Communication

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Ex Vivo Lentiviral *VPS33B* Gene Therapy Restores Platelet α-Granule Number in a Murine Model of ARC Syndrome

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Abstract

Arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome is a rare, usually fatal autosomal recessive multisystem disorder caused by biallelic mutations in *VPS33B* or *VIPAS39*, which encode components of a vesicular trafficking complex required for organelle biogenesis. Loss of function causes a platelet α-granule deficiency and a severe bleeding diathesis, and no curative therapy exists. Building on the efficacy of ex vivo lentiviral gene therapy in inherited haematological disorders, we evaluated haematopoietic stem cell (HSC) gene delivery in a tamoxifen-inducible *Vps33b* knockout mouse model (*Vps33b*$^{fl/fl}$-CreER^{T2}). Knockout mice tolerated myeloablative conditioning and supported efficient donor reconstitution after transplantation of healthy lineage-negative cells. We then transduced lineage-negative cells from knockout donors ex vivo with a third-generation lentiviral vector encoding codon-optimised human *VPS33B* under an elongation factor 1α short (EFS) promoter and transplanted them into conditioned knockout recipients. Transmission electron microscopy showed that platelet α-granule numbers were restored to wild-type levels after both bone marrow transplantation and ex vivo gene therapy. These findings demonstrate the feasibility of haematopoietic *VPS33B* gene delivery and support further preclinical evaluation of gene therapy for the bleeding phenotype of ARC syndrome.

Keywords: ARC syndrome; *VPS33B*; platelet α-granules; haematopoietic stem cell gene therapy; lentiviral vector; storage pool disorder; thrombopoiesis; rare disease

1. Introduction

Arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome is a severe autosomal recessive multisystem disorder characterised by progressive cholestatic liver disease, renal tubular dysfunction, congenital arthrogryposis, ichthyosis, and a haematological phenotype that combines platelet dysfunction with susceptibility to severe infection [1–3]. Most affected children die in the first year of life, commonly from gastrointestinal haemorrhage or infection, and no disease-specific therapy exists [1]. ARC syndrome is caused by biallelic loss-of-function mutations in *VPS33B* or *VIPAS39*, encoding VPS33B and VIPAR (also termed VPS16B), which form a cytosolic complex that regulates SNARE-dependent vesicular trafficking required for the biogenesis of specialised

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organelles in epithelia, megakaryocytes and immune cells [1,2,4–7,16]. The bleeding tendency reflects a profound platelet storage-pool disorder caused by defective α-granule biogenesis in megakaryocytes, with markedly impaired platelet aggregation [4,9]. Importantly, individuals with hypomorphic *VPS33B* alleles exhibit attenuated phenotypes [2], indicating that even partial restoration of VPS33B function may be beneficial.

Ex vivo lentiviral transduction of autologous haematopoietic stem and progenitor cells (HSPCs) has produced durable clinical benefit across an expanding range of inherited haematological and immunological disorders [10–14], enabling stable transgene integration without the alloimmune complications of allogeneic transplantation. In parallel work, we have developed liver-directed *in vivo* lentiviral gene therapy that corrects the hepatic phenotype of ARC syndrome [17], addressing a disease compartment distinct from the haematopoietic defect considered here. In the present study we tested whether *ex vivo* haematopoietic gene therapy could increase platelet α-granule number in a tamoxifen-inducible *Vps33b* knockout mouse model (*Vps33b*$^{fl/fl}$-CreER^{T2}), evaluating the feasibility of haematopoietic transplantation and *ex vivo VPS33B* gene delivery.

2. Results

Because haematopoietic stem cell transplantation (HSCT) had not previously been evaluated in the ARC murine model, we first tested whether tamoxifen-induced knockout (KO) mice tolerate myeloablation. Adult KO mice received split-dose total body irradiation (6 + 5 Gy) followed by intravenous transplantation of 0.5×10^6 lineage-negative (Lin$^-$) cells from healthy male donors (Figure 1A). All recipients (n = 3) survived the early post-transplant period and engrafted efficiently, with donor-derived chimerism approaching 100% in peripheral blood (Figure 1B). Spleen weights trended lower after HSCT but remained elevated relative to wild-type (WT), as previously reported [9] (Figure 1C). These data establish the feasibility of haematopoietic transplantation in this model and provided the basis for the gene therapy experiments.

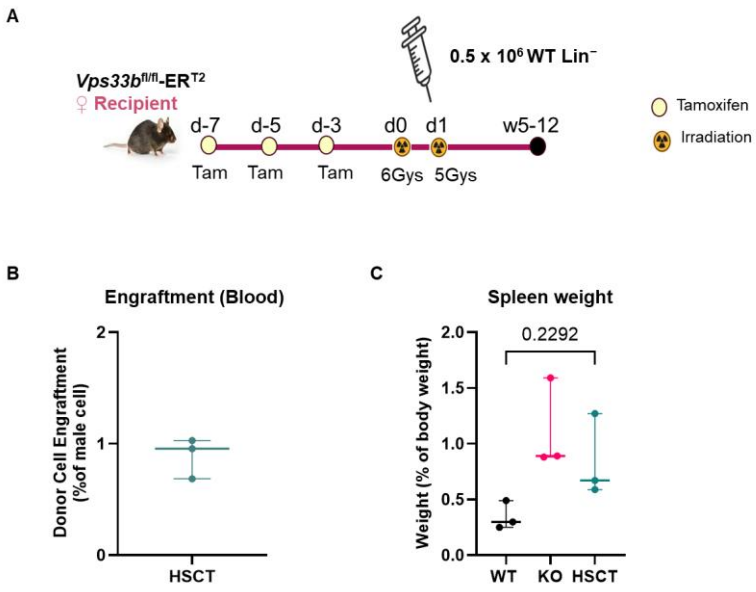

Figure 1. *Vps33b*$^{fl/fl}$-CreER^{T2} mice tolerate myeloablation and support efficient haematopoietic reconstitution. (A) Experimental schematic: *Vps33b*$^{fl/fl}$-CreER^{T2} mice received tamoxifen (d−7, d−5, d−3) to induce *Vps33b* knockout, split-dose total body irradiation (6 Gy on d0 and 5 Gy on d1) and intravenous transplantation of 0.5×10^6 Lin$^-$ cells from healthy male wild-type donors on d1; recipients were analysed between weeks 5 and 12. (B) Donor-derived chimerism in recipient peripheral blood, quantified by Y-chromosome qPCR. (C) Spleen weight (percentage of body weight) in wild-type (WT), untreated knockout (KO) and haematopoietic stem cell transplantation (HSCT) groups. Graphs show individual values, medians and interquartile ranges (IQRs); n = 3; p-value by one-way ANOVA with Tukey post-hoc correction.

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We next transduced Lin⁻ cells from KO donors *ex vivo* with a third-generation, VSV-G–pseudotyped, self-inactivating lentiviral vector encoding codon-optimised human *VPS33B* under the EFS promoter (EFS.coh*VPS33B*), and transplanted them into conditioned KO recipients (Figure 2A,B). At six weeks, gene therapy (LV-GT) recipients showed near-complete donor chimerism in bone marrow (Figure 2C) and stable gene marking (mean vector copy number ≈ 0.4 vg/dg; Figure 2D). Codon-optimised *VPS33B* transcript was readily detected in bone marrow from LV-GT recipients but not in WT controls, confirming transgene expression (Figure 2E). Spleen weight remained elevated relative to WT (Figure 2F). Serum procalcitonin, markedly raised in untreated KO mice ($p = 0.0002$ vs WT), was not significantly different from WT after gene therapy ($p = 0.10$), consistent with partial correction of the systemic inflammatory phenotype (Figure 2G).

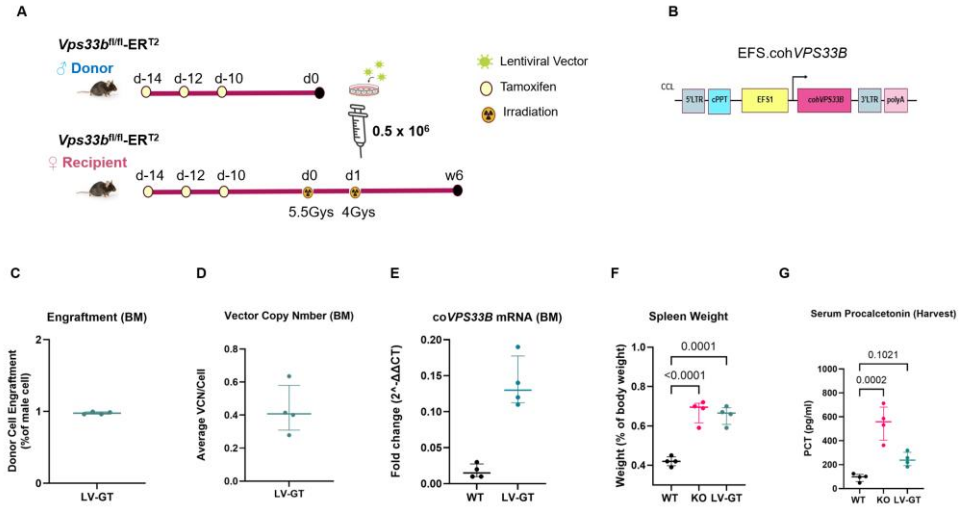

Figure 2. *Ex vivo* lentiviral *VPS33B* gene therapy enables stable engraftment and transgene expression. (A) Schematic of the LV-GT strategy: male donor and female recipient *Vps33b*$^{fl/fl}$-CreER^{T2} mice received tamoxifen (d−14, d−12, d−10); on d0 donor Lin⁻ bone marrow cells were transduced *ex vivo* with the EFS.coh*VPS33B* lentiviral vector and 0.5×10^6 cells were transplanted intravenously on d1 into conditioned recipients (5.5 Gy on d0 and 4 Gy on d1); mice were analysed at 6 weeks. (B) Lentiviral vector schematic (5′LTR, central polypurine tract [cPPT], EFS promoter, codon-optimised human *VPS33B* [coh*VPS33B*], 3′LTR and polyA). (C) Donor chimerism in recipient bone marrow by Y-chromosome qPCR. (D) Mean vector copy number per cell. (E) Codon-optimised *VPS33B* expression in bone marrow by RT-qPCR, normalised to *β-actin* ($2^{-\Delta\Delta Ct}$). (F) Spleen weight (percentage of body weight) in WT, KO and LV-GT mice. (G) Serum procalcitonin (PCT). Graphs show individual values, medians and IQRs; n = 4 per group; p-values by one-way ANOVA with Tukey post-hoc correction.

Transmission electron microscopy (TEM) of platelets from untreated ARC mice showed the characteristic ultrastructural defect: markedly reduced α-granule numbers, frequent empty vacuoles and smaller residual α-granule-like structures (Figure 3A). α-Granules were quantified manually in 20 platelets per animal by three investigators blinded to genotype, using established morphological criteria [9]. Both HSCT and *ex vivo VPS33B* gene therapy significantly increased α-granule counts relative to untreated ARC mice (HSCT vs KO $p = 0.0113$; LV-GT vs KO $p = 0.0104$), restoring numbers to levels indistinguishable from WT (HSCT vs WT $p = 0.6220$; LV-GT vs WT $p = 0.4523$; Figure 3B). The mean number of α-granules per platelet increased from ≈ 1 in untreated KO mice to 3.0 (HSCT) and 3.5 (LV-GT), comparable to the WT mean of ≈ 4.

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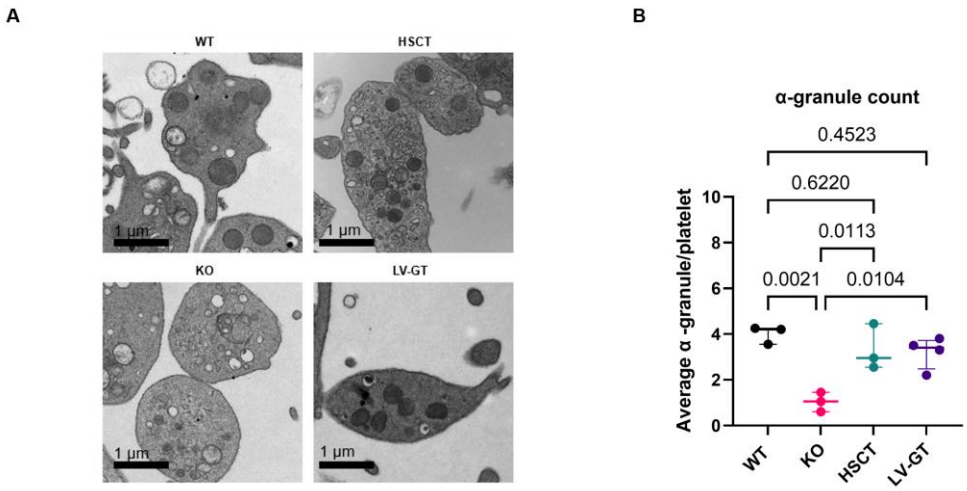

Figure 3. Restoration of *VPS33B* expression restores platelet α-granule number. (A) Representative transmission electron micrographs of platelets from wild-type (WT), untreated knockout (KO), haematopoietic stem cell transplantation (HSCT) and gene therapy (LV-GT) mice; platelets from KO mice show markedly reduced α-granule content, with empty vacuoles and smaller α-granule-like structures. Scale bars, 1 µm. (B) Platelet α-granule number per platelet, averaged across 20 platelets per animal by three blinded investigators. Graph shows individual values, medians and IQRs; n = 3 per group (n = 4 for the LV-GT group); p-values by one-way ANOVA with Tukey post-hoc correction.

3. Discussion

These findings provide proof-of-concept that re-establishing VPS33B expression within the haematopoietic compartment increases platelet α-granule number and ameliorates the systemic proinflammatory phenotype in a mouse model of ARC syndrome. The near-complete restoration of α-granule number was achieved at a modest mean vector copy number (≈ 0.4 copies/cell), suggesting that low transgene dosing is sufficient to influence megakaryocyte organelle number. The EFS promoter, an internal mammalian promoter associated with reduced genotoxicity relative to viral long terminal repeats and used in several *ex vivo* gene therapy trials [10], supports a translationally favourable profile.

Several considerations temper the interpretation of these results and define the next phase of work. First, our readout is the number of platelet α-granules by TEM; we did not assess α-granule cargo content or platelet function, so we describe restoration of α-granule number rather than full correction of the α-granule deficiency or of haemostasis. Functional confirmation (platelet aggregation, secretion and bleeding time) together with biochemical quantification of α-granule cargo will be required. Of note, only minor changes in platelet aggregation and bleeding time were detectable in this model previously [9], so these murine assays are relatively insensitive here. Second, the study establishes feasibility; formal confirmation of the extent and kinetics of *Vps33b* ablation in the transplanted donors and recipients, and the use of longer induction intervals as in earlier work [9], would further validate the model. Third, longer follow-up and larger, sex-balanced cohorts will be needed to assess durability and any insertional-mutagenesis risk. Finally, ARC syndrome is a multisystem disorder with hepatic, renal and neurological involvement and severe failure to thrive; haematopoietic gene therapy would address only the platelet/bleeding phenotype, and any clinical application would require careful consideration of patient selection and of the feasibility of stem-cell harvesting and myeloablative conditioning in affected infants. These results should therefore be interpreted as an early feasibility step rather than a complete therapeutic solution.

In principle, the same *ex vivo*, EFS-driven platform could be explored for other inherited platelet α- or δ-granule disorders, such as *VIPAS39*/*VPS16B*-related ARC, Gray platelet syndrome (*NBEAL2*) and Hermansky–Pudlak syndrome, by substitution of the therapeutic transgene, recognising that

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disorders with additional non-haematopoietic manifestations would require complementary approaches.

4. Materials and Methods

All animal work was conducted in compliance with UK Home Office regulations under project licence PP9223137. A tamoxifen-inducible conditional *Vps33b* knockout model (*Vps33b*$^{fl/fl}$-CreER^{T2}) was used as previously described [9]. Tamoxifen dosing, conditioning regimens, transplanted cell doses and analysis time points are detailed in the figure legends. Female recipients received lineage-negative bone marrow (Lin$^-$) cells from male donors to enable Y-chromosome–based chimerism assessment. *Vps33b*$^{fl/fl}$-CreER^{T2} mice were allocated to WT (no tamoxifen exposure) or tamoxifen-induced knockout (KO) groups; KO mice were further assigned to untreated, HSCT or *ex vivo* lentiviral gene therapy (LV-GT) arms. Lin$^-$ cells were isolated by magnetic lineage depletion (Miltenyi Biotec, Bergisch Gladbach, Germany). For LV-GT, Lin$^-$ cells were transduced *ex vivo* with a third-generation, VSV-G–pseudotyped, self-inactivating lentiviral vector (EFS.coh*VPS33B*) encoding codon-optimised human *VPS33B* under the elongation factor 1α short (EFS) promoter at a multiplicity of infection of 2 by spinoculation (600 × g, 60 min). Cells were cultured overnight in StemSpan SFEM with mSCF (100 ng/mL), mFlt3L (100 ng/mL) and hTPO (25 ng/mL) before intravenous transplantation. Donor chimerism and vector copy number (VCN) were quantified by multiplex quantitative PCR (Y-chromosome and proviral targets, respectively). Codon-optimised *VPS33B* expression was measured by RT-qPCR normalised to *β-actin*. Blood was collected by cardiac puncture into acid-citrate-dextrose; platelets were processed for transmission electron microscopy and α-granule quantification as described [9], with α-granules counted manually in 20 platelets per animal by three investigators blinded to genotype. Haematopoietic populations were analysed by flow cytometry (BD FACSymphony A5). Statistical comparisons used unpaired Student's t-test or one-way ANOVA with Tukey post-hoc correction (GraphPad Prism v10); $p < 0.05$ was considered significant.

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Institutional Review Board Statement: All animal procedures were conducted in compliance with UK Home Office regulations under project licence PP9223137.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author. Lentiviral plasmid maps will be deposited in Addgene upon acceptance.

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Conflicts of Interest: The authors declare no competing financial interests. P.G. is a Guest Editor of the Special Issue "Individualised Therapies for Rare Disorders" in which this article is submitted; the manuscript was

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