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Prevention of Cholestatic Liver Disease and Reduced Tumorigenicity in a Murine Model of PFIC Type 3 Using Hybrid AAV-*piggyBac* Gene Therapy

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Recombinant adeno-associated viral (rAAV) vectors are highly promising vehicles for liver-targeted gene transfer, with therapeutic efficacy demonstrated in preclinical models and clinical trials. Progressive familial intrahepatic cholestasis type 3 (PFIC3), an inherited juvenile-onset, cholestatic liver disease caused by homozygous mutation of the ABCB4 gene, may be a promising candidate for rAAV-mediated liver-targeted gene therapy. The *Abcb4*$^{-/-}$ mice model of PFIC3, with juvenile mice developing progressive cholestatic liver injury due to impaired biliary phosphatidylcholine excretion, resulted in cirrhosis and liver malignancy. Using a conventional rAAV strategy, we observed markedly blunted rAAV transduction in adult *Abcb4*$^{-/-}$ mice with established liver disease, but not in disease-free, wild-type adults or in homozygous juveniles prior to liver disease onset. However, delivery of predominantly nonintegrating rAAV vectors to juvenile mice results in loss of persistent transgene expression due to hepatocyte proliferation in the growing liver. *Conclusion:* A hybrid vector system, combining the high transduction efficiency of rAAV with *piggyBac* transposase-mediated somatic integration, was developed to facilitate stable human ABCB4 expression *in vivo* and to correct juvenile-onset chronic liver disease in a murine model of PFIC3. A single dose of hybrid vector at birth led to life-long restoration of bile composition, prevention of biliary cirrhosis, and a substantial reduction in tumorigenesis. This powerful hybrid rAAV-*piggyBac* transposon vector strategy has the capacity to mediate lifelong phenotype correction and reduce the tumorigenicity of progressive familial intrahepatic cholestasis type 3 and, with further refinement, the potential for human clinical translation. (Hepatology 2019;0:1-15).

Biliary phosphatidylcholine (PC) is vital for mitigating bile salt–induced toxicity by the formation of mixed micelles with bile salts and cholesterol.[1] An adenosine triphosphate binding cassette transporter, ABCB4, also known as class III multidrug resistant P-glycoprotein (MDR3), is a hepatocanalicular floppase that mediates PC translocation into bile.[2] The importance of ABCB4 to bile homeostasis is exemplified by a rare hereditary cholestatic disorder, progressive familial intrahepatic cholestasis type 3 (PFIC3, OMIM #602347[3]), in which ABCB4 mutations result in chronic liver disease, commonly of childhood onset.[4] Individuals with PFIC3 have reduced or absent biliary PC excretion,[5]

Abbreviations: ABCB4, adenosine triphosphate binding cassette, subfamily B, member 4; ALP, alkaline phosphatase; ALT, alanine aminotransferase; bp, base pair; cDNA, complementary DNA; eGFP, enhanced green fluorescent protein; hABCB4, human ABCB4; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin; LP1, liver-specific promoter; mGAPDH, murine genomic glyceraldehyde 3-phosphate dehydrogenase; ns, not significant; PC, phosphatidylcholine; PFIC3, progressive familial intrahepatic cholestasis type 3; rAAV, recombinant adeno-associated virus; TRsh, short piggyBac terminal repeats.

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and therefore unmitigated bile salt–induced canalicular and cholangiocyte damage resulting in a progressive cholangiopathy and biliary fibrosis. Affected individuals are also at risk of hepatocellular and biliary malignancies.[6,7] Approximately 50% of patients progress to cirrhosis, portal hypertension, and end-stage liver failure, requiring liver transplantation at a median age of 7.5 years.[8] Although survival from liver transplant has improved, paucity of donor organs, surgical morbidity, immunological rejection, and lifelong immunosuppression remain major issues for transplant recipients.

Gene therapy may offer an alternative and definitive treatment for PFIC3 patients. Among contemporary gene transfer vehicles, recombinant adeno-associated viral vectors (rAAVs) show exceptional promise for liver-targeted applications.[9,10] This vector system possesses several properties that make it ideally suited to liver gene transfer. These include the ability to transduce nondividing cells such as quiescent hepatocytes, and a low propensity to induce liver inflammation and high tropism of selected serotypes for primary human hepatocytes *in vivo*.[9,11-14] Therapeutic promise in the liver is exemplified by multiyear therapeutic efficacy following a single infusion of rAAV-expressing Factor IX in hepatocytes of adult patients with severe hemophilia B.[15]

In the process of devising a conventional rAAV-mediated strategy for treatment of PFIC3, we observed markedly blunted rAAV transduction in adult *Abcb4*$^{-/-}$ mice with established liver disease, but not in disease-free wild-type adults, or in *Abcb4*$^{-/-}$ juveniles prior to liver disease onset. Because this *Abcb4*$^{-/-}$ murine model on an FVB background has severe and progressive liver disease with juvenile onset, early initiation of therapy was essential. However, we and others have previously demonstrated loss of efficacy following treatment of juvenile mice due to the loss of rAAV vector episomes that accompany hepatocellular proliferation in the growing liver.[11,16]

To address these challenges, we exploited a hybrid rAAV-*piggyBac* transposon vector system to stably express human ABCB4 in neonatal *Abcb4*$^{-/-}$ mice. *PiggyBac* transposons are mobile genetic elements that integrate their genetic cargo into the host genome, but lack an inherent capacity for efficient and specific *in vivo* delivery.[17] Thus, this approach combines the highly hepato-tropic and efficient liver-targeting properties of rAAV with the integrative capacity of *piggyBac* transposons. A single treatment with this hybrid system in the newborn period resulted in sustained restoration of bile composition, prevention of biliary cirrhosis, and markedly reduced liver tumorigenesis.

Materials and Methods

ADENO-ASSOCIATED VIRAL VECTOR CONSTRUCTION AND PRODUCTION

A conventional rAAV construct was engineered to facilitate hepatocyte-specific expression of human ABCB4 (hABCB4) *in vivo*. The coding sequence for hABCB4 (Genbank Acct. No. NM_018849.2) was amplified by PCR from a pBluescript R plasmid clone obtained from the Mammalian Gene Collection.[18] Efficient initiation of translation was facilitated by the inclusion of an optimized Kozak consensus sequence.[19] Amplified hABCB4 complementary DNA (cDNA) was subcloned into a plasmid

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containing AAV serotype 2 inverted terminal repeats (ITRs) and a liver-specific transcriptional control unit (LP1), amplified from ssAAV.LP1.hFIXco,[20] which has been demonstrated to mediate liver-specific expression in clinical trial patients and animal models.[15,20]

For therapeutic evaluation of the hybrid rAAV-*piggyBac* transposon vector system, a previously reported rAAV construct encoding *piggyBac* transposase under the transcriptional control of a liver-specific promoter (pAAV2-LSP1.PBase)[21] was used to mediate integration of rAAV-delivered transposons into hepatocyte genomes *in vivo*. The dose and ratio between transposon and transposase rAAV vectors were based on previously reported work.[21]

To accommodate a hABCB4 coding sequence and *piggyBac* transposable elements required for transposition within rAAV packaging capacity, a hybrid vector was engineered with short *piggyBac* terminal repeats (TRsh), which mediate efficient transposition into mammalian cells *in vivo*.[22] The short *piggyBac* terminal repeats (5′TRsh, 67 base pair [bp]; 3′TRsh, 40 bp) were amplified by PCR from pPB-CA-GFP,[23] inserted immediately internal to AAV2 ITR to flank an expression cassette containing LP1 and a custom-synthesized, codon-optimized human ABCB4 coding sequence of transcript variant A (Acc. No. NM_000443.3; GenScript USA Inc., Piscataway, NJ). The resultant construct was designated as pAAV2-TRsh.LP1.cohABCB4-varA.

