunohistochemistry. We used a mouse anti-human CD105 antibody to identify human CD105, a specific marker expressed on the membrane of HUCMSCs, at both 2 weeks and 5 months post transplantation. At 2 weeks post transplantation, both tr-HUCMSCs and untr-HUCMSCs were observed in the spleen, liver, and bone marrow, with a particularly high concentration in the bone marrow compared to the control (Fig. 7). At 5 months post transplantation, weakly positive cells were detected in the spleen, liver, and bone marrow of both tr-HUCMSCs and untr-HUCMSCs groups compared to the control (Fig. 8).

Discussion

AAV-mediated Gene therapy offers great potential for curing Hemophilia B. Recently, the FDA approved the use of AAV5 as the vector to deliver the FIX variant padua for the treatment of adult Hemophilia B. Targets the liver cells, which are primarily responsible for producing FIX, the traditional gene therapy has its advantages. However, the relatively high dosage of pure virus injection and exorbitant cost may limit its widespread application in real-world patients.

In this study, we developed a novel AAV vector expressing hFIXco, designated DJ/8-hFIX, which has potential applications in the treatment of hemophilia B. Our results demonstrate that tr-HL7702, expressing DJ/8-hFIX exhibits robust hFIXco secretion in vitro. The design of LP1, which includes a liver-specific promoter and locus control region (LCR), plays a crucial role in achieving sustained and efficient gene expression in liver cells. This is achieved through targeted deletions based on an analysis of liver transcription factor binding sites. We employed recombinant AAV-DJ vectors with a heparin binding domain (HBD) mutation (AAV-DJ/8) and observed superior in vitro transduction efficacies compared to other wild-type serotypes[22].

While the regular gene therapy utilizing viral vectors to deliver genes for hemophilia B has made significant strides in treating this genetic disorder, it still raises concerns. A substantial injection of the virus may lead to a inconsistent distribution of the virus across different cell types and cause harm to other vital organs, including the heart and brain. Although AAV vectors have been demonstrated to be harmless in humans, additional research is required to evaluate the safety of the virus as it transits to different cells and ultimately enters liver cells. The ultimate determinant of the success of traditional gene therapy may be the dose-dependent liver function toxicity. The higher the vector dose, the higher the success rate of gene therapy; however, it also increases the likelihood of liver function toxicity.

MSCs are fibroblastic and plastic-adherent cells that possess the ability to differentiate into various mesenchymal lineages, both in vitro [23, 24]and in vivo[25,26]. Currently, the commercial source of HUCMSCs infusion has been utilized in clinical practice, particularly in the prevention and treatment of GVHD, as well as the stimulation of hematopoiesis recovery following hematopoietic stem cell transplantation. It has been observed that HUCMSC infusion results in slow recovery of hematopoiesis with minimal side-effects[19]. Sung Jin Kim et al. observed that scAAV2 and scAAV5 were effective in transferring the GFP gene to human bone marrow-derived MSCs and umbilical cord blood-derived MSCs[27]. AAV-DJ/8 demonstrates efficient transduction across various cell types derived from diverse species and tissues, including primary human hepatocytes, melanoma cells, and embryonic stem cells[22]. Given the immunogenic properties and self-renewal potential of HUCMSCs as one of the stem cells, we propose that HUCMSC could serve as an excellent host cell for delivering FIX. To date, there are no known reports that have demonstrated the use of HUCMSCs as host cells in gene therapy applications.

In this study, we introduced the utilization of HUCMSCs as host cells, which were transduced with DJ/8-hFIX, as a groundbreaking substitute for high-dose vector injection. We observed successful transduction of DJ/8-hFIX into HUCMSCs. The activity assays revealed that the hFIXco proteins secreted by tr-HUCMSCs exhibited comparable stability and continuity to those secreted by tr-HL7702. Similar results

were observed in both NSG and F9-KO mice, indicating consistent performance across different animal models. Our experimental results robustly demonstrate the efficacy of this approach both in vitro and in vivo. Although this result is still preliminary, it holds significant promise for potential clinical applications due to its preliminary yet promising nature. The ultimate utility of this approach necessitates further development through rigorous and extensive pre-clinical studies prior to clinical trial verification.

When adding supernatant from tr-HUCMSCs at various ratios to Hemophilia B patients' sera, there is a prompt reduction in APTT by approximately 10 seconds and a surge in hFIX activity to over 40%. Compared to the tr-HL7702 group, the supernatant from tr-HUCMSCs showed equivalent therapeutic efficacy in Hemophilia B patients' sera. this finding suggests that the activation of hFIXco secretion in tr-HUCMSCs is on par with that of HL7702. Although the observed increase in hFIX activity was relatively not very high and potentially was attributed to the dilution of the cell supernatant, the elevation in FIX activity is remarkable. Additionally, during this study, serum from a single Hemophilia B patient was obtained, and the results of coagulation time correction were promising. Despite the limited number of patients' sera used, preliminary findings suggest that this study demonstrates, at least in part, that both tr-HUCMSCs and tr-HL7702 have the capacity to secrete hFIXco with coagulation potential.

In the animal study, we used a dosage of 1×10^3 HUCMSCs cells /g of mouse tail injection, which was consistent with the safety infusion dose for humans. This approach allowed us to investigate the relationship between dosage and side-effects. By co-incubating HUCMSCs at a ratio of 1:1000, the total DJ/8-hFIX dose for cell-based gene therapy amounted to 10^9 vg /kg. The total vector dosage used in this cell-based gene therapy was significantly lower than the traditional vector dosage, which ranges from approximately 10^{11} to 10^{12} vg /kg. Although this cell-based gene therapy has not yet been applied in humans, the significantly smaller total vector dosage used in this approach appears to offer greater safety compared to traditional gene therapy. Through transducing the vector into HUCMSCs in vitro without exposing them to other human tissue cells, we aim to avoid transducing other tissue cells in the body, thereby ensuring safety. HUCMSCs have been demonstrated to have minimal side effects in clinical trials. Our approach has the potential to avoid potential liver, brain, and heart toxicity that can be induced by high-dose vector injection.

