

# Preclinical studies of efficacy thresholds and tolerability of a clinically ready lentiviral vector for pyruvate kinase deficiency treatment

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**Pyruvate kinase deficiency (PKD) is a rare autosomal recessive disorder caused by mutations in the *PKLR* gene. PKD is characterized by non-spherocytic hemolytic anemia of variable severity and may be fatal in some cases during early childhood. Although not considered the standard of care, allogeneic stem cell transplantation has been shown as a potentially curative treatment, limited by donor availability, toxicity, and incomplete engraftment. Preclinical studies were conducted to define conditions to enable consistent therapeutic reversal, which were based on our previous data on lentiviral gene therapy for PKD. Improvement of erythroid parameters was identified by the presence of 20%–30% healthy donor cells. A minimum vector copy number (VCN) of 0.2–0.3 was required to correct PKD when corrected cells were transplanted in a mouse model for PKD. Biodistribution and pharmacokinetics studies, with the aim of conducting a global gene therapy clinical trial for PKD patients (RP-L301-0119), demonstrated that genetically corrected cells do not confer additional side effects. Moreover, a clinically compatible transduction protocol with mobilized peripheral blood CD34<sup>+</sup> cells was optimized, thus facilitating the efficient transduction on human cells capable of repopulating the hematopoiesis of immunodeficient mice. We established conditions for a curative lentiviral vector gene therapy protocol for PKD.**

## INTRODUCTION

Pyruvate kinase (PK) deficiency (PKD) is a rare anemia syndrome caused by mutations in the *PKLR* gene. Despite its rarity, PKD is the most frequent congenital anemia caused by a hereditary defect of the glycolytic pathway. PKD exhibits autosomal recessive inheritance, and, to date, more than 300 different *PKLR* mutations have been identified causing PKD, which indicates a very high molecular and clinical heterogeneity.<sup>1–3</sup> The frequency of PKD is not well

defined, and prevalence estimates have ranged between 3:1,000,000 and 1:20,000.<sup>4</sup> Genotype-phenotype correlative and PKD natural history studies<sup>5</sup> have suggested that patients with severe hemolytic anemia more commonly have deleterious mutations or missense pathogenic variants that affect the active site or stability of the RPK protein.<sup>6</sup> Severity of symptomatology is variable and involves several organs, although the predominant clinical manifestation is anemia, which is present in 90%–95% of cases, varying from mild to transfusion dependent. Splenomegaly is also present in the majority of PKD patients (80%–85%) with a variable degree of enlargement. Jaundice (40%–70%) and gallstones (30%–45%) are also common complications.<sup>7</sup> Aplastic crises (2%–14%), bone deformities (9%), extramedullary erythropoiesis (9%), delayed puberty (8%), hyperpigmentation (6%), and leg ulcers and pulmonary hypertension (2%–3%) are less common manifestations.<sup>2,6–8</sup> *In utero* complications and neonatal jaundice are also quite frequent (59%–90%), although some newborns show no evidence of jaundice and/or only mild anemia. However, significant pediatric anemia may result in decreased appetite and growth and fatigue and lethargy, requiring management with regular transfusions.<sup>7</sup> Additionally, hemolysis is typically exacerbated by acute infections, stress, and pregnancy; thus the quality of life of PKD patients is seriously affected.

No specific therapy for severe PKD patients is available to date, and supportive treatments, such as frequent blood transfusions, iron chelation therapy, and, in some cases, splenectomy, are primarily palliative and do not address the underlying pathophysiology.

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Allogeneic hematopoietic stem cell transplantation (HSCT) has been shown to correct the disorder; however, there is little experience in applying HSCT in PKD. An understanding of HSCT in PKD is mostly based on animal studies. One recent publication has described the worldwide experience of allogeneic HSCT in PKD, which has enabled initial steps regarding guideline development for HSCT in this disease.<sup>9</sup> In addition to the limited experience regarding the clinical use of HSCT, for which there is not a defined conditioning regimen and is not the considered standard therapy in PKD treatment, this procedure is associated with extensive toxicity and incomplete engraftment. Fortunately, potential changes in the PKD treatment landscape are on the horizon. A small molecule (AG-348) that works as an activator of the endogenous RPK enzyme is currently being evaluated in several clinical trials worldwide (ClinicalTrials.gov: NCT02476916, NCT03548220, and NCT03559699).<sup>10</sup> Although this agent confers hemoglobin increases of at least 1.5 g/dL in PKD patients who are transfusion independent and is well tolerated in most patients (as has been observed in a phase II trial), patients with marked loss-of-function or non-missense *PKLR* variants do not appear to benefit, and there have been limited evaluations to date in PKD patients with more extensive anemia or transfusion requirements.<sup>7</sup>

Gene therapy has emerged as a new and beneficial treatment for non-malignant hematopoietic disorders with results potentially favorable relative to those associated with allogeneic HSCT from alternative donors. Several clinical studies in different monogenic diseases, including X-linked severe combined immunodeficiency (X1-SCID), adenosine deaminase deficiency-induced SCID (ADA-SCID), Wiskott-Aldrich syndrome (WAS), X-linked chronic granulomatous disease (CGD),  $\beta$ -thalassemia ( $\beta$ -Tal), sickle cell disease (SCD), and Fanconi anemia (FA), have previously demonstrated that gene therapy with lentiviral vectors is both efficacious and safe.<sup>11</sup> In addition to the improved clinical status observed in most treated patients, no adverse events due to gene therapy-related insertional oncogenesis have been observed to date with currently utilized self-inactivated vectors. Over recent years, we have demonstrated proof of concept for gene therapy efficacy in PKD with both retroviral- and lentiviral-mediated correction in a PKD mouse model.<sup>12,13</sup> Consequently, a therapeutic approach utilizing the PGK-codon-optimized (co)RPK-woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) lentiviral vector (LV) received the orphan drug designation (ODD) by the European Medicines Agency (EMA; EU/3/14/1330) and the US Food and Drug Administration (FDA; #DRU-2016-5168).<sup>12</sup> In the current study, non-clinical data to establish thresholds for dose efficacy, pharmacokinetics, and biodistribution have been collected to facilitate the clinical applicability of lentiviral-mediated correction, thus enabling the first clinical evaluation in PKD patients in accordance with EMA and FDA regulations. Based on these studies and on the licensing of the therapeutic medicinal product, a phase I clinical trial to evaluate the safety of the infusion of autologous CD34<sup>+</sup> cells transduced with a lentiviral vector carrying the red cell coRPK gene in adult and pediatric subjects with PKD (ClinicalTrials.gov:

