Stem Cell Research & Therapy

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Engineering biomimetic bone marrow niche with gene modified mesenchymal stromal cells for ex vivo culture of human hematopoietic stem and progenitor cells

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Abstract

Background Hematopoietic Stem and Progenitor Cells (HSPCs) gene therapy has shown significant progress, with commercial approval for at least four distinct haematological disorders, and poised for a rapid expansion in the upcoming years. Despite these advancements, the ex vivo culture of HSPCs continues to present significant challenges. The stress induced by ex vivo culture can negatively impact transplantation outcomes, while the need for exogenous cytokine supplementation contributes to the high costs associated with gene therapy products.

Methods We developed genetically modified human bone marrow MSCs (GM-MSCs) secreting cytokines such as Stem cell factor (SCF), Thrombopoietin (TPO), FMS-like tyrosine kinase-3-ligand (FLT3L), and Interleukin-3 (IL3), closely resembling bone marrow cellular niche to augment HSPCs culture.

Results HSPCs proliferate on GM-MSCs akin to standard conditions, devoid of external cytokine supplementation and these HSPCs retain their stem cell characteristics, colony-forming potential, stemness gene signatures, and capacity for long-term multilineage reconstitution in NBSGW mice. We demonstrate that our biomimetic feeder layer supports and alleviates stress associated with Homology Directed Repair (HDR) mediated gene-editing of HSPCs for fetal haemoglobin reactivation for a potential application in β-hemoglobinopathies gene therapy.

Conclusion Our GM-MSCs offer a compelling alternative to traditional cytokine supplementation by establishing a biomimetic bone marrow niche that fosters HSPC expansion while maintaining their stemness. These findings underscore the potential of engineered MSCs to revolutionize ex vivo HSPCs culture, ultimately enhancing their therapeutic value for gene therapy applications.

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Keywords Mesenchymal stromal cells, Hematopoietic stem and progenitor cells, Gene therapy, Homology-directed gene editing, B-hemoglobinopathies

Introduction

Over the past decade, hematopoietic stem and progenitor cells (HSPCs) gene therapy has made remarkable progress, driven by the advancement of cutting-edge technologies and methodologies that enhance its safety and precision [1]. The approval of Casgevy, the first CRISPR/Cas9-based HSPCs gene therapy for sickle cell disease (SCD), marks a significant milestone [2]. HSPCs possess a unique ability to be mobilized from the bone marrow, undergo ex vivo manipulation, and successfully re-engraft, where they self-renew and differentiate into all hematopoietic lineages. This remarkable versatility positions HSPCs gene therapy at the forefront of ex vivo genetic engineering approaches. Despite its potential, ex vivo manipulation of HSPCs remains challenging. Key obstacles include culture-induced cellular stress, cytotoxic effects from the manipulation process, reduced engraftment efficiency of modified cells, and the presence of a large fraction of committed progenitors in the graft, which can lower the in vivo frequency of gene-modified cells in the long term [3, 4]. These limitations necessitates the retrieval and manipulation of large quantities of HSPCs from patients, which increases the overall cost of the production process [5, 6]. Additionally, the requirement for various manipulation reagents and cytokines from commercial vendors further complicates the logistics and expense associated with HSPCs gene therapy.

The stem cell niche in human bone marrow provides essential scaffolds, cellular frameworks, and soluble cues that support the maintenance of HSPCs. Mesenchymal stromal cells (MSCs) play a critical role in maintaining HSPCs homeostasis primarily through the release of paracrine factors and also through HSPC-MSC contact, which collectively foster a supportive environment for haematopoiesis [7, 8]. Several pre-clinical and clinical studies have highlighted the significant role of MSCs when cocultured or co-transplanted with adult HSPCs or umbilical cord blood (UCB) HSPCs. This approach has been shown to enhance engraftment, accelerate hematopoietic recovery, and effectively mitigate the risk of graft-versus-host disease (GvHD) [9–15]. Research has demonstrated that MSCs obtained from various sources such as bone marrow (BM), adipose tissue (AT), and umbilical cord matrix effectively promote the ex vivo expansion and maintenance of UCB HSPCs, while preserving a primitive subpopulation. Furthermore, a recent study showed that bone marrow-derived MSCs (BM-MSCs) in mitigating the toxicity associated with gene manipulation enhancing both the numbers and fitness of CRISPR-Cas9 gene-edited HSPCs [16, 17], leading to improved engraftment outcomes of gene modified cells [16]. Additionally, ex vivo co-culture of MSCs with HSPCs under physiological oxygen levels has been shown to preserve the stemness of HSPCs, further enhancing their therapeutic potential [18, 19].

Given the beneficial role of MSCs in supporting the functionality of in vitro cultured HSPCs, and the need to overcome barriers to the accessibility of gene therapy, innovative approaches are essential to optimize ex vivo culture conditions and improve therapeutic outcomes. Thereby, we developed genetically modified mesenchymal stromal cells (GM-MSCs) that secrets key growth factors— SCF, FLT3L, TPO, and IL3 to support the expansion of HSPCs without exogenous cytokine supplementation. GM-MSCs enhanced $CD34^{+}CD90^{+}$ cell expansion and maintained HSPCs functionality, leading to successful engraftment in bone marrow, spleen, and peripheral blood. Furthermore, the coculture system significantly reduces stress associated with HDR gene editing, thereby enhancing HDR efficiency, and HSPCs viability. Overall, our findings suggest that GM-MSCs can effectively maintain the HSPCs ex vivo, offering a cost-effective and efficient alternative to conventional cytokine supplementation for HSPCs culture. This approach shows promise for therapeutic applications in HSPCs transplantation.

Materials and methods

Isolation, culture and characterisation of BM-MSCs and wharton's jelly-derived MSCs (WJ-MSCs)

The ethical approval for the use of human umbilical cord in this study was granted by the Institutional Review Board (IRB) of Christian Medical College, Vellore, India. Human umbilical cord samples were collected after obtaining written consent from patients undergoing elective caesarean sections at full term. Under sterile conditions, the cord lining and blood vessels were removed, and the remaining jelly-like tissue was treated with Dispase I protease for enzymatic digestion. WJ-MSCs were then isolated from the processed tissue, following the procedures described in previous studies [20]. Bone marrow aspiration was collected from healthy donors after receiving written informed consent and approval in accordance with Institutional Review Board (IRB) of Christian Medical College, Vellore, India. MSCs were isolated by using the density gradient centrifugation as described previously [21]. Isolated BM-MSCs and WJ-MSCs Cells were expanded in DMEM-Alpha modification (AL080A) with 10% FBS and 1X Pen-Strep. The isolated BM-MSCs and WJ-MSCs were assessed for the