Based on the previously reported normal AAV vector baseline used in gene therapy[28], we arbitrarily selected a dosage of 1×10^{11} vg/kg of pure LP1-hFIX for intravenous injection into mice tail veins as positive control. Fortunately, all *F9-KO* mice that received this dosage survived, and their coagulation activity was observed to improved. When compared to wild-type mice, minimal significance was observed between the F9-KO mice and wild-type mice. For future studies, we plan to increase the LP1-hFIX dosage for pure gene therapy and prepare for clinical trials.

A notable disparity was observed in the activity of hFIX detected in NSG mice that received injections of DJ/8-hFIX or 0.9% NS. This finding suggests that the detection of hFIX activity is specific to human FIX and does not cross-react with mouse FIX. The results of human FIX activity in the sera of F9-KO mice 60

days post-injection were consistent with the findings obtained from NSG mice. To date, we are unaware of any published studies addressing this particular issue.

The study involving F9-KO mice found that, following a 90-day injection period, the clotting times of both the DJ/8-hFIX group and the group receiving tr-HUCMSCs were similar to those of the wild-type mice. Furthermore, these clotting times were significantly shorter than those of mice treated with untr-HUCMSCs or 0.9% NS injection group. This suggests that the transgene was effective in both direct injection and tr-HUCMSCs in F9-KO mice. As a result, hFIXco secretion in tr-HUCMSCs was similar to that of direct injection. The mice treated with untr-HUCMSCs and those in the control group experienced severe bleeding and ultimately succumbed. In contrast, mice that received direct injections of DJ/8-hIX or tr-HUCMSCs were able to survive minor bleeding episodes lasting approximately 200 seconds, similar to wild-type mice. This suggests that the transgenes exert their therapeutic effects by minimizing bleeding episodes.

Human MSCs were xenotransplanted into fetal sheep and integrated into different tissues such as bone marrow, spleen, and liver[25]. In our experiments, after a 2-week period of transplantation, HUCMSCs were detected in bone marrow, spleen and liver, especially in bone marrow, which was consistent with the literature reports[25]. This suggests that HUCMSCs have retain their functional properties regardless of whether they have been transduced or not. Additionally, the tr-HUCMSCs may establish themselves in the bone marrow microenvironment and exert their secretory function of hFIXco. After five months of transplantation, only weakly CD105-positive cells were detected in the tissues using immunohistochemical analysis. Hematoxylin-eosin staining showed that the tissue characteristics were similar to those of the control group. This indicates that the distribution of HUCMSCs or their transduced forms does not have any significant adverse effects such as tumorigenic activity on the tissue. The observed phenomenon might be explained by the multilinear differentiation properties of HUCMSCs, which enable them to differentiate into cells already present in the surrounding tissue.

In this study, we observed successful transduction of HUCMSCs by the novel DJ/8-hFIX. Additionally, stable and consistent secretion of hFIXco was observed in the tr-HUCMSCs. The biological activity of the secreted hFIXco was evaluated both in vivo and in vitro. Interestingly, our experiment did not reveal any significant differences when comparing direct injection of the DJ/8-hFIX. This cell-based gene therapy has been shown to be safe in animal models, and the next steps involve its translation to clinical trials. Although the disease-free survival at five months post-injection may suggest the safety of HUCMSCs based DJ/8-hFIX gene therapy, and the hematoxylin-eosin stain reveals normal tissue at this stage, it remains unclear whether the DJ/8-hFIX gene has integrated into the genomic DNA of host cells. Further investigation is needed to determine the presence of anti-DJ/8-hIX antibodies in patients during clinical trials. The precise mechanism by which HUCMSCs internalize the DJ/8-hFIX vector and secrete active hFIXco remains elusive. More comprehensive research is required to address this issue.

Conclusion: our study introduces a groundbreaking approach for gene therapy in hemophilia B. The use of HUCMSCs as a cell-based gene therapeutic intervention offers a safer alternative to traditional high-

dose viral injection therapy. This potential represents a significant advancement in the therapeutic applications of HUCMSCs, marking the beginning of a new era in cell-based gene therapy.

Abbreviations

MSCs	mesenchymal stem cells
HUCMSCs	human umbilical cord blood derived mesenchymal stem cells
FDA	the U.S. Food and Drug Administration
FIX	coagulation factor IX
hFIXco	codon-optimized human FIX
AAV	adenovirus-associated virus
scAAV-DJ/8	self-complementary adeno-associated virus vector DJ/8
DJ/8-hFIX	ScAAV-DJ/8-LP1-hFIXco vector
HEK293T	The human embryonic kidney 293T cell line
CHO	Chinese hamster ovary cell line
HL7702	normal human liver cell line
FL	human amnion cell line
ARDS	acute respiratory distress syndrome
DMD	Duchenne's muscular dystrophy
GVHD	graft versus host disease
HAPO-HCR	the human apolipo-protein hepatic control region
hAAT	the human alpha-1-antitrypsin
HAPO-HCR-hAAT	Consecutive segments of HAPO HCR and hAAT
SV40	modified SV40 small t antigen intron
vg	vector genomes
Vg/Kg	vector genomes per Kilogram
APTT	Activated Partial Thromboplastin Time
HBD	heparin binding domain
tr-HUCMSCs	the DJ/8-hFIX transduced HUCMSCs
untr-HUCMSCs	the DJ/8-hFIX untransduced HUCMSCs
0.9% NS	0.9% Normal Saline
tr-HL7702	The DJ/8-hFIX transduced HL-7702 cells
F9-KO	*F9*-Knock out mice
tr-CHO	the DJ/8-hFIX transduced CHO

tr-FL	the DJ/8-hFIX transduced FL cell lines
HRP	horseradish peroxidase
NSG	NOD-SCID gamma mice

