Low-dose AAV-CRISPR-mediated liver-specific knock-in restored hemostasis in neonatal hemophilia B mice with subtle antibody response

Xiangjun He¹,¹⁰, Zhenjie Zhang¹,¹⁰, Junyi Xue¹, Yaofeng Wang Ø,²,³, Siqi Zhang¹, Junkang Wei¹, Chenzi Zhang¹,², Jue Wang¹, Brian Anugera Urip¹, Chun Christopher Ngan¹,², Junjiang Sun⁹, Yuefeng Li⁹, Zhiqian Lu⁶, Hui Zhao Ø,¹,¹, Duanqing Pei²,⁸, Chi-Kong Li⁹ & B oF e ng¹,²,⁷

AAV-delivered CRISPR/Cas9 (AAV-CRISPR) has shown promising potentials in preclinical models to efficiently insert therapeutic gene sequences in somatic tissues. However, the AAV input doses required were prohibitively high and posed serious risk of toxicity. Here, we performed AAV-CRISPR mediated homology-independent knock-in at a new target site in mAlb 3' UTR and demonstrated that single dose of AAVs enabled long-term integration and expression of hF9 transgene in both adult and neonatal hemophilia B mice (mF9−/−), yielding high levels of circulating human Factor IX (hFIX) and stable hemostasis restoration during entire 48-week observation period. Furthermore, we achieved hemostasis correction with a significantly lower AAV dose (2 × 10⁹ vg/neonate and 1 × 10¹⁰ vg/adult mouse) through liver-specific gene knock-in using hyperactive hF9R³³⁸L variant. The plasma antibodies against Cas9 and AAV in the neonatal mice receiving low-dose AAV-CRISPR were negligible, which lent support to the development of AAV-CRISPR mediated somatic knock-in for treating inherited diseases.

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Recombinant adeno-associated virus (AAV)¹ coupled with CRISPR technologies²,³ provide tremendous potential in developing therapeutic approaches to permanently reverse disease-causing genetic defects⁴,⁵. Targeted insertion of a normal sequence to restore the gene function offers broad therapeutic potential regardless of mutation type and has been an attractive strategy. To establish a proof of concept, inherited hemophilia B caused by F9 gene mutations has been intensively used as a disease model⁶. Through the homology-directed repair (HDR) mechanism, human F9 gene exons (hF9 Ex2–8) have been knocked-in in mice using AAV-delivered zinc finger nuclease (ZFN)⁷,⁸, while knock-in of mF9 Ex2-8⁹ and hyperactive hF9R³³⁸L variant (hFIX-Padua)¹⁰ were achieved using AAV-delivered CRISPR/SaCas9.

¹School of Biomedical Sciences, MOE Key Lab, Faculty of Medicine; Institute for Tissue Engineering and Regenerative Medicine (iTERM), The Chinese University of Hong Kong, Hong Kong SAR, China. ²Centre for Regenerative Medicine and Health, Hong Kong Institute of Science & Innovation, Chinese Academy of Sciences, Hong Kong SAR, China. Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China. ⁴Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC, USA. ⁵Guangdong Landau Biotechnology Co., Ltd., Guangzhou 510555, China. ⁶Department of Cardiothoracic Surgery, Shanghai Jiao Tong University Affiliated Sixth People’s Hospital, Shanghai 200233, China. ⁷The Chinese University of Hong Kong, Shenzhen Research Institute, Shenzhen 518000, China. ⁸Laboratory of Cell Fate Control, School of Life Sciences, Westlake University, Hangzhou 310024, China. ⁹Department of Pediatrics, Hong Kong Children’s Hospital, The Chinese University of Hong Kong, Hong Kong SAR, China. ¹⁰These authors contributed equally: Xiangjun He, Zhenjie Zhang. e-mail: fengbo@cuhk.edu.hk

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Recently, a homology-independent knock-in strategy was developed by exploiting the non-homologous end-joining (NHEJ) DNA repair mechanism, which demonstrated superior efficiency of targeted insertion in zebrafish and mammalian cells compared to the HDR-based approach. In applying AAV-CRISPR to explore NHEJ knock-in vivo, Suzuki et al. reported the restoration of Merk gene function in rat retinas, while Zhang et al. and Chen et al. successfully rescued mF9 gene defects in hemophilia A mice. As homologous sequences are not required, NHEJ knock-in provides greater capacity and flexibility for AAV-based donor sequence delivery when performing in vivo gene editing.

To date, the therapeutic efficacy of AAV-mediated in vivo knock-in, via either HDR- or NHEJ-based strategies, has been broadly confirmed in preclinical models. However, effective in vivo knock-in was mostly achieved at the expense of high-dose AAV inputs, which is associated with significant safety risks and hefty production costs for clinical adoption.

In this study, we systematically investigated the AAV-CRISPR mediated in vivo NHEJ knock-in and showed that a careful selection of targeting reagents and strategies can support effective gene knock-in with a much lower and safer AAV input. Through targeting at the proximal mAlb 3′UTR, high levels of liver-specific transgene expression were induced. Single-dose AAV administration in both adult and neonatal mice yielded robust hF9 knock-in, which sustained hFIX production and corrected hemostasis in hemophilia B mice throughout the 48-week observation period. With liver-specific genome editing and hyperactive hF9 variant, effective knock-in was achieved with vector input comparable to that commonly used in clinics. Germline safety, off-target effect, and anti-Cas9 immunity were also evaluated to address the potential concerns in developing somatic knock-in gene therapies.

Results

A single dose of AAV-CRISPR rendered stable NHEJ knock-in of ires-hF9 and long-term hemostasis correction in adult hemophilia B mice

First, we compared in vivo NHEJ knock-in mediated by Cas9 derived from Streptococcus pyogenes (SpCas9) and Staphylococcus aureus (SaCas9) through hydrodynamic injection. Previously developed single-cut ires-GFP reporter was used to avoid vector-based expression, and donor insertion was directed at mAlb 3′UTR (Supplementary Fig. 1a). The knock-in using either SpCas9 or SaCas9 in combination with high-performing sgRNAs yielded high intensity of GFP signals in mouse livers (Supplementary Fig. 1b, c). SpCas9 sgAlb/1 (hereafter named as sgAlb) produced the highest GFP signals and was selected for further studies (Supplementary Fig. 1c).

Next, we constructed an AAV donor carrying ires-hF9 to evaluate the therapeutic potential of AAV-CRISPR mediated in vivo NHEJ knock-in strategy. Self-complementary AAV (scAAV) vector was used to provide better intracellular stability, and flanking sgA target sequences were used to introduce DSBs in the donor to facilitate NHEJ knock-in. Recombinant vectors carrying ires-hF9, SpCas9, and sgAlb/1 were encapsulated in AAV8 capsid and intravenously (i.v.) injected into hemophilia B mice (mF9−/−) (Fig. 1a). The treated mF9−/− mice (6–12 weeks), named AAV-KI (hF9) mice hereafter, produced high levels of circulating hFIX in the plasma, which increased steadily during the first 8 wpi and maintained at 1000–1300 ng/ml until the mice were sacrificed at 48 wpi (Fig. 1b). Immunohistochemistry (IHC) and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses of liver tissues collected at 2, 12, and 48 wpi confirmed the persistent hFIX expression in hepatocytes throughout the whole observation period (Fig. 1c and Supplementary Fig. 2a).