To compare the relative therapeutic potential of hybrid rAAV-*piggyBac* transposon encoding each hABCB4 transcript variant, custom-synthesized, codon-optimized hABCB4 transcript variants B and C (NM_018849.2 and NM_018850.2, respectively) were inserted in place of the transcript variant A coding sequence in the plasmid construct.

All rAAV vectors were pseudo-serotyped with the AAV8 capsid and produced in human embryonic kidney 293 cells using a helper virus-free triple plasmid transfection method, as described.[24,25] Vector genome titers were assigned by real-time quantitative PCR targeting the hABCB4 sequence.

ANIMAL PROCEDURES

Abcb4-deficient mice (FVB.129P2-Abcb4^{tm1Bor}/J; stock number 002539) were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed under specific pathogen-free conditions with 12-hour light/dark cycles and maintained on standard rodent chow (Specialty Feeds, Glen Forrest, Western Australia) with water provided *ad libitum*. All experimental procedures were approved by the Children's Hospital at Westmead and Children's Medical Research Institute Animal Care and Ethics Committee. Recombinant AAV vectors were administered to newborn (up to 3 days), juvenile (up to 4 weeks), and adult mice through intraperitoneal injection in volumes of 20 μL, 50 μL, and 100 μL, respectively. Unless otherwise stated, mice were used with an approximate equal sex distribution within groups.

BLOOD AND BILE COLLECTION AND ANALYSES

Blood was collected by cardiac puncture from anesthetized mice, as a terminal procedure, and placed in lithium heparin–coated vessels. Plasma was separated by centrifugation and stored at −20°C prior to analyses. Plasma alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bile acids were analyzed using a Vitros Chemistry Analyzer (Ortho Clinical Diagnostics, Raritan, NJ).

Bile was collected from mice, and they were fasted for 4 hours prior to the procedure. Water was provided *ad libitum* throughout. Under isoflurane inhalational anesthesia, a midline laparotomy incision was performed to expose the liver and gallbladder. The common bile duct was occluded using a surgical clamp, and bile was aspirated from the gall bladder into prechilled, uncoated collection tubes (Eppendorf, Hamburg, Germany) prior to storage at −20°C until ready for analyses.

For biliary phosphatidylcholine (PC) quantification, bile was diluted 100-fold in 0.05% (vol/vol) Triton X-100, and PC concentration was determined using the EnzyChrom Phospholipid Assay kit (Bioassay Systems, Hayward, CA). These were assayed against dilutions of a supplied fixed concentration standard, according to the manufacturer's instructions. Total biliary bile acids (TBAs) were quantified using a colorimetric assay based on an enzymatic cycling method (Diazyme Laboratories, Poway, CA), according to supplied instructions.

Bile specimens, diluted 200-fold in 0.9% (wt/vol) NaCl, were measured against a supplied 50-μM bile acid standard. Biliary cholesterol concentration was quantified on bile samples diluted 10-fold in water using the EnzyChrom Cholesterol Assay kit (Bioassay Systems), according to the manufacturer's instructions, and concentrations were determined based on a standard curve made from serial dilutions of supplied fixed concentration cholesterol. Biliary PC, TBAs, and cholesterol were determined by change in absorbance at 570 nm, 405 nm and 340 nm, respectively, measured using a spectrophotometric plate reader (VersaMax Microplate Reader; Molecular Devices, San Jose, CA).

VECTOR COPY NUMBER ANALYSIS

Using standard phenol-chloroform and ethanol precipitation methods,[26] DNA was extracted from 30-50 mg of snap-frozen liver. Vector copy numbers were determined by quantitative real-time PCR targeting the hABCB4 sequence, using the Quantitect Sybr Green Kit (Qiagen, Germantown, MD). The PCR reactions were normalized to murine genomic glyceraldehyde 3-phosphate dehydrogenase (mGAPDH) copies using the Quantitect Sybr Green Kit. Each reaction contained 150 ng of genomic DNA. Standard curves were generated using serial dilutions of linearized plasmid containing either hABCB4 or mGAPDH. To determine the relative abundance of vector copies and to account for a proportion of hepatocytes that are multinucleated, vector copies are expressed per diploid cell, assuming that each diploid cell contains two copies of mGAPDH.

VECTOR-DERIVED mRNA EXPRESSION

Total RNA was extracted from 50 mg of snap-frozen liver tissue using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, and treated with DNAse I (Life Technologies). Complementary DNA was synthesized by reverse transcription using the SuperScript III First-Strand Synthesis SuperMix (Life Technologies) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the Quantitect Sybr Green Kit, targeting reverse-transcribed hABCB4 cDNA. Normalization of mRNA transcript quantitation was performed by amplifying signals to murine β-actin cDNA with the two-step quantitative real-time PCR procedure described previously.

FLUOROMETRY

Fluorometric analyses to quantify enhanced green fluorescent protein (eGFP) from liver lysates were performed as described.[27]

HISTOLOGY AND IMMUNOFLUORESCENCE

Paraffin-embedded liver sections of 4-μm thickness were prepared from liver tissue, fixed in 10% (vol/vol) neutral-buffered formalin. Sections were stained with hematoxylin & eosin (H&E) and sirius red/fast green for histological examination.

To detect eGFP expression, liver tissues were fixed in 4% (wt/vol) paraformaldehyde, cryopreserved in 10%-30% (wt/vol) sucrose, frozen in optimal cutting temperature compound (Tissue-Tek O.C.T.; Sakura Finetek, Torrance, CA). Direct eGFP fluorescence of frozen section (5 μm) was detected using filters D480/303 (excitation) and D535/40m (emission) on an Olympus BX-51 TRF microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Images were captured with a SPOT camera using SPOT software version 4.01 (Diagnostic Instruments, Sterling Heights, MI).

QUANTITATIVE ANALYSIS OF LIVER FIBROSIS

Digital microscope images were acquired from liver sections stained with sirius red using a Zeiss Axioimager microscope (Carl Zeiss Microscopy, Germany). To determine the percentage of fibrotic area, the positive staining area in 10 FOVs at ×100 magnification per animal were analyzed using ImageJ software (Rasband, W.S.; National Institutes of Health, Bethesda, MD).[28]

STATISTICS

Mann-Whitney U tests were applied to all continuous data sets. Fisher's exact test was applied to categorical data. Results were analyzed using

GraphPad Prism (GraphPad Software, La Jolla, CA). *P* values of at least 0.05 were considered statistically significant.

Results

BLUNTED rAAV LIVER TRANSDUCTION IN *ABCB4*$^{-/-}$ ADULT, BUT NOT IN DISEASE-FREE WILD-TYPE ADULT OR JUVENILE *ABCB4*$^{-/-}$ MICE

To evaluate gene therapy in the *Abcb4*$^{-/-}$ chronic cholestatic liver disease model, a conventional rAAV vector was constructed encoding a human ABCB4 cDNA (subsequently identified as isoform B) under the control of a hepatocyte-specific promoter (LP1) and pseudo-serotyped with the highly murine hepatotrophic AAV8 capsid (Fig. 1A).