Declarations

Ethics approval and consent to participate:

The study was conducted in accordance with The animal use protocol approved by the Institutional Animal Care and Use Committee (IACUC), Zhejiang Center of Laboratory Animals(ZJCLA). ("Mesenchymal Stem Cell-Based Gene Therapy for Hemophilia B using scAAV-DJ/8-LP1-hFIXco Transduction" Approval No. ZJCLA-IACUC-20020133, approved September 15th, 2020)

Consent for publication: Not applicable

Availability of data and materials: The data used in this article are available from the corresponding author upon appropriate request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions

Z.B.B and Y.M.T formulated the project ideas and designed the experiment. Z.B.B conducted the cell and animal experiments, collected and analyzed data, and drafted and revised the manuscript. Y.M.T, as the corresponding author, had overall responsibility for the project, including revising and approving the final version of the manuscript. J.T.L performed the determination of human FIX activity in both in vitro experiments and mouse models. W.Q.X and L.Y.Z provided project-related suggestions and assistance in conducting some of the experiments.

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Figures

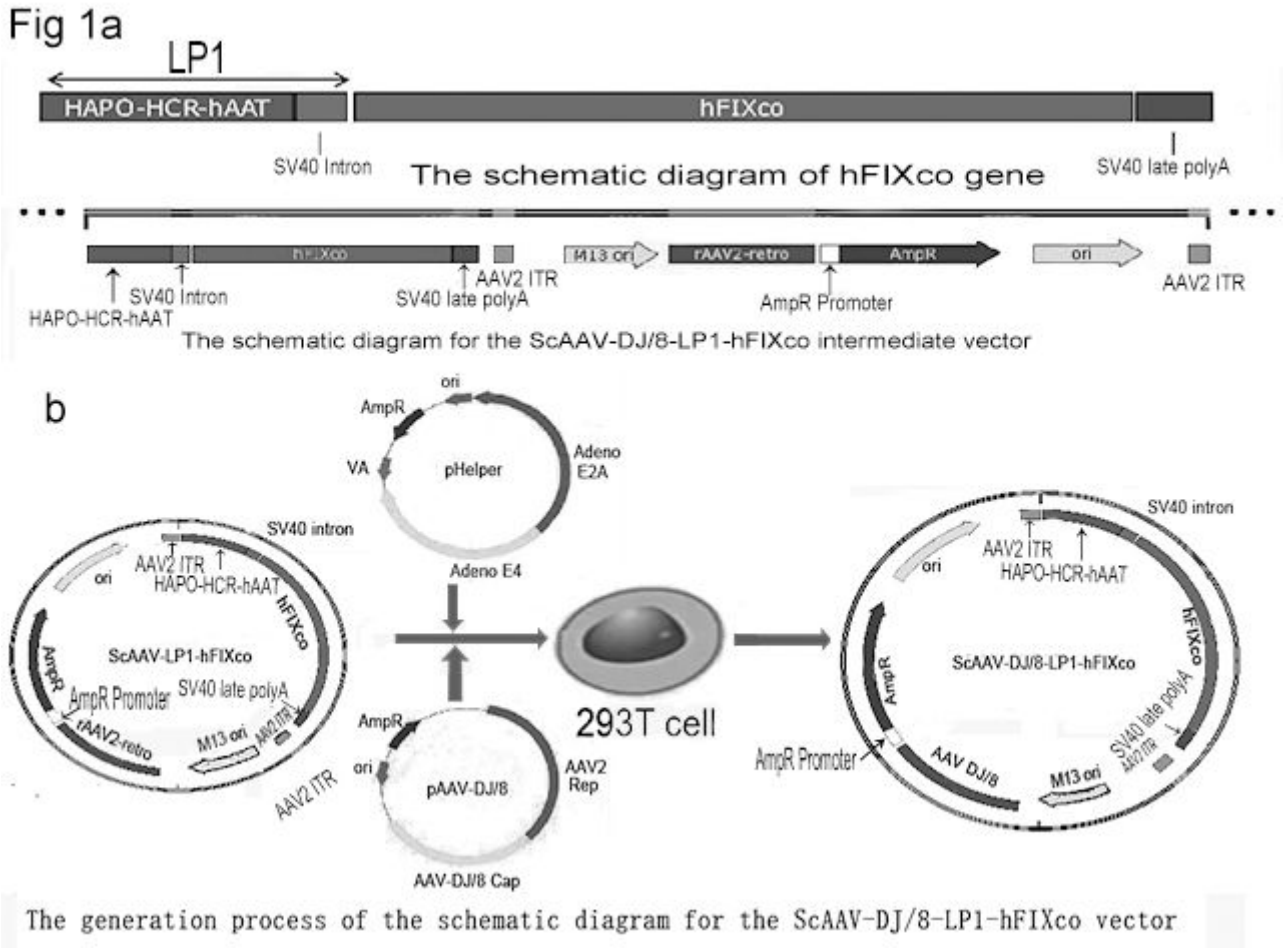

Figure 1

Gene structure of the designed hFIXco and schematic diagram illustrating the construction of DJ/8-hFIX vector. (a) Gene structure map of hFIXco carrying HAPO-HCR-hAAT promoter; hFIXco carrying HAPO-HCR-hAAT promoter is packaged into ScAAV to generate SCAAV-LP1-hFIXco. (b) The DJ/8-hFIX vector was successfully generated using SCAAV-LP1-hFIXco, pAAV-DJ/8, and pHelper in 293T cells.