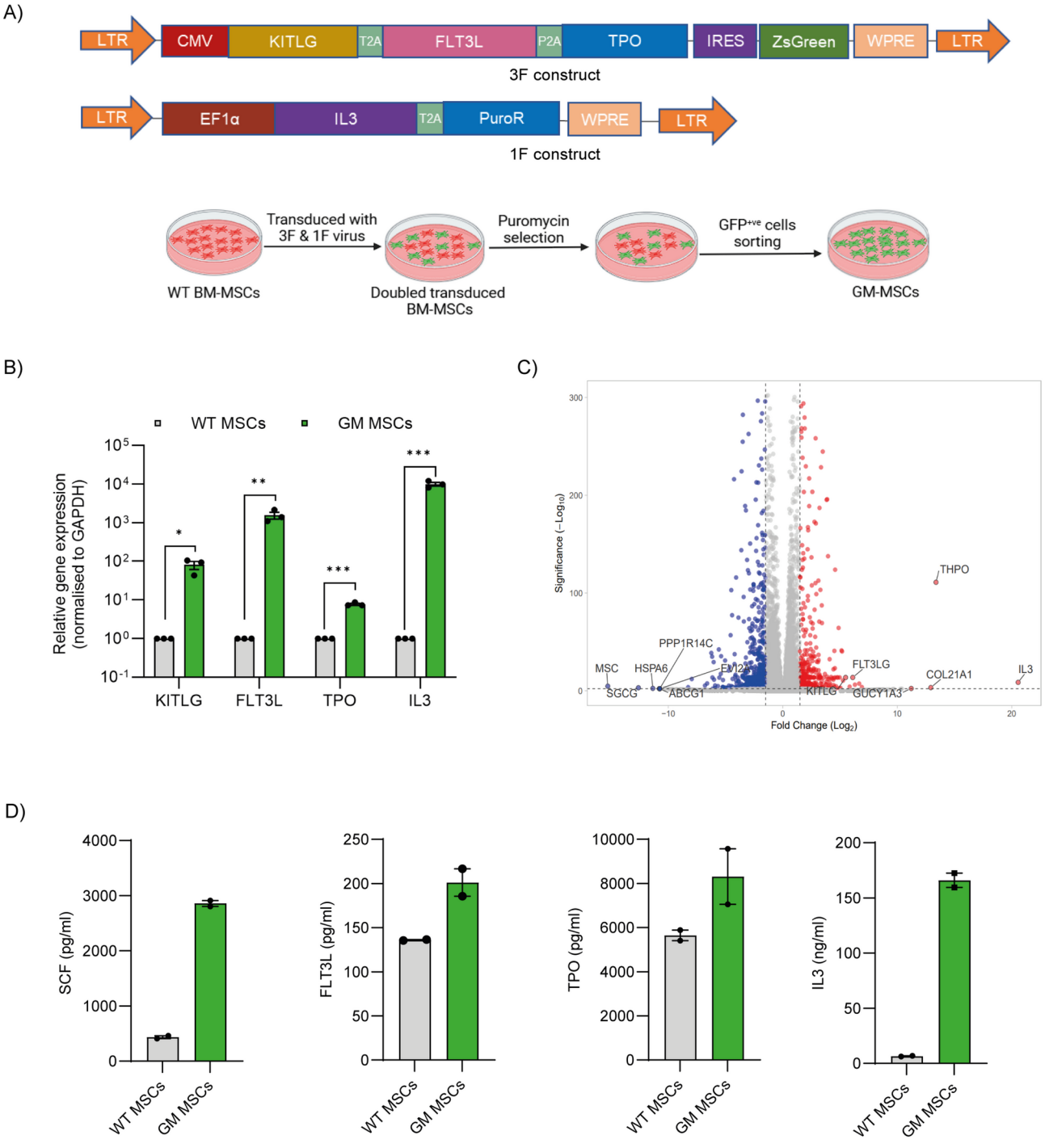

Fig. 1 Generation and characterisation of genetically modified MSCs (GM-MSCs). (**A**) Schematic representation of polycistronic Lentiviral vector contains KITLG, FLT3L, TPO, in one vector with Zsgreen as expression marker and IL3 in another vector with puromycin resistance gene as selection marker for double transduction. (**B**) Analysis of mRNA expression of KITLG, FLT3L, TPO, and IL3 by qPCR. (mean ± SEM for $n = 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; unpaired t test). (**C**) Volcano plot displaying differentially expressed genes in GM-MSCs compared to WT-MSCs. Genes with a statistically significant (padj < 0.01) and substantial (≥ 1.5 log2 fold change) difference in expression are shown in colour: red for upregulated genes and blue for downregulated genes in GM-MSCs. Thrombopoietin represented as THPO. (**D**) Protein expression levels determined by ELISA (mean ± SEM for $n = 2$). (Schematic illustrations were created with BioRender.com)

immunophenotypic expression of the surface marker including CD34, CD14, CD73, CD90, CD105, and HLA-DR. To assess the trilineage differentiation potential, WT-MSCs and GM-MSCs were subjected to Osteogenic, chondrogenic and adipogenic differentiation. For adipogenic differentiation, 5×10^4 cells were seeded in 24-well

plates containing standard media one day prior to induction. The following day, adipogenesis was induced using StemPro™ Adipogenic Differentiation Medium (Cat# A1007001). These differentiation media were maintained for a period of 21 days. After the adipogenic differentiation, the cells were fixed with 4% paraformaldehyde and washed with distilled water. Then, stained with Oil Red O (Cat# O1391-250 ml, Sigma). Following staining, images were captured using a Leica microscope. For osteogenic differentiation 5×10^4 cells were seeded in 24-well plates containing standard CD34 expansion medium. The following day, osteogenic induction media (StemPro™ Osteogenesis Differentiation Medium - A1007201) was added and maintained for over 21 days. Following the differentiation period, the cells were fixed with 10% neutral buffered formalin and stained with Alizarin Red solution (Cat# 2003999, Sigma). Following staining, images were captured using a Leica microscope. For chondrogenic differentiation, 1 x 10^5 cells were seeded in 96-well plates with standard media. The next day, chondrogenesis induction media (StemPro™ - A1007101) was added, and the media were replaced every two days. During the differentiation process, the cells aggregated and formed pellets. After 21 days of differentiation, the pellets were fixed using 4% paraformaldehyde, paraffin-embedded, and sectioned at 4 µm thickness. These sections underwent staining with Alcian blue, Safranin O, Toluidine blue, and Picro Sirius red. Following staining, all slides were mounted with DPX for imaging using an Olympus BX43f microscope.

Purification and expansion of CD34$^+$ HSPCs and coculture with MSCs

The leftover mobilized peripheral blood (mPB) bags following allogeneic stem cell transplantation were sourced from the transplantation unit of Christian Medical College, Vellore. This was conducted with prior approval from the institutional review board. Peripheral blood mononuclear cells (PBMNCs) were isolated using Ficoll density gradient centrifugation with Lymphoprep™ (#07801). These PBMNCs were then cryopreserved. Depending on experimental requirements, the PBMNCs were thawed, and CD34$^+$ HSPCs were enriched using the EasySep™ CD34 Positive Selection Kit II (Cat No: 17896, STEMCELL Technologies). The detailed methodology for PBMNC isolation and CD34$^+$ cell purification has been described in our previous publication [22]. Prior to initiating co-culture experiments, the purified HSPCs were pre-cultured in basal serum-free essential medium (SFEM-II) without cytokines for 8–12 h. This step was undertaken to enhance recovery and enrich the population of cells that had successfully overcome stress from thawing and purification. Flow cytometry (FACS) analyses were performed on the cells using specific marker panels, including CD34 and CD90 or CD34, CD90, CD38, CD45RA, and CD49f, depending on experimental requirements. For the co-culture experiments, the purified CD34$^+$ cells were maintained in SFEM supplemented with hematopoietic stem cell-specific cytokines: TPO (100 ng/ml), FLT3 (300 ng/ml), SCF (300 ng/ml), and IL-3 (50 ng/ml) [23]. Cells were cultured under standard conditions or co-cultured with BM-MSCs or WJ-MSCs. For initial screening experiments, MSCs were seeded at a density of 10^4 cells/cm^2 to serve as a supportive feeder layer. 1×10^5 CD34$^+$ HSPCs were cultured under various conditions, including standard culture (SC), co-culture with BM-MSCs, WJ-MSCs, and GM-BMMSCs, with or without the addition of exogenous cytokines as specified for each experiment. Media replenishments were performed on day 3, and cells were harvested on day 6. A harsh pipetting technique was used to dislodge any HSPCs adhering to the MSC feeder layer. The collected cells were then passed through a 0.4 µm cell strainer to minimise the contamination of MSCs during the flow cytometric analysis. These harvested cells were counted and subjected to flow cytometry analysis using marker panels including CD34 and CD90, or CD34, CD90, CD38, CD45RA, and CD49f, depending on experimental requirements. Additionally, colony-forming unit (CFU) assays were performed to assess colony forming potential of the cultured HSPCs. For experiments investigating hypoxic conditions, cells were cultured at a physiological oxygen concentration (1% O_2 termed as hypoxia). In these settings, a cocktail of small molecules RUS [3] was added to the culture medium as specified in the experimental design.

Cloning of the construct, lentiviral production, and generation of genetically modified MSCs (GM MSCs)

The plasmid construct expressing the three hematopoietic cytokines (3 F: KITLG, FLT3L, TPO), as illustrated in Fig. 1A, was generated by cloning the coding sequences of KITLG (NM_000899.3), FLT3L (NM_001459.3), and TPO (NM_000460) into the backbone of Addgene plasmid #52,516, replacing the RFP cassette via Gibson Assembly (GA). The cytokine sequences were linked using self-cleaving T2A and P2A peptides to enable their co-expression under the control of a CMV promoter. A separate vector was similarly constructed for IL-3 (NM_000588) by cloning the gene under the EF1α promoter into Addgene plasmid #112675, replacing the Cas9 sequence using GA. The gene sources are listed in Supplementary Table 1. GA products were transformed into competent bacterial cells (Stable3 strain), and positive clones were screened by colony PCR. Recombinant plasmids were purified and verified for correct orientation and sequence integrity via Sanger sequencing.

Lentiviral vectors were produced in HEK-293T cells cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were co-transfected with the envelope plasmid pMD2.G (Addgene #12259), packaging plasmid psPAX2 (Addgene #12260), and either the 3 F–1 F expression plasmid using the inhouse liposome [24] transfection protocol. Viral supernatants were collected at 48 and 72 h post-transfection, pooled, and concentrated using Lenti-X™ Concentrator (Takara Bio). The 100X concentrated viral stocks were aliquoted and stored at − 80 °C until further use. The titres of the lentiviral vectors were determined to be 2.61×10^7 IU/mL for the 3 F vector and 3.09×10^7 IU/mL for the 1 F vector (Lenti-X qRT-PCR Titration Kit, Takara, Cat. No. 631235). MSCs were co-transduced at passage 3 with both vectors at a multiplicity of infection (MOI) of 30. After transduction, cells were subjected to puromycin selection, followed by GFP-based fluorescence-activated cell sorting to isolate the 9% double-positive populations. The successfully modified MSCs were then expanded and cryopreserved for downstream applications.