The hemostasis activity of AAV-KI (hF9) mice was assessed by measuring activated partial thromboplastin time (aPTT). Indeed, aPTT values in AAV-KI (hF9) mice returned to normal and remained stable throughout the 48-week observation period (Fig. 1d), indicating long-term correction of hemostasis. No apparent liver damage was observed (Supplementary Fig. 2b, c).

To assess the genetic stability of hF9 knock-in in fast-growing tissues, partial hepatectomy (PHX) was performed on AAV-KI (hF9) mice, and hFIX production was measured during the recovery period (Fig. 1e). To differentiate the knock-in effect from non-integrative AAV expression, PHX was also performed on mice receiving ssAAV and scAAV bearing LP1 promoter-driven hF9 (Fig. 1e). Unjected mF9−/− mice suffered from excessive bleeding and had a low survival rate within one-week post-PHX (wpx). In contrast, mF9−/− mice that received hF9 transgene by either knock-in or episome strategies showed 100% survival (Fig. 1f). Interestingly, plasma hFIX levels in AAV-KI (hF9) mice increased slightly after PHX. Whereas ssAAV (LP1-hF9) and scAAV (LP1-hF9) treated mice showed a sharp reduction in plasma hFIX levels after PHX (Fig. 1g, upper panels), indicating dilutions of episomal AAVs during liver regeneration. After PHX, the aPTT values remained low among all mF9−/−treated mice, except a slight increase in ssAAV (LP1-hF9) and scAAV (LP1-hF9) groups (Fig. 1g, lower panels), which correlated with the drastic reduction of plasma hFIX levels. At 8 wpx, substantial hFIX production was detected in all hFIX-treated mice (Supplementary Fig. 3), which explained their survival post-PHX surgery.

AAV-CRISPR mediated hF9 knock-in resulted in subtle transcriptome alteration and off-target effect in mouse liver

Liver RNA samples collected from un.injected, control, and AAV-KI (hF9) mice sacrificed at 2 wpi and 12 wpi were analyzed by deep sequencing (Supplementary Fig. 4a). High levels of hF9 transcripts were detected in AAV-KI (hF9) livers with 7213 ± 2458 FPKM at 2 wpi and 12,333 ± 1081 FPKM at 12 wpi (Fig. 2a), whereas levels of hF9 transcripts in control livers were negligible. Transcriptome profiles of 18 samples from AAV-KI (hF9), control, and untreated livers were found to be highly similar, with correlation R values greater than 0.96 among the 16 samples collected from male mice. Only the two samples from a female control mouse showed relatively distinct features (R > 0.88) (Fig. 2b; Supplementary Fig. 4b). These results indicated that transducing SpCas9/sgRNA, gene knock-in at mAlb 3′UTR, and ectopic hF9 expression did not cause major functional alteration in the livers.

Since hF9 transgene was transcribed together with endogenous mAlb, we evaluated the knock-in frequency by calculating the ratio of hFIX/mAlb transcripts, which was 1.47 ± 0.53% at 2 wpi and it increased to 2.58 ± 0.24% at 12 wpi (Fig. 2c). Consistently, RNA-seq reads from AAV-KI (hF9) livers can be mapped to the integration junction site in mAlb-ires-hF9 chimeric transcript template, which was absent in control and un injected mice (Fig. 2d, upper panels). Importantly, many of these reads carried precise fusion sequences of donor and genome at the CRISPR-cleaveage sites (Fig. 2d, lower panels). Based on the junction data, 1.04 ± 0.14% mAlb-ires-hF9 chimeric transcripts were observed at 2 wpi, and the percentage increased to 3.26 ± 0.09% at 12 wpi (Fig. 2e; Supplementary Fig. 5).

As NHEJ knock-in is nondirectional, reverse insertions of ires-hF9 were also detected specifically in AAV-KI (hF9) livers, at comparable levels to those with forwarding insertions, around 1.41 ± 0.15% at 2 wpi and 5.25 ± 0.81% at 12 wpi (Fig. 2e; Supplementary Fig. 5). Whereas AAV vector integrations at sgAlb target site were detected at much lower rates, around 0.38 ± 0.14% and 0.72 ± 0.12% at 2 wpi and 12 wpi, respectively (Fig. 2e; Supplementary Fig. 5). Restricted by the 150-bp length for paired reads, the AAV vector types and integration orientations could not be assessed. These data indicated that cleaved AAV donors are more suitable for NHEJ knock-in.

Consistently, the occurrence of indels was solely observed among sgAlb cleavage site across the entire mAlb transcript in AAV-KI (hF9) livers. Approximately, 14.91 ± 2.85% indels were detected at 2 wpi and the rates increased to 42.3 ± 0.89% at 12 wpi (Fig. 2f). Mismatch analysis...
at individual nucleotide positions indicated that on-target indels largely occurred at SpCas9/sgAlb cutting site between the third and fourth nucleotide positions from protospacer adjacent motif sequence (Fig. 2g).

Furthermore, we assessed the off-target effect using the RNA-seq data. While extensive in silico prediction identified 129 candidate off-target sequences with ≤4 mismatches to the sgAlb target sequence (Supplementary Figs. 6 and 7a), only three were located in exons or in proximity (±50 bp) to exons (Supplementary Fig. 7b). The corresponding coding genes showed low expressions (<2 FPKM) and no correlation with AAV or knock-in treatments (Supplementary Fig. 7c).

We also examined the top ten off-target sequences predicted with Cas-OFFinder by low-throughput genome PCR-sequencing, followed by Inference of CRISPR Edits (ICE) analysis (Supplementary Fig. 8a). Among four liver samples examined, no editing event was observed at any of the candidate off-target sites. In contrast, 13.5 ± 4.5% and 25.5 ± 3.5% indels were observed at sgAlb cleavage site in AAV-KI (hF9)
liver DNAs collected at 12 wpi and 48 wpi, respectively (Supplementary Fig. 8b, c).

**NHEJ knock-in of hF9 in neonatal hemophilia B mice corrected hemostasis without altering the germline genome**

To investigate the efficacy of NHEJ knock-in in neonatal mice, AAVs at a total dose of 1 × 10¹⁰ vg/neonate were delivered intraperitoneally (i.p.) into mF9−/− pups at P3, and blood samples were collected every 4 weeks (Fig. 3a). The AAV-KI (hF9) pups robustly produced hFIX in plasma, which reached 1000–1500 ng/ml by 20 wpi and remained stable until 48 wpi (Fig. 3b). The presence of hFIX was also confirmed by IHC analyses and no sign of liver damage was observed with H&E staining (Fig. 3c). The hFIX-positive hepatocytes in AAV-KI (hF9) pups appeared in larger colonies than those in adult mice, implying a greater proliferation rate in young liver cells. Consistently, hemostasis correction was confirmed among the AAV-KI (hF9) pups and remained stable throughout the observation period (Fig. 3d).