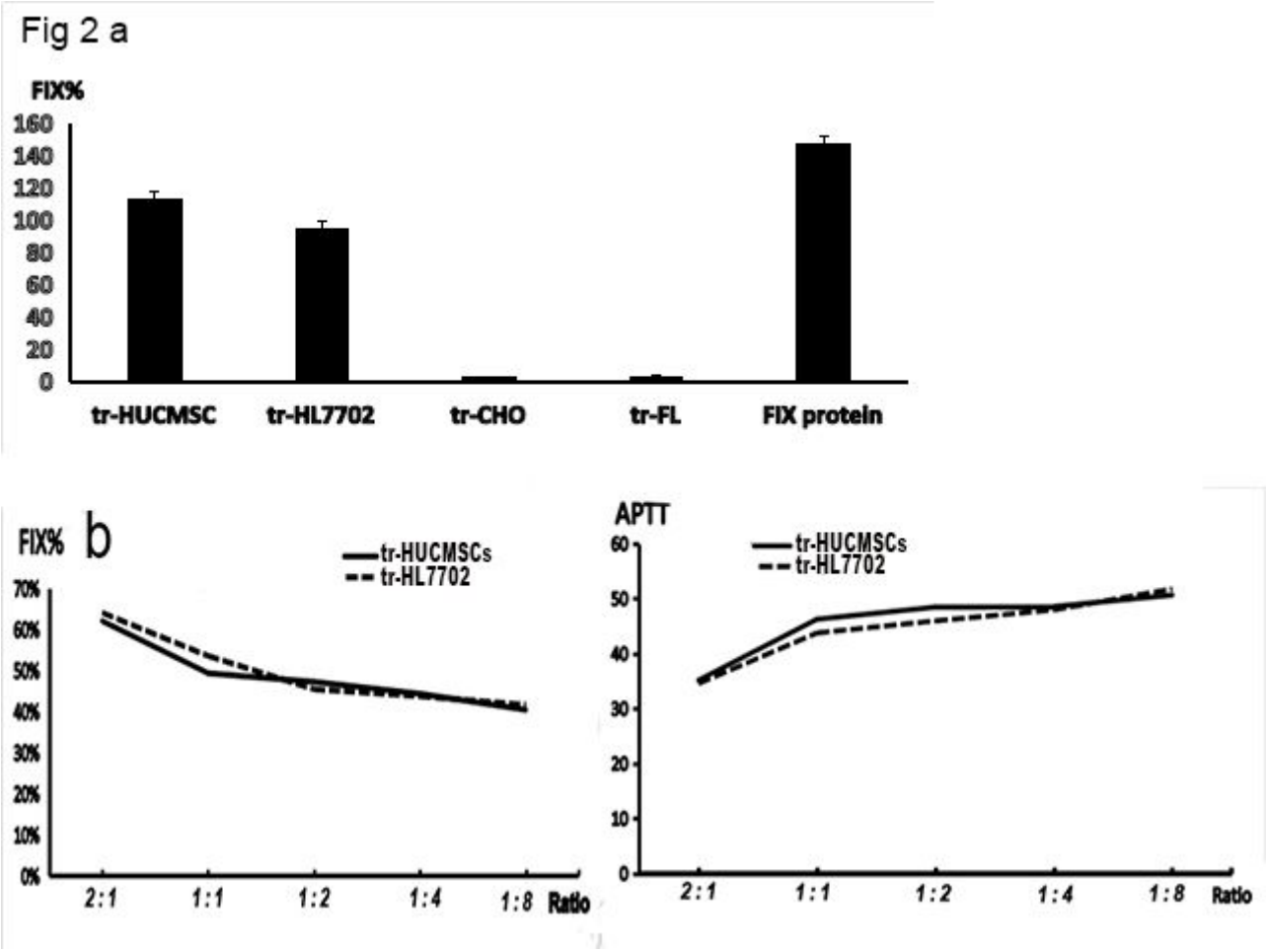

Figure 2

hFIXco secretion in different transduced cell types and its activity in serum of Hemophilia B patient. (a) After transducing HUCMSCs, HL7702, CHO, and FL cell lines with DJ/8-hIX for 24 hours, we detected the activity of hFIXco in the supernatants. For comparison, a pure hFIX protein sample was used as a positive control.(b) The assessment of the activity of hFIXco produced by the tr-HUCMSCs and tr-HL7702 in the blood serum of a Hemophilia B patient.