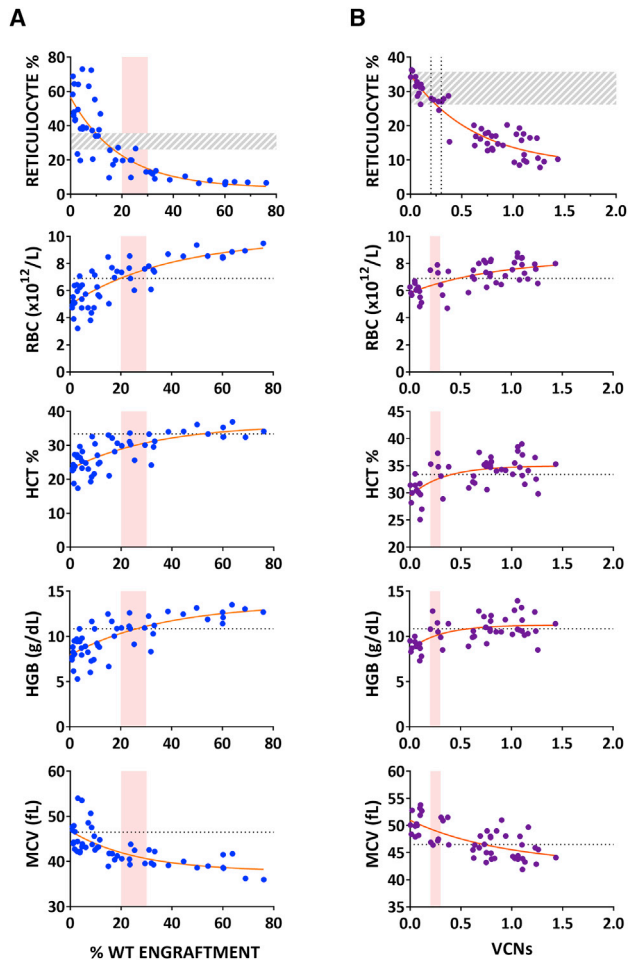
NCT04105166) has recently been approved by the Spanish Medicaments Agency (“Agencia Española de Medicamentos y Productos Sanitarios” [AEMPS]) and also by the US FDA (RP-L301).

## RESULTS

### Therapeutic level of wild-type (WT) or lentiviral-corrected hematopoietic cells to correct the phenotype of PKD mice

In order to define the level of healthy cells required to correct the PKD phenotype, we transplanted different ratios of WT and PKD bone marrow (BM) cells into lethally irradiated PKD mice (referred to originally as AcB55 mice), which are homozygous for a loss-of-function mutation that causes the 269T > A substitution in the *Pklr* gene. The consequent isoleucine-to-asparagine substitution at residue 90 of the PKLR protein (I90N) causes hemolytic anemia, acute splenomegaly, and a high number of circulating reticulocytes, which result in a mouse model of PKD.<sup>14</sup> Different ratios of male WT, female PKD-deficient hematopoietic cells (5%, 15%, 30%, 40%, 60%, and 100%) were transplanted into lethally conditioned PKD females (n = 3/group) with the remainder (95%, 85%, 70%, 60%, 40%, and 0%) comprised of male PKD BM cells. Five,  $7 \times 10^6$  cells of the different cell mixes were transplanted per recipient mouse. Recipients were followed for 9 months. Correction of essential erythroid parameters in transplanted mice (namely reticulocytes, hematocrit, erythrocyte count, hemoglobin, and mean corpuscular volume) was monitored at different time points after transplantation. As shown in Figure 1, each of the erythroid parameters indicated normalization when WT hematopoietic contribution was above 20%, with comprehensive normalization observed when levels exceeded 30%. Therefore, we estimated that correction of the PKD phenotype was proportionally related to the percentage of WT hematopoiesis analyzed in each mouse at each time point of sample collection and demonstrated that 30% WT-engrafted cells were sufficient to correct the PKD phenotype (Figure 1A).

Next, based on our previous studies described by Garcia-Gomez et al.,<sup>12</sup> we addressed the same question with lentivirally corrected mouse PKD hematopoietic progenitors. Hematopoietic progenitors from male PKD mice were transduced with a clinically applicable PGK-coRPK-WPRE LV (see Supplemental text for additional production details) at different multiplicities of infections (MOIs), ranging from 0.3 to 50 and then transplanted into lethally irradiated female PKD mice. As a control group, cells were transduced with a vector comprised of an identical backbone, except for an EGFP cDNA substituting the coRPK therapeutic transgene. In a first attempt, we used high MOIs, 25 and 50 transduction units per cell (TUs/cell). Reticulocyte percentages in mice transplanted with the therapeutic vector showed normal values through 215 days post-transplant. Normal values were also observed in all other erythroid parameters analyzed, whereas values observed in the animals transplanted with cells transduced with the EGFP control vector resembled the PKD phenotype (Figure S1A). Vector copy number (VCN) was evaluated by qPCR from peripheral blood (PB) samples. Stable VCN values at approximately 1 copy per cell were observed when the therapeutic LV was used and around 2–3 copies/cells when the EGFP LV was used (Figure S1A).



**Figure 1. Analysis of the therapeutic levels of wild-type (WT) and LV-corrected PKD hematopoietic cells required to correct the phenotype of PKD mice**

The correlation between hematological red blood parameters and hematopoietic engraftment in (A) and VCN in (B). Quantification of reticulocytes, red blood cell count, hematocrit, hemoglobin level, and erythroid mean corpuscular value (MCV) is shown. Each dot represents either the engraftment or VCN of a single animal at different time points of analysis. The orange line shows the non-linear correlation. The dashed, horizontal interval represents standard deviation of non-transplanted PKD mice, and data below the dashed interval are considered non-diseased. Dotted, horizontal lines are the thresholds set based on receiver operating characteristic (ROC) curves performed in non-transplanted healthy and PKD mice. Orange line, non-linear correlation. Pink columns highlight the interval from 20% to 30% of WT cells among all engrafted cells or among 0.2 to 0.3 VCNs in case of lentivirally corrected cells. Each dot represents either the engraftment or VCN of a single animal in a point of analysis. Analyses were performed at different time points, from 30 to 215 days post-transplant.