Eight-week-old *Abcb4*$^{-/-}$ and *Abcb4*$^{+/-}$ males were injected intraperitoneally with 1×10^{12} vg/mouse. To determine efficacy, biliary PC concentration was assessed 2 weeks after treatment and compared with a saline-injected group. In contrast to *Abcb4*$^{+/-}$ adult mice, *Abcb4*$^{-/-}$ mice did not exhibit increased biliary PC concentrations compared with saline-injected controls (Fig. 1B). Analysis of vector genome copies and human ABCB4-specific mRNA levels in the liver correlated with the observed effects of treatment on PC levels, thereby confirming markedly impaired liver transduction in *Abcb4*$^{-/-}$ mice (Fig. 1C,D). Despite receiving an equivalent vector dose, *Abcb4*$^{-/-}$ mice had 59-fold less vector genome copies 2 weeks after treatment compared with age-matched *Abcb4*$^{+/-}$ mice. Given the presence of established liver disease in young adult *Abcb4*$^{-/-}$ mice,[2] this result is consistent with previous studies reporting reduced rAAV transduction in the presence of fibrotic liver disease.[29] We therefore hypothesized that gene delivery to younger *Abcb4*$^{-/-}$ mice, prior to significant liver disease progression, would permit more efficient liver transduction and achieve desired therapeutic effects.

To test this hypothesis and determine the optimal age at which to initiate gene therapy, liver transduction of *Abcb4*$^{-/-}$ and age-matched *Abcb4*$^{+/+}$ juvenile mice was compared at birth, 2, 4, 6, and 8 weeks of age, using a previously described rAAV2/8 vector encoding an eGFP reporter under the transcriptional control of a liver-specific promoter.[11] Analysis of liver transduction was performed 1 week after treatment (Fig. 2A).

Significantly lower vector copies were detected in the livers in *Abcb4*$^{-/-}$ mice compared with age-matched *Abcb4*$^{+/+}$ mice at all ages evaluated, except for mice treated at birth, in which a similar trend was observed without statistical significance (Fig. 2B).

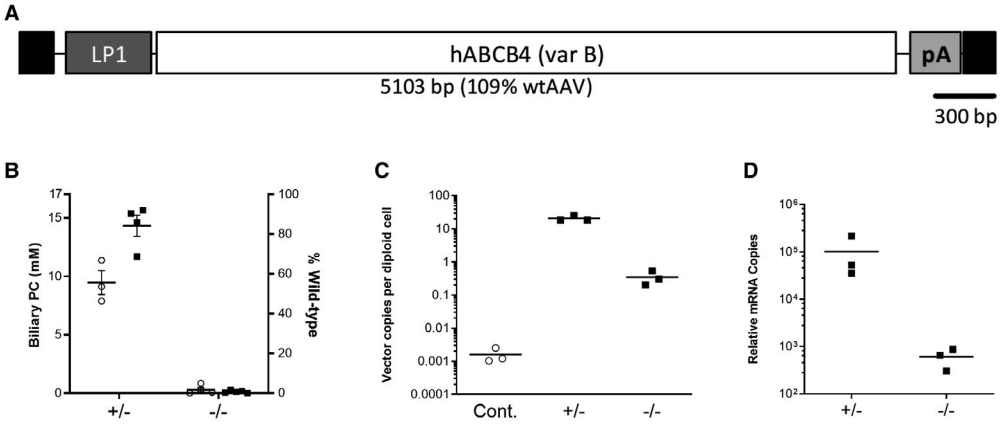

FIG. 1. Blunted rAAV transduction and transgene expression in 8-week-old *Abcb4*$^{-/-}$ mice compared with *Abcb4*$^{+/-}$. (A) Schematic diagram of AAV vector construct encoding the hABCB4 (transcript variant B) cDNA. (B) Biliary PC concentration in rAAV-transduced *Abcb4*$^{+/-}$ and *Abcb4*$^{-/-}$ (filled squares) compared with phosphate-buffered saline–injected controls (open circles) at 2 weeks following injection. Vector copies per diploid cell in liver tissue detected by quantitative real-time PCR (C) and relative mRNA copies detected in liver specimens from rAAV-transduced *Abcb4*$^{-/-}$ and *Abcb4*$^{+/-}$ that received an equivalent vector dose (D) ($n \geq 3$ per group). Error bars represent SEM. Abbreviations: pA, bovine growth hormone polyadenylation sequence; +/-, heterozygotes; -/-, homozygotes.

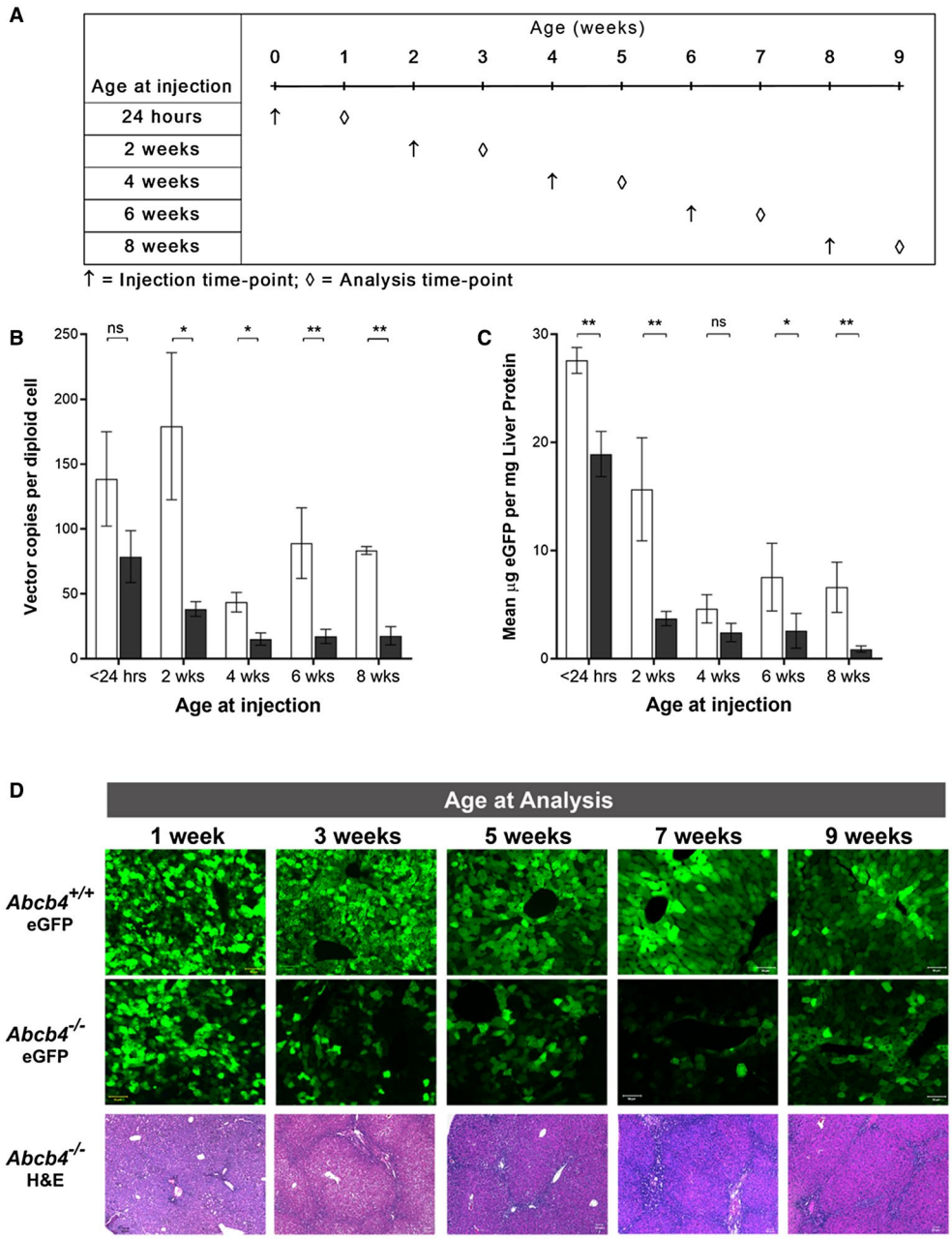

FIG. 2. Comparison of rAAV transduction and eGFP expression between age-matched *Abcb4*$^{+/+}$ and *Abcb4*$^{-/-}$ juvenile mice. (A) Experiment design: groups of age-matched *Abcb4*$^{+/+}$ and *Abcb4*$^{-/-}$ mice were injected with eGFP encoding rAAV at 24 hours and 2, 4, 6, and 8 weeks of age (arrows) and liver tissue analyzed 1 week following injection (diamonds). Vector copies per diploid cell in liver tissue (B) and eGFP expression analyzed by fluorometry (C) in *Abcb4*$^{-/-}$ juvenile mice treated with eGFP encoding rAAV (black bars) compared with age-matched wild-type mice that received an equivalent vector dose (white bars). (D) Representative immunofluorescence and histology images at 1 week following injection (scale bar = 50 µm) (n ≥ 6 per group). Statistical significance was determined using Mann-Whitney U tests. **P* ≤ 0.05; ***P* ≤ 0.01. Error bars represent mean ± SEM.