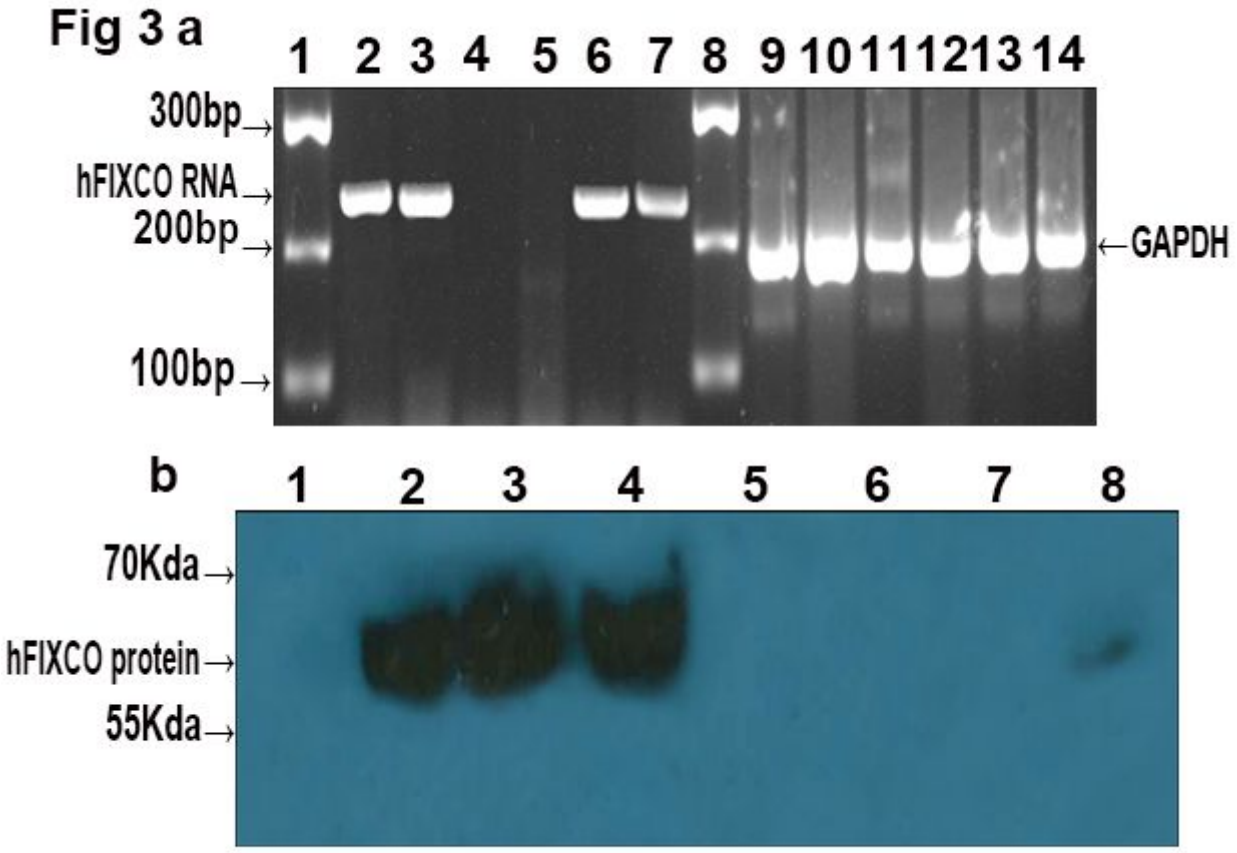

Figure 3

The expression of hFIXco in tr-HUCMSCs and tr-HL7702 by PCR and Western blot. (a) hFIXco RNA was detected in tr-HUCMSCs and HL7702 using RNA detection techniques (the figure presented is the cropped gel. Full-length gels are presented in Supplementary Figure 1). The gel lanes were labeled as follows: Lane 1: marker, Lane 2 and Lane 3: tr-HUCMSCs cultured for 24 hours and 5 months respectively, Lane 4 and Lane 5: untr-HUCMSCs cultured for 24 hours and 5 months,respectively, Lane 6 and Lane 7: tr-HL7702 cultured for 24 hours and 5 months respectively. Lane 8: marker. Lanes 9-14: GAPDH served as an internal control in the above-mentioned samples. (b) the hFIXco protein detection in the supernatants and cell lysates of tr-HUCMSCs(the figure presented is the cropped blot. Full-length blot are presented in Supplementary Figure 2). The samples were loaded as follows: Lane 1: marker, Lane 2 and Lane 3:supernatant of tr-HUCMSCs after 24 hours and 5 months culture, Lane 4: cell lysate of tr-HUCMSCs after 5 months of transduction, Lane 5 and Lane 6:supernatant and cell lysate of untr-HUCMSC for 24 hours, Lane 7:blank medium. Lane 8: pure FIX protein (8ng/lane) .

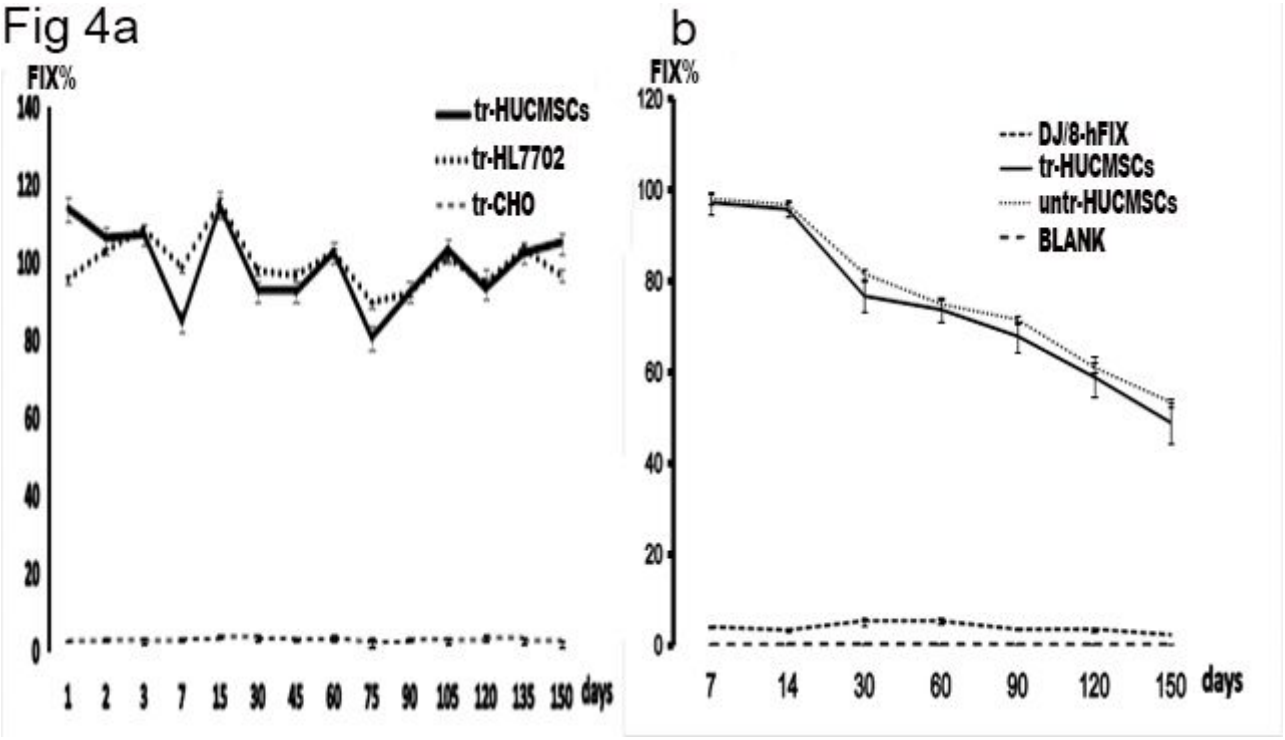

Figure 4

The stability and continuity of hFIXco secretion in tr-HUCMSCs in vitro and in vivo. (a) From 24 hours to five months, The stability and continuity of hFIXco activity secreted by the tr-HUCMSCs, tr-HL7702, and tr-CHO cells were observed. (N=4, $P < 0.05$). (b) From 7 days to 5 months, the activity of hFIXco remained consistent but gradually decreased in the NSG mice injected with tr-HUCMSCs and directly with DJ/8-hIX (N=4, $P < 0.05$). Notably, mice injected with untr-HUCMSCs or blank (0.9% NS injection) exhibited minimal hFIXco activity.