In contrast, scAAV (LP1-hF9) injection triggered high plasma hFIX production at 1 wpi, followed by a dramatic decline to 4 wpi, suggesting rapid dilution of episomal scAAVs in fast-growing livers (Supplementary Fig. 9a, b). The decline in plasma hFIX corresponded to slightly higher aPTT values observed from 2 to 4 wpi (Supplementary Fig. 9c). The observations were further supported by IHC staining (Supplementary Fig. 9d, e).

AAV2/8 displays a remarkably broad tissue tropism; yet its ability to infect testicular germ cells remains controversial22-23. To address this issue, testicular DNA samples from six male AAV-KI (hF9) pups were examined by genome PCR. While AAV-CRISPR mediated hF9 knock-in was robustly confirmed in all six livers, no hF9 knock-in or AAV integration was observed in testes (Fig. 3e). Furthermore, four pairs of mice grown from AAV-KI (hF9) pups were bred and their offspring were examined for possible generational transmission (Supplementary Fig. 10a). Genome PCR analysis showed that F1-generation pups carried neither hF9 knock-in nor AAV integration (Fig. 3f and Supplementary Fig. 10b). Consistently, their aPTT values were the same as uninjected mF9−/− mice (Supplementary Fig. 10c). These data indicated that AAV-CRISPR mediated NHEJ knock-in was restricted to somatic tissues, which posed a minimal risk for germline modification and unintended vertical transmission.

**Liver-specific knock-in via AAV-CRISPR eliminated unintended genome editing in off-target tissues**

In preceding experiments, liver-specific expression was established in mice by knocking-in promoterless transgenes at mAlb locus (Figs. 1–3), where AAV2/8-delivered nEF-SpCas9 could silently edit the host genome in other tissues. We then replaced the universal nEF promoter with a liver-specific LP1 promoter to restrict knock-in activity to the liver. To visualize the tissue-specific knock-in, AAV donor carrying luciferase reporter was knocked-in at the ubiquitously expressed mActb at 3’UTR using either LP1-SpCas9 or nEF-SpCas9 (Fig. 4a). Gene knock-in using nEF-SpCas9 produced broad luciferase signals throughout the body (Fig. 4b, left). In contrast, LP1-SpCas9 mediated tissue-specific editing, yielding signals exclusively restricted to the liver (Fig. 4b, right). Noticeably, the high promoter activity of LP1 also contributed to higher luciferase activity in AAV-KI (Luc) mouse livers (Fig. 4c, d).

Next, we compared LP1-SpCas9 and nEF-SpCas9 mediated hF9 knock-in in mAlb 3’UTR in mF9−/− mice. Similarly, higher hFIX production was detected in both adult and neonatal AAV-KI (hF9) mice treated with LP1-SpCas9 (Fig. 4e). Although site-specific knock-in at mAlb resulted in liver-specific hFIX expression in both LP1-SpCas9 and nEF-SpCas9 edited mice (Fig. 4f), genome PCR analysis confirmed that targeted insertion of hF9 was confined to the liver in mice edited using LP1-SpCas9, whereas those treated with nEF-SpCas9 received hF9 knock-in in multiple organs including heart and kidney (Fig. 4g).

**Liver-specific NHEJ knock-in corrected hemostasis in neonates and adults with low-dose AAV**

To determine the lowest AAV dose needed for effective hF9 knock-in in neonatal mice, the therapeutic AAV mixture consisted of ires-hF9 donor, LP1-SpCas9, and sgAlb/sgA at 2:1:1 ratio was successively diluted in 1:5 with PBS and delivered into mF9−/− neonates at P1 through i.p. or i.v. routes (Fig. 5a, left). Blood assays at 2 wpi showed that the lowest dose which yielded significant hFIX production and corrected hemostasis was 1 × 10¹⁰ vg/neonate via i.p injection and 2 × 10⁹ vg/neonate via i.v. injection. More than 100 ng/ml of plasma hFIX was detected and a significant reduction in aPTT value was observed in these mice (Fig. 5a, middle and right).

Next, we constructed an AAV donor carrying hF9EKA24 mutant, which encodes hyperactive hFIX variant Padua24, to explore a further reduction of AAV dose needed for therapeutic knock-in. The ires-hF9EKA24 donor was delivered alongside LP1-SpCas9 and sgAlb/sgA at 2:1:1 ratio into mF9−/− neonates at P1 through i.p. or i.v. routes (Fig. 5a, left). Blood assays at 2 wpi showed that the lowest dose which yielded significant hFIX production and corrected hemostasis was 5 × 10¹⁰ vg/neonate via i.p. injection and 10⁹ vg/neonate via i.v. injection, approximatively fivefold lower than those using normal hF9 (Fig. 5a). Notably, with the same AAV dose at 2 × 10⁹ vg/neonate, i.v. administration resulted in a higher plasma Fadu level and significantly lower aPTT value than those of i.p. injection (Fig. 5b), yielding robust hFIX activity and persistent hemostasis correction till 20 wpi (Supplementary Fig. 11).

To further evaluate the dose effect, we conducted a dose-response assay for hF9EKA24 knock-in in adult mF9−/− mice. The AAV mixture containing ires-hF9EKA24, LP1-SpCas9, and sgAlb/sgA was successively diluted to yield six different doses: 2 × 10¹⁰, 4 × 10⁹, 8 × 10⁹, 4 × 10⁸, 2 × 10⁷, 1 × 10⁶ vg/mouse (Fig. 5c, upper). 6–12-week mF9−/− mice were divided into six groups to receive the different doses of AAVs through i.v. injection. Blood samples were collected weekly or biweekly up to 32 wpi. Notably, mice in all six treatment groups

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**References**

[1] Nature Communications | (2022) 13:7275

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[2] Nature Communications | (2022) 13:7275

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[3] Nature Communications | (2022) 13:7275
produced plasma hFIX-Padua at significant levels. Similar to the observations in adult AAV-KI (hF9) mice (Fig. 1b), a steady increase of plasma hFIX-Padua in the first 4–6 wpi was evident in all six groups (Fig. 5c, lower left). Effective hemostasis correction was achieved with AAV dose as low as 1 × 10^10 vg/mouse, which produced plasma hFIX-Padua at around 350 ng/ml (Fig. 5c, lower, red circle). The relative hFIX activities among all treatment groups were restored significantly since 2 wpi, with the lowest AAV dose at 1 × 10^10 vg/mouse yielding approximately 30% of the normal hFIX activity (Fig. 5d).

In vivo knock-in with low-dose AAV-CRISPR greatly reduced indels at the target site among neonatal mice

To elucidate the in vivo gene editing profiles yielded with low-dose AAV-CRISPR, liver RNAs extracted from fifteen AAV-KI (hF9^R338L) mice

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receiving various AAV doses at either adult or neonatal stage were analyzed by deep sequencing (Supplementary Fig. 12). Regardless of treatment time points and AAV input doses, the transcriptome profiles were highly similar (R ≥ 0.8) among all the samples examined (Fig. 6a). The levels of hF9R338L transcripts in both adult and neonatal AAV-KI (hF9R338L) were AAV dose-dependent (Fig. 6b), which were concordant with the plasma hFIX-Padua concentrations (Fig. 3b, c).

Subsequently, the frequencies of indels, forward and reverse insertions of mAlb-hF9R338L and AAV integrations at the sgAlb target site were assessed. Based on RNA reads mapped to knock-in/integration junctions, ires hF9R338L insertion in either orientation decreased with reduced AAV input (Fig. 6c). In the adult mouse receiving AAV input at 1 × 109 vg/mouse, the forward and reverse knock-in was 0.49% and 0.48%, respectively. Whereas in the neonatal mouse treated with the lowest effective AAV dose at 2 × 109 vg/ neonate, 0.077% forward and 0.052% reverse insertions were detected. The AAV integrations were around 0.037% and 0.004% in the corresponding adult and neonatal AAV-KI (hF9R338L) mouse examined (Fig. 6c).

Interestingly, rates of indels at sgAlb target site were significantly lower in neonatal AAV-KI (hF9R338L) mice, compared to their adult counterparts while AAV dose-dependent reduction was observed in both groups (Fig. 6c). The adult AAV-KI (hF9R338L) mouse receiving AAV input at 1 × 109 vg/mouse yielded approximate 21.55% of mAlb transcripts carrying target indels. Distinctly, 6.43%–11.39% indels were observed in the neonatal mice treated with high AAV doses at 1 × 1010 and 5 × 109 vg/neonate, while only around 1.41% indels were detected when AAV input was reduced to 2 × 109 vg/neonate.

AAV-CRISPR induced heterogeneous anti-Cas9 immunities in adult mice, but low immunity to Cas9 in neonatal mice

To address the immunogenicity of AAV-delivered Cas9, we determined the presence of anti-Cas9 antibodies after AAV-CRISPR treatment in various mouse models. Adult ICR mice injected with AAV2/8 carrying either nEF-Cas9 or LPI-Cas9 were sampled weekly or bi-weekly to determine the levels of plasma anti-Cas9 antibody using homemade ELISA (Fig. 7a, upper). Three out of four mice developed antibodies against Cas9, and the levels increased dramatically after 3 wpi. The remaining one showed negligible anti-Cas9 activity throughout the 16-week observation period (Fig. 7a, lower). Plasma analysis for adult AAV-KI (hF9) mice edited with nEF-Cas9 or LPI-Cas9 (Fig. 4e) also showed diverse levels of anti-Cas9 activities at 6 wpi, without significant difference between the two groups (Fig. 7b).

Retro-analysis of adult AAV-KI (hF9) mice (Fig. 1b-d) showed detectable plasma Cas9 antibodies in all five mice during 6–20 wpi. While four mice showed the highest anti-Cas9 activity around 10–12 wpi, one mouse responded slowly and its anti-Cas9 activity peaked at 20 wpi. The overall levels of anti-Cas9 activities declined in all five mice after 20 wpi and remained at lowest levels after 28 wpi (Fig. 7c).

Consistently, heterogeneous anti-Cas9 activities were observed in adult AAV-KI (hF9R338L) mice receiving different AAV doses (Supplementary Fig. 13a). Levels of anti-Cas9 antibodies detected at 6 wpi showed no correlation with AAV inputs, hFIX-Padua production, or Cas9 expression levels (Supplementary Fig. 13b, c). In contrast, anti-AAV2/8 antibodies were robustly developed in all treated mice (Supplementary Fig. 14). Collectively, these findings indicated that SpCas9 provoked heterogeneous immune responses in adult mice, which did not cause dramatic elimination of gene-edited hepatocytes.

Interestingly, retro-analysis of AAV-KI (hF9) pups (Fig. 3b–d) demonstrated slower development of immune responses and lower anti-Cas9 activities than those in adult mice (Fig. 7d). Similarly, AAV-KI (hF9) pups receiving lower AAV doses (Fig. 5a) showed sporadic anti-Cas9 activities, regardless of the administration routes (i.p. or i.v.) (Supplementary Fig. 15a). Markedly, AAV-KI (hF9) pups also developed anti-AAV2/8 antibodies at much lower levels than their adult counterparts. The anti-AAV activities were negligible in pups receiving AAVs at 1 × 108 vg/neonate via i.p. or 2 × 108 vg/neonate via i.v. (Supplementary Fig. 15b). These results suggested that administration at the neonatal stage might bypass the pre-existing immunity and is promising for AAV-CRISPR-based gene-editing therapy.

Pre-existing anti-Cas9 immunity had a subtle impact on in vivo knock-in by AAV-CRISPR

To evaluate the impact of host anti-Cas9 immunity on AAV-CRISPR-mediated in vivo knock-in, we performed hF9R338L knock-in in pre-immunized mice. Adult mF9−/− mice at 6–12 weeks were pre-immunized with SpCas9 protein at 25 μg/mouse via subcutaneous (s.c.) injection. Neonatal mF9−/− pups at P1 were treated with SpCas9 protein at 10 μg/pup by either i.v. or i.p. injection. Heterogeneous anti-Cas9 activities were detected in SpCas9-immunized adult mice, which reached the highest levels after two weeks and decreased substantially at the fifth-week post-immunization (Fig. 7e). In pre-immunized neonatal mice, low anti-Cas9 activities were detected throughout the 5-week observation, regardless of injection routes (Fig. 7f, Supplementary Fig. 16a).

The Cas9-immunized mice and controls were then i.v. injected with AAV mixture containing ires-hF9R338L donor, LPI-SpCas9, and sgAlb/sgA at 2:1 ratio, at a total dose of 4 × 1011 vg/mouse. Notably, all mice treated for AAV-KI (hF9R338L) produced plasma hFIX-Padua at significant levels with a steady increase at early stages (Fig. 7e, f). No significant difference was observed between the controls and SpCas9-immunized mice, and similar levels of plasma hFIX-Padua were detected in adult and neonatal pre-immunized mice (Fig. 7e, f). The anti-Cas9 analysis detected no immunological memory in both adult and neonatal mice after SpCas9 immunization (Fig. 7e, f). No reverse correlation was observed between the levels of anti-Cas9 antibodies and plasma hFIX-Padua concentrations, either before or after hF9R338L knock-in (Supplementary Fig. 16b, c). Collectively, these data suggested that the host anti-Cas9 immunity posed a subtle impact on in vivo gene knock-in outcomes through systemic delivery.
Fig. 3 | NHEJ knock-in of hF9 in neonatal mice corrected hemophilia B without modifying germline genome. a Schematics for i.p. injection of AAVs carrying SpCas9, sgAlb/sgA and ires-hF9 into neonatal mF9−/− pups at postnatal day 3 (P3) (upper), AAV vector carrying ires-hF9 (middle) and the sgAlb target site at mAlb 3′UTR (lower). b hFIX protein levels in pup plasma after i.p. injection of AAVs carrying SpCas9 (1 × 1011 vg/mouse), sgAlb/sgA (1 × 1011 vg/mouse), and ires-hF9 donor (2 × 1011 vg/mouse) (n = 7). The Control group was injected with ires-hF9 donor only (n = 5), and un.injected mF9−/− mice were examined as reference (n = 5). ELISA was performed using an anti-hFIX antibody. c DNA fragment sizes were identified using agarose gel electrophoresis. Two representative samples are shown per group. d Schematics for injection method and tissue collection after male AAV-KI (hF9) pups grew into adults (left). Genome PCR analysis on livers (red) and testes (blue) collected from individual AAV-KI (hF9) pups (right). Primer sequences for detecting S′ junctions (Alb-F1/R1) and hF9 integration (F2/R2) were listed in Supplementary Table 2. The binding sites of primers Alb-F1/R1 and hF9 integration (F2/R2) were listed in Supplementary Table 2. The binding sites of primers Alb-F1/R1 and hF9 integration (F2/R2) were listed in Supplementary Table 2. The binding sites of primers Alb-F1/R1 and hF9 integration (F2/R2) were listed in Supplementary Table 2. The binding sites of primers Alb-F1/R1 and hF9 integration (F2/R2) were listed in Supplementary Table 2. The binding sites of primers Alb-F1/R1 and hF9 integration (F2/R2) were listed in Supplementary Table 2. The binding sites of primers Alb-F1/R1 and hF9 integration (F2/R2) were listed in Supplementary Table 2. The binding sites of primers Alb-F1/R1 and hF9 integration (F2/R2) were listed in Supplementary Table 2. 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transcriptome analysis showed no evident alteration of liver functions. Furthermore, using LP1-SpCas9 could confine the transgene knock-in to the liver, achieving tissue-specific gene editing. Notably, employing LP1-SpCas9 to knock-in hyperactive hF9R338L could reduce the AAV input dose to $2 \times 10^9$ vg/neonate and $1 \times 10^{10}$ vg/adult for hemostasis correction in mF9$^{-/-}$ mice. These AAV inputs were 10- to 100-fold lower than those reported$^{7-10,13-15}$, and the treatment in neonates yielded low levels of anti-AAV and anti-Cas9 antibodies. Collectively, our results suggested that therapeutic knock-in can be achieved with low and safe AAV inputs in clinics, and administration
The mice were sacrificed at 2 weeks post-infection (wpi) with 7.0 × 10^{12} vgs/mouse, SGActb/SGAl (5.0 × 10^{11} vgs/mouse), together with nEF-mediated by AAV2/8-delivered nEF-SpCas9 (left) or LP1-SpCas9 (right), shown in Luminescence images of treated ICR mice at 2 wpi. Targeted insertion was compared to other strategies reported7,10,27,30.

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Fluorescence imaging was used to confirm that AAV knock-in at Alb locus was achieved. As shown in the Supplementary Fig. 6, nEF-mediated knock-in at the Alb was observed, as indicated by the green fluorescence in the liver section of the treated mice. Moreover, the use of LP1 promoter significantly improved the targeting efficiency, as evidenced by the increased fluorescence intensity compared to the nEF-mediated knock-in (Supplementary Fig. 6).

The large sizes of Cas9 proteins and a small payload of AAV often result in low transduction efficiency. To address this challenge, we employed a bicistronic expression strategy involving the viral 2A peptide14,15 and splice targeting sequences27,31. This approach allowed for efficient delivery of Cas9 and sgRNA, facilitating targeted modification at the Alb locus.

To further investigate the feasibility of this approach, we performed a series of experiments to optimize the delivery vectors and Cas9/sgRNA combination. We found that a higher expression level of Cas9 was achieved by using the hF9 promoter, which is specifically expressed in the liver. This resulted in improved targeting efficiency at the Alb locus.

In conclusion, our study demonstrates the potential of AAV-based gene delivery for targeted modification at specific loci. The bicistronic expression strategy and improved targeting efficiency achieved by using the hF9 promoter represent promising avenues for future development of AAV-based gene editing strategies.
reverse knock-in and AAV integration. The underlying molecular mechanisms are largely unexplored, and further reduction of the unintended knock-in/integration through new vector designs would be desired.

Finally, default NHEJ repair generates background indels at the CRISPR cleavage site. Our data showed that the indel frequencies increased over time (Fig. 2e, f) and correlated with AAV inputs (Fig. 6c), ranging from 12.06% to 46.22% at sgAlb target sites among adult hF9 and hF9R338K knock-in mice. Interestingly, we observed much lower indel frequencies (1.41–11.39%) in neonatal knock-in mice producing comparable levels of plasma hFIX-Padua (Fig. 6c). We speculated that the persistent expression of AAV-delivered Cas9 and sgRNAs in the adult liver may yield excessive indels at the target site, whereas rapid dilution of AAV episomes in neonatal pups reduced indel occurrence.
These data revealed a previously unrecognized advantage of applying somatic gene knock-in at the neonatal stage.

A recent report of anti-Cas9 immunity in human [44, 45] raised new concerns for clinical translation of AAV-CRISPR-based gene editing therapy [46, 47]. Host immunity to Cas9 was associated with potential harms, such as immune attacks to Cas9-expressing cells or induced tissue damage. Moreover, pre-existing or even post-dosing anti-Cas9 immunity might diminish gene editing outcomes by destroying the edited cells. Similarly to the previous report [48], heterogeneous anti-Cas9 activities were detected among adult mice treated with AAV-CRISPR or SpCas9 protein in our study (Fig. 7a–c; e). Interestingly, our retro-analysis and longitudinal data supported that host anti-Cas9 activities were transient and disappeared eventually (Fig. 7c, e). Concordantly, the hFIX-Fadua productions were similar in the mock and SpCas9 and sgAlb/sgA at 2:1:1 ratio. Plasma hFIX levels (middle) and aPTT values (right) were measured at 2 wpi. Total AAV doses (vg/mouse) used in each group were indicated in the x-axis. The number of mice per group (n) was indicated in the brackets above the data bars. b Knock-in of hFIXP in SpCas9 and sgAlb/sgA at 2:1:1 ratio. Plasma hFIX-Fadua levels (left) and aPTT values (right) were measured at 2 wpi. Total AAV doses (vg/mouse) used in each group were indicated in the x-axis. The number of mice per group (n) was indicated in the brackets above the data bars. c Knock-in of hFIX in SpCas9 in adult mF9−/− mice with various AAV doses. mF9−/− mice at 6–12 weeks were injected with AAV2/8 carrying ires-hFIXP donor, LPI-SpCas9, and sgAlb/sgA at 2:1:1 ratio. Control mice were injected with donors only. The schematics for i.v. injection and workflow were shown in the upper left. The AAV doses (vg/mouse) tested were shown in the graph legend (upper right). The number of mice per group (n) was indicated in the brackets after the doses. Data shown were plasma hFIX-Fadua levels (lower left) and aPTT values (lower right) measured at 0, 1, 2, 4, 24, and 32 wpi for individual mouse groups. d Relative hFIX activities at 2 wpi among different dose groups in (e). The AAV doses (vg/mouse) used in each group were indicated in the x-axis. The standard curve was generated using recombinant human factor IX (MonofIX). The number of mice per group (n) was indicated in the brackets after the doses in (e). All data are mean ± SD. Statistical analyses were performed using two-tailed unpaired t-tests. n.s. not significant.
AAV-CRISPR for NHEJ knock-in. AAV mixtures were prepared to consist of AAV-donor (ires-hF9, ires-Luc or ires-hF9R338L), AAV encoding SpCas9 (driven by either nEF or LP1 promoter), and AAV carrying sgRNAs (sgAlb/sgA or sgActb/sgA) at 2:1:1 ratio prior injections. To deliver NHEJ knock-in of hF9 in adult mF9−/− mice (8–12 weeks) (Figs. 1, 4e–g), 300 µl AAV mixture containing ires-hF9 donor, nEF- or LP1-SpCas9 and U6-sgAlb/sga at 2:1:1 ratio was administered via tail vein at a total dose of 2 × 1012 vg/mouse. To knock-in of Luciferase reporter in adult ICR mice (8–12 weeks) (Fig. 4a), 300 µl AAV mixture of ires-Luc donor, nEF- or LP1-SpCas9 and U6-sgActb/sga at 2:1:1 ratio was administered via tail vein at a total dose of 2 × 1012 vg/mouse. To deliver NHEJ knock-in of hF9 in

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**Fig. 6** | RNA-seq analysis for liver-specific knock-in of hF9R338L with various AAV input doses revealed much-reduced indel rates at a target site in neonatal mice. a Transcriptomic correlation heat map built with R values based on RNA-seq data of 15 liver samples collected from adult and neonatal AAV-KI (hF9R338L) mice receiving various AAV doses. Data are Pearson correlation coefficients. Samples and treatment conditions were shown in Supplementary Fig. 12. b Levels of hF9R338L transcripts detected by RNA-seq among the adult (upper) and neonatal (lower) AAV-KI (hF9R338L) liver samples examined in (a). Each dot represents one mouse sample. Data shown are mean ± SD for adult mice receiving 8 × 106 vg/mouse (n = 3) and neonates receiving 5 × 106 and 5 × 105 vg/pup (n = 2). Data at other doses were from one mouse (n = 1). FPKM fragments per kilobase million. #: the minimum effective AAV input doses yielding relative hFIX activity above 20% of the normal (Fig. 5d; Supplementary Fig. 11b). c Percentages of mAlb transcripts carrying indels, forward or reverse ires-hF9R338L, and AAV integration at sgAlb target site, among the adult (upper left) and neonatal (upper right) AAV-KI (hF9R338L) mice examined in (a). The AAV doses (vg/mouse) used in each group were indicated in the x-axis. #: mice treated with minimum effective AAV input doses. The representative data in the table (lower) are from adult and neonatal AAV-KI (hF9R338L) mice treated with minimum effective AAV input doses (marked with # in upper panels).
neonatal mF9−/− pups at P1–P3 (Fig. 3), 50 µl AAV mixture containing ires-hF9 donor, nEF-SpCas9 and U6-sgAlb/sgA at 2:1:1 ratio was administered at a total dose of 4 × 10¹¹ vg/mouse, either intraperitoneally (i.p.) or intravenously (i.v.) via a facial vein.

To perform dose–response analysis in Fig. 5, an AAV mixture consisting of ires-hF9 (or ires-hF9R338L) donor, LP1-SpCas9 and sgAlb/sgA at 2:1:1 ratio was successively diluted to yield different doses as indicated. Fifty microlitre AAV mixtures at various doses were injected to neonatal mF9−/− mice (P1) via i.v. or i.p. injection, while 300 µl AAV mixtures at various doses were injected to adult mF9−/− mice (6–12 weeks) via i.v. injection. Donor control mice in Fig. 5 only received the ires-hF9R338L donor at a dose of 4 × 10¹⁰ vg/mouse. Both male and female mice were used in these AAV-CRISPR-mediated NHEJ knock-in experiments.
AAV-based overexpression. The ssAAV or scAAV vectors carrying LPHf9 were injected to adult m9f9/− mice at 6–12 weeks via tail vein at the dose of 5 × 10^11 vg/mouse (Fig. 1e–g). scAAV vectors carrying LPHf9 were i.p. injected to neonatal m9f9/− mice at P3 at the dose of 1 × 10^12 vg/mouse (Fig. 1e–g). The AAV2/8 vectors carrying either nEF-SpCas9 or LPI-SpCas9 were injected to adult ICR mice at 6 weeks via tail vein at the dose of 5 × 10^11 vg/mouse (Fig. 7a).

spCas9 pre-immunization. Twenty-five microgram SpCas9 protein (Invitrogen, #A36499) was subcutaneously (s.c.) injected to adult m9f9/− mice (6–8 weeks). Ten microgram SpCas9 protein was i.v. or i.p. injected into neonatal m9f9/− mice at P1. The control mice were injected with PBS. Five weeks later, AAV mixture containing either ires-hF9R338L donor, LPI-SpCas9, and sgAlb/sga at a 2:1:1 ratio was i.v. injected into both SpCas9-immunized or PBS-treated mice, at a total input dose of 4 × 10^11 vg/mouse.

Bioluminescence in vivo imaging. Mice were weighed, anesthetized using Ketamine/Xylazine (100 mg/kg and 10 mg/kg body weight respectively), and i.p injected with α-luciferin (GoldBio, #LUCK-100) at 0.15 mg/g body weight, followed by immediate bioluminescence in vivo imaging (Bruker In-vivo Xtreme Imaging System).

Partial hepatectomy. Mice were anesthetized using Ketamine/Xylazine (100 mg/kg and 10 mg/kg body weight, respectively) via i.p. injection and placed in the supine position. Ligation and excision of left and medium lobes were performed. The excised liver tissues were fixed immediately in cold 4% paraformaldehyde or embedded in Tissue-Tek™ O.C.T. compound (Sakura Finetek™ 4583) through snap freezing in liquid nitrogen for histological analysis.

Plasma hFIX assay
Blood specimens were collected by retro-orbital bleeding from adult mice or adolescent mice at 2–4 weeks. From pups younger than 2 weeks, blood specimens were collected by decapitation after anesthesia using Ketamine/Xylazine (100 and 10 mg/kg body weight, respectively). To collect plasma, blood specimens obtained were immediately mixed with 3.8% sodium citrate at 9:1 ratio to stop coagulation and subsequently spun down. The upper transparent plasma was then transferred into a new tube and kept at −80 °C for further use. The levels of plasma hFIX were determined by ELISA using Human Factor IX ELISA Kit (Abcam, ab188393).

Activated Partial thromboplastin time (aPTT) test
aPTT was measured using STart 4 Hemostasis Analyzer (Diagnostica Stago). Briefly, plasma samples collected were diluted 5 times with Owren-Koller buffer (Diagnostica Stago, #TSGO0360) and then mixed with human FX-deficient plasma (George King Biomedical, Inc. #FIX-ID) and C.K.Prest reagent (Diagnostica Stago, #00398) at 1:1 ratio, followed by 37 °C incubation for 180 s. Coagulation was triggered by the addition of 25 mM calcium chloride, and clot formation time was measured by STArt 4 Hemostasis Analyzer (Diagnostica Stago).

Histology and flow cytometry
For histological and flow cytometry analyses, fresh liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin, or directly snap frozen in Tissue-Tek™ O.C.T. compound (Sakura Finetek™ 4583) for cryosectioning. HE and IHC staining was performed on 5 or 10 μm frozen sections.
Genomic PCR analysis
Mouse genomic DNA was extracted using TIANamp Genomic DNA Kit (Tiangen, #4992254), and Phusion High-Fidelity DNA Polymerase (New England Biolabs, #M0530L) was used for each PCR reaction.

Indel detection via low-throughput PCR and sequencing. In silico prediction of off-target sites by sgAlb was performed using “Cas-OffFinder v2.4” (http://www.rogenome.net/cas-offinder/). Genomic fragments covering sgAlb target sequence or the top ten off-target sites were amplified from AAV-KI (hF9) mice. The amplicons were subjected to Sanger sequencing and the data obtained were analyzed using IGE (www.igenes.com) or STAGE (www.stage.com.au) to determine indel rates. The primers used were listed in Supplementary Table 3.

RNA extraction and qRT-PCR
Total RNA was extracted from fresh or frozen tissues using TRizol reagent (Thermo Fisher Scientific, #15596026), followed by reverse-transcription into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, #4368814). PCR was then performed using the Phusion High-Fidelity DNA polymerase kit (New England Biolabs, #M0530L), and quantitative real-time RT-PCR was performed using the SYBR® Premix Ex Taq kit (Takara, #RR039A) in 7900HT Fast Real-Time PCR system (Applied Biosystems).

RNA-seq analysis
RNA-seq libraries were constructed using VAHTS mRNA-seq V3 Library Prep Kit for Illumina (Vazyme, #NR611) and sequenced in illumina HiSeq X by IGE BioTechnology Ltd. (Guangzhou, China). The total sequencing depths of all samples were more than 6 million in PE150 mode. RNA-seq clean reads were mapped to mouse transcript annotation of Gencode vM25 version on mm10 genome using RSEM (v1.0). The mapping rate is over 95%, and more than 5000 genes were detected from RNA-seq in all samples. The RNA-seq data were deposited into NCBI, and the accession number is PRJNA782865.

Fragments Per Kilobase Million (FPKM) values were used for the normalization and evaluation of gene expression levels. Data analysis and visualizations were performed in the R environment. Mutations and Indels were analyzed by HSA2 (v2.2.1)10, HTSeq (v2.0.7)11, and SAMtools (v1.9)12, then were visualized and calculated in Integrative Genomics Viewer (IGV) (v2.11.4).

Statistics and reproducibility
All data are presented as means ± SD of at least three independent experiments. Fluorescent-imaging analysis and animal studies were performed blinded and randomized. Representative experiments are shown after being repeated at least three times independently with similar results. Calculations were done using Microsoft Excel 2019, and graphs were plotted in GraphPad Prism 9 version 9.4.1. The sample sizes and p values are indicated in the figure graphs or the figure legends. Comparisons between the two groups were measured by two-tailed unpaired Student’s t-test. Differences with p values <0.05 were considered statistically significant.

Data availability
The authors confirm that the data supporting the findings in this study are available within the article and its supplementary materials. Source data are provided in this paper. The RNA-seq data generated in this study have been deposited in the NCBI Gene Expression Omnibus database under accession code PRJNA782865. Source data are provided in this paper.

References
1. Wang, D., Tai, P. W. L. & Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. Nat. Rev. Drug Discov. 18, 358–376 (2019).
2. Swiech, L. et al. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nat. Biotechnol. 33, 102–106 (2015).
3. Ran, F. A. et al. In vivo genome editing using Staphylococcus aureus Cas9. Nature 520, 186–191 (2015).
4. Cox, D. B., Platt, R. J. & Zhang, F. Therapeutic genome editing: prospects and challenges. Nat. Med. 21, 121–131 (2015).
5. Fellmann, C., Gowen, B. G., Lin, P. C., Doudna, J. A. & Corn, J. E. Cornerstones of CRISPR-Cas in drug discovery and therapy. Nat. Rev. Drug Discov. 16, 89–100 (2017).
6. Geodee, A. C. Hemophilia B: molecular pathogenesis and mutation analysis. J. Thromb. Haemost. 13, 1184–1195 (2015).
7. Li, H. et al. In vivo genome editing restores haemostasis in a mouse model of haemophilia. Nature 475, 217–221 (2011).
8. Anguela, X. M. et al. Robust ZFN-mediated genome editing in adult hemophilic mice. Blood 122, 3283–3287 (2013).
9. Ohmori, T. et al. CRISPR/Cas9-mediated genome editing via postnatal administration of AAV vector cures haemophilia B mice. Sci. Rep. 7, 4159 (2017).
10. Wang, L. et al. CRISPR/Cas9-mediated in vivo gene targeting corrects hemostasis in newborn and adult factor IX-knockout mice. Blood 133, 2745–2752 (2019).
11. Auer, T. O., Duroire, K., DeClan, A., Concordet, J. P. & Del Bene, F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Res. 24, 142–153 (2014).
12. He, X. et al. Knock-in of large reporter genes in human cells via CRISPR/Cas9-induced homology-dependent and independent DNA repair. Nucleic Acids Res. 44, e85 (2016).
13. Suzuki, K. et al. In vivo genome editing via CRISPR/Cas9-mediated homology-independent targeted integration. Nature 540, 144–149 (2016).
14. Chen, H. et al. Hemophilia A ameliorated in mice by CRISPR-based in vivo genome editing of human Factor VIII. Sci. Rep. 9, 16838 (2019).
15. Zhang, J. P. et al. Curing hemophilia A by NHEJ-mediated ectopic F8 insertion in the mouse. Genome Biol. 20, 276 (2019).
16. Wang, D., Zhang, F. & Gao, G. CRISPR-based therapeutic genome editing: strategies and in vivo delivery by AAV vectors. Cell 181, 136–150 (2020).
17. He, X., Uri, B. A., Zhang, Z., Negan, C. C. & Feng, B. Evolving AAV-delivered therapeutics towards ultimate cures. J. Mol. Med 99, 593–617 (2021).
18. McCarty, D. M., Monahan, P. E. & Samulski, R. J. Self-complementary recombinant adeno-associated virus (scAAV)
vectors promote efficient transduction independently of DNA synthesis. Gene Ther. 8, 1248–1254 (2001).
19. Lin, H. F., Maeda, N., Smithies, O., Straight, D. L. & Stafford, D. W. A coagulation factor IX-deficient mouse model for human hemophilia B. Blood 90, 3962–3966 (1997).
20. Cunningham, S. C., Dane, A. P., Spinoulas, A., Logan, G. J. & Alex- jander, I. E. Gene delivery to the juvenile mouse liver using AAV2/8 vectors. Mol. Ther. 16, 1081–1088 (2008).
21. Pipe, S., Leebeek, F. W. G., Ferreira, V., Sawyer, E. K. & Pasi, J. Clinical considerations for capsid choice in the development of liver-targeted AAV-Based gene transfer. Mol. Ther. Methods Clin. Dev. 15, 170–178 (2019).
22. Wang, Z. et al. Adeno-associated virus serotype 8 efficiently delivers genes to muscle and heart. Nat. Biotechnol. 23, 321–328 (2005).
23. Rajasekar, S. et al. Infectivity of adeno-associated virus serotypes in mouse tests. BMC Biotechnol. 18, 70 (2018).
24. VandenDriessche, T. & Chuah, M. K. Hyperactive factor IX padua: a game-changer for hemophilia gene therapy. Mol. Ther. 26, 14–16 (2018).
25. Simhadri, V. L. et al. Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population. Mol. Ther. Methods Clin. Dev. 10, 105–112 (2018).
26. De Caneva, A. et al. Coupling AAV-mediated promoterless gene targeting to SaCas9 nuclease to efficiently correct liver metabolic diseases. JCI Insight 5, e128863 (2019).
27. Wang, Q. et al. CRISPR-Cas9-mediated in vivo gene integration at the albumin locus recovers hemostasis in neonatal and adult hemophilia B mice. Mol. Ther. Methods Clin. Dev. 18, 520–531 (2020).
28. Ginn, S. L. et al. Efficient in vivo editing of OTC-deficient patient-derived human hepatocytes. JHEP Rep. 2, 100065 (2020).
29. Miller, D. G., Petek, L. M. & Russell, D. W. Adeno-associated virus vectors integrate at chromosome breakage sites. Nat. Genet. 36, 767–773 (2004).
30. Sharma, R. et al. In vivo genome editing of the albumin locus as a platform for protein replacement therapy. Blood 126, 1777–1784 (2015).
31. Chen, X. et al. Long-term correction of haemophilia B through CRISPR/Cas9 induced homology-independent targeted integration. J. Genet. Genomics 6, 2022.06.001. Online ahead of print.
32. Landau, D. J. et al. In vivo zinc finger nuclease-mediated targeted integration of a glucose-6-phosphatase transgene promotes survival in mice with glycosgen storage disease type IA. Mol. Ther. 24, 697–706 (2016).
33. Hinderer, C. et al. Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. Hum. Gene Ther. 29, 285–298 (2018).
34. High-dose AAV gene therapy deaths. Nat. Biotechnol. 38, 910 (2020).
35. Srivastava, A. et al. Guidelines for the management of hemophilia. Haemophilia 19, e1–e47 (2013).
36. Zheng, N., Li, L. & Wang, X. Molecular mechanisms, off-target activities, and clinical potentials of genome editing systems. Clin. Transl. Med. 10, 412–426 (2020).
37. Vakulskas, C. A. & Behlke, M. A. Evaluation and reduction of CRISPR off-target cleavage events. Nucleic Acid Ther. 29, 167–174 (2019).
38. Liu, G., Zhang, Y. & Zhang, T. Computational approaches for effective CRISPR guide RNA design and evaluation. Comput. Struct. Biotechnol. J. 18, 35–44 (2020).
39. Manghwar, H. et al. CRISPR/Cas systems in genome editing: methodologies and tools for sgRNA design, off-target evaluation, and strategies to mitigate off-target effects. Adv. Sci. 7, 1902312 (2020).
40. Nelson, C. E. et al. Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. Nat. Med. 25, 427–432 (2019).
41. Kappel, C. et al. A largely random AAV integration profile after LPLD gene therapy. Nat. Med. 19, 889–891 (2013).
42. Park, K. J. et al. Adeno-associated virus 2-mediated hepatocellular carcinoma is very rare in Korean patients. Ann. Lab. Med. 36, 469–474 (2016).
43. Berns, K. I. et al. Adeno-associated virus type 2 and hepatocellular carcinoma? Hum. Gene Ther. 26, 779–781 (2015).
44. Hanlon, K. S. et al. High levels of AAV vector integration into CRISPR-induced DNA breaks. Nat. Commun. 10, 4439 (2019).
45. Ibraheim, B. et al. Self-inactivating, all-in-one AAV vectors for precision Cas9 genome editing via homology-directed repair in vivo. Nat. Commun. 12, 6267–6267 (2021).
46. Zhang, C. et al. Homology-independent multicellular disruption via CRISPR/Cas9-based knock-in yields distinct functional outcomes in human cells. BMC Biol. 16, 151 (2018).
47. Charlesworth, C. T. et al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nat. Med. 25, 249–254 (2019).
48. Li, A. et al. AAV-CRISPR gene editing is negated by pre-existing immunity to Cas9. Mol. Ther. 28, 1432–1441 (2020).
49. Gough, V. & Gersbach, C. A. Immunity to Cas9 as an obstacle to persistent genome editing. Mol. Ther. 28, 1389–1391 (2020).
50. Moreno, A. M. et al. Immune-orthogonal orthogonals of AAV capsids and of Cas9 circumvent the immune response to the administration of gene therapy. Nat. Biomed. Eng. 3, 806–816 (2019).
51. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823 (2013).
52. Lock, M. et al. Rapid, simple, and versatile manufacturing of recombinant adeno-associated viral vectors at scale. Hum. Gene Ther. 21, 1259–1271 (2010).
53. Mitchell, C. & Willenbring, H. A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. Nat. Protoc. 3, 1167–1170 (2008).
54. Conant, D. et al. Inference of CRISPR edits from Sanger trace data. CRISPR J. 5, 123–130 (2022).
55. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12, 323 (2011).
56. Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nat. Biotechnol. 37, 907–915 (2019).
57. Putri, G. H., Anders, S., Pyl, P. T., Pimpana, J. E. & Zanini, F. Analysing high-throughput sequencing data in Python with HTSeq 2.0. Bioinformatics 38, 2943–2945 (2022).
58. Danecic, F. et al. Twelve years of SAMtools and BCFtools. Giga- Science 10, giab008 (2021).

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**Author contributions**

X.H. and Z.Z. performed most of the experiments and data analysis; J.X., S.Z., N.I., T.W. to N.I.; and T13-402/17-N to L.Q. X.H. and B.F. conceived the project, designed experiments and data analysis; Y.W. and Junkang W. performed bioinformatics analysis. X.H. and Z.Z. performed most of the experiments and data analysis; J.X., S.Z., N.I., T.W. to N.I.; and T13-402/17-N to L.Q. X.H. and B.F. conceived the project, designed experiments and data analysis; Y.W. and Junkang W. performed bioinformatics analysis. X.H. and Z.Z. performed most of the experiments and data analysis; J.X., S.Z., N.I., T.W. to N.I.; and T13-402/17-N to L.Q. X.H. and B.F. conceived the project, designed experiments and data analysis; Y.W. and Junkang W. performed bioinformatics analysis.
experiments, interpreted results, and wrote the paper. Z.L., H.Z., D.P., and C.L. revised the paper.

**Competing interests**
The authors declare no competing interests.

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**Correspondence** and requests for materials should be addressed to Bo Feng.

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