There was correspondingly reduced eGFP expression in the *Abcb4*$^{-/-}$ livers (Fig. 2C,D), demonstrating that the optimal age at which to initiate therapy in *Abcb4*$^{-/-}$ mice is the newborn period. However, rAAV-mediated gene transfer this early poses the challenge of rapid AAV episome loss from the growing liver.[11]

HYBRID rAAV-*PIGGYBAC* TRANSPOSON VECTOR STRATEGY AND hABCB4 SPLICE VARIANT EVALUATION FOR VECTOR DESIGN OPTIMIZATION

To overcome rapid loss of transgene expression following neonatal vector delivery, our previously reported hybrid rAAV-*piggyBac* transposon vector strategy was used with the intent of stabilizing transgene expression by transposition of the expression cassette out of AAV vector episomes and into the host hepatocyte genome before episome loss occurs.[21] Although earlier experiments confirmed the functionality of hABCB4 splice variant B, the phosphatidylcholine floppase activity relative to the two other known hABCB4 splice variants (A and C) had not been previously studied.[30] Accordingly, a comparative analysis of all three splice variants was undertaken to ensure optimal hybrid rAAV-*piggyBac* vector efficacy. *PiggyBac* transposons encoding cDNAs for each of the three hABCB4 splice variants, under the transcriptional control of the LP1 promoter, were subcloned into rAAV proviral constructs designated pAAV2-TRsh.LP1.cohABCB4-varA to C (Fig. 3A). To facilitate somatic integration of the encoded hABCB4-*piggyBac* transposons, vectors stocks prepared from these hybrid constructs, pseudo-serotyped with the type 8 capsid, were co-administered with a rAAV vector–encoding *piggyBac* transposase (rAAV2/8.LSP.pBase) (Fig. 3A).[21] Groups of newborn *Abcb4*$^{-/-}$ pups were injected with rAAV2/8 vector encoding each of the variant hABCB4 transposons at a dose of 5×10^{11} vg/mouse co-administered with 5×10^{10} vg/mouse of rAAV2/8.LSP.pBase (n = 4 per group). Liver, blood, and bile samples were collected from each mouse at 4 weeks of age.

However, there was less than a 2-fold difference in the normalized vector copy numbers between each variant isoform treatment group; mice that received variant A had a biliary PC concentration 4.9-fold and 7.0-fold higher than mice that received vector encoding variants B or C, respectively (Fig. 3B). Furthermore, compared with variant B and C treatment groups, variant A recipients had at least 2-fold lower levels of serum ALT and ALP, greater than 5-fold lower serum bile acid levels (Fig. 3B), and normal liver histology (Fig. 3C). Isoform A was therefore selected for subsequent phenotype correction studies.

SUCCESSFUL PREVENTION OF CHRONIC LIVER DISEASE WITH A HYBRID rAAV-*PIGGYBAC* TRANSPOSON VECTOR ENCODING hABCB4 DELIVERED AT BIRTH

Newborn *Abcb4*$^{-/-}$ mice were injected at less than 24 hours of age with 5×10^{11} vg/mouse of rAAV2/8-TRsh.LP1.cohABCB4-varA, co-administered with 5×10^{10} vg/mouse of rAAV2/8.LSP.pBase. To evaluate the efficacy of the hybrid vector strategy, the analysis of liver, bile, and blood samples was performed at 4, 8, 12, and 16 weeks of age (pBase-positive, $n \geq 4$ per group) (Fig. 4A). To assess whether the transposon vector alone conferred therapeutic benefit, *Abcb4*$^{-/-}$ mice were injected at less than 24 hours of age with 5×10^{11} vg/mouse of rAAV2/8-TRsh.LP1.cohABCB4-varA without the *piggyBac* transposase vector, and samples harvested for analysis at 4 and 12 weeks of age.

Compared with the untreated *Abcb4*$^{-/-}$ controls, mice that received rAAV encoding the hABCB4 transposon in combination with rAAV encoding *piggyBac* transposase had significantly elevated biliary PC concentrations at all time points, equivalent to 55%-75% wild-type levels (Fig. 4B). This was sufficient to prevent onset of liver pathology including fibrosis, and to maintain normal liver biochemistry (Fig. 4C,D). Mice treated in the newborn period also exhibited normal liver architecture with absence of inflammatory infiltration and fibrosis (Fig. 4E). Other constituents of bile also approached wild-type composition (Supporting Fig. S1).

Abcb4$^{-/-}$ mice that received rAAV encoding the hABCB4 transposon alone had a small but significant elevation of biliary PC concentration at 4 and 12 weeks of age, equivalent to 3%-5% wild-type levels. However, liver histology showed abnormal cholestatic changes with fibrosis, indicative of bile toxicity (Supporting Fig. S2).

DELAYING THERAPY RESULTS IN INCREMENTAL LOSS OF EFFICACY

To model the likely context of human therapy, we next evaluated therapeutic efficacy in juvenile *Abcb4*$^{-/-}$ mice with varying degrees of liver pathology at the

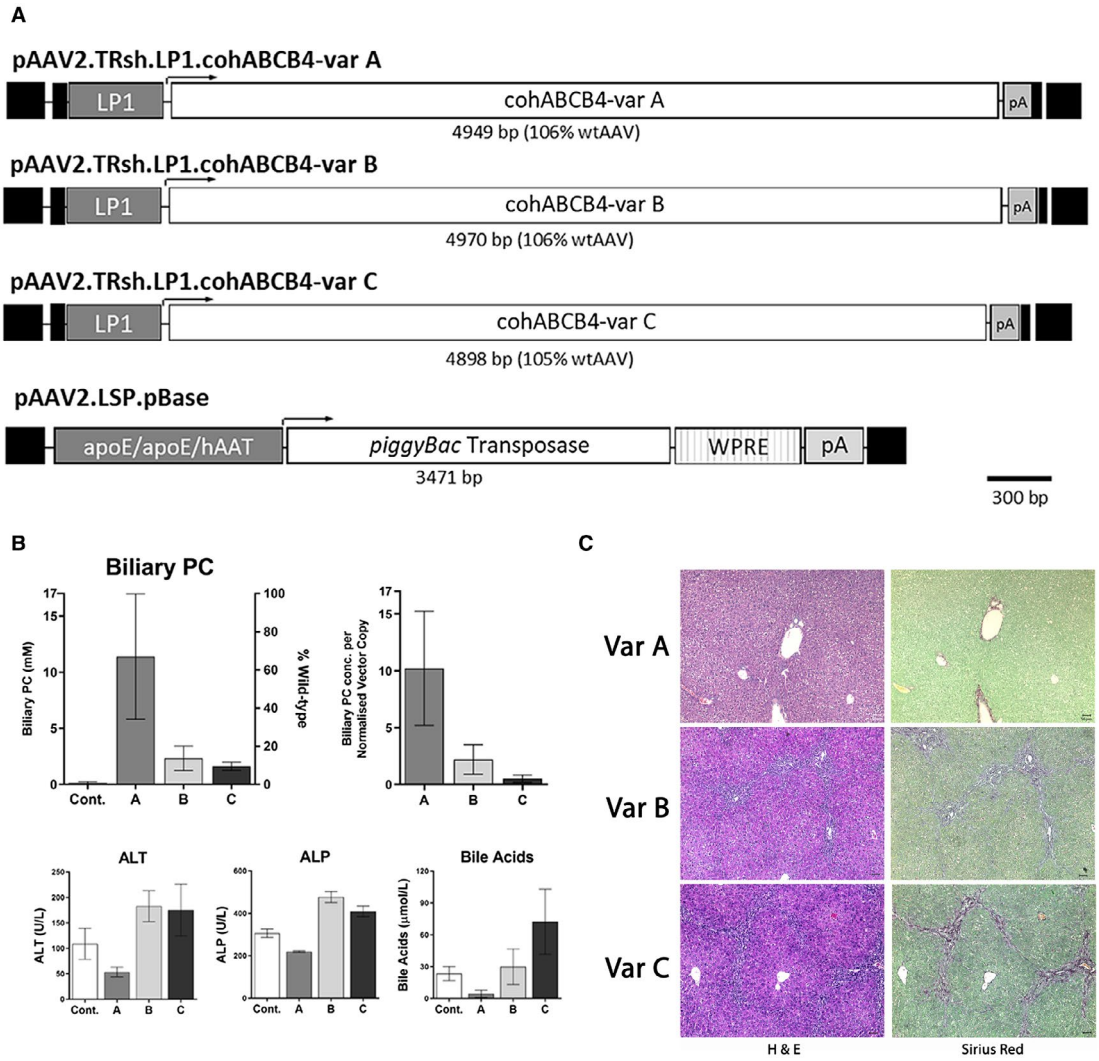

FIG. 3. Comparative treatment efficacy using different hABCB4 splice variants. (A) Schematic diagram of rAAV expression cassettes encoding each of the known hABCB4 transcript variants and an rAAV expression cassette encoding a *piggyBac* transposase. (B) Comparison between biliary PC, relative biliary PC normalized to vector copies per diploid cell in liver, serum ALT, ALP, and bile acids measured at 4 weeks of age in *Abcb4*$^{-/-}$ treated with equivalent doses of hybrid vector encoding either hABCB4 transcript variant A, B, or C at birth, and 4-week-old untreated *Abcb4*$^{-/-}$ controls. (C) Representative H&E and sirius red stained images from 4-week-old *Abcb4*$^{-/-}$ mice from each of the transcript variant groups. Scale bar = 50 μm; n = 4 per group. Error bars represent mean ± SEM. Abbreviations: apoE, apolipoprotein E enhancer; hAAT, human alpha-1 antritrypsin promoter; pA, simian virus 40 polyadenylation sequence; WPRE, woodchuck hepatitis posttranslational regulatory element.

time of intervention. To deliver an equivalent dose relative to body weight, *Abcb4*$^{-/-}$ mice were injected intraperitoneally with 0.5-2 × 10^{12} vg/mouse of rAAV2/8 vector encoding the hABCB4 transposon, co-administered with 0.5-2 × 10^{11} vg/mouse of rAAV2/8 encoding *piggyBac* transposase at 1, 2, 3, or 4 weeks of age (Fig. 5A). Liver, bile, and blood samples were collected for analysis at 8 weeks of age.

All groups had significantly elevated biliary PC concentrations above that of untreated controls (Fig. 5B). However, intergroup comparisons revealed an incremental loss of efficacy with increasing age, from 76% wild-type biliary PC concentrations when treated in the newborn period to less than 30% when treated at 4 weeks of age. This was associated with increased liver fibrosis, as well as rises in serum ALT, ALP, and

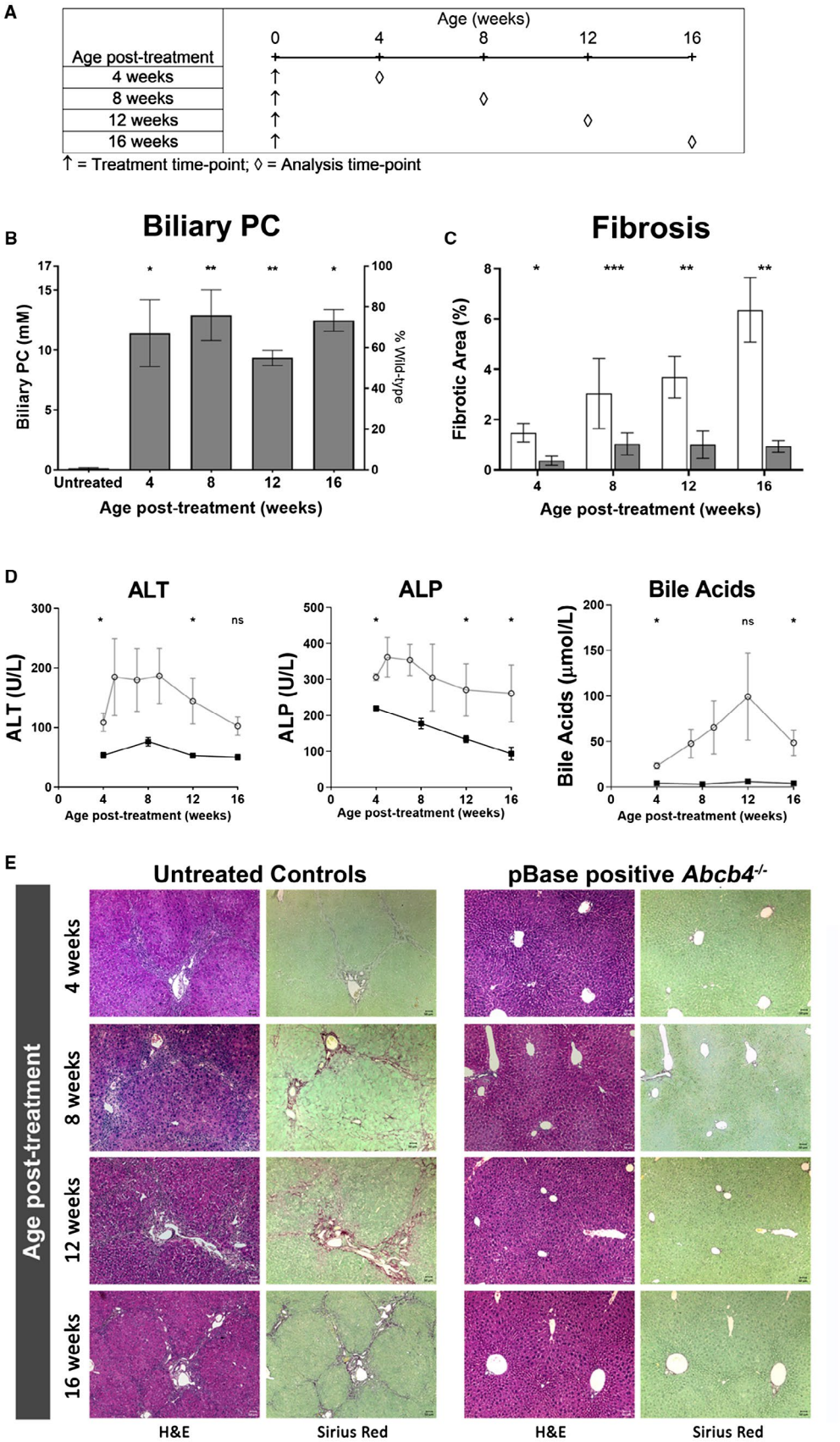
A
Age (weeks)
0
4
8
12
16
Age post-treatment
4 weeks
8 weeks
12 weeks
16 weeks
↑ = Treatment time-point; ◊ = Analysis time-point
B
Biliary PC
Biliary PC (mM)
% Wild-type
Untreated
Age post-treatment (weeks)
C
Fibrosis
Fibrotic Area (%)
Age post-treatment (weeks)
D
ALT
ALT (U/L)
Age post-treatment (weeks)
ALP
ALP (U/L)
Age post-treatment (weeks)
Bile Acids
Bile Acids (μmol/L)
Age post-treatment (weeks)
ns
E
Untreated Controls
pBase positive Abcb4-/-
Age post-treatment
4 weeks
8 weeks
12 weeks
16 weeks
H&E
Sirius Red
H&E
Sirius Red

FIG. 4. Phenotype correction of *Abcb4*$^{-/-}$ mice treated in the newborn period. (A) Experiment design: *Abcb4*$^{-/-}$ mice were treated with hybrid rAAV-transposon vector encoding hABCB4 variant A co-administered with *piggyBac* transposon vector at birth (arrows). Bile, blood, and liver tissue were analyzed at 4, 8, 12, and 16 weeks after treatment (diamonds) (n ≥ 4 per group). (B) Biliary PC concentration in hybrid vector–treated *Abcb4*$^{-/-}$ mice compared with age-matched untreated *Abcb4*$^{-/-}$ controls. (C) Percentage of liver occupied by fibrosis in hybrid rAAV-transposon treated mice (gray columns) compared with age-matched untreated controls (white columns). (D) Serum ALT, ALP, and bile acids in hybrid vector treated mice (filled squares) compared with age-matched untreated controls (open circles). Statistical significance is shown for age-matched groups. (E) Representative H&E and sirius red stained images from treated *Abcb4*$^{-/-}$ and untreated controls at 4 to 16 weeks following treatment (scale bar = 50 μm). Statistical significance was determined using Mann-Whitney U tests. $^{*}P < 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. Error bars represent mean ± SEM.

bile acids (Fig. 5C,D). Notably, young *Abcb4*$^{-/-}$ mice treated prior to 3 weeks of age with hABCB4 transposon and *piggyBac* transposase rAAV vectors had near-complete phenotype correction with normal liver histology (Fig. 5E).

REDUCED LIVER TUMOUR INCIDENCE OBSERVED IN LONG-TERM COHORT

To assess the long-term efficacy of the hybrid vector strategy, newborn *Abcb4*$^{-/-}$ mice were injected with 5×10^{11} vg/mouse of rAAV2/8-TRsh.LP1.cohABCB4-varA, co-administered with 5×10^{10} vg/mouse of rAAV2/8.LSP.pBase (n = 56). Liver and bile from cohorts of mice were analyzed at 6, 9, 12, 16, 20, and 24 months of age, or if they exhibited clinical signs of deterioration (weight loss, labored breathing, and reduced activity). These were compared with age-matched untreated controls (n = 63). Newborn *Abcb4*$^{+/+}$ mice were also injected with hybrid vectors and analyzed at 24 months of age to independently evaluate possible vector-related genotoxicity. A single injection of hybrid vectors at birth led to stable, sustained hABCB4 expression in *Abcb4*$^{-/-}$ mice up to 24 months of age (Supporting Fig. S3).

Of mice aged 6 months or older, markedly fewer hybrid rAAV-hABCB4 transposon-treated *Abcb4*$^{-/-}$ developed frank liver tumors (> 1 mm protuberant or invasive lesions) compared with age-matched untreated *Abcb4*$^{-/-}$ mice (Fig. 6A). Histological examination of these tumors revealed steatotic dysplastic lesions with pleomorphic hyperchromatic nuclei similar to hepatocellular carcinoma previously described in this mouse model (Fig. S6B).[31] None of the treated *Abcb4*$^{+/+}$ mice developed liver tumors (Supporting Table S1). Small, nonprotruding nodules (≤ 1 mm) were observed in 27% of treated *Abcb4*$^{-/-}$ and 43% of *Abcb4*$^{+/+}$ mice that were given hybrid vectors (Fig. 6B and Supporting Table S1). Formalin-fixed, paraffin-embedded liver tissue containing nodules from treated *Abcb4*$^{-/-}$ mice were carefully sectioned for examination, revealing heterogeneous histology: 11.8% of specimens showed hepatocyte dysplasia and 29.4% appeared to be regenerative nodules. No abnormality was identified in 58.8% (data not shown).

Because hepatocarcinogenesis in *Abcb4*$^{-/-}$ mice is driven by liver inflammation and bile toxicity,[31] we postulated that tumors may arise in mice that had a lower level of hybrid vector transduction. *Abcb4*$^{-/-}$ mice express a mutant murine abcb4 protein that fails to localize to the canalicular membrane, so we were unable to definitively demonstrate specific staining of the vector-derived human transgene product by immunohistochemistry using anti-human ABCB4 antibodies (data not shown). The biliary PC concentration was measured to assess the liver-wide hABCB4 expression in hybrid vector-treated *Abcb4*$^{-/-}$ mice that developed liver tumors, compared with those without liver tumors and those that exhibited macroscopic liver nodules. Treated *Abcb4*$^{-/-}$ mice that had pristine livers without lesions had significantly higher biliary PC concentrations than mice that exhibited either frank tumors or liver nodules (Fig. 6C). Of the specimens from treated mice with identified tumors, the adjacent nonmalignant tissue did not have cirrhosis. Furthermore, similar biliary PC concentrations were observed in *Abcb4*$^{-/-}$ mice that did not develop fibrosis when analyzed at earlier time points.

Discussion

This study demonstrates that liver-targeted gene therapy can effectively treat chronic cholestatic liver disease in a murine model of PFIC3. Using a hybrid rAAV-*piggyBac* transposon vector system to express

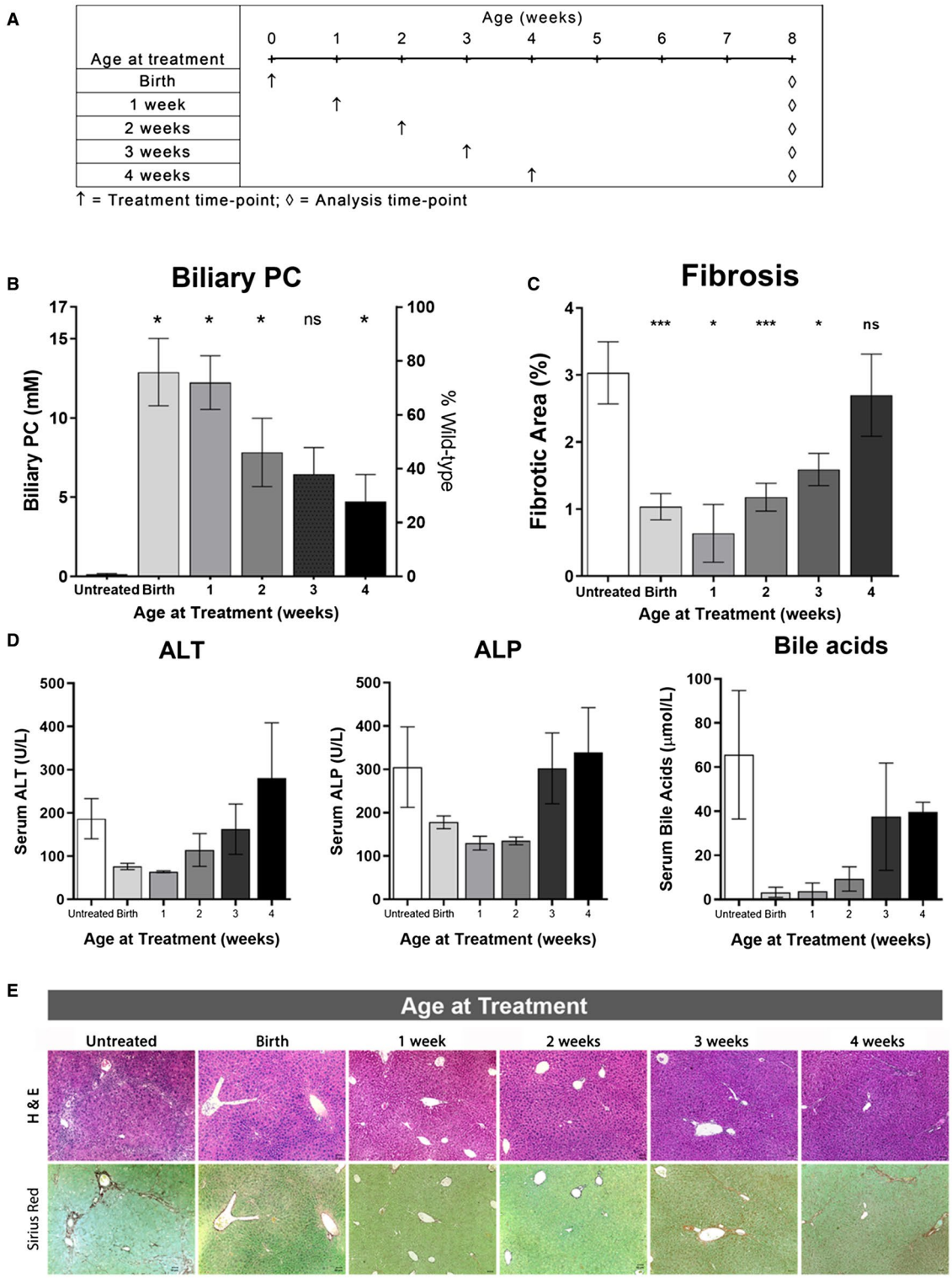

FIG. 5. Delaying therapy results in incremental loss of therapeutic efficacy. (A) Experiment design: *Abcb4*$^{-/-}$ mice treated with hybrid vector between birth and 4 weeks of age at similar doses relative to body weight, compared with 8-week-old untreated *Abcb4*$^{-/-}$ controls (arrows). Liver, blood, and bile analyses were performed at 8 weeks of age (diamonds) (n ≥ 4 per group). Biliary PC concentration (B), amount of liver fibrosis (C), serum ALT, ALP, and bile acids (D), and representative liver sections, stained with H&E and sirius red, were analyzed to demonstrate degree of pathology and fibrosis (scale bar = 50 μm). Statistical significance was determined using Mann-Whitney U tests. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$. Error bars represent mean ± SEM.

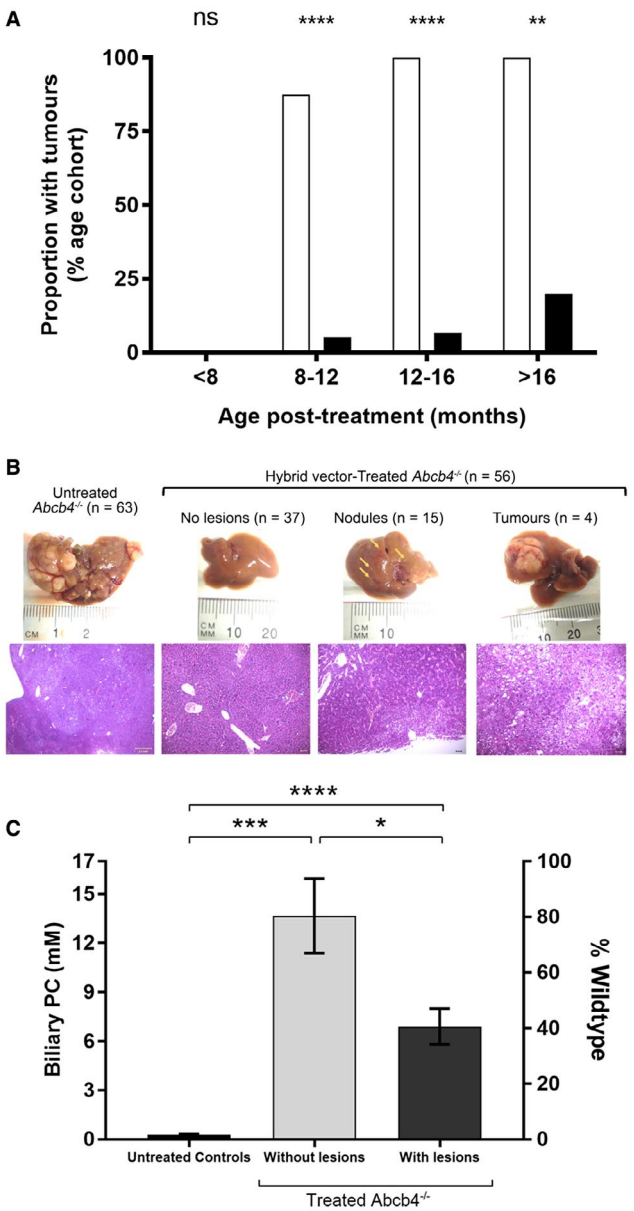

FIG. 6. Reduced incidence and delayed onset of liver tumors observed in *Abcb4*$^{-/-}$ mice treated with hybrid rAAV-transposon vector at birth compared with untreated *Abcb4*$^{-/-}$ mice. (A) Proportion of treated and control *Abcb4*$^{-/-}$ mice in each age cohort that had macroscopically visible hepatocellular tumors (≥ 1 mm diameter and protruberant lesions) (untreated, white columns; treated, black columns). Comparison within each cohort was performed using Fisher's exact test. $^{**}P \leq 0.01$; $^{****}P \leq 0.0001$. (B) Representative macroscopic and histological appearance of frank liver tumors, nodules (yellow arrows), and normal mouse livers from hybrid rAAV-transposon treated and untreated *Abcb4*$^{-/-}$ mice (number in each cohort in parentheses). Histology images are stained with H&E. (C) Biliary PC concentrations of treated mice that developed lesions (tumors or nodules), had livers without lesions, and untreated controls. Statistical significance was determined using Mann-Whitney U test. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. Error bars represent mean ± SEM.

hABCB4 in *Abcb4*$^{-/-}$ mice *in vivo*, a single treatment administered in the newborn period was sufficient to confer life-long phenotype correction with absence of liver pathology and a significantly reduced incidence of hepatocellular tumors.

This hybrid vector system was developed to facilitate long-term transgene expression in the context of neonatally administered rAAV vectors through *piggyBac* transposase-mediated integration of the therapeutic expression cassette *in vivo*. Blunted rAAV transduction of hepatocytes in young adult *Abcb4*$^{-/-}$ mice with established liver disease was a significant barrier to therapeutic efficacy using conventional rAAV. For a given input vector dose, maximum therapeutic benefit to *Abcb4*$^{-/-}$ mice was conferred by treatment in the newborn period, whereas liver transduction was markedly less effective in older *Abcb4*$^{-/-}$ animals compared with age-matched *Abcb4*$^{+/+}$ controls.