Subsequently, low MOIs ranging from 0.3 to 25 were tested, and phenotypical behavior was analyzed (Figure S1B). Efficacy of the treatment depends on the MOI used but also on the percentage of engraftment achieved with the corrected cells transplanted. Thus, in order to define the number of corrected cells required to correct the deficiency, a correlation of the phenotypic correction with the VCN

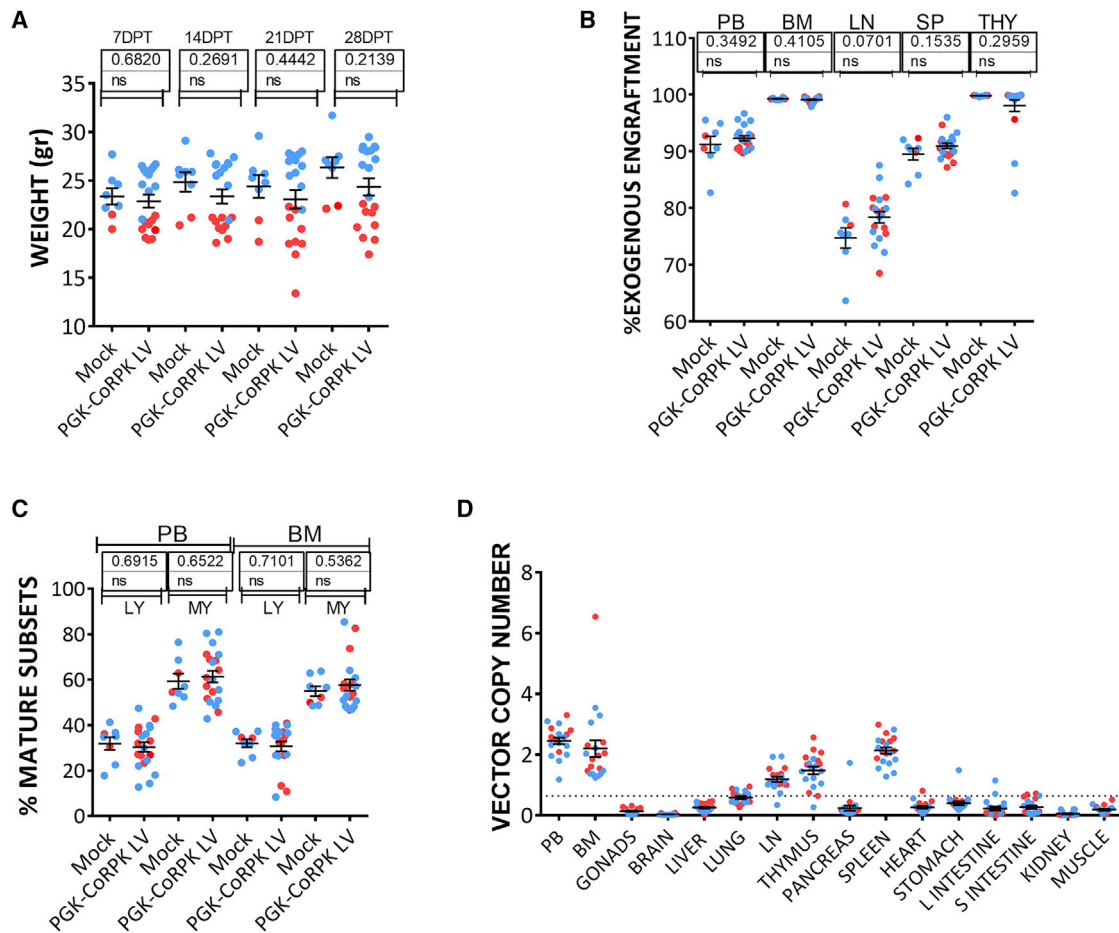
was performed as shown in Figure 1B. VCNs obtained at all transduction MOIs were plotted against the different hematological parameters. Reticulocyte percentage showed a clear correlation with VCN (coefficient of determination [ $R^2$ ] = 0.868), with VCNs between 0.2 and 0.3 constituting the threshold below which the murine PKD phenotype could not be rescued. The remaining erythroid parameters were consistent, with PKD phenotypic correction at levels above 0.3 VCN as well, but the dispersed distribution of the values did not provide a good correlation ( $R^2 < 0.5$ ) (Figure 1B).

#### Safety studies and biodistribution analysis did not indicate toxicity in hematopoietic cells or recipient animals upon lentiviral transduction with PGK-coRPK-WPRE LV

In order to study if treatment with transduced cells could generate any physical, behavioral, biochemical, or morphologic abnormalities in transplanted mice, a toxicity and biodistribution study was undertaken to evaluate any potential persistence or shedding of the ODD. BM-purified lineage negative ( $Lin^-$ ) cells from either male or female P3D2F1 WT donors, in which hematopoietic cells express  $CD45.1^+$ , were transduced at MOI 100 TUs/cell with a good manufacture practices (GMP)-like batch of the therapeutic PGK-coRPK-WPRE LV. Transduced cells (defined as the ODD product) were washed, and  $4 \times 10^5$  cells were transplanted intravenously into lethally irradiated B6D2F1 WT congenic recipients of both sexes (male into female and female into male) (Figure S2). A total of 20 B6D2F1 mice (9 females and 11 males) were transplanted with P3D2F1  $Lin^-$  cells transduced with the PGK-coRPK-WPRE therapeutic vector. As control groups, 6 male and 2 female B6D2F1 WT mice were transplanted with untransduced  $Lin^-$  BM cells from female or male P3D2F1 mice, respectively.

An aliquot of transduced  $Lin^-$  cells was maintained in liquid culture for 14 days to determine the VCN of the cells transplanted into the recipient animals. Transduction efficiency was similar in both male and female mice. Transduced cells from female animals showed a VCN of 1.82, whereas transduced cells from male animals presented, on average, a VCN of 1.91. The number of cells transplanted per mouse would therefore be equivalent to the injection of between  $1.5 \times 10^7$  and  $2.7 \times 10^7$  progenitor cells/kg body weight, well above what is anticipated for patient doses in a PKD gene therapy trial. Considering a mean of 1.87 integrations per cell, animals had received, on average, a dose of  $4.2 \times 10^7$  integrations/kg body weight, which is much greater than is expected in PKD patients transplanted with the ODD. Thus, these studies involved doses approximately 5-fold above those anticipated for humans in optimal scenarios (VCN 1–3 with cell doses  $4 \times 10^6$   $CD34^+$  cells/kg). Transplanted animals were monitored daily and followed for up to 1 month after transplantation. All animals survived with no significant weight changes between the group transplanted with transduced cells and their counterparts that received non-transduced cells (Figure 2A).