Fig 5

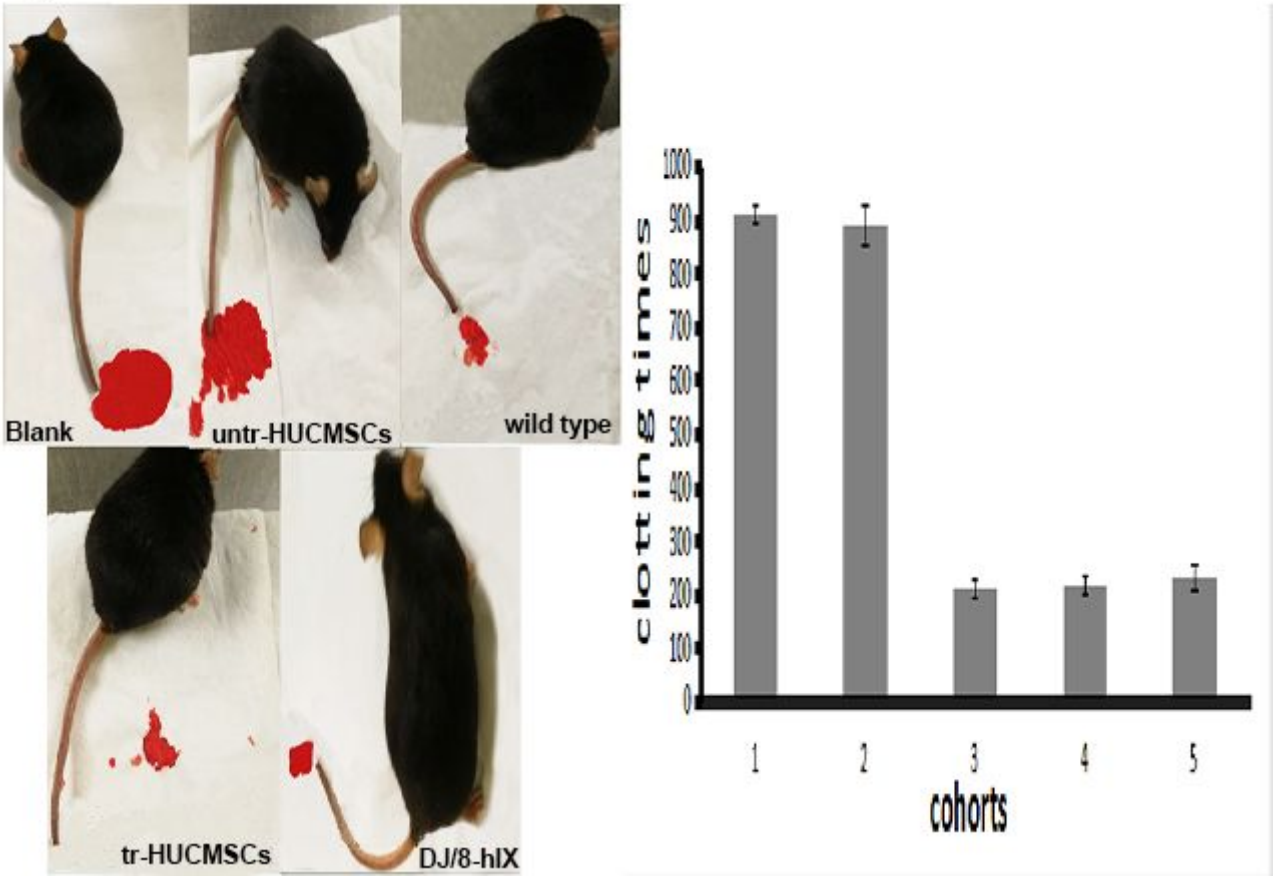

Figure 5

Clotting time of all the experiment cohorts at 90 days after transplantation(N = 4 per group). 1. Blank (*F9*-KO mice with 0.9% NS injection). 2. untr-HUCMSCs (1×10^3 cells/g) injection. 3. wild type (same strain as *F9*-KO mice, but without the *F9* gene knocked out). 4. tr-HUCMSCs(1×10^3 cells/g) injection. 5.DJ/8-hIX(1×10^{11} vg/kg) injection. The *F9*-KO mice were anesthetized using tribromoethanol. The tail was cut by 1cm, and photographs were taken 30 seconds post-incision. For test Clotting time, the filter paper was used to touch the wound at regular intervals until blood cessation was observed.

