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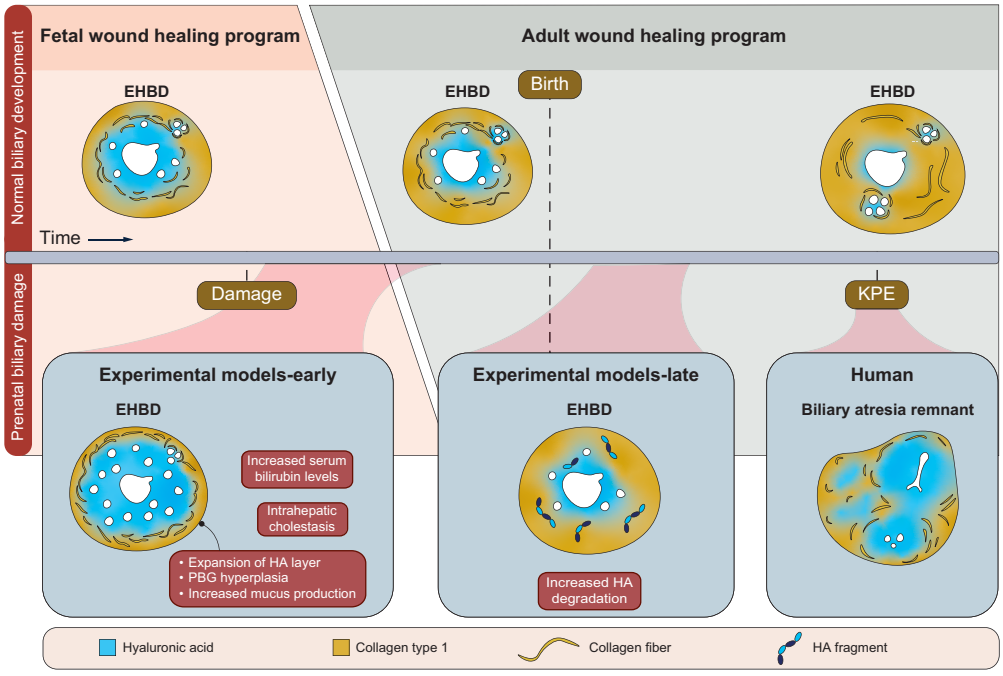
Graphical abstract



Highlights

- Fetal and neonatal extrahepatic bile ducts have high levels of hyaluronic acid, located directly around the lumen.
- Damage to the fetal extrahepatic bile duct is followed by increased hyaluronic acid deposition.
- Biliary atresia remnants show increased hyaluronic acid deposition around epithelial structures compared to controls.
- Hyaluronic acid in neonatal extrahepatic bile ducts is degraded after injury.
- Hyaluronic acid polymers of different sizes have distinct biological effects on cholangiocytes.

Impact and implications

Biliary atresia is a pediatric cholangiopathy associated with high morbidity and mortality rates; although multiple etiologies have been proposed, the fetal response to bile duct damage is largely unknown. This study explores the fetal pathogenesis after extrahepatic bile duct damage, thereby opening a completely new avenue to study therapeutic targets in the context of biliary atresia.

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A fetal wound healing program after intrauterine bile duct injury may contribute to biliary atresia

Iris E.M. de Jong[1,2], Mallory L. Hunt[3], Dongning Chen[2,4,5], Yu Du[2,6], Jessica Llewellyn[2], Kapish Gupta[2], David Li[2], Dorothea Erxleben[7], Felipe Rivas[7], Adam R. Hall[7,8], Emma E. Furth[9], Ali Naji[10], Chengyang Liu[10], Abhishek Dhand[4], Jason A. Burdick[11], Marcus G. Davey[12], Alan W. Flake[12], Robert J. Porte[1], Pierre A. Russo[13], J. William Gaynor[3], Rebecca G. Wells[2,4,5,9,*]

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Background & Aims: Biliary atresia (BA) is an obstructive cholangiopathy that initially affects the extrahepatic bile ducts (EHBDs) of neonates. The etiology is uncertain, but evidence points to a prenatal cause. Fetal tissues have increased levels of hyaluronic acid (HA), which plays an integral role in fetal wound healing. The objective of this study was to determine whether a program of fetal wound healing is part of the response to fetal EHBD injury.
Methods: Mouse, rat, sheep, and human EHBD samples were studied at different developmental time points. Models included a fetal sheep model of prenatal hypoxia, human BA EHBD remnants and liver samples taken at the time of the Kasai procedure, EHBDs isolated from neonatal rats and mice, and spheroids and other models generated from primary neonatal mouse cholangiocytes.
Results: A wide layer of high molecular weight HA encircling the lumen was characteristic of the normal perinatal but not adult EHBD. This layer, which was surrounded by collagen, expanded in injured ducts in parallel with extensive peribiliary gland hyperplasia, increased mucus production and elevated serum bilirubin levels. BA EHBD remnants similarly showed increased HA centered around ductular structures compared with age-appropriate controls. High molecular weight HA typical of the fetal/neonatal ducts caused increased cholangiocyte spheroid growth, whereas low molecular weight HA induced abnormal epithelial morphology; low molecular weight HA caused matrix swelling in a bile duct-on-a-chip device.
Conclusion: The fetal/neonatal EHBD, including in human EHBD remnants from Kasai surgeries, demonstrated an injury response with prolonged high levels of HA typical of fetal wound healing. The expanded peri-luminal HA layer may swell and lead to elevated bilirubin levels and obstruction of the EHBD.

Introduction

Biliary atresia (BA) is a devastating disease of newborns primarily affecting the extrahepatic bile ducts (EHBDs). Patients with BA are born seemingly healthy but develop obstruction of the EHBD days to weeks after birth followed by the rapid development of hepatobiliary fibrosis and cirrhosis. Although the etiology of BA remains under investigation, compelling evidence points to a prenatal insult.[1,2] Several causes of bile duct injury in BA have been suggested, including environmental toxins, a viral infection, or a genetic defect; although the preponderance of data suggest that it is a primary environmental insult, it is likely that BA is multifactorial.[2–4]

Regardless of the cause of biliary damage in BA, mechanisms of fetal EHBD repair are not known. Repair of the injured adult EHBD requires peribiliary glands (PBGs), which are mucus-producing glands in the submucosa that harbor a cholangiocyte stem/progenitor cell population. With severe damage to the duct, these cells proliferate, migrate towards denuded luminal surfaces, and differentiate to mature cholangiocytes.[5,6] Additionally, this may be associated with extracellular matrix (ECM) deposition and scarring or fibrosis, as per a typical adult wound healing program.

Healing during much of the fetal period, however, is scarless, as demonstrated in multiple organs including the heart, skin, lung, and tendon.[7–10] Although not previously studied in the EHBD, fetal wound healing in general is dominated by deposition of the glycosaminoglycan hyaluronic acid (HA) rather than the type I collagen typical of adult wound healing.[11] HA is a highly charged polymer consisting of numerous repeats of D-glucuronic acid and N-acetylglucosamine and is a component of the normal ECM of the EHBDs at all stages of development, at least in mice.[12] HA is synthesized in high molecular weight form (HMW, >1 MDa) by hyaluronic acid synthases (HAS) and degraded from a high to a low molecular

Keywords: biliary atresia; hyaluronic acid; peribiliary glands; cholangiocyte; cholangiopathy.
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weight (LMW) form by hyaluronidases (HYAL); a dynamic process of production and degradation regulates the average molecular mass of HA in tissues.[13,14] HMW HA is critical in embryogenesis, facilitating proliferation, cell migration, growth, and development. Indeed, fetal wound healing is characterized by the accumulation of HMW HA, which is implicated in scarless, regenerative wound healing. In contrast, LMW HA (<250 kDa) is associated with pro-inflammatory properties and increased fragmentation and is typical of pathological conditions including adult wound healing.[11]

We hypothesized that the injured fetal EHBD initially follows a program of fetal wound healing. The goal of this study was to define the fetal response to EHBD injury and in particular to determine whether fetal EHBD repair follows the fetal wound healing paradigm and whether this has implications for the development of BA.