In vivo engraftment analysis

All in vivo studies involving NBSGW mice were performed in accordance with the guidelines set by the Institutional Animal Ethics Committee (IAEC) of Christian Medical College, Vellore, India. 8 to 9 weeks old mice (both male and female) were preconditioned with busulfan at a dose of 12.5 mg/kg body weight, administered 48 h before the infusion. Day 0 equivalent cell numbers of 0.25×10^6 cells/mice were cultured in SC, WT-MSCs and GM-MSCs for five days and infused into mice via tail vein injection. Fourteen weeks post transplantation, peripheral blood (PB), bone marrow (BM), and spleen were collected from mice following an intraperitoneal lethal dose of ketamine and xylazine. Analysis of human cells engraftment were assessed in PB, BM and spleen by incubating the cells with mouse Fc block and stained with hCD45 and mCD45 antibodies after RBC lysis. Engraftment percentage is calculated using the formula (hCD45%/hCD45%+mCD45%) x100. In addition, multilineage markers of differentiation including CD3, CD13, CD19, and CD235a in BM were analysed. Cells were acquired using Cytoflex LX (Beckmann Coulter) and analysed using Flow Jo. The work has been reported in line with the ARRIVE guidelines 2.0.

Quantitative real-time PCR analysis

A total of 1×10^6 million WT and Dual selected transduced MSCs (GM-MSCs) were harvested for RNA isolation using Macherey-Nagel™ NucleoSpin™ RNA Kit (Cat. no. 740955.50). cDNA was synthesised from total RNA using an iScript cDNA Synthesis Kitc (Cat. no. 6110 A). Quantitaive realtime PCR analysis were performed using SYBR Premix Ex Taq II (Takara Bio Cat. no. RR820A) for quantifying the specific transcripts and analysed with QuantStudio 6 Flex (Life Technologies). Primers used for qPCR analysis in this study are mentioned in Supplementary Table 2.

Protein quantification by ELISA

Culture condition media (CCM) were collected from WT-MSCs and GM-MSCs condition cultured with STEMin1™ xenofree media (Himedia - AL520). SCF, FLT3L, TPO, and IL3 were quantified from CCM with respective ELISA kit as per instruction. ELISA kit used in protein quantification are mentioned in Supplementary Table 3.

Colony-forming unit analysis

After six days of culture, 5×10^2 HSPCs from SC and coculture conditions were seeded in 1.5 mL of Methocult Optimum (STEMCELL Technologies Cat. no.04034) and 14 days after plating the colonies were scored using inverted microscope. Based on the morphology by microscopic images, colonies were categorized as CFU-GEMM, CFU-GM, and CFU-E.

Analysis of ROS levels

The HSPCs that co-cultured with SC and GM-MSCs were harvested. 2×10^5 cells washed twice with 1X PBS and then stained with 10µM of 2′,7′-Dichlorofluorescin diacetate (H2DCFDA) dye (Invitrogen, Cat. no. D399) and incubated at 37 °C in the dark for 40 min. Following the incubation, the cells were washed with PBS and measured the Mean Fluorescence Intensity (MFI) by using flow cytometry.

Apoptosis assay

The HSPCs were harvested from culture condition SC and GM-BMMSCs. $2\text{-}5 \times 10^5$ cells washed twice with ice-cold PBS and then resuspended in 1X Annexin V binding buffer from the BD apoptosis kit (Cat no. 556547). The cells were stained with APC-annexin V (Cat no. 550475) and 7-AAD simultaneously for 15 min, following the manufacturers protocol and then analysed by using flow cytometry.

Gene editing of HSPCs

To perform HDR-mediated genome editing in HSPCs, we utilized an sgRNA and corresponding ssODN targeting the HBG promoter, as previously reported in our published study (gRNA sequence: UAUCUGUCUGAA ACGGUCCC) [17]. A total of 2×10^5 HSPCs were resuspended in supplemented P3 primary cell solution and nucleofected using a Lonza 4D nucleofector (Pulse code: DZ100) with 25 pM of RNP, comprising 50 pM sgRNA

(Synthego) and 25 pM Cas9 (Takara), along with 80 pM of ssODN. Following electroporation, cells were collected and cultured for downstream analyses. Gene editing efficiency was assessed 72 h post-electroporation through DNA extraction, Sanger sequencing, and ICE knock-in analysis (Synthego). The ssODN sequence is available in Supplementary Table 4.

Erythroid differentiation

A three-phase erythroid differentiation protocol was employed to differentiate HSPCs that had been gene-edited at HBG promoter for incorporating the HbF inducing mutations, -175 T > C and − 158 C > T as previously described in published studies [25]. 72 h post-electroporation, HSPCs were cultured for 7 days in Phase I erythroid differentiation medium, consisting of IMDM GlutaMax Supplement media, supplemented with 5% AB serum, 20 mg/mL insulin, 2 U/mL heparin, 3 U/mL EPO, 330 mg/mL Holotransferrin, 100 ng/mL SCF, 50 ng/mL IL3, and 1 mg/mL hydrocortisone (cell density 5×10^4 cells/ml). During Phase II (days 8–12), cells were maintained at a density of 2×10^5 cells/ml in media containing all the components of Phase I, except hydrocortisone and IL3. Phase III (days 12–20) involved culturing the cells at a density of 5×10^5 cells/ml in media containing all the Phase II components, excluding SCF, with a medium change on day 16. The cells were harvested on 20th day and HbF^{+ve} cells and differentiation markers were analysed.

Transcriptome analysis

Total RNA was isolated using the Qiagen RNA isolation kit and quantified with the Qubit RNA HS Assay. RNA purity was evaluated using the QIAxpert system, and RNA integrity was assessed on the Agilent TapeStation with RNA HS ScreenTapes (Cat# 5067–5579). Library preparation for total RNA sequencing (RNA-seq) was performed according to the NEB Ultra II Directional RNA-Seq Library Prep Kit protocol. The libraries were quantified using the Qubit High Sensitivity Assay (Invitrogen, Cat# Q32852). RNA sequencing was conducted on the Illumina HiSeq 4000 platform, yielding 60 million paired-end reads of 100 base pairs. Read counts were obtained from mapped reads using FeatureCounts. Differential gene expression analysis was carried out using DESeq2. Gene Set Enrichment Analysis (GSEA) was performed with the Broad Institute's GSEA software, where a ranked list of differentially expressed genes from the RNA-seq data was tested against reference gene sets from the literature. Gene ontology analysis was conducted using Enrichr [26]. Volcano plots were generated using VolcaNoseR [27], and a heatmap of differentially regulated genes was visualized using Heat-mapper [28].

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM), and a p-value of < 0.05 was considered statistically significant. The specific statistical tests used for each experiment are indicated in the respective figure legends. For comparisons between two groups, an unpaired two-tailed Student's t-test was employed. For comparisons involving more than two groups, ordinary one-way ANOVA followed by Sidak's multiple comparisons test was used to account for multiple testing. All statistical analyses were performed using GraphPad Prism version 10.

Results

Genetically modified MSCs secrete cytokines required for *ex vivo* culture of HSPCs

We first investigated whether the source of feeder MSCs influences the HSPC proliferation rates in the presence of regular cytokines such as SCF, FLT3L, TPO and IL6 used for HSPC culture (Supplementary Fig. 1A). We used absolute number of $CD34^+CD90^+$ cells as the readout, as it takes the percentage of primitive cells, fold expansion and the viability into the consideration. We observed both BM-MSCs and WJ-MSC feeder systems produced absolute number of $CD34^+$ $CD90^+$ cells, comparable to the standard conditions of HSPCs culture (SC) that is without a feeder layer (Supplementary Fig. 1B).

The HSPCs are usually cultured ex vivo by supplementing the culture medium with recombinant SCF, FLT3L, TPO and IL3 cytokines [23]. Given the well documented and extensively characterized interactions between BM-MSCs and HSPCs in the previous literature [16, 17], BM-MSCs were selected for genetic engineering to overexpress the cytokines. We transduced the BM-MSCs with viral vectors co-expressing three cytokine factors (3 F) —KITLG (SCF), FLT3L, TPO and ZsGreen reporter alongside IL3 (hereafter described as 4 Factors- 4 F) and puromycin in another vector. The transduced cells positive for both Zsgreen and puromycin were selected and 4 F genetically modified MSCs (GM-MSCs) were expanded for one passage, before being subjected to characterization (Fig. 1A). The GM-MSCs showed a significant increase in the expression of KITLG, FLT3L, TPO, and IL3 compared to wild-type MSCs as analysed using qPCR (Fig. 1B). The transcriptomic analysis of the GM-MSCs also showed a significant increase in the expression of 4 F cytokines (Fig. 1C). Next, to confirm 4 F cytokines are secreted, we quantified these cytokines in the culture medium using ELISA. The supernatant from GM-MSC cultures exhibited elevated levels of 4 F secretory factors compared to non-transduced WT-MSCs (Fig. 1D and Supplementary Fig. 1C), indicating successful cytokine secretions from the GM-MSCs. Furthermore, the immunophenotype of the GM-MSCs and their

differentiation potential towards adipogenic, osteogenic, and chondrogenic lineages remained similar to that of the parental MSCs confirming the genetic over-expression of cytokines are not altering the characteristics of the MSCs (Supplementary Fig. 1D and E).