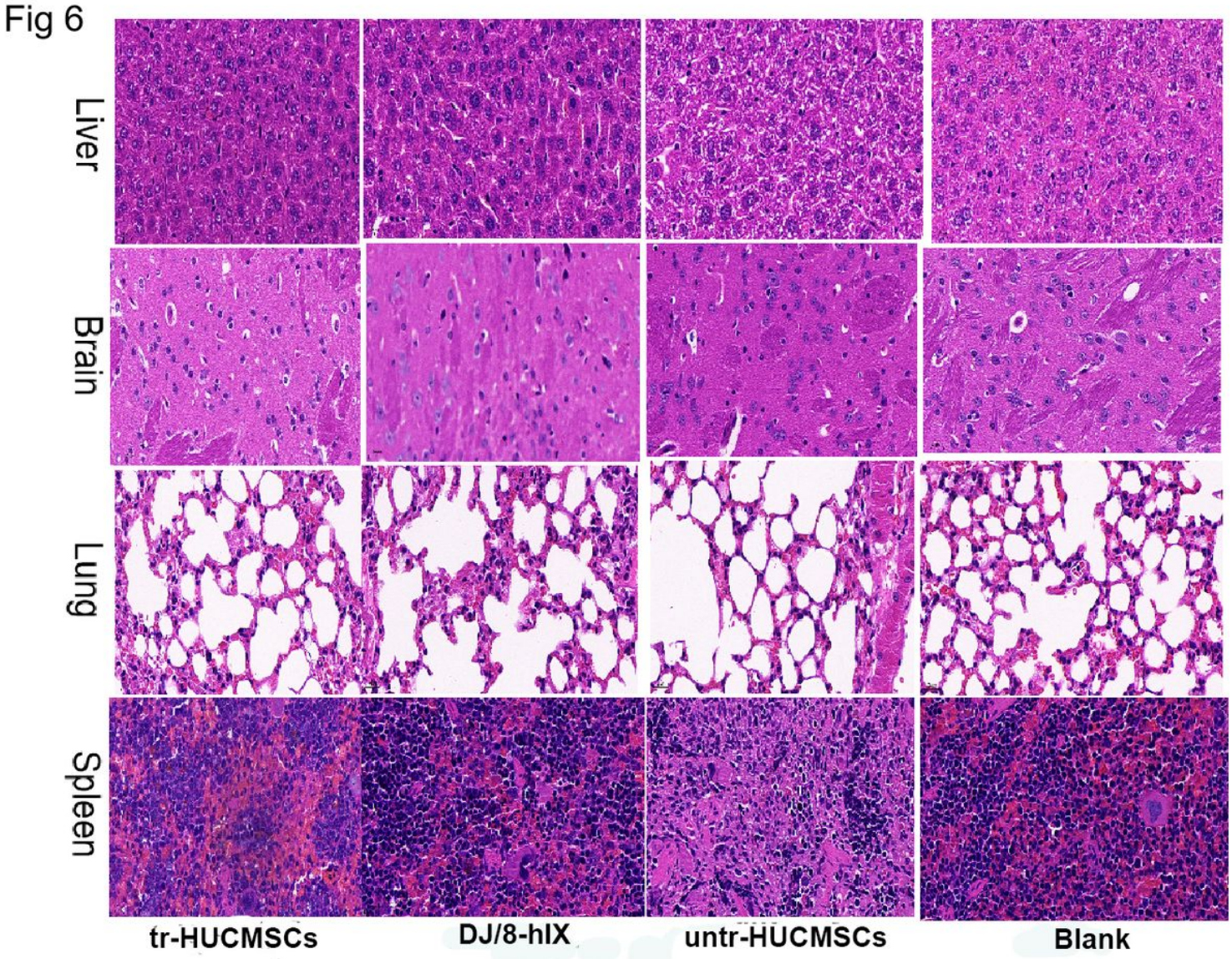

Figure 6

hematoxylin-eosin stain in the tissue of NSG mice after 5 months of transplantation. Animal tissues such as liver, spleen, lung and brain were collected for hematoxylin-eosin stain (40×).