Materials and methods

Animals

Handling and care of rodents was carried out according to protocols that were approved by the University of Pennsylvania Institutional Care and Use Committee, as per the National Institutes of Health Guide for the Care and Use of Laboratory Animals (protocol 804862). Animals were selected for use in experiments by age, regardless of sex. EHBDs were dissected from the distal end just before entering the pancreas to the proximal end just below the cystic duct. EHBD and bile samples from sheep were collected as excess tissue as part of previously described experiments whereby fetuses were maintained *in utero* ("normal", 20-25 ml/kg/min oxygen) or in an external biobag and supplied with 14-16 ml/kg/min oxygen ("hypoxia").[15,16] The handling and care of sheep complied with the ethical standards of the National Institutes of Health Guide for the Care and Use of Animals and protocols were approved by the Institutional Animal Care and Use Committee of The Children's Hospital of Philadelphia (protocol #1212).

Human samples

Anonymized human adult EHBD sections were collected as part of the Human Pancreas Procurement and Analysis Program, which was granted exemption by the University of Pennsylvania Institutional Review Board (protocol 826489). Participants (seven adults, 23-67 years old) were otherwise healthy individuals who died unexpectedly, with consent obtained from next of kin. Formalin-fixed paraffin embedded human EHBD tissue sections from four stillborn fetuses (22, 34, 36, and 39 weeks of gestation), three infants (2, 5, and 6 months old), and 16 BA remnants derived from the porta hepatis were obtained from the Anatomic Pathology archives of the Children's Hospital of Philadelphia, with the approval of the Institutional Review Board (IRB protocol 1371). Of the BA remnant sections, nine included epithelial structures. The study also included formalin-fixed paraffin-embedded samples that corresponded to the large intrahepatic bile ducts from three adults with primary sclerosing cholangitis (PSC), obtained from the pathology archives of the University of Pennsylvania (IRB protocol 831726).

Further details regarding the materials and methods are reported in supplementary information.

Results

Fetal and neonatal EHBDs have higher HA than adult ducts, localized in a peri-luminal ring

We first determined the differences in HA and collagen composition between the fetal and adult EHBD. As has been reported for other organs, staining using HA binding protein showed that HA in the EHBD is high in the fetus and neonate compared to the adult[17] across a range of species (humans, sheep, rats, and mice) (Fig. 1A and Fig. S1). When we quantified EHBD HA content biochemically, we found a decrease in percent HA content from neonates to adults (Fig. 1B). This was accompanied by a gradual increase in type I collagen content (Fig. S2). Surprisingly, HA in fetal and neonatal EHBDs was almost entirely located in a dense layer directly around the lumen, while collagen was peripheral; as development progressed, HA around the lumen gradually decreased and was replaced by collagen, which, as we previously reported and also observed here, was deposited from the outside in (Fig. 1A, D and Fig. S2).[12] Accordingly, the ratio of collagen to HA increased over time (Fig. 1C). Second harmonic generation imaging and Sirius Red staining for collagen showed a clear distinction between the HA layer and the more peripheral collagen layer in the fetus, especially in the larger species (human/sheep) (Fig. 1D,E). Note that the human fetal EHBD sample was obtained at a gestational age of 36 weeks, whereas the sheep fetus EHBD sample corresponded to a gestational age of 121 days (mean gestation in sheep is ~147 days) (Fig. 1D,E). Much of the embryological development of the EHBD is completed after birth in rodents, comparable with the last trimester in humans.[18–20] This is in line with our HA staining showing a wide layer of HA in the EHBD wall on postnatal day 1 in rodents resembling the HA staining of the sheep fetus and human EHBD (Fig. 1A).

Taken together, these data show that ECM content and distribution in fetal/neonatal EHBDs is fundamentally different from the adult EHBD, providing a substantially different environment – HA is predominant in the fetus, whereas collagen is predominant in adults – for bile duct regeneration.

Prenatal EHBD damage increases HA

HA is a major mediator of scarless wound healing in the fetus.[11] We therefore asked whether there are changes in the HA layer after EHBD damage. To study fetal EHBD wound healing, we used a sheep model of extra-uterine gestation (Fig. S3A-C). After 14-21 days under hypoxic conditions, there were significant differences between the EHBDs of sheep maintained *ex utero* compared to sheep of the same gestational age that remained *in utero*. This included patchy detachment of the surface epithelium with mural bleeding, increased leukocyte infiltration, and a statistically significant expansion in the width of the HA layer directly around the lumen (Fig. 2A, top row, and Fig. S3D, E). We observed a similar significant increase in the thickness of the peri-luminal HA layer in biliary remnants removed at the time of Kasai hepatoportoenterostomy from patients with BA, compared to controls of similar age (2-6 months of age) (Fig. 2A, middle row), although no increase in myofibroblasts was found (Fig. S4). In contrast, large bile ducts of adults with PSC did not show a significant expansion of the HA layer compared to adult controls (Fig. 2A, lower row).