Animals were sacrificed 1 month after transplantation, and hematopoietic and non-hematopoietic organs were analyzed. White blood cell (WBC) counts both in PB and in BM revealed no significant differences between groups transplanted with transduced versus



**Figure 2. Absence of side effects in mice transplanted with HSPCs transduced with high multiplicities of infection (MOIs) of the therapeutic PKD-LV**

Transduced cells do not generate any physical, behavioral, biochemical, or morphologic abnormalities in transplanted mice. (A) Weight variation within different groups. (B) Level of donor engraftment in different organs measured as percentage of CD45.1-positive donor cells. (C) Multilineage distribution in peripheral blood (PB) and BM at 1 month post-transplantation in mice from biodistribution experiments. Lymphocyte percentage (LY %) includes B220<sup>+</sup> and CD3<sup>+</sup> cells, whereas Myeloid percentage (MY %) refers to Gr1<sup>+</sup> and Mac1<sup>+</sup> subpopulations. (D) Biodistribution of the lentiviral vector in transplanted mice. Each symbol represents the VCN of a single animal in the referred organ. Data from female (red) (PGK-coRPK-WPRE LV transduced, n = 9; mock n = 2) and male (blue) (PGK-coRPK-WPRE LV transduced, n = 11; mock n = 6) recipients are shown. Boxes define the statistical differences among mice transplanted with either untransduced or LV-transduced cells.

non-transduced cells (Figure S2B). Additionally, no significant differences were observed between male and female recipients. Blood samples were collected from each animal to analyze plasma chemistry parameters. Total protein, urea nitrogen, lipase, alkaline phosphatase, alanine transaminase/glutamic-pyruvic transaminase (ALT/GPT), aspartate aminotransferase/glutamic oxaloacetic transaminase (AST/GOT), calcium, phosphates, glucose, or albumin was analyzed. No significant changes were found in any of the analyzed parameters between animals that received transduced cells and their counterparts that received non-transduced cells (Figure S2C).

When engraftment of donor cells was evaluated by the presence of CD45.1<sup>+</sup> cells, all animals (males and females) showed between 82.7% and 95.6% engrafted cells in PB and between 97.9% and 99.6% in BM with respect to total hematopoietic cells (Figure 2B).

When other hematopoietic organs were analyzed, a high level of donor engraftment (77.29% ± 4.83% in lymph nodes [LNs], 90.53% ± 2.44% in spleen, and 98.57% ± 3.93% in thymus) was also observed in concordance with the high hematopoietic reconstitution observed in BM and PB, which was independent of recipient gender (Figure 2B). When non-hematopoietic organs (liver and gonads) were analyzed, a very low level of hematopoietic cells was observed, which was also independent of recipient gender (Figure S2B). Donor hematopoietic engraftment in hematopoietic subsets (myeloid and lymphoid cells) was also analyzed to study potential lineage differentiation bias emanating from the lentiviral transduction. No differences were observed in either PB or BM, indicating that no harmful effects in hematopoietic differentiation were induced in the short term due to the procedure (Figures 2A–2C).



Biodistribution studies were also conducted to evaluate if the presence of the lentiviral vector was restricted to *ex vivo*-transduced hematopoietic cells and to ascertain if proviral sequences were detected in other tissues. VCN was determined in multiple hematopoietic and non-hematopoietic organs from the transplanted mice. VCN values were similar in all hematopoietic organs in which hematopoietic engraftment was previously confirmed (PB, BM, LN, thymus, and spleen). When non-hematopoietic organs were analyzed, nearly undetectable VCN values were obtained; residual values were ascribed to the presence of hematopoietic extravasated cells that may reside in the different tissues (Figure 2D). Notably, these results demonstrate that there is no unexpected presence of the vector in the gonads, implying that there is no risk of germline transmission.

Serum obtained from transplanted animals was also tested for the presence of vector-derived HIV p24 capsid protein to confirm the absence of replication-competent lentiviruses (RCLs) from the viral vector used. No p24-positive signal was detected in any of the animals transplanted with transduced cells (Figure S2D).

Cell doses and viral concentrations used in the biodistribution assay are well above those specifications anticipated for human evaluation (likely at least 5-fold above the number of cells and amount of virus to be administered per patient in the clinical trial). No premature deaths were observed during the analysis period. Considering again an average of  $4 \times 10^6$  CD34<sup>+</sup> cells/kg in humans with likely VCN in the 1–3 range, we have exceeded 5-fold the viral dose that would be administered in humans without detectable toxic effects.

To obtain additional toxicology data, histological analyses were performed in multiple organs obtained from the transplanted animals. Organs (testis/ovaries, liver, kidneys, spleen, stomach, large intestine, brain, lungs, heart, muscle, thymus, LNs, and pancreas) were collected and fixed, and histologic sections of each organ were evaluated. All analyzed tissues were considered to be within normal histologic limits for mice at this age, with the exception of the marked degeneration identified in gonads in both transduced and untransduced groups and presumed to be caused by irradiation of animals prior to transplant. No evidence of any malignancy was seen in any of the tissues examined. There were no noteworthy differences in the histologic appearance of lentiviral-transduced versus untransduced specimens (Figure S2E).

#### **Human hematopoietic progenitors subjected to the transduction protocol using the PGK-coRPK-WPRE LV maintain their engraftment and repopulation ability when assayed in NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/SzJ (NSG) mice**

We assessed the hematopoietic engraftment of the ODD product, PGK-coRPK-WPRE LV-transduced human hematopoietic stem and progenitor cells (HSPCs), using optimized conditions including transduction enhancers (Figure S4). mPB-CD34<sup>+</sup> cells from a healthy donor (HD) were transduced with PGK-coRPK LV in the presence of transduction enhancers.<sup>15,16</sup> Transduction with the PGK-coRPK-WPRE LV did not affect the *in vitro* hematopoietic progenitor poten-

tial of mPB-CD34<sup>+</sup> (Figure S3A). Up to 50% of the hematopoietic progenitors were transduced with an average of  $0.99 \pm 1.54$  VCN per colony ( $3.01 \pm 0.77$  VCN per transduced colony, considering a transduced colony-forming unit [CFU] when its VCN is over 0.3) (Figure S3B).

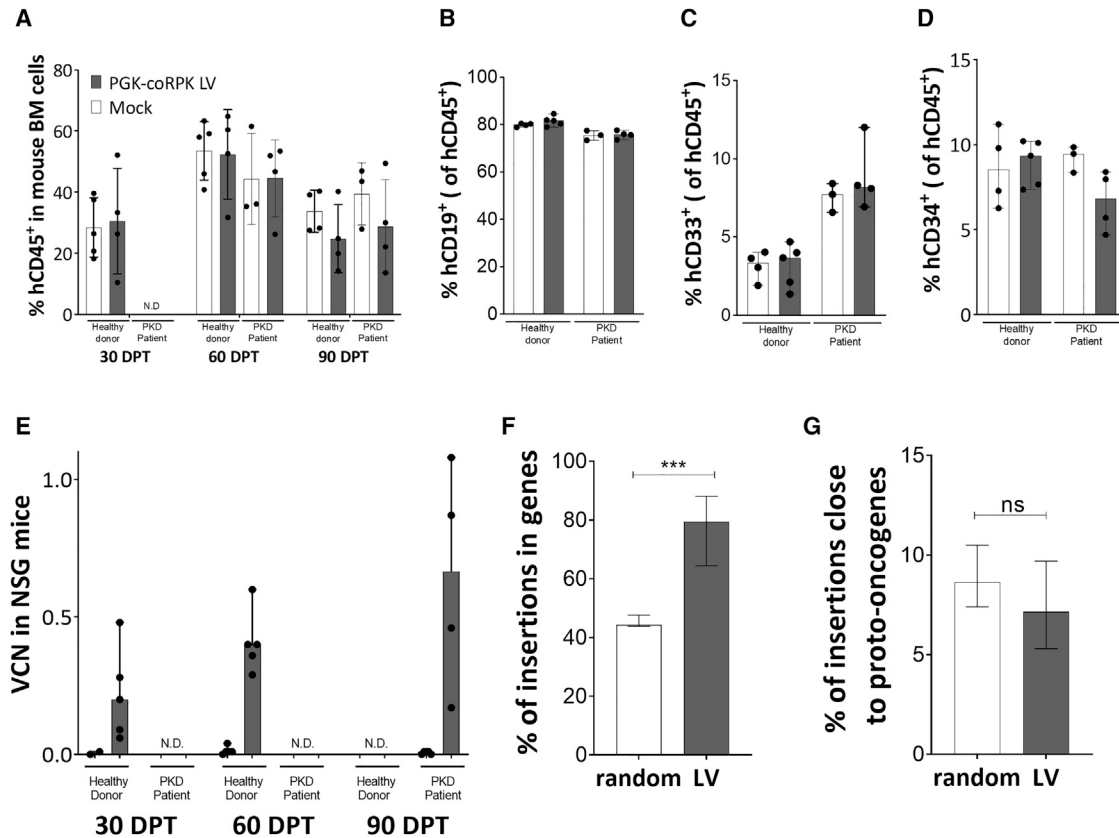
Next, we evaluated the NSG mouse-repopulating ability of transduced mobilized PB (mPB) CD34<sup>+</sup> cells, from HDs and from a PKD patient. As shown in Figure 3A, no statistical differences between mice transplanted with HD- and PKD-transduced cells were observed. Additionally, animals transplanted with LV-transduced HSPCs did not display any alteration of the hematopoietic lineages within human engraftment (Figures 3B–3D). Engraftment of transduced human cells was confirmed by qPCR as indicated in Figure 3E. VCN in the animals was followed over time. A variable VCN, probably due to the human engraftment variability, with an average of  $0.65 \pm 0.4$  per cell was observed at 90 days post-transplant in human cells coming from PKD patient-transduced cells, indicating the transduction of the human HSPCs at these time points.

In order to determine if the vector-integration pattern harbored any bias toward potentially harmful genome loci, we performed an integration site analysis of BM and spleen cells from recipient mice. As expected, a preferential integration of the LV provirus within genes was observed (Figure 3F). No bias toward integration in proximity to proto-oncogenes was observed (Figure 3G).

#### **Pre-validation of PGK-coRPK-WPRE good manufacturing procedures GMP-grade LV**

In order to optimize the transduction conditions using a GMP-grade LV, mPB-CD34<sup>+</sup> samples from HDs were transduced. In order to facilitate a clinically applicable transduction protocol suitable for the manufacture of a therapeutic product to be used in a global PKD gene therapy trial (RP-L301-0119), transductions were carried out at two different preclinical sites with different HD samples to evaluate the variability in transduction with identical transduction procedures. Cells were pre-stimulated and transduced according to the protocol developed (see Figure S3) at a concentration of  $1 \times 10^6$  cells/mL and at increasing MOIs (20, 50, 100, and 200). Clinically applicable LV produced under GMP conditions did not result in toxicity at the level of committed progenitors, since the content of both granulomacrophage and erythroid progenitor colonies did not show significant differences between the pre-stimulated mock-transduced and the transduced ones (Figure 4A).

In addition, the objective of enabling sufficient therapeutic vector copies per cell (target range 1–3) was achieved at the lowest MOI evaluated (MOI 20;  $5 \times 10^6$  viral particles (VP)/mL) (Figure 4B). These results were obtained by qPCR in the expanded CD34<sup>+</sup> cells in liquid culture for 14 days. Although there was an apparent increase in the VCN conferred by an increase in MOI from 25 to 50, no further VCN increases were evident with MOIs of greater than 50 (Figure 4B).



**Figure 3. Development of an efficient transduction protocol of human HSPCs with preserved repopulating ability**

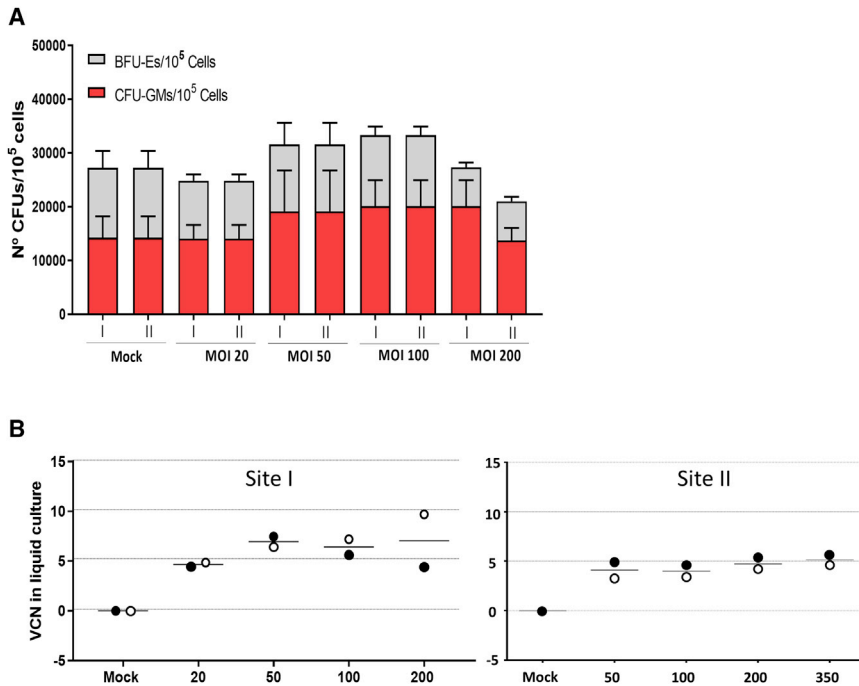
(A) Engraftment ability of mock-transduced or transduced CD34<sup>+</sup> cells from mobilized PB ([mPB]-CD34<sup>+</sup> cells) from healthy donors (HDs) and from one PKD patient in immunodeficient NSG mice over time. (B–D) Multilineage engraftment of human-transduced cells analyzed 90 days post-transplant. (E) VCN in human cells obtained from the BM of NSG mice engrafted with transduced cells. Values are represented as the mean  $\pm$  SD of the mean. Dots represent each mouse transplanted with the HD sample for mock (n = 5) or transduced condition (n = 4) and for the PKD sample for mock (n = 3) or transduced condition (n = 4). (F) Percent of all integrations inside of transcriptional units. (G) Percent of integrations within 100 kb of proto-oncogenes. Results are compared to matched random control (mrc) sites. \*\*\*p < 0.001, unpaired t test. A total of 1,351 unique insertion sites were identified.

## DISCUSSION

Autologous transplant of LV-corrected HSPCs is currently being evaluated with the intent of finding a definitive cure for PKD, in particular, for the more severe clinical variants of this disorder. Consequently, we conducted studies in a mouse model mimicking PKD to enable clinical evaluation of our previously described PGK-coRPK-WPRE LV.<sup>12</sup> In our previous work with this PKD mouse model, we demonstrated that retrovirally derived human RPK expression was capable of fully correcting the PKD phenotype when over 25% genetically corrected cells were transplanted.<sup>13</sup> Now, we have identified the therapeutic threshold for hematopoietic progenitors transduced with PGK-coRPK-WPRE LV, which is required to correct the PKD phenotype in a mouse model that closely resembles the human disease. Moreover, we show that an excess of transduced hematopoietic progenitors was not associated with side effects seen in previous long-term studies<sup>12</sup> and in biodistribution and toxicology studies performed in the current study, which is consistent with a favorable safety profile of the PGK-coRPK-WPRE LV. Furthermore,

we established a clinically applicable gene therapy protocol for transduction of human hematopoietic progenitors that achieved transduction levels above the minimum established threshold. Additionally, this transduction protocol was highly reproducible among different HSPC donors and investigative sites. Altogether, the transduction of PKD-HSPCs in combination with their autologous transplantation is likely to be a safe and effective therapeutic alternative for PKD patients.

HSPC-directed gene therapy has been increasingly recognized as a safe and definitive therapy for several hematopoietic inherited diseases. To date, LV gene therapy has been employed in several successful clinical trials to correct the hemoglobinopathies,  $\beta$ -Tal, and SCD.<sup>17–19</sup> Indeed, Zynteglo, a gene therapy product and procedure for the treatment of  $\beta$ -Tal, has been recently recommended by the EMA for marketing authorization (<https://www.ema.europa.eu/en/news/new-gene-therapy-treat-rare-inherited-blood-condition>). The clinical program made feasible by the results described is focused



**Figure 4. Transduction of mPB CD34<sup>+</sup> cells from HDs with the PGK-coRPK-WPRE vector in GMP-grade quality**

(A) Number of hematopoietic progenitors expressed in colony-forming units (CFUs) per  $1 \times 10^5$  cells referring to granulomacrophage (GM [CFU-GM]) progenitors and erythroid colony-bursting units (BFU-Es), performed in two different sites: site I and site II. (B) Number of vector copies per cell at the different MOIs post-transduction determined in the CD34<sup>+</sup> cells expanded in liquid culture during 14 days in site I (left) and site II (right). Gray and white circles correspond to two PB HD different samples.

on the cure or substantive reversal of PKD by autologous gene therapy, which eliminates the underlying iron overload and need for chronic and frequent blood transfusions and entails fewer of the many side effects associated with allogeneic HSCT.<sup>9</sup>

Recently, a new small molecule allosteric pyruvate kinase (PK) enzyme activator (AG-34, Mitapivat) has been demonstrated as a viable alternative for the treatment of PKD patients who are not transfusion dependent. This agent has been associated with increased hemoglobin in approximately 50% of the patients who had at least one missense *PKLR* mutation.<sup>20</sup> This drug stabilizes RPK tetramer and increases PKD activity in red blood cells. However, some degree of RPK protein synthesis is required to enable efficacy.<sup>21</sup> Thus, for those PKD patients who suffer from severe anemia with very low or absent PK protein, enzyme activators are unlikely to alleviate the underlying disease.<sup>20</sup> Autologous transplant of LV-corrected HSPCs will hopefully prove to be a crucial therapeutic option for these more severely afflicted patients and will hopefully be feasible at junctures prior to end-organ damage resulting from disease progression and iron overload.

We have assessed the pre-clinical safety profile of transplantation with PGK-coRPK-WPRE-transduced autologous HSPCs (Figure 2). We did not identify any side effects after transplant of viral and cell doses that are likely 5-fold higher than those anticipated for clinical administration. In addition, no recombination events resulting in the generation of RCL were observed, as measured by the analysis of new capsids in serum of transplanted animals using the p24 ELISA quantification. This suggests that the generation of the PKD viral vector and the transduction of HSPC are safe. A PGK-coRPK-WPRE-

based gene therapy approach for PKD is likely to be associated with a safety profile similar to other lentiviral vector-based approaches in non-malignant hematologic conditions; long-term follow-up studies are ongoing and will hopefully confirm the safety of LV-based gene therapy, especially for RBC-inherited diseases.<sup>11</sup> A favorable safety profile will be essential if LV-based gene therapy is to become a viable therapeutic option for severe PKD patients for whom current palliative measures neither address the

underlying pathophysiology nor mitigate many of the more severe disease-related effects.

In addition, we have demonstrated that the transduction protocol for human HSPCs using PGK-coRPK-WPRE LV is highly reproducible among different sites (Figure 4). This will facilitate the activation of a global standardized procedure in which manufacture of the medicinal product at a large scale and under highly controlled conditions is mandatory. This is a necessary step to enable the accessibility of gene therapy for PKD patients across geographic regions.

Altogether, our objective is to evaluate the autologous transplant of LV-transduced HSPCs as a potentially safe and definitive therapeutic alternative to enable correction of severe PKD. In fact, a first-in-human gene therapy clinical trial for the treatment of PKD (ClinicalTrials.gov: NCT04105166) is currently active, and the initial patients have been treated as of mid-2020; preliminary clinical data were recently updated, and the efficacy and safety profile have, to date, been encouraging and consistent with our preclinical results.<sup>22</sup>

## MATERIALS AND METHODS

### Mice and animal procedures

Recombinant congenic PKD mice (AcB55 mice) were initially obtained from Emerillon Therapeutics (Montreal, QC, Canada), maintained, and backcrossed with a C57BL/6 donor parental strain at the Laboratory Animal Facility of Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) until pure background was obtained by inbreeding (registration number ES280790000183). WT littermates were used as controls. All mouse strains

(see Table S1) were then housed and bred at the CIEMAT Animal Facility (registration number ES280790000183) from breeding pairs originally obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were routinely screened for pathogens, in accordance with FELASA (Federation of European Laboratory Animal Science Associations) procedures. All animal experiments were performed in compliance with European and Spanish legislations and institutional guidelines: Directive 2009/41/CE, Spanish Law 9/2003, and R.D 178/2004. The mouse protocol was approved by “Consejería de Medio Ambiente y Ordenación del Territorio” (protocol number PROEX 073/15), and all procedures were approved by the CIEMAT Animal Experimentation Ethical Committee according to external and internal biosafety and bioethics guidelines.

Routine bleeding for PB collection was carried out by lateral tail-vein puncture and sampling into commercially EDTA-treated tubes (Microvette CB 300 K2E; SARSTEDT, Numbrecht, Germany). PB hematological counts were analyzed using an Abacus Junior Vet Haematological Analyzer (CVM Diagnóstico Veterinario, Tudela, Spain).

To perform BM transplantation, different doses of total BM were transplanted into lethally irradiated (two doses of 4–5 Gy spaced 24 h using X-ray equipment MG324 [300 kV, 12.8 mA; Philips, Hamburg, Germany]) mice. For human progenitor transplantation, human CD34<sup>+</sup> cells were transplanted into immunodeficient NSG mice.

Lin<sup>-</sup> cells were purified by cell sorting using lineage-specific antibodies, and mouse cytometry analyses were performed using antibodies detailed in Table S2. 4',6-diamidino-2-phenylindole (DAPI; Roche)-negative staining was used as a marker of cell viability. Analyses were performed in the LSRFortessa cell analyzer (Becton Dickinson [BD]).

Transduced CD34<sup>+</sup> cells from HD and PKD samples were injected intravenously into NSG mice previously irradiated with 1.5 Gy. To evaluate the level of human hematopoietic engraftment, cells were collected by femoral BM aspiration at 4 to 12 weeks post-transplant and stained with hCD45 APC-cy7 (Cy7) (eBioscience) and CD34 APC (BD Biosciences) antibodies according to the manufacturer's instructions. Analyses of multilineage engraftment were performed using CD45 APC-Cy7 (BioLegend), CD34 APC (BD Biosciences), CD33 PE (BD Biosciences), CD19 PE-Cy7, and CD3 fluorescein isothiocyanate (FITC; BioLegend), according to the manufacturer's instructions. Fluorochrome-matched isotypes were used as controls. DAPI-positive cells were excluded from the analysis. All flow cytometric analyses were performed on the LSRFortessa (BD Biosciences) and analyzed with FlowJo software version (v.7.6.5).

The number of mice per group required to obtain statistical significance was estimated using the online GRANMO v.7.12. software, which allows the identification of the sample size and power calculator.

#### Transduction of mouse hematopoietic progenitors

Donor mice were sacrificed by cervical dislocation. Both tibiae and femora were surgically extracted, and BM was harvested by flushing bones under sterile conditions. Whole BM was stained by using lineage-specific PE-conjugated antibodies, and mouse hematopoietic progenitors (Lin<sup>-</sup> cells) were sorted using a BD Influx Cell Sorter (BD Biosciences). Purities of Lin<sup>-</sup> cells were always above 92%.

Lin<sup>-</sup> cells were cultured at  $0.5 \times 10^6$  cells/mL overnight in X-Vivo20 (Lonza) medium plus cytokines (50 ng/mL human [h]-interleukin [IL]-11 + 50 ng/mL murine stem cell factor [SCF] and 0.5% penicillin/streptomycin [all from Thermo Fisher Scientific]) on 5  $\mu\text{g}/\text{cm}^2$  retronectin (Takara Bio, Otsu, Japan)-coated, 24-well, non-treated tissue-culture plates. Cells were transduced for an additional 24 h with lentiviral supernatants MOI = 100;  $5 \times 10^8$  vp/mL). Afterward, cells were collected, washed, resuspended in fresh medium, and transplanted through the tail vein of lethally irradiated recipient mice. A fraction of transduced Lin<sup>-</sup> cells was cultured *in vitro* for 15 days for VCN determination.

#### Transduction of human hematopoietic progenitors

mPB samples were provided by Hospital Universitario Infantil Niño Jesús (HUINJ) or commercially supplied by Hemacare. Human cord blood samples were provided by Centro de Transfusión de la Comunidad de Madrid. All samples were collected under written consent and Fundación Jiménez Díaz Institutional Review Board agreement (number PKDefin [SAF2017-84248-P]). Human mononuclear cells were obtained by Ficoll-Paque PLUS (GE Healthcare) density gradient isolation, according to the manufacturer's recommendations. Purified CD34<sup>+</sup> cells were obtained by immunoselection using the CD34 Micro-Bead Kit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. Purities above 90% hCD34<sup>+</sup> cells were routinely obtained. Cells were viably frozen in 10% dimethyl sulfoxide solution and stored at liquid nitrogen or used fresh.

Fresh or thawed mPB-CD34<sup>+</sup> cells were pre-stimulated at a concentration of  $1 \times 10^6$  cells/mL in stem cell growth medium [SCGM] 20 (CellGenix) supplemented with 100 ng/mL recombinant human [rh] thrombopoietin [TPO]/100 ng/mL rhSCF/100 ng/mL rhTPO/20 ng/mL IL-3 for  $22 \pm 4$  h on 2  $\mu\text{g}/\text{cm}^2$  retronectin-coated plates. Cells were then transduced at  $0.5 \times 10^6$  cells/mL, MOI 100 ( $5 \times 10^7$  vp/mL) of PGK-coRPK-WPRE LV for  $16 \pm 4$  h with the addition of 4  $\mu\text{g}/\text{mL}$  protamine sulfate (Hospira Invicta) in the absence or presence of the two transduction enhancers. After transduction, cells were washed and seeded for clonogenic assays or *in vitro* expansion for 12–14 days.

#### *In vitro* culture

For clonogenic studies, mouse cells,  $1.5 \times 10^3$  BM-derived Lin<sup>-</sup> mouse cells, or 150 mPB-CD34<sup>+</sup> human cells were seeded in methylcellulose-based media with recombinant cytokines (Methocult M3534, H4435, and H4034 Optimum; STEMCELL Technologies, Vancouver, BC, Canada). On day 7 (mouse) or 14 (human), resulting



colonies were scored using an inverted microscope to identify the different hematopoietic colony types. Liquid expansion of transduced cells was also maintained in SCGM 20 supplemented with 100 ng/mL rhTPO/100 ng/mL rhSCF/100 ng/mL rhTPO/20 ng/mL IL-3 for 12–14 days.

#### Determination of VCN

In experiments in which colony forming cells were evaluated for VCN, individual colonies were isolated and suspended in 1 mL PBS (Merck KGaA, Darmstadt, Germany) for 1 h at room temperature (RT). Genomic (g) DNA was extracted from the cells using the protocol described previously<sup>23</sup> while in liquid-cultured cells.

DNA extraction was performed by using the commercial kit DNeasy Blood & Tissue Kits (QIAGEN, catalogue number 69506; and Genomic DNA PureLink, Thermo Fisher Scientific) according to the manufacturers' instructions. qPCR amplification was performed in a 7500 Fast Real-Time PCR System and QuantStudio Real-Time PCR (Applied Biosystems/Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA) using TaqMan with the use of fluorescent probes (Table S3).

For VCN determination, proviral genome and host cell genome were amplified and quantified by TaqMan-based multiplex qPCR. The psi packaging sequence was used to quantify viral genomes, whereas the albumin (*Alb*) or titin (*Ttn*) genes were used to quantify human or murine genome, respectively. Quantification of viral and cell genomes was performed as previously described.<sup>23</sup> Plasmids for quantification were kindly provided by Dr. Sabine Charrier (Genethon, Évry, France). qPCR was conducted in an Applied Biosystems 7500 Fast Real-Time PCR system (Thermo Fisher Scientific).

#### Biodistribution studies

For *in vivo* biodistribution studies,  $4 \times 10^5$  Lin<sup>-</sup> BM cells from male and female P3D2F1 mice transduced with PGK-coRPK-WPRE LV were intravenously administered into lethally irradiated B6D2F1 congenic recipients of both sexes. Results were compared with a control group that received non-transduced cells ( $n = 6$  male, and  $n = 2$  females). 1 month after BM transplantation (see Table S1), mice were sacrificed by anesthetic overdose with Avertin (15  $\mu$ L of a 2.5% solution/1 g weight). Animals were perfused with PBS/EDTA solution through a cardiac cannula and hematopoietic (PB, BM, spleen, thymus, LNs), and non-hematopoietic (liver, lung, kidney, pancreas, large and small intestine, stomach, gonads, brain, muscle, heart) organs were extracted and processed for histology, flow cytometry, analysis of VCN, and serology for the presence of HIV p24 protein, measured according to the manufacturer's instructions (Alliance HIV-1 P24 Antigen ELISA Kit [96 Test] [NEK050001KT]; PerkinElmer) (see Supplemental text).

#### Flow cytometry

Cells were stained by using specific antibodies and washed with flow cytometry buffer (PBS containing 0.5% BSA and 0.05% sodium azide). Flow cytometry analyses were performed in the LSRFortessa

cell analyzer (BD, Franklin Lakes, NJ, USA). Offline analysis was performed with the FlowJo Software v.X (Tree Star, Ashland, OR, USA).

#### Statistics

Data from all experiments were represented as the average  $\pm$  standard error of the mean (mean  $\pm$  SEM). For the analyses, non-parametric data were analyzed with Mann-Whitney test or Kruskal-Wallis, and for parametric data, Student's *t* test was performed (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001). All of the statistical analyses were performed using GraphPad Prism v.5.00 for Windows (GraphPad Software, San Diego, CA, USA; <https://www.graphpad.com/>).

#### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2021.07.006>.

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#### AUTHOR CONTRIBUTIONS

S.N., O.Q.-B., and R.S.-D. designed and performed experiments and wrote the manuscript. S.L.-M., K.L., and B.C.B. designed and performed experiments. A.G.-T., O.A., M.V., K.L., S.F.-B., V.N.-R., A.M.-V., I.O.-P., A.B., M.R., and M.G.-B. performed experiments. J.C.R. provided GMP-like viral batches. J.A.B. and J.D.S. suggested procedures and reviewed the manuscript. J.-C.S. designed and performed experiments, wrote the manuscript, and provided grants support.

#### DECLARATION OF INTERESTS

J.D.S., K.L., B.C.B., and S.G. are employees of Rocket Pharmaceuticals. J.A.B. and J.-C.S. are consultants and receive funding from Rocket Pharmaceuticals.

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