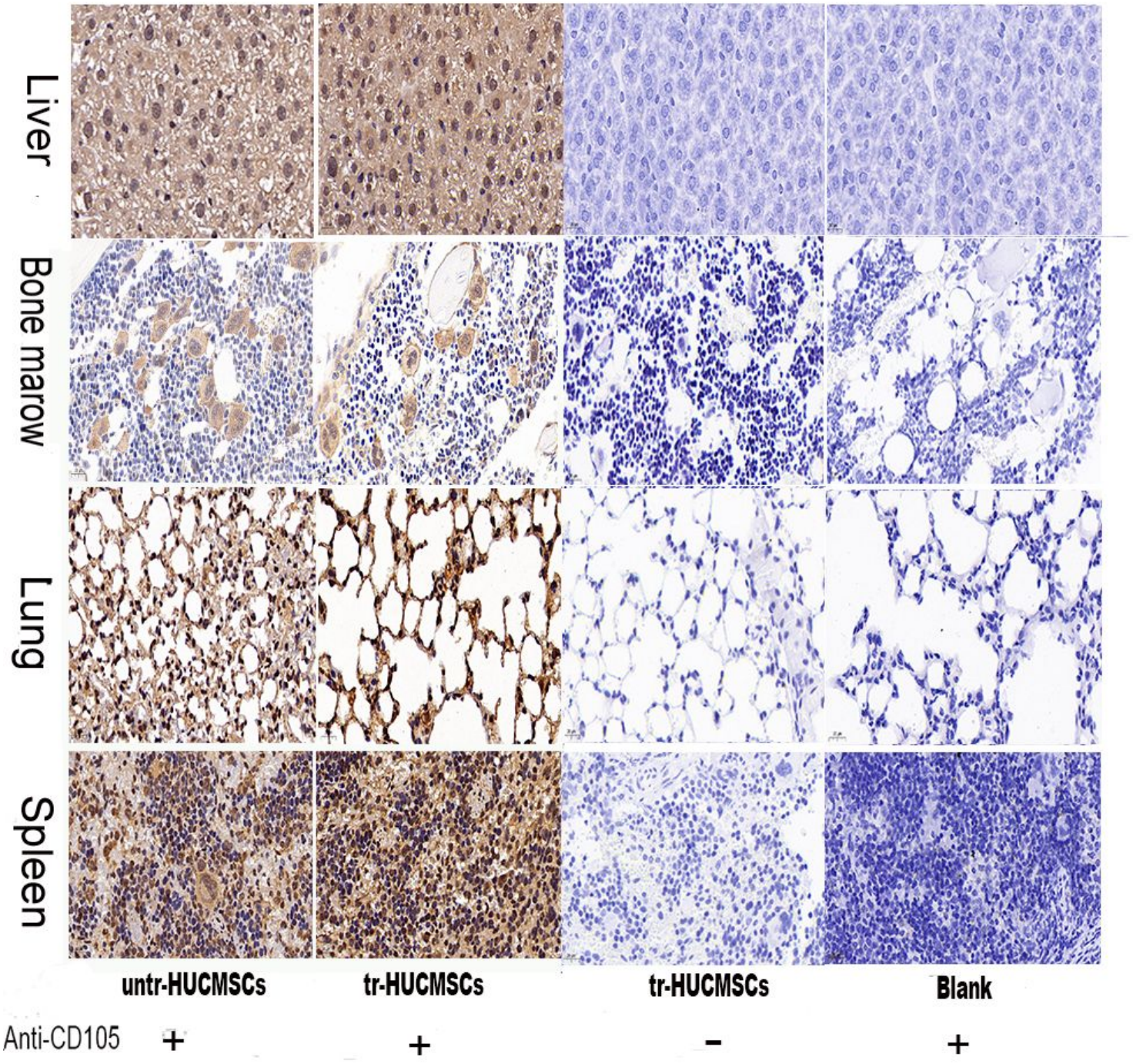

Figure 7

immunohistochemical analysis of the distribution of tr-HUCMSCs or untr-HUCMSCs in NSG mice after 2 weeks transplantation. Selected animal tissues such as liver, spleen, lung and bone marrow were collected for immunohistochemical analysis(40×). (Blank: injected with 0.9% NS. Anti-CD105 +: adding anti-CD105 antibody; anti-CD105 -: no adding anti-CD105 antibody).

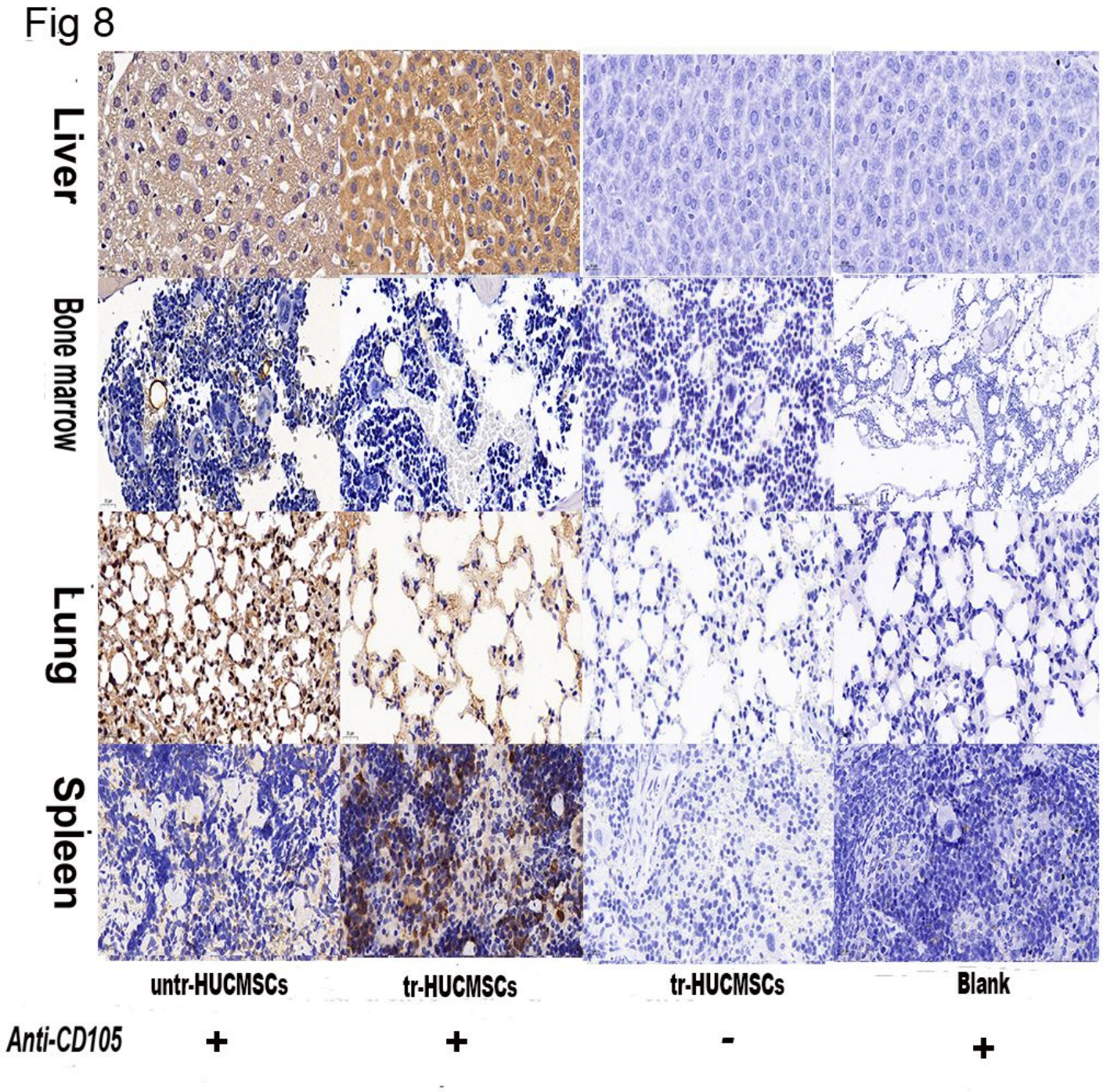

Figure 8

immunohistochemical analysis of the distribution of tr-HUCMSCs or untr-HUCMSC in NSG mice after 5 months transplantation. Selected animal tissues such as liver, spleen, lung and bone marrow were collected for immunohistochemical analysis (40×). (Blank: injected with 0.9% NS. Anti-CD105 +: adding anti-CD105 antibody; anti-CD105 -: no adding anti-CD105 antibody).

Supplementary Files

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