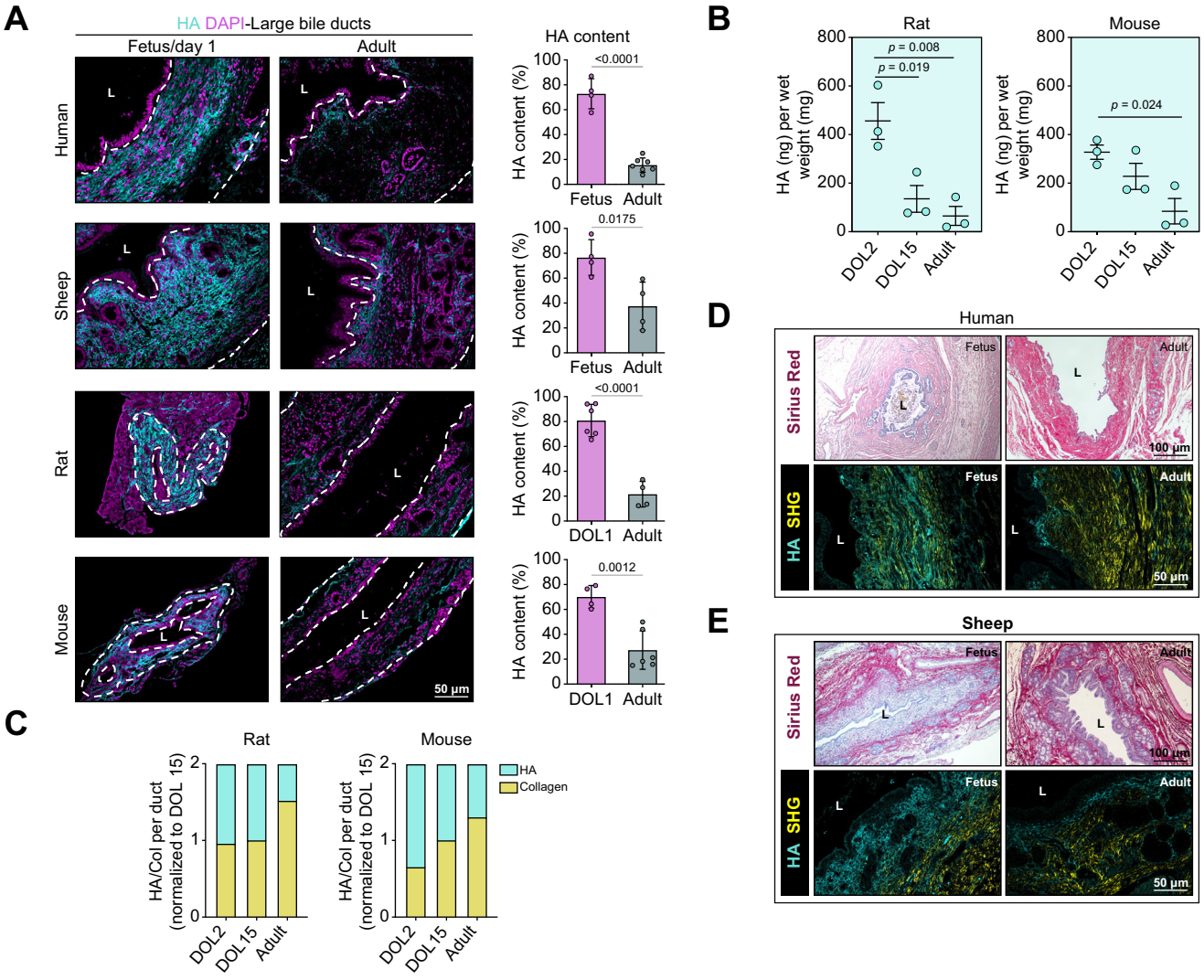

Fig. 1. Normal mammalian fetal and neonatal EHBDs have high HA and low collagen compared to adult EHBDs. (A) HA content of adult or fetal/neonatal human (GA: 22-39 weeks), sheep (GA: 121 and 128 days), and rat and mouse EHBDs (DOL 1). HA content was defined as % coverage of wall by HA binding protein signal (n ≥3 individuals). (B) Concentration of HA (ng) per mg wet weight EHBD. n = 3 homogenates, each containing 2-15 EHBDs. (C) Ratio of HA and collagen in the EHBD, normalized to DOL 15. (D) Human fetus (GA: 36 weeks) and adult EHBD. SHG imaging includes both forward and backward scatter. (E) Sheep fetus (GA: 121 days) and adult EHBD. All graphs show mean±SD. Significance determined by Student's *t* test (A) or one-way ANOVA with Tukey's *post hoc* test (B). EHBD, extrahepatic bile duct; HA, hyaluronic acid; SHG, second harmonic generation.

Viewed in another way, the distance between the lumen and the collagen layer appeared greater in the injured fetal sheep EHBDs and human BA remnants compared to age-appropriate controls (Fig. 2B and Fig. S5A, B). Although the BA remnant samples typically lacked a lumen, any epithelial structures visualized were surrounded by HA, with collagen outside of the HA ring (Figs 2B, 3A, and Fig. S5A, C). The main HA-producing enzymes are HAS1–3.[11] Interestingly, we observed an accumulation of HAS1-3-expressing mesenchymal cells near the lumen in fetal sheep EHBDs, mostly after hypoxia, but no differences in the total number of HAS+ mesenchymal cells were found between normal and hypoxic ducts (Fig. S6).

BA remnant collagen fibers differ from controls

Given that HA can disrupt collagen organization,[21] we asked whether there were organizational differences between the collagen in EHBD remnants from patients with BA and controls. The structure of the collagen around the HA layers in BA remnants was determined using the ImageJ plugin TWOMBLI to identify matrix patterns. It showed that collagen fibers in Kasai remnants from patients with BA were shorter and had more variable curvature but did not differ in density compared to controls (Fig. 3A-E). This is consistent with the collagen organization that is observed in adult pathology.[22]

Fetal EHBD injury leads to a robust progenitor cell response

We hypothesized that the differences between the ECM of the normal adult and the fetal EHBD reflect different programs of wound healing. Epithelial regeneration after injury of the adult EHBD has been described in the context of adult cholangiopathies.[23] The injured EHBDs of fetal sheep showed

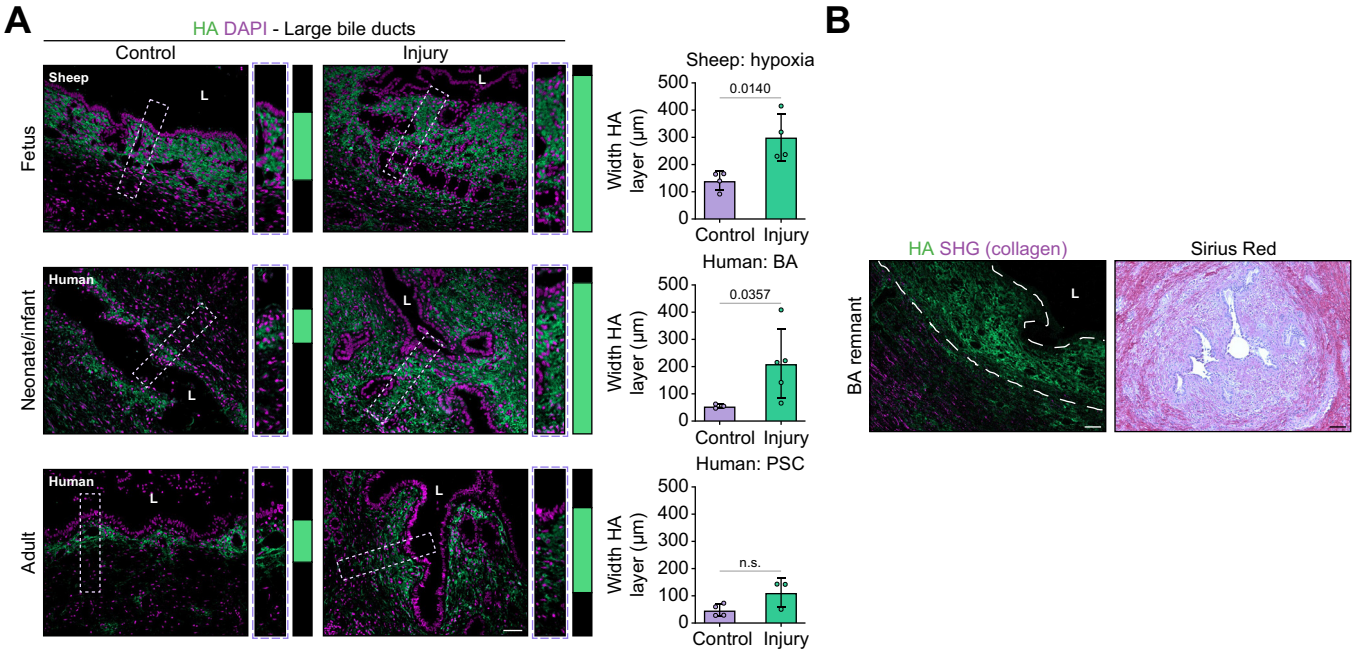

Fig. 2. Prenatal EHBD injury causes an increase in HA. (A) HA layer of damaged/diseased and normal fetal sheep (GA: 128 days) and infant and adult human EHBDs. Significance determined by Student's *t* test (fetal sheep) and Mann-Whitney test (infant and adult human). (B) Representative images of BA EHBD remnants (n = 9). Overlay of immunohistochemistry for HA (green) and visualization of collagen by SHG imaging (forward and backward scatter are both magenta) and Sirius Red staining. Graphs show mean±SD of n ≥3 individuals for each condition. All scale bars = 50 µm. BA, biliary atresia; EHBD, extrahepatic bile duct; GA, gestational age; HA, hyaluronic acid; SHG, second harmonic generation.