As the development of liver pathology is progressive with age, we hypothesized that pathophysiological changes associated with liver disease may detrimentally affect rAAV delivery and transduction. Reduction in rAAV and adenoviral transduction and transgene expression have been described previously in other models of liver disease, including those induced by carbon tetrachloride and bile duct ligation.[29,32] Our data confirm these observations in a chronic cholestatic model. Reduction in detectable vector genome copies and comparably lower hepatocyte-specific transgene expression in *Abcb4*$^{-/-}$ livers 1 week after treatment is consistent with a block in rAAV delivery to hepatocytes, and a possible small contribution from AAV episome dilution/degradation in concert with increased hepatocellular turnover. Others have demonstrated that tissue inflammation also affects intracellular processing of vectors, leading to reduced transcription and transgene silencing.[33,34] However, because reduced vector copy numbers was accompanied by lower eGFP expression levels in *Abcb4*-deficient liver, blocks in transcription are unlikely to be dominant factors. We speculate that potential mechanisms involve disease-related alterations in hepatic architecture, detrimentally affecting multiple points along the hepatocyte transduction pathway, including reduced vector access to the hepatocytes, alterations in the efficiency of endocytosis, and impaired intracellular trafficking. Sequestration of rAAV into inflammatory or nonparenchymal cells with subsequent degradation is also a possibility.

Recombinant AAV biology in a disease-specific context has important implications for developing a therapeutic gene transfer strategy for PFIC3 and other chronic liver diseases. These observations underscore the importance of evaluating vector performance in models that best recapitulate disease pathology and progression. In this model, delaying the initial age of hybrid vector administration resulted in incremental loss of therapeutic efficacy. Importantly, when hybrid vectors were delivered prior to the onset of fibrosis, normal liver architecture was preserved into adulthood, whereas treatment after the onset of fibrosis failed to result in reversal of pathology.

Lack of phenotype correction with rAAV-transposon vector alone confirmed that *piggyBac* transposase-mediated integration of the hABCB4 expression cassette was required to reach the threshold of persistent transgene expression necessary to prevent onset of liver disease in mice treated in the newborn period. Because administering the rAAV-transposon vector alone is effectively conventional rAAV gene therapy, a small subset of transposon-bearing vector genomes was likely to have spontaneously integrated in a transposase-independent manner, while the remaining episomal rAAV-transposon vector genomes would have been lost as a consequence of hepatocellular proliferation during liver growth. Hence, the small but significant increase in biliary PC concentration was demonstrated in mice treated with the hABCB4 encoding transposon vector alone (3.9% wild-type concentrations), in keeping with the reported low rate of spontaneous rAAV integration.[11] This was insufficient, however, to prevent liver pathology.

The low rate of conventional rAAV integration was strategically exploited to minimize persistent *piggyBac* transposase expression, which could cause multiple transposition cycles, leading to eventual loss of transposons and therefore therapeutic transgene expression, and additionally could increase damage to the genome through aberrant chromosomal break repair and recombination events.[35,36] Thus, to add to the biosafety and efficacy of this hybrid vector system, a further advancement would be to deliver a more labile source of transposase expression, such as mRNA using lipid nanoparticles.

In the present study, the hybrid system also permitted assessment of the relative PC floppase function of 3 naturally occurring in-frame splice variants of the human ABCB4 gene on a functionally null *Abcb4*$^{-/-}$ background. Isoform A conferred higher phosphatidylcholine concentration in bile than isoforms B or C, which is consistent with anticipated differences in protein structure based on *in silico* modeling.[30] Relative to transcript variant A, variant B uses an alternate in-frame splice site in the 3′ coding region that is predicted to insert an additional 21 bp in a region encoding a nucleotide binding domain, whereas variant C lacks an alternate in-frame exon that encodes a 47 amino acid sequence involving segment 11 of a transmembrane domain.[30] The effects of these structural variations on isoforms B and C are yet to be fully elucidated, but may confer unidentified functions independent of PC floppase activity.

Together with previous work using other murine models,[21] our data demonstrate that rAAV technology can be used to efficiently deliver transposons to facilitate long-term transgene expression following delivery to the growing liver. Although markedly extending the utility of AAV vectors in the liver, minor costs include a slightly reduced packaging capacity[37,38] that is required to accommodate minimal transposase recognition sites and the need for two-hit kinetics, given that the transposable element and *piggyBac* transposase are delivered in separate virions. Thus, the relative dosage ratio of transposon to transposase virions, which herein was based on our previous published work in other mouse models,[21] could be further optimized to maximize transposition efficiency. This will also be aided by the ongoing development of highly human liver tropic capsids, such as LK03 and NP59.[13,14]

Another consideration with integrating vector systems is the risk of genotoxicity caused by disruption of endogenous gene function and/or regulation.[39] Although most integration events do not carry risk, rare events in the vicinity of proto-oncogenes or tumor suppressor genes have oncogenic potential. Integration site analyses indicate that *piggyBac* transposon integration behavior is nonrandom, preferentially occurring within the coding regions of actively transcribed genes in close proximity of transcriptional start sites and CpG islands and with affinity for open chromatin structures.[21,22,40-42] Of relevance, we recently reported an analysis of 127,396 unique integration sites in the livers of mice treated with hybrid rAAV-*piggyBac* vectors and found no evidence of hotspots for integration near genes known to be drivers of hepatocellular carcinogenesis.[21] Nevertheless, the risk of insertional

mutagenesis remains and must be balanced against the benefits of disease intervention.

Such risk benefit is evident in the current study, in which the hybrid rAAV-*piggyBac* system not only corrected the underlying disease, but also significantly reduced the risk of hepatocellular carcinoma (HCC) associated with untreated chronic cholestatic PFIC-related liver disease.[8] Although liver transplantation to treat end-stage biliary cirrhosis would also diminish this HCC risk, posttransplant immunosuppression is associated with the risk of other malignancies, including lymphoproliferative disease, which affects 4%-15% of pediatric liver transplant recipients.[43,44] Additionally, there is the need for lifelong immunosuppression and risk of other morbidities, including susceptibility to infection, renal impairment, cardiovascular abnormalities, surgical complications, and allograft rejection, along with graft loss.[45,46]

Achieving adequately efficient rAAV transduction for human application remains one of the biggest challenges of translating preclinical efficacy observed in small animal models to the bedside. Studies based on nonhuman primates predict that lower efficiency of gene transfer and lower expression for a given dosage may be reasonably expected.[47] In the face of two-hit kinetics, this hybrid vector system places even greater demands on rAAV gene transfer efficiencies. Furthermore, based on findings in this *Abcb4*$^{-/-}$ murine model, a relatively high level of liver transduction in homozygotes, sufficient to increase biliary PC concentrations to at least 40% wild-type levels, is necessary to prevent chronic cholestatic disease and fibrosis. These findings concur with that of a previously reported human MDR3-expressing transgenic *Abcb4*$^{-/-}$ model.[48] Our data also indicate that an even higher level of liver-wide transduction may be necessary to prevent tumorigenesis, as HCC may possibly arise from focal areas of untransduced hepatocytes where bile remains toxic.

Therefore, optimizing vector design and delivery to maximize transduction and transgene expression will be vital. Important considerations include (1) refining vector design to maximize transgene expression within rAAV packaging constraints, (2) choosing capsid serotypes that efficiently transduce human hepatocytes, (3) optimizing vector doses as well as determining the ideal transposon-to-transposase vector ratio, to avoid transposase overexpression inhibition, and (4) minimizing persistent *piggyBac* transposase expression by delivering a more labile source of transposase.

In summary, this powerful hybrid rAAV-transposon vector system has the potential to offer infants and children with PFIC3 a relatively safe, effective, and reliable alternative treatment to liver transplantation. Future work should be directed toward evaluating and addressing safety issues and surmounting translational challenges, to improve efficacy in the human setting and to refine vector design prior to clinical application.

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