GM-MSCs supports the HSPCs culture without exogenous cytokines

Next, we aimed to investigate whether the cytokines secreted by GM-MSCs are functional and contribute to the culture of HSPCs. To answer this, we cocultured HSPCs with both wild-type MSCs and GM-MSCs, with and without cytokine supplementation, to assess their impact on HSPCs viability and function (Fig. 2A). While standard condition (SC) devoid of cytokine supplementation and the wild type MSCs failed to induce any improvement in the absolute number of $CD34^+$ $CD90^+$ HSCs and total nucleated cells (TNC), GM-MSCs cultured without cytokine supplementation demonstrated a modest increase. However, this was significantly lower than the SC with supplemented conditions (Fig. 2B and C and Supplementary Fig. 2A). This suggests that GM-MSC-derived cytokines contribute to HSPCs support but may require enhanced production to achieve optimal proliferation. Therefore, we explored whether increasing the culture density of MSC cultures could produce sufficient cytokines to support HSPCs expansion at levels comparable to the standard culture conditions supplemented with exogenous cytokines. To this end, we co-cultured various densities of GM-MSCs (10,000, 20,000, 30,000, and 40,000 cells) with a constant number of HSPCs. We observed a gradual increase in total cell numbers proportional to the MSC density, ultimately reaching levels comparable to those obtained under SC (Supplementary Fig. 2B). Furthermore, we noted a comparable increase in the absolute numbers of human $CD34^+$ $CD90^+$ cells (Fig. 2D) across different GM-MSC ratios. The proportion of HSPCs subsets (Supplementary Fig. 2C) remained unchanged under all these conditions. Importantly, our findings revealed that a 4:10 ratio of MSCs to HSPCs achieved expansion levels like those attained in standard cultures, without the necessity for exogenous cytokine supplementation. Based on these results, we chose 4:10 ratio for further experiments.

In the bone marrow niche, HSPCs are exposed to hypoxic conditions, which are vital for their maintenance and functionality [29]. We cultured HSPCs under SC (with cytokines), wild type MSCs (with cytokines) and GM-MSCs (without external cytokines supplementation) under varying oxygen levels and observed that the absolute HSC numbers, fold expansion of $CD34^+$ $CD90^+$ cells, CFU counts and the proportion of CFU in the GM-MSCs condition matched the SC and the wild type MSC conditions with the cytokines both at normoxia and hypoxia (Fig. 2E, and Supplementary Fig. 2D-F).

In our previous studies, we demonstrated that culturing HSPCs with a small molecule cocktail comprising Resveratrol, UM729, and SR1 (collectively referred to as RUS) enhances the stemness and functionality of gene-modified HSCs [3]. While the RUS cocktail significantly increased the absolute number of $CD34^+$ $CD90^+$ HSCs, under SC, it had no effect on the HSPCs cultured under GM-MSCs (Fig. 2F).

MSCs impose a regulatory influence that limits the absolute expansion of these cells, likely mimicking in vivo niche environment. Conversely, in standard culture conditions, the absence of such regulatory mechanisms allows RUS to drive a more pronounced expansion of $CD34^+$ $CD90^+$ cells. To further confirm the stemness of HSPCs cultured in the cytokine-free GM-MSCs, we evaluated the absolute number of primitive HSCs marked by $CD34^+CD90^+CD45RA^-CD38^-CD49f^+$ cells and observed a similar number of primitive HSCs in the GM-MSCs and the SC, with RUS added in both conditions (Fig. 2G).

Further characterization showed GM-MSCs had a mild effect on reducing the ROS levels and apoptosis associated with in vitro culture (Supplementary Fig. 3D and 3E) and had no effect on the cell cycle (Supplementary Fig. 3F). Collectively, these findings confirm that GM-MSCs can support the HSPC culture without external cytokine supplementation.

GM-MSCs improve the stemness signatures of HSPCs through their secretory factors

RNA sequencing analysis revealed differential expression of 1,210 genes, with 911 genes upregulated and 299 genes downregulated in HSPCs cocultured with GM-MSCs compared to SC (Fig. 3A, Supplementary Table 5). Heatmap and volcano plot analyses highlighted an increased expression of genes involved in cell adhesion and stem cell maintenance, particularly ITGA3 and ITGA11 (Fig. 3B-C). Gene Ontology (GO) analysis of the differentially expressed genes revealed significant enrichment in pathways related to extracellular matrix organization, structural organization, collagen fibril formation, regulation of cell migration, and cell population proliferation (Fig. 3D). Conversely, the downregulated genes were primarily associated with functions such as calcium influx, molecular mediators of immune response and negative regulators of oxidoreductase activity (Fig. 3E). Gene set enrichment analysis (GSEA) using hallmark gene sets revealed a normalized enrichment score (NES) of 1.64 with a false discovery rate (FDR) q-value of 0.001 for the Hallmark Epithelial-Mesenchymal Transition (EMT) gene set (Fig. 3F). Notably, previous studies have demonstrated that extracellular vesicles (EVs) derived from BM-MSCs are enriched in proteins and mRNA associated

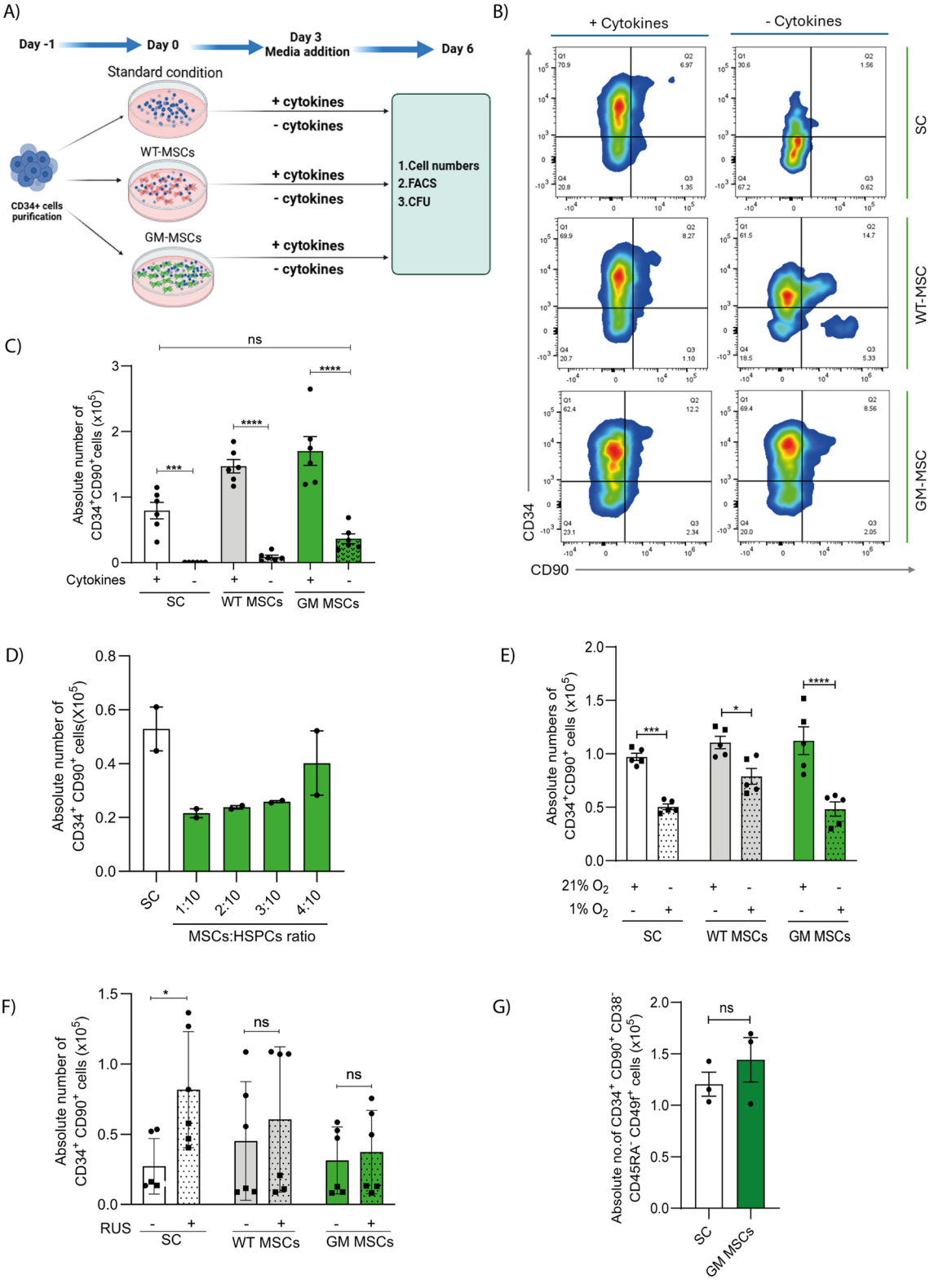

Fig. 2 (See legend on next page.)

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Fig. 2 GM-MSCs support the HSPCs without exogenous cytokines supplementation. (**A**) Schematic representation of the HSPCs co-culture with MSCs as feeder layer with and without cytokine supplementation. The purified $CD34^+$ cells were co-cultured with wild type BM-MSCs and GM-MSCs or cultured with standard condition. After 6 days, cells were analysed for the colony forming potential, flow cytometric analysis for stemness markers and the cell count. (**B**) Representative flow cytometry plots of gating strategy of $CD34^+$ $CD90^+$ cells co-cultured with WT-MSCs and GM-MSCs with and without cytokines supplementation. (**C**) Absolute number of human $CD34^+$ $CD90^+$ cells co-cultured with WT-MSCs and GM-MSCs with and without cytokines supplementation. (mean ± SEM for $n = 6$ and donor = 2; Ordinary one-way ANOVA (Sidak's multiple comparison test)). (**D**) Absolute number of human $CD34^+$ $CD90^+$ cells co-culture with different GM-MSCs: HSPCs ratios without cytokines supplementation. (mean ± SEM for $n = 2$ and donor = 1). (**E**) Absolute number of human $CD34^+$ $CD90^+$ cells with 21% and 1% oxygen concentrations in SC, WT-MSCs and GM-MSCs conditions. (mean ± SEM for $n = 6$ and donor = 2; Ordinary one-way ANOVA (Sidak's multiple comparison test)). (**F**) Absolute number of human $CD34^+$ $CD90^+$ cells co-culture with and without RUS treatment in SC, WT-MSCs and GM-MSCs. (mean ± SEM for $n = 6$ and donor = 2; Ordinary one-way ANOVA (Sidak's multiple comparison test)). (**G**) Absolute number of $CD34^+CD90^+CD45RA^-CD38^-$ $CD49f^+$ cells culture with RUS under hypoxia condition. (mean ± SEM for $n = 3$ and donor = 1; Unpaired t-test). ns = non-significant: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (Schematic illustrations were created with BioRender.com)

with extracellular matrix organization, positive regulation of cell migration, and the transforming growth factor beta receptor (TGFBR) signalling pathway, highlighting their potential role in modulating cellular dynamics [30]. Given the similar enrichment observed in our transcriptome, we compared our dataset with the ExoCarta database derived from BM-MSCs [31], and observed that among the 908 upregulated genes, 249 were specifically enriched in BM-MSC-derived exosomes (Supplementary Fig. 3G). These findings support the hypothesis that GM-MSCs may exert beneficial effects on the cultured HSPCs through the extracellular vesicles.

To further explore whether the upregulated genes share commonalities with HSPCs stemness signatures, we analyzed gene sets enriched in $ITGA3^+$ HSPCs, previously shown to possess both long-term (LT) and short-term repopulation potential [32]. GSEA revealed a NES of 1.37 with an FDR q-value of 0.03 (Fig. 3G). These results suggest that HSPCs cocultured with GM-MSCs retain long-term repopulating HSCs (LT-HSC) characteristics during expansion.

HSPCs cultured on GM-MSCs retain multilineage engraftment characteristics *in vivo*

To further evaluate the functional potential of HSPCs cocultured with GM-MSCs in the absence of exogenous cytokine supplementation, we conducted transplantation experiments. $CD34^+$ HSPCs were expanded for five days under standard culture conditions, BM-MSC coculture, and GM-MSC coculture, followed by transplantation into NBSGW mice. Hematopoietic reconstitution was assessed after 14 weeks in the bone marrow (BM), peripheral blood (PB), and spleen, along with an analysis of multilineage markers in BM (Fig. 4A). HSPCs cocultured with GM-MSCs demonstrated comparable engraftment efficiency in BM (Fig. 4B-C), PB (Fig. 4D), and spleen (Fig. 4E). Additionally, the frequency of $CD34^+$ cells remained consistent across BM, PB, and spleen (Fig. 4F-H). Further analysis of multilineage differentiation from the $hCD45^+$ fraction in BM, including T cells (CD3), B cells (CD19), myeloid cells (CD13), and erythroid cells (CD235a) from the $hCD45^-$ population, revealed robust and unbiased reconstitution across all lineages (Fig. 4I). Collectively, these findings indicate that HSPCs cocultured with GM-MSCs retain their in vivo engraftment potential without requiring cytokine supplementation.

GM-MSCs supports HDR gene editing of HSPCs for improved fetal hemoglobin expression

HDR gene editing in HSPCs is hindered by cell toxicity caused by HDR donors [17]. We earlier reported that wildtype BM-MSCs, combined with cytokines, mitigated the toxicity associated with ssODN and DNA-PK inhibitor AZD-7648. This approach allowed for efficient introduction of -175T > C and − 158 C > T mutations in the HBG promoter of HSPCs, activating fetal hemoglobin expression in erythroid cells [17]. We now examined whether GM-MSCs and their secretory factors could overcome HDR gene editing toxicity.

To test this, HSPCs cells were cultured with either GM-MSCs or WT-MSCs (along with cytokines) and edited for − 175T > C and − 158 C > T conversions in the HBG promoter. The RNP-ssODN-electroporated cells were immediately seeded onto GM-MSCs or WT-MSCs or cultured under standard conditions. AZD-7648 was added in all the conditions and removed after 24 h. The HSPCs were further expanded on the MSC stromal layer for 48 h (Fig. 5A).

Genotyping performed 72 h post-electroporation revealed that overall HDR efficiency in the HSPCs cocultured with GM-MSCs was close to the HSPCs cocultured with BM-MSCs (71.6 ± 0.88% vs. 80.3 ± 0.33%) (Fig. 5B) and the absolute HDR positive cells were 2-fold higher than the SC (Fig. 5C).

This underscores the ability of GM-MSCs to support gene-edited cell expansion without cytokines while mitigating toxicity associated with RNP-ssODN-mediated HDR editing and AZD-7648 treatment. The GM-MSCs not affected erythroid differentiation, as the proportion of erythroid subsets remained unchanged relative to SC and controls (Fig. 5D, Supplementary Fig. 4A). Importantly, erythroblasts originated from HSPCs cultured on GM-MSCs showed significant upregulation of

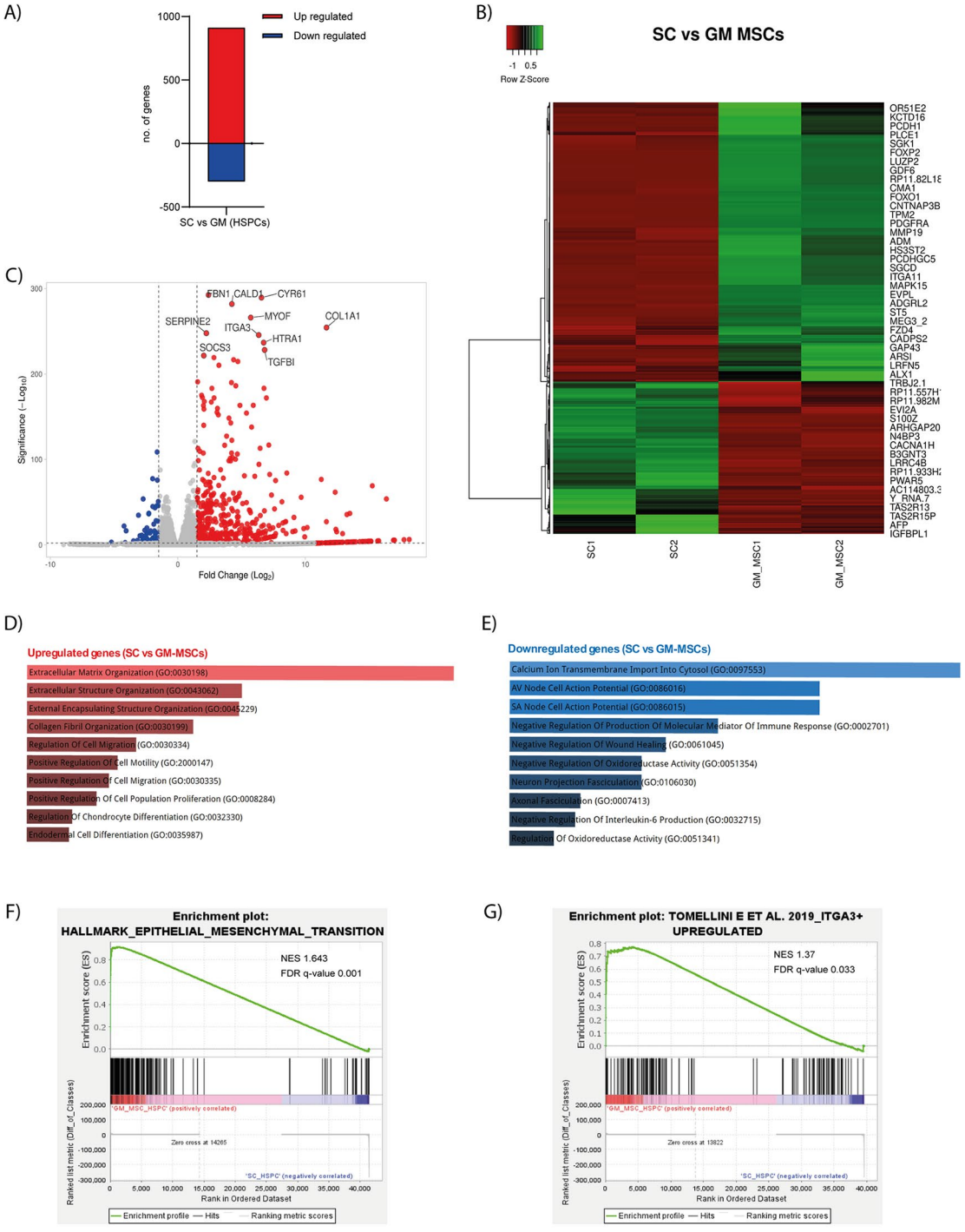

Fig. 3 Transcriptomic analysis of HSPCs cultured in SC and GM-MSCS. (**A**) Bar graph represents the number of genes up regulated and down regulated in HSPCs cocultured with GM-MSCs versus SC ($n = 2$ and donor = 1). (**B**) Heat map shows the top differentially expressed genes in HSPCs cocultured with GM-MSCs versus SC ($n = 2$ and donor = 1). (**C**) Volcano plot displaying differentially expressed genes in HSPCs cultured with GM-MSCs compared to SC. Genes with a statistically significant (padj < 0.01) and substantial (≥ 1.5 log2 fold change) difference in expression are shown in colour: red for upregulated genes and blue for downregulated genes in GM-MSCs ($n = 2$ and donor = 1). (**D**) Gene ontology analysis of upregulated genes highlights enriched biological processes associated with the gene sets ($n = 2$ and donor = 1). (**E**) Gene ontology analysis of downregulated genes highlights enriched biological processes associated with the gene sets ($n = 2$ and donor = 1). (**F**) GSEA analysis of Hallmark Epithelial-Mesenchymal Transition gene set in GM-MSC co-cultured HSPCs (NES = 1.643) compared to SC ($n = 2$ and donor = 1). (**G**) GSEA analysis performed on enriched gene sets in ITGA3^{+} HSPCs fraction obtained from Tomellini E. et al., ($n = 2$ and donor = 1)

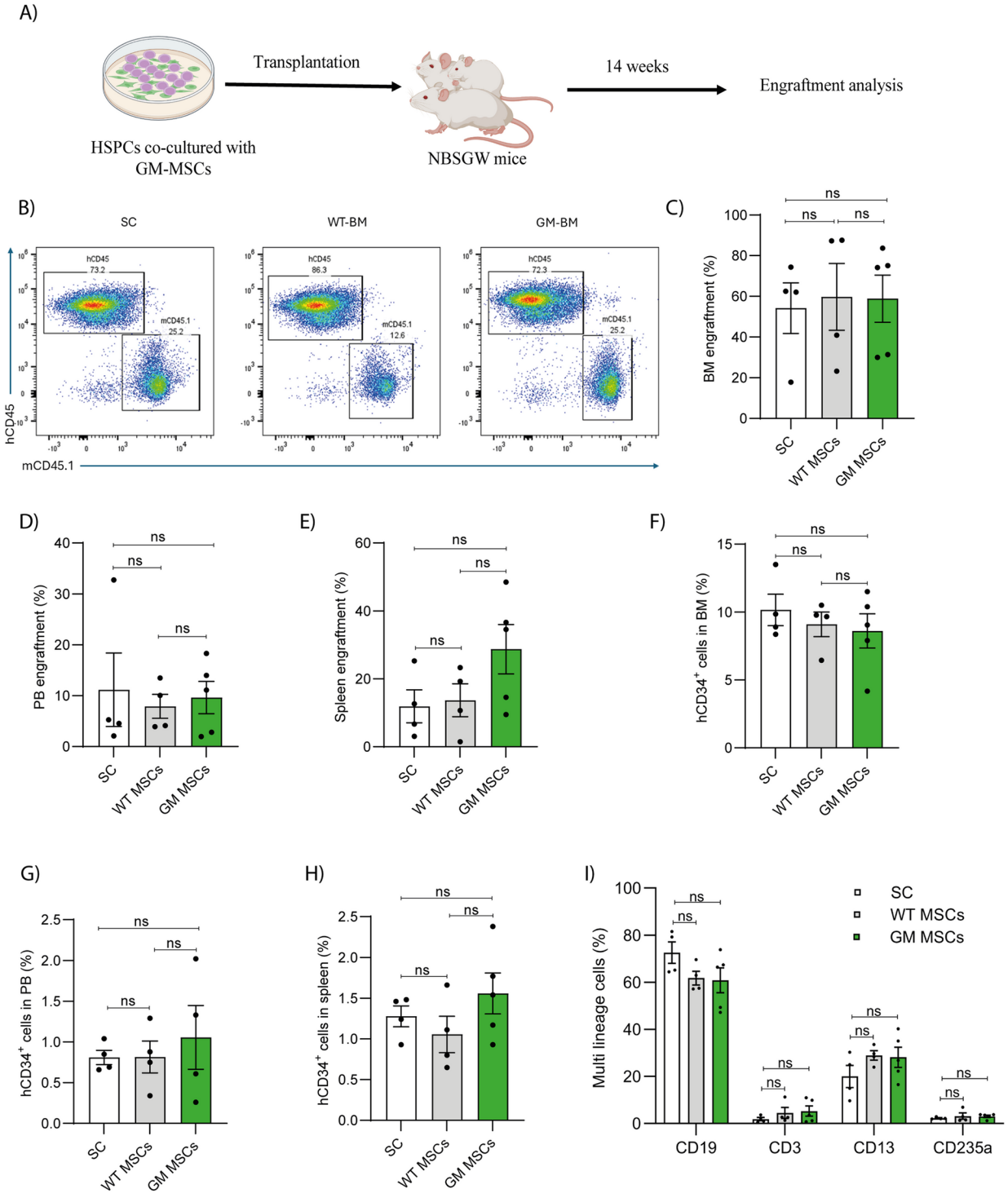

Fig. 4 GM-MSCs cocultured HSPCs maintains engraftment potential *in vivo*. (**A**) representative image of the *in vivo* experimental plan. (**B**) Representative flow cytometry plots of gating strategy of hCD45+ cells 14th week in the mouse bone marrow. (**C**) Percentage of human cells engraftment in the bone marrow 14 weeks post infusion. (**D**) Percentage of human cells engraftment in the peripheral blood 14 weeks post infusion. (**E**) Percentage of human cells engraftment in the spleen 14 weeks post infusion. (**F**). Percentage of hCD34+ cells present in the engrafted hCD45+ cells in the BM 14 weeks after transplantation. (**G**) Percentage of hCD34+ cells present in the engrafted hCD45+ cells in the PB 14 weeks after transplantation. (**H**) Percentage of hCD34+ cells present in the engrafted hCD45+ cells in the spleen 14 weeks after transplantation. (**I**) The multilineage reconstitution of human cells in the mouse BM 14 weeks after transplantation. Lymphoid cells (CD3 and CD19), myeloid cells (CD13), erythroid cells (CD235a) and HSPCs (CD34). Error bars represent mean ± SEM. Each dot represents individual mice. (Donor =1). (n=4-5; mean±SEM; ordinary one-way ANOVA (Sidak's multiple comparison test). (Schematic illustrations were created with BioRender.com)

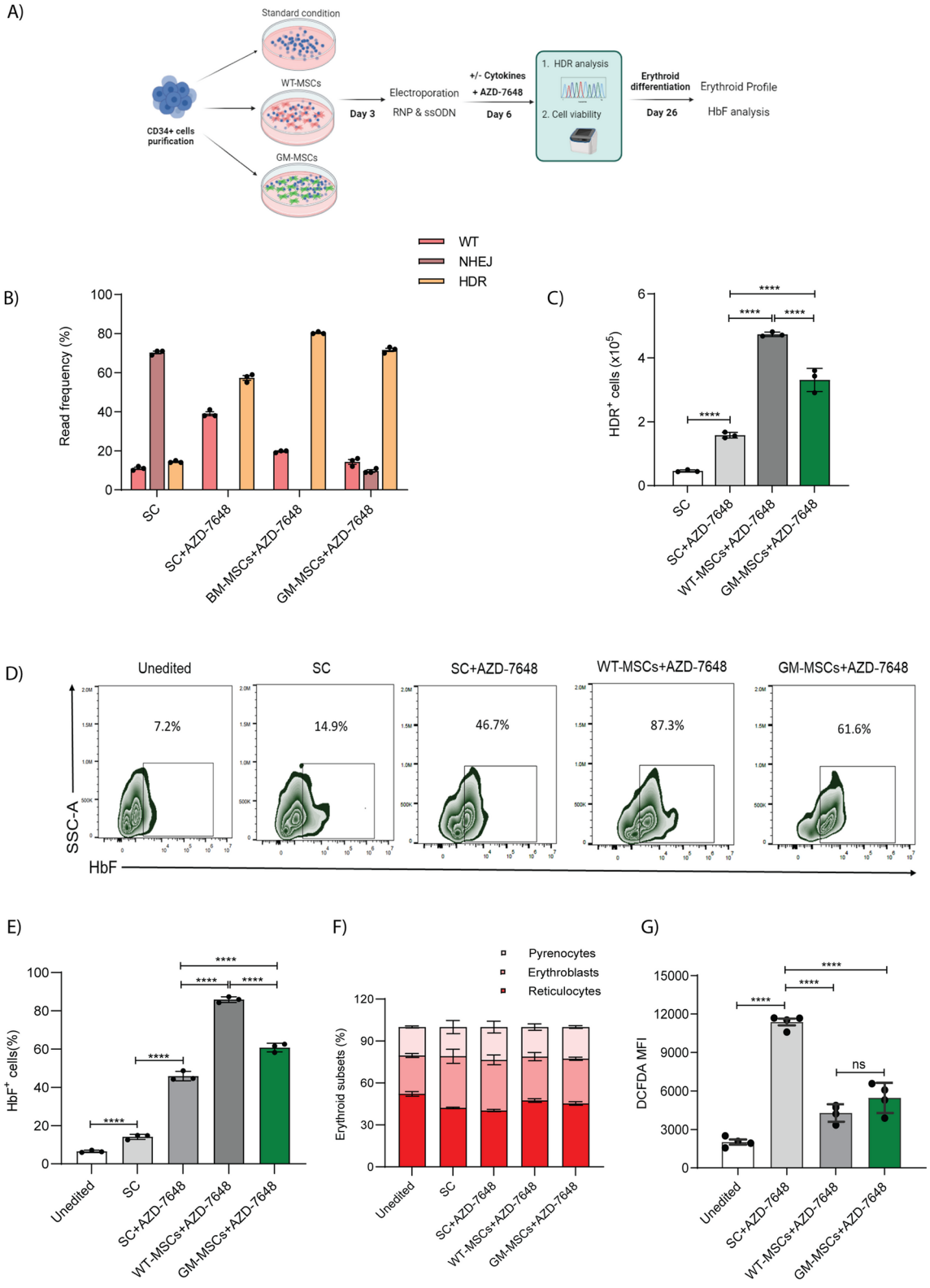

Fig. 5 (See legend on next page.)

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Fig. 5 +Figure 5. GM-MSCs expanded edited HSPCs enhances the fetal hemoglobin production (**A**) Schematic representation of the experimental workflow for evaluating the gene-editing efficiency of HSPCs co-cultured with WT-MSCs and GM-MSCs and erythroid differentiation analysis of edited HSPCs. The purified CD34++ cells were co-cultured with WT-MSCs and GM-MSCs or cultured under regular protocol for three days. After 3 days, Cells were electroporated using RNP and ssODN in lonza nucleofector. The edited cells were treated with AZD-7648 for 24 hours and further expanded on WT-MSCs and GM-MSCs or cultured under regular protocol. During 72 hours, cell viability analysis and Cells were collected for sequencing. Further cells were seeded in erythroid differentiation medium and differentiated 20 days for HbF analysis. (**B**) Read frequency corresponding to HDR, NHEJ indels and WT sequences in the genotyped samples analysed by sanger sequencing and synthego ICE-Knock-in analysis. The standard condition (SC) refers to regular culture condition where HSPCs were cultured in cytokine culture medium. The SC condition was tested with or without AZD-7648. Edited HSPCs were cultured with WT-MSCs and GM-MSCs in cytokine culture medium or without cytokines both in presence of AZD-7648. (mean±SEM for n=3). (**C**) Absolute HDR positive cells was measured on 72 hours post electroporation for different conditions. (mean±SEM for n=3; donor=1; ordinary one-way ANOVA (Sidak's multiple comparison test)). (**D**) Representative flow cytometry plots of gating strategy of HbF+ cells of erythroid differentiated cells from edited cells. (**E**) Percentage of HbF+ cells generated on in vitro erythroid differentiation of edited HSPCs cultured using standard protocol or with WT-MSCs and GM-MSCs. (mean±SEM for n=3; donor=1; ordinary one-way ANOVA (Sidak's multiple comparison test)). (**F**) Proportion of erythroid subsets generated on day 20 of erythroid differentiation for different co-culture conditions. The populations are gated as reticulocytes, erythroblast and pyrenocytes. (mean±SEM for n=3). *** p<0.001; **** p<0.0001. (**G**) The mean fluorescence intensity of DCFDA+ staining was used to detect reactive oxygen species (ROS) in edited HSPCs after 48 hours co-culture with WT-MSCs and GM-MSCs. (mean±SEM for n=4). **** p<0.0001. (Schematic illustrations were created with BioRender.com)

HbF^+ cells over the SC (60.8 ± 1.33% vs. 45.8 ± 1.43%). However, it could not outcompete the WT-MSCs group (85.86 ± 0.86%) (Fig. 5E and F). The ROS levels in the edited HSPCs co-cultured with GM-MSCs post-editing were lower compared to the SC (Fig. 5G). This could be a potential reason for the improved viability of HDR positive cells. Collectively, these findings highlight that GM-MSC-based coculture conditions provide an effective platform for ssODN-mediated HDR editing by mitigating the toxicity associated with ssODN and AZD-7648 treatment. Furthermore, GM-MSC coculture with AZD-7648 represents a promising universal platform for HDR-based genome-editing strategies.

Discussion

The functional fitness of HSCs determines the success of the transplantation. The current HSPCs gene manipulation protocols rely on ex vivo culture, where any compromise of their stemness hamper the success of the gene therapy. Here, we report the development of GM-MSCs engineered to secrete hematopoietic supportive growth factors – SCF, FLT3L, TPO and IL3 in addition to its characteristic paracrine stemness promoting factors, direct cell-cell interactions and extracellular matrix components mimicking the natural bone marrow niche for ex vivo culture of HSPCs.

Our results demonstrated that GM-MSCs secreted the four cytokines, typically supplemented in ex vivo culture of HSPCs, and augmented their proliferation in a five-day culture period. Prevalent supplementation of these factors in HSPCs clinical studies, signifies their importance in the ex vivo culture. Owing to their natural supportive cellular niche of HSCs, BM MSCs are being explored for HSPCs cultures for a superior transplantation outcome [33, 34]. However, these findings explored MSCs as feeder layers with externally supplemented cytokines for proliferation of HSPCs. The exploration of MSCs, intrinsically secreting 4 F essential cytokines not only ease the preparation of the HSPCs culture medium but also improves the stemness, recapitulating the bone marrow niche (Fig. 2C-G). Although a comprehensive long-term stability analysis was not within the scope of this study, all functional assays were performed using GM-MSCs at passages 4 to 5 post-transduction. During this window, we consistently observed stable cytokine secretion and robust HSPC support, indicating that the engineered MSCs retain their functional properties during early expansion.

In addition to supportive role, these GM-MSCs modulate the stemness of the cells through multiple pathways including extracellular matrix reorganisation, migration and calcium ion transport (Fig. 3D). Notably, recent findings indicate that reduced calcium levels in the culture media lead to lower intracellular calcium levels in HSCs compared to progenitors. Moreover, culturing HSPCs under these conditions enhances stemness, as evidenced by single-cell RNA sequencing and transplantation studies [35–37]. Based on our RNA-sequencing analysis, we observed that numerous factors typically attributed to MSCs are also robustly expressed in cocultured HSPCs. This overlap is likely mediated by intercellular communication through exosomes, highlighting the potential influence of MSC-derived exosomal cargo on HSPC biology. These findings underscore a compelling, yet incompletely understood, mechanism of MSC-HSPC crosstalk. Elucidating how exosome-delivered factors modulate HSPC fate decisions will be a critical focus for future studies, with significant implications for understanding stem cell maintenance, self-renewal, and lineage commitment. In addition, our RNA seq experiments on the HSPCs show a clear downregulation of genes involved in calcium signalling, indicating one of the mechanisms by which GM-MSCs influence stemness (Fig. 3E). The increased expression of ITGA3 in the GM-MSC cultured HSPCs is a strong indicator of the stemness of the HSCs (Fig. 3G). This observation was consistent with the in vivo

transplantation assays where HSPCs expanded in co-culture with GM-MSCs demonstrated robust engraftment in bone marrow, spleen, and peripheral blood, accompanied by multilineage differentiation potential equivalent to that of standard cytokine-supplemented controls. The ability of GM-MSC-expanded HSPCs to retain their engraftment and differentiation capacities underscores the therapeutic relevance of this platform.

HDR gene editing of HSPCs is limited by the efficiency and toxicity of the HDR donors. In our prior findings, we showed that ssODN donors and DNA-PK inhibitors imparted significant toxicity on the HSPCs and this effect could be mitigated with co-culturing MSCs [17]. We extended that observation here to show that GM-MSCs could be explored as a feeder layer to prepare the HDR-edited HSPCs for gene therapy applications. We demonstrate the co-introduction of -175T > C and −158 C > T conversions in the HBG promoter at an efficiency of around 70% and these resulted in the activation of HbF in the erythroblasts (Fig. 5B-G) suggesting the application of this approach for the gene therapy for beta-hemoglobinopathies. While the sgRNA used in this study is free from off-target editing [17], the impact of global suppression of NHEJ DNA repair by AZD-7648 needs detailed investigation [38, 39].

Exploring GM-MSCs also address the high costs and limitation of traditional HSPCs expansion methods that relay on cytokine cocktail. This alternative strategy, devoid of external supplementation of cytokines makes cell therapy manufacturing process economically viable and scalable. Similar cost-effective strategies are increasingly recognized as a pre-requisite for the widespread adoption of advanced cell therapies [25, 40].

Importantly, this opens up an area where additional HSCs influencing factors can be overexpressed to mitigate the editing associated concerns. AZD-7648 has a strong influence in HDR gene editing, but also introduces large apparent deletions [38]. Expressing antagonists of DNA-PKs along with the cytokines may overcome the reliance on AZD-7648, and its unwarranted side effects. Also, such GM-MSCs could be an ideal candidate to expand UCB HSPCs for a longer duration.

Despite the efforts, multiple limitations need to be addressed in this study. A key limitation lies in the inability to precisely define or modulate the concentration of cytokines secreted by GM-MSCs. While prior studies have demonstrated a wide range of effective

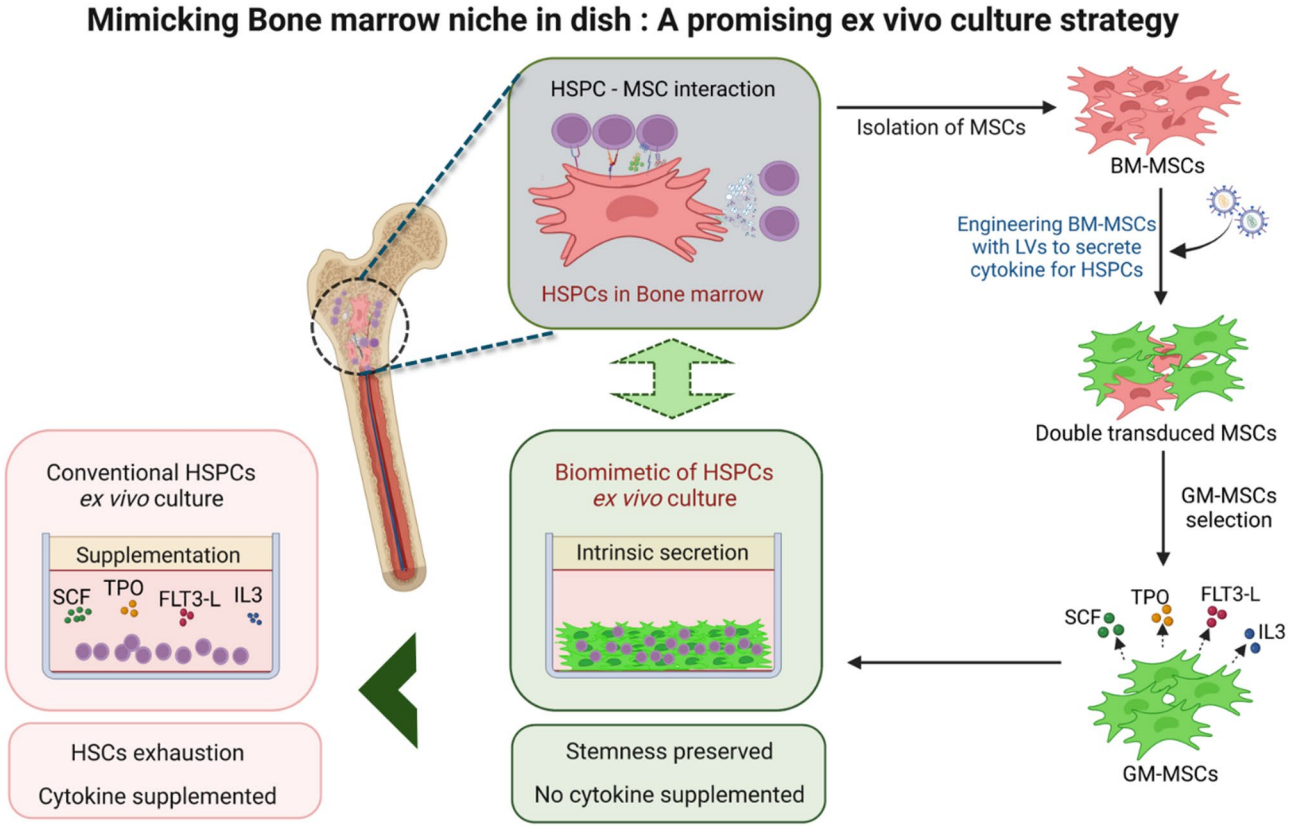

Fig. 6 Graphical abstract of this study. Our GM-MSCs express key growth factors, including SCF, FLT3L, TPO, and IL3, to support HSPC expansion through endogenous cytokine supplementation. Co-culturing with engineered MSCs supplies essential growth factors, effectively mimicking the bone marrow niche and further enhancing HSPC expansion while maintaining stemness. (Schematic illustrations were created with BioRender.com)

concentrations for cytokines such as SCF and FLT3L (50–300 ng/mL) [34, 41], IL-3 presents a particular challenge due to its narrow optimal range (20–50 ng/mL) [23, 42], where deviations may negatively impact long-term HSC maintenance. Recent findings also suggest that cytokine-free expansion systems using small molecule agonists may offer a viable alternative for sustaining primitive HSPC populations [43]. Moreover, as primary cells, MSCs gradually lose their proliferative capacity with increasing passage number. To support scalable and long-term HSPC culture, immortalization of GM-MSCs represents a compelling strategy. Approaches such as ectopic expression of human telomerase reverse transcriptase (hTERT), in combination with shRNA-mediated knockdown of cell cycle regulators like $p16^{INK4a}$ or p53, have been shown to significantly prolong MSC lifespan while preserving their hematopoietic support and immunomodulatory properties [44, 45]. Concurrently, the establishment of standardized cell banking systems including master and working cell banks will be essential to ensure batch-to-batch consistency, minimize donor-to-donor variability, and align with GMP standards for clinical-grade applications. Furthermore, although our data suggest that GM-MSCs mitigate cellular stress associated with HDR-mediated gene editing, a more detailed mechanistic analysis—including evaluation of p53 activation, γH2AX accumulation, chromosomal integrity at the target site, and potential off-target effects are necessary to strengthen the translational relevance of this platform. Finally, we acknowledge that the use of HSPCs from a single donor in the in vivo transplantation experiments represents a limitation of this study. While the engraftment outcomes are consistent with our previous work, incorporating additional donors in future experiments could help address potential donor-dependent variability and further strengthen the robustness of our conclusions.

Conclusions

In summary, our study addresses one of the critical challenges in culturing adult human HSPCs ex vivo with preserving the stemness (Fig. 6), in turn facilitating gene therapy applications for haematological disorders. The potential of GM-MSCs to address various challenges in HSPCs manipulation and expansion presents exciting avenues for future research and clinical applications.

Abbreviations

HSPCs	Hematopoietic stem and progenitor cells
SCF	Stem cell factor
TPO	Thrombopoietin
FLT3L	FMS–like tyrosine kinase–3–ligand
IL3	Interleukin–3
BM	Bone marrow
WJ	Wharton's jelly
MSCs	Mesenchymal stem cells
GM	MSCs–genetically modified mesenchymal stromal cells
ssODN	Single–stranded oligonucleotide
HDR	Homology–directed repair
NHEJ	Nonhomologous end joining
HbF	Fetal hemoglobin
RNP	Ribonucleoprotein
NBSGW	NOD. Cg–$Kit^{W–41J}$ Tyr^{+} $Prkdc^{scid}$ $Il2rg^{tm1Wjl}$/ThomJ
TNC	Total nucleated cells

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-025-04474-4.

Supplementary Material 1

Acknowledgements

ST acknowledges the funding support from the Department of Biotechnology, Ministry of Science and Technology, Government of India through the grants BT/PR26901/MED/31/377/2017, BT/PR31616/MED/31/408/2019, BT/PR38267/GET/119/348/2020, and BT/PR45683/MED/31/465/2022. S.M. thanks Dr. Alok Srivastava (Former head), CSCR for providing CSCR core-funding. V.V. thanks ICMR-New Delhi, India for the ICMR-SRF fellowship and P.B. thanks CSIR-New Delhi, India for JRF fellowship. We thank Porkizhi Arjunan and Ramya Arumugam for their help in this study. We thank the staff of the flow cytometry, animal facility, and core facilities of CSCR for their technical support.
Use of AI: We used chatGPT and Perplexity for English language editing.

Author contributions

Conceptualization: S.M., S.K., and S.T. Experimental execution and analysis: S.S., V.V., M.K.A., G.M., and P.B. Technical supervision: M.K.K., S.M., and S.T. Manuscript – review & editing: S.S., V.V., M.K.A., P.B., S.M., M.K.K and S.T. Funding acquisition: S.M., and S.T.

Funding

The authors thank the funders of the Department of Biotechnology, Ministry of Science and Technology, Government of India.

Data availability

RNA sequence datasets are available in the Gene Expression Omnibus repository (GEO: GSE292072). The datasets used and/or analysed during the current study can be available upon reasonable request from the corresponding author.

Declarations

Ethics approval

The title of study "In vivo efficacy and safety studies of CSCR-ST04, the gene edited autologous hematopoietic stem cells for the gene therapy of β-hemoglobinopathies". This study was approved by the IRB committee of the Christian Medical College Vellore (Approval number: 11807) dated: 30.01.2019. The study titled "Therapeutic applications of extracellular vesicles-derived from 3D-cultured human Wharton's Jelly Mesenchymal stromal cells (hWJ-MSCs)". was approved by the IRB committee of Christian Medical College, Vellore (Approval Number: 14495), dated 23.02.2022.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 17 March 2025 / Accepted: 18 June 2025
Published online: 01 July 2025

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