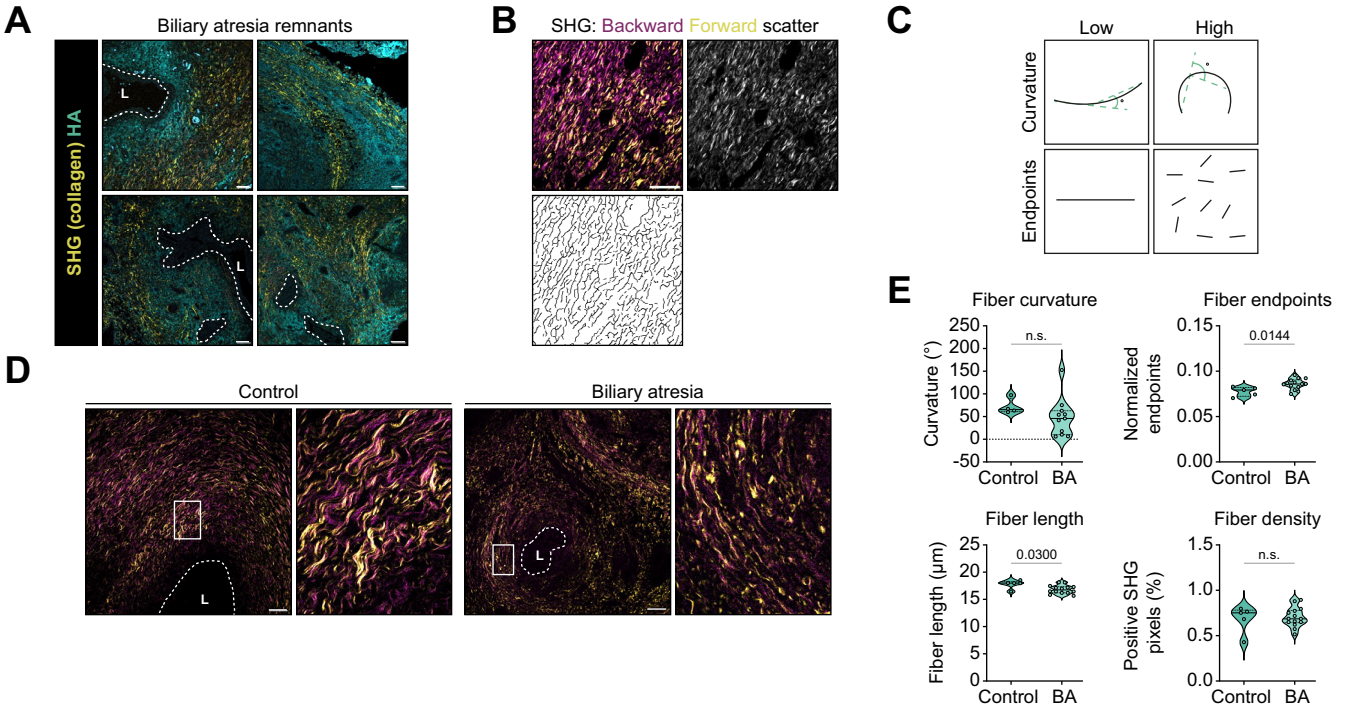

Fig. 3. Collagen in BA is organized around HA areas and the fibers are shorter. (A) Representative images of BA remnants (n = 14), with collagen visualized by SHG imaging and HA by HA binding protein. (B) Mask rendering from SHG signals (both forward and backward scatter) on a control sample (age: 2 months). (C) Schematic of curvature and endpoint calculations. (D) Representative SHG imaging of EHBDs from human controls (n = 5; age: 2-6 months) and BA remnants (n = 14) taken at the Kasai procedure. (E) Fiber curvature, endpoints, length, and density. Significance determined by Student's *t* test. All scale bars = 50 µm. BA, biliary atresia; EHBD, extrahepatic bile duct; HA, hyaluronic acid; SHG, second harmonic generation.

marked regeneration with the presence of a double-layered surface epithelium, PBG hyperplasia (*i.e.*, increased PBG area), and increased proliferation of epithelial and mesenchymal cells (Fig. 4A,B). HA has been shown to increase proliferation in cholangiocytes through CD44;[24] although no differences in CD44 expression were observed in the EHBDs of control *vs.* hypoxic animals (Fig. S7), it is likely that the increased amount of HA is sufficient to trigger CD44 to increase cholangiocyte proliferation. PBGs were distributed regularly throughout the submucosa in both the control and the hypoxia group in fetal EHBDs (Fig. 4A), in contrast to adult EHBDs in which the PBGs generally appear in compact clusters.[5] In fetal sheep incubated in the external womb, the area occupied by PBGs expanded in proportion to the duration of time in hypoxic conditions and increased in proportion to the increase in width of the EHBD HA layer (Fig. 4C-E).

High percentages of Sox9+ and PDX1+ cholangiocytes were observed in the BA remnants, resembling the PBGs of the control neonatal/infant (age: 2-6 months) EHBDs (~100% of the PBG cells were PDX1+ and Sox9+) (Fig. 4F). Anion exchanger 2 marks mature and functional cholangiocytes and was generally located in the surface epithelium of control EHBDs, but no cholangiocytes in the BA remnants expressed anion exchanger 2 (Fig. 4F, middle panel). The surviving cholangiocytes in BA remnants showed high proliferation rates compared to controls (p <0.01) (Fig. 4F, lower panel). In fetal sheep EHBD samples, there was also marked expression of Sox9 although it was found in PBGs as well as in the surface epithelium, suggesting that in fetal life the surface epithelium is still immature (Fig. S8).

Thus, although we are unable to induce similar injuries in fetal and adult EHBDs to compare their responses directly, the fetal biliary regenerative response appears to be distinct from that of the adult[5] and is characterized by marked PBG hyperplasia, marked proliferation, and increased HA (rather than collagen) deposition immediately around the lumen.

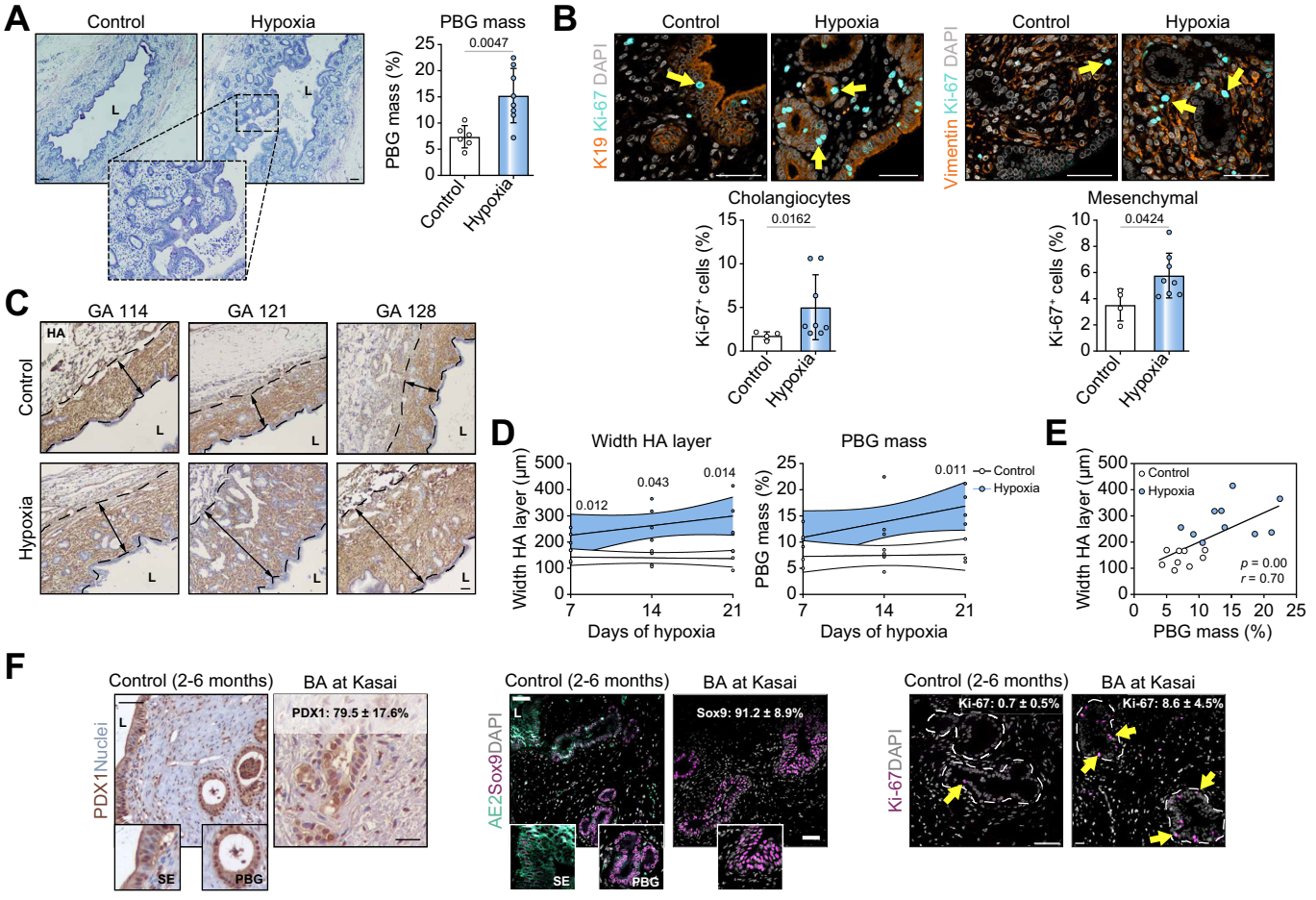

Fig. 4. Prenatal hypoxia causes a marked epithelial regenerative response in EHBDs of fetal sheep. (A) H&E staining of EHBDs from control (n = 6) and hypoxia (n = 8) groups (GA: 121 and 128 days) and graphs representing PBG area. (B) Immunofluorescence for Ki-67 (proliferation), K19 (cholangiocytes), and vimentin (mesenchymal cells) on sheep fetus EHBDs (GA 121 and 128). n = 4 (controls) and n = 8 (hypoxia). (C) Representative HA stains (brown) in control and hypoxia groups (n ≥3 for each timepoint). (D) HA layer width (from C) and PBG area (from A). Graphs show mean±SE of the best curve to fit the data. (E) Correlation between the HA layer width and PBG area (as plotted in D). (F) Human control EHBDs (n = 5; age: 2-6 months) and BA remnants (n = 14) stained for PDX1, AE2, Sox9, and Ki-67. % positive cells is provided. Significance determined by Student's *t* test (A-D), Mann-Whitney (B: cholangiocytes) or Spearman correlation (E). Graph shows mean±SD (all panels). All scale bars = 50 µm. BA, biliary atresia; EHBD, extrahepatic bile duct; GA, gestational age; HA, hyaluronic acid; PBG, peribiliary gland.

HA deposition by the fetal EHBD after injury predisposes the duct to obstruction

The width of the peri-luminal HA layer as well as the water-retaining (and therefore swelling) qualities of HA led us to hypothesize that the HA deposition in the fetal ducts predisposed to duct obstruction.[25] We therefore examined the fetal sheep in the hypoxia group for markers of biliary obstruction. First, we found that all liver samples taken from the animals in the hypoxia group (n = 11) showed bile plugs in the intrahepatic bile ducts while none of those from the control group had plugs. Additionally, serum bilirubin was significantly increased in the hypoxia group (Fig. 5A). Second, we measured the density and width of the outer collagen layer in control *vs.* injured EHBDs and showed that it was narrower in injured ducts compared to controls, and that there was a trend towards increased collagen density, consistent with compression of the collagen bundles by an expanding HA layer (Fig. 5B). To determine whether swelling of HA around a duct-like structure could lead to luminal narrowing, we used a microfluidic device that was previously developed in our lab[26] whereby collagen or collagen plus HMW or LMW HA was gelled around a needle, forming a channel (Fig. 5C). At baseline after needle removal, the diameters of the channels ranged between 97–144 μm and were comparable between the three conditions. We then added PBS to the system and determined changes in luminal diameter. The HA-filled ECM swelled, leading to a decrease in luminal diameter, particularly in the LMW HA-containing devices (Fig. 5C, D).

Thus, there is compelling evidence for obstruction in the ducts of injured fetal sheep, even in the absence of liver or duct fibrosis; *in vitro* modeling as well as the matrix structure of the EHBDs are consistent with HA-associated swelling as a cause.

EHBD regeneration in fetal sheep promotes mucus production

We also considered the properties of bile after fetal EHBD injury. In addition to there being increased numbers of PBGs in EHBDs from hypoxic fetal sheep (Fig. 4A,D), the periodic acid-Schiff-positive area of the PBGs was consistently higher in the hypoxia group compared to controls, indicating increased mucin production (Fig. 6A). In the hypoxia group, mucin-positive cells were evident in both PBGs and the surface epithelium, which contained goblet cells (Fig. 6B and Fig. S9A), in line with reports of goblet cells in human fetal EHBDs at later points of gestation.[27] Mucus produced by PBGs, like all mucus, has a high viscosity and contributes to the viscosity of bile.[28] Based on the number of mucin-containing cells in EHBDs in the hypoxia group, we hypothesized that the viscosity of bile from these animals would be increased. However, there was no bile visible in the gallbladders of the hypoxic animals. We therefore compared the viscosity of bile from the control fetal sheep with bile from two adult sheep. Although bile viscosity was higher in a subset of the fetal samples, we were unable to collect enough to determine significance (Fig. S9B).

We hypothesized that if mucus-containing bile accumulated in the EHBD and led to increased obstruction, we would be able to detect mucus in the intrahepatic bile plugs.

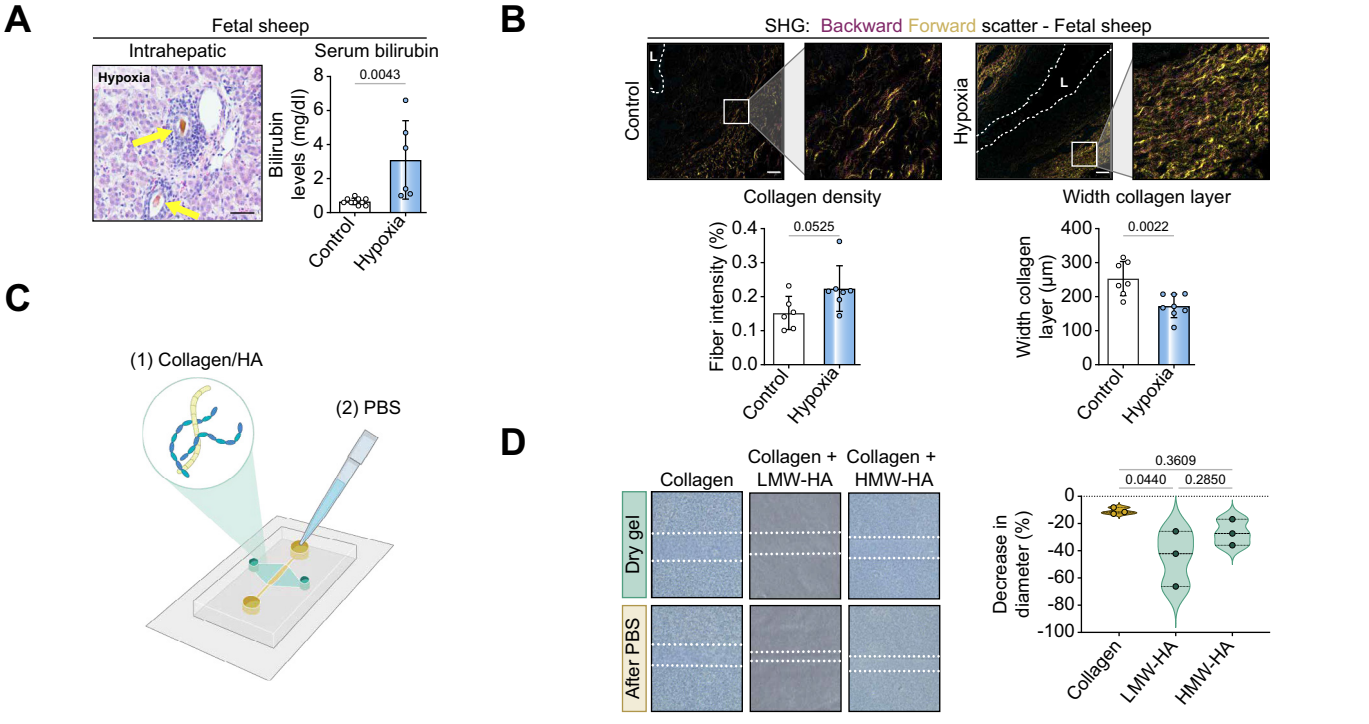

Fig. 5. HA deposition predisposes to obstruction. (A) H&E staining of a representative fetal sheep liver in the hypoxia group showing bile plugs. Serum bilirubin levels of fetuses in the control (n = 10) and hypoxia (n = 6) groups (GA: 121 and 128 days). (B) SHG imaging of EHBDs from control (n = 7) and hypoxic fetal sheep (n = 8) to determine collagen density and width of collagen layers. (C) Setup of the microfluidic device. (D) (left) Devices with the three types of matrices (n = 3, each), before and after swelling; (right) Violin plot of % change in luminal diameter for each group. Significance determined by Student's *t* test or one-way ANOVA with Tukey's *post hoc* test (D). Graphs show mean±SD. All scale bars = 50 μm. EHBD, extrahepatic bile duct; GA, gestational age; HA, hyaluronic acid; SHG, second harmonic generation.

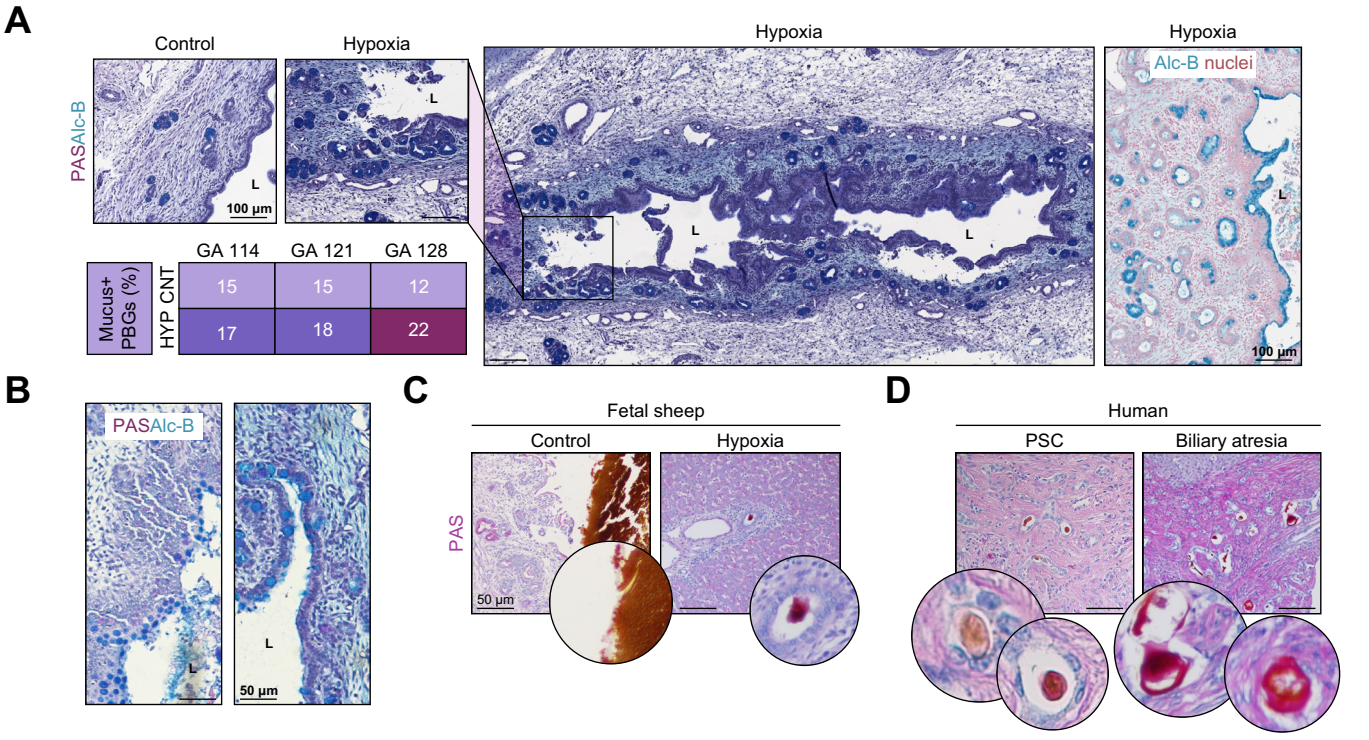

Fig. 6. The fetal EHBD regenerative response involves an increase in mucus production. (A) PAS and alcian blue-stained EHBDs from fetal sheep in control and hypoxia groups. Table indicates % PBGs showing mucus production for the indicated GA (n ≥3 for each timepoint). Colors, from light to dark purple, correspond to increasing percentages (<15%, <20%, and >20%). (B) Combined PAS and alcian blue staining of goblet cells in the surface epithelium of the hypoxia group. (C) PAS staining of bile in the gallbladder (controls did not show intrahepatic bile plugs) and bile plug in the intrahepatic bile ducts. (D) PAS staining of intrahepatic bile plugs of patients with PSC or BA (n = 3, each). BA, biliary atresia; EHBD, extrahepatic bile duct; GA, gestational age; PAS, periodic acid-Schiff; PSC, primary sclerosing cholangitis.

Bile plugs were only seen in the hypoxia group; they stained positive for periodic acid-Schiff, indicating the presence of mucus, in contrast to bile in the gallbladder of controls (Fig. 6C). Pretreating livers with bilirubin oxidase to minimize potential interference from bilirubin pigments in bile confirmed the positive staining (Fig. S10). Similar treatment of livers from patients with BA also showed mucus staining of bile plugs. Normal human livers do not contain bile plugs so cannot be used for comparison, but liver sections from patients with PSC showed less prominent mucus in bile plugs than did BA livers (Fig. 6D). The mechanism of increased mucus is not known; HA did not induce mucus production in HA-treated spheroids, suggesting that the mechanism is HA independent (Fig. S9C).

Collectively, these results suggest that PBG hyperplasia and mucus production, part of the regenerative response of the fetal EHBD, may result in increased bile viscosity, potentially contributing to EHBD obstruction. The damage-repair response of fetal EHBDs includes expansion of the HA layer, which swells, compressing both the collagen layer and the lumen. The decreasing diameter of the lumen may impede bile flow, especially if the bile is already viscous.

Neonatal EHBDs contain higher molecular weight HA than adult EHBDs, which is degraded after injury

In order to better understand the dynamics of HA during development and after injury, we studied the expression of the enzymes that synthesize and degrade HA. Neonatal mesenchymal cells showed increased numbers of HAS1-3-positive cells by immunostaining compared to adults ($p = 0.01$; $p = 0.01$; $p < 0.01$, respectively) (Fig. 7A). These results suggest that production of HA in the EHBD submucosa of neonates is higher than in adults, consistent with our data showing that neonatal ducts have increased HA compared to adult ducts, including on a per weight basis, across species (Fig. 1). Staining for the two major HA degradation enzymes, HYAL1 and HYAL2, similarly demonstrated higher expression in neonates compared to adults ($p < 0.01$; $p < 0.01$) (Fig. 7A). We used solid-state nanopore technology[29] to determine the size of HA polymers in neonates and adults, and we found that the molecular weight of HA was significantly higher in neonates compared to adults, consistent with an ongoing process of degradation after synthesis (Fig. 7B, Table S2). Next, we asked whether injury to neonatal EHBDs led to increased HA degradation. Treatment of neonatal rat EHBD explants with biliatresone, a toxin that causes a BA-like phenotype in animal models and in explants,[30] resulted in increased breakdown of fluorescein-labelled HA (Fig. 7C and Fig. S11). There was some damage in the DMSO-treated EHBDs, which was likely the result of the explant culture system.

Together with Figs 1 and 2, these results indicate that production, degradation, and average molecular weight of HA in the submucosa of neonatal bile ducts is higher than in adult ducts, and that injury leads to both increased production and degradation of HA.

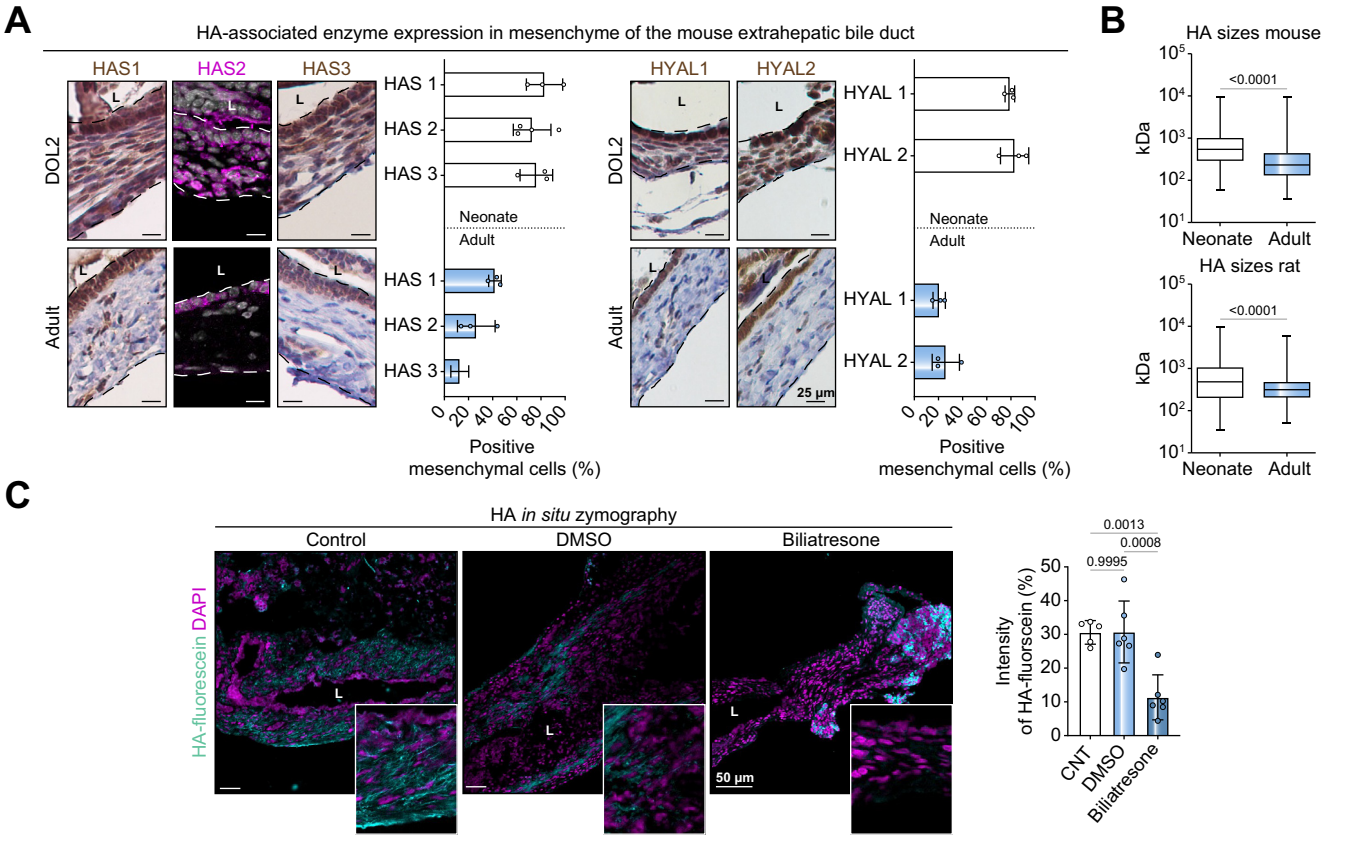

Fig. 7. HA size distribution and production/degradation enzymes differ between neonates, adults, and after injury. (A) Mouse neonatal EHBDs at day 2 after birth and adult EHBDs stained for HAS1-3 and HYAL1-2. Percentage positive mesenchymal cells for n = 3 for each stain shown in graph. (B) HA size distribution in mouse and rat neonatal and adult EHBDs, as measured by solid-state nanopore technology. (C) HA *in situ* zymography using fluorescein-labelled HA, overlaid on 4/5-day-old rat neonatal EHBDs after immediate harvest (control; n = 5) or after an additional 5-day treatment with DMSO (n = 6) or biliatresone (n = 6). Significance determined by Student's *t* test or one-way ANOVA with Tukey's *post hoc* test (C). Graphs show mean ± SD (all panels). EHBD, extrahepatic bile duct; HA, hyaluronic acid; HAS, hyaluronic acid synthase; HYAL, hyaluronidase.

The size of HA influences EHBD cell behavior in 2D and 3D cultures

Our EHBD data are consistent with extensive literature reporting that adult wound healing is associated with an increased degradation of HA, yielding a high concentration of LMW HA, whereas fetal wound healing is characterized by an accumulation of HMW HA.[11,14,31] To determine the cellular response of the EHBD to LMW and HMW HA, we co-cultured primary neonatal mouse cholangiocytes and EHBD mesenchymal cells in spheroids. Cells were embedded in droplets containing a mixture of Matrigel/collagen or Matrigel/collagen/HA. Cells cultured in mixtures with HMW HA demonstrated increased spheroid growth, diameter, and proliferation rates compared to cells cultured in mixtures with LMW HA (Fig. 8A,B). Epithelial architecture differed between the groups – in LMW HA, 19% of the spheroids showed an aberrant morphology, whereas in HMW HA or Matrigel/collagen alone, significantly more spheroids appeared circular with a clear lumen (1% and 2% with aberrant morphology, respectively) (Fig. 8C,D); yet no differences in cholangiocyte markers were noted (Fig. S12).

To further study the effects of HMW HA on cholangiocyte growth, we cultured human cholangiocytes in a bile duct-on-a-chip microfluidic device[26] with an ECM of collagen alone or collagen with HMW HA. Initially, the cholangiocytes in both conditions formed a monolayer. This was followed, however, by the formation of outpouchings exclusively in the HMW HA ECM (Fig. 8E, leftmost panels). This phenomenon was paralleled by an increase in cholangiocyte proliferation (Fig. 8E, middle and rightmost panels). To assess the influence of LMW and HMW HA on collagen production in the EHBD, we employed a 2D co-culture system. We seeded a mixture of cholangiocytes and mesenchymal cells isolated from neonatal mouse EHBDs and assessed collagen deposition and organization after treatment with media supplemented with water (control), LMW HA, or HMW HA (Fig. 8F). After 2 weeks of culture, the monolayers comprised cholangiocyte clusters surrounded by dense layers of fibroblasts (Fig. 8F, leftmost panels). Second harmonic generation imaging showed that collagen deposition was increased by treatment with both LMW and HMW HA, but particularly by HMW HA (Fig. 8F, middle panels). Collagen fibers were more aligned (higher peak around the 0° position) in the HMW HA-treated group compared to the control and LMW HA groups (p <0.01 and p = 0.01, respectively) (Fig. 8F, rightmost panel). Note that cholangiocytes were directly surrounded by HA suggesting HA production (Fig. S13); in Fig. S13, spheroids were cultured without HA. The addition of HA to collagen gels decreased stiffness and increased viscosity, consistent with a malleable environment that enhances tissue development and regeneration (Fig. S14).[32]

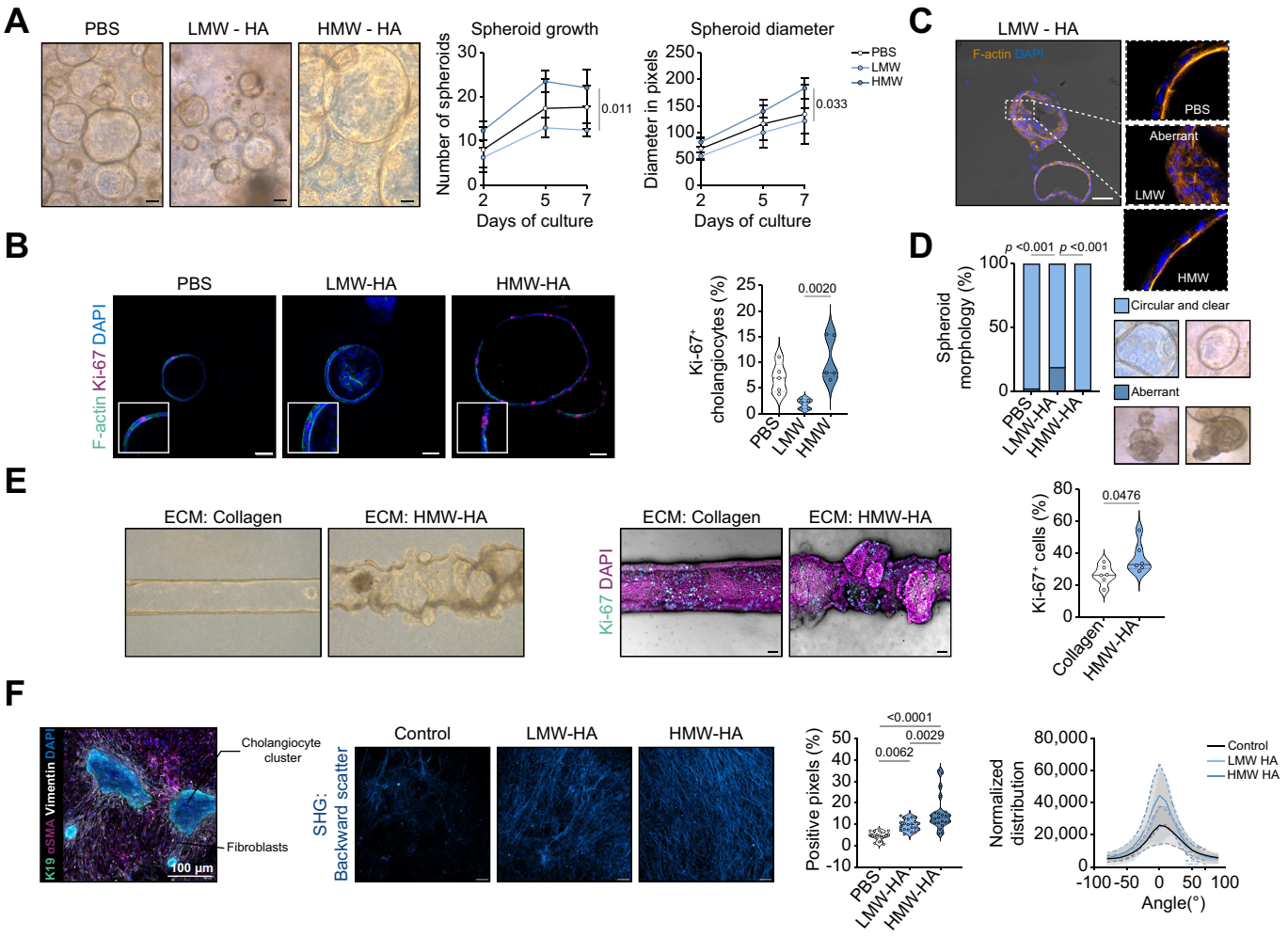

Fig. 8. LMW and HMW have different effects on EHBD cholangiocytes and mesenchymal cells. (A) Spheroids derived from mouse primary cholangiocytes cultured in collagen with PBS, LMW HA, or HMW HA (n = 5 biological repeats with 2-3 technical replicates per condition). (B) Proliferation (Ki-67) of spheroids in each condition. (C) Spheroids stained with F-actin with bright field background. (D) Morphological scoring of spheroids in each condition. (E) Human cholangiocytes were seeded in the bile duct-on-a-chip device with a mixture of collagen alone or HMW HA with collagen in the ECM compartment. Immunofluorescence for Ki-67 on the right (n = 3 biological repeats with 2 technical replicates per condition). (F) (left) K19, αSMA, and vimentin staining of 2D co-cultures; (middle) SHG imaging (back scatter) of the cholangiocyte/fibroblast-derived matrices in each condition (n = 3 biological repeats with 3 technical replicates per condition). Significance determined by area under the curve (A), Student's *t* test (B, E), Chi square test (D) and one-way and two-way ANOVA for SHG intensity and collagen alignment, respectively (F). Graphs show mean±SD (all panels). All scale bars = 50 μm. ECM, extracellular matrix; EHBD, extrahepatic bile duct; HA, hyaluronic acid; HMW, high molecular weight; LMW, low molecular weight; SHG, second harmonic generation.

Together, these data show that HMW HA stimulates cholangiocyte spheroid formation and proliferation and suggest that it may provide a favorable environment for PBG organization *in vivo*. In contrast, LMW HA promotes aberrant spheroid morphology and impedes spheroid growth. This may have important implications for cholangiocyte structures surrounded by HA that become fragmented in pathological conditions. In addition, both LMW and HMW HA stimulate collagen production and alignment; this is essential for EHBD development as well as for both regenerative (healing) and pathological (fibrotic) wound repair.

Discussion

We demonstrate here that fetal EHBD injury initially leads to a vigorous program of fetal wound healing. Specifically, 1) the fetal/neonatal EHBD had high levels of HA compared to the adult EHBD; 2) in the fetus and neonate, HA was localized circumferentially immediately adjacent to the lumen, with a relatively thin layer of collagen at the periphery of the duct, while in adults the collagen layer reached from the periphery to just below the mucosal layer; 3) EHBD injury in fetal sheep and in human BA resulted in an expansion of the HA layer encircling the bile duct lumen, with luminal narrowing and potential compression of the peripheral collagen layer; 4) sheep fetuses with EHBD injury demonstrated elevated bilirubin levels, consistent with EHBD obstruction, although no liver or EHBD fibrosis was noted; 5) sheep fetuses with EHBD injury showed increased levels of biliary mucus production, potentially contributing to increased bile viscosity and EHBD obstruction; 6) the molecular weight of HA was significantly higher in the EHBDs of neonates compared to adults, and HA degradation in the neonate increased after injury; 7) LMW HA in 3D *in vitro* systems promoted an aberrant epithelial architecture, whereas

HMW HA stimulates proliferation and the formation of PBG-like structures; and 8) both LMW and HMW HA stimulated collagen production in co-cultures of EHBD-derived fibroblasts and cholangiocytes. Collectively, these data not only show that fetal EHBD injury stimulates a fetal-type wound healing response, but also suggest that the increased deposition of HA, while enhancing regeneration, leads to swelling and obstruction – a potential example of a beneficial response (fetal wound healing) "going bad", and a new mechanism that may contribute to the pathophysiology of BA.

Fetal wound healing is characterized by regenerative, scarless repair. This pro-regenerative microenvironment features high levels of HMW HA and collagen-3 instead of the pro-fibrotic LMW HA and collagen-1 seen in scarring adult wounds.[11,14,25] Fetal wound healing across tissues – including the lung, heart, tendon, and skin – is characterized in rodent and sheep models by prolonged increases in HA levels compared to adults, lasting up to 3 weeks after the initial injury.[33] In our fetal sheep model, EHBD injury lasting as long as 21 days was accompanied by marked expansion of the HA layer and increases in bilirubin levels. Although we were unable to monitor the consequences of injury longer than 21 days in this model, the nature of the fetal wound healing program in other tissues suggests that expansion of the EHBD HA layer and subsequent narrowing of the lumen (as described below) could persist for weeks after the initial injury, potentially aggravating cholestasis and thereby EHBD wall damage.

In humans, transition to a so-called adult program of wound healing typically begins in the third trimester of pregnancy. Adult wounds contain predominantly LMW HA[31] and in a fetal rabbit model of skin wounds, experimental fragmentation of HA with hyaluronidase was associated with more 'adult-like' scarring, defined by excessive collagen production and neovascularization.[34] We showed here that damage to the neonatal rat EHBD led to increased HA degradation, reflecting a transition to adult wound healing. We propose that fetal EHBD injury (especially if it is persistent) or a subsequent regenerative response in late gestation could lead to increased HA degradation, consistent with features of adult wound healing (Fig. S15). Thus, initial prolonged HA deposition (a component of fetal wound healing) could lead to bile duct obstruction early in gestation, followed by scarring/fibrosing (a hallmark of BA) when the transition to an adult wound healing program takes place.

Our results suggest that LMW HA in the EHBD wall during the transitional period and during adult wound healing could promote aberrant epithelial morphology and decreased cholangiocyte growth. The BA EHBD remnants (from Kasai surgeries) we examined had features of both fetal and adult wound healing, with an expanded peri-epithelial HA layer and epithelial structures that were phenotypically immature and morphologically distinct from normal PBGs and/or surface epithelium, consistent with fetal wound healing, but collagen organization reflecting an adult pathological process, as would be expected based on the age of patients with BA at the time of performing the Kasai procedure. The remarkable distribution of HA and collagen in both fetal EHBDs and BA remnants suggests that BA should be viewed in light of age-appropriate fetal-adult wound healing programs and that drawing parallels between BA and adult fibrosing cholangiopathies should be undertaken with caution.

The high concentrations of HA in the fetal EHBD may have implications beyond regenerative healing. An HA-rich ECM is a porous meshwork that exerts pressure on its surroundings due to repulsion between and within molecules.[32] Furthermore, owing to its hygroscopic properties, HA can swell up to 1,000 to 10,000 times, thereby shaping an environment in which cells and cell structures are distanced from each other, avoiding inhibitory contacts.[25,35] This malleable HA-rich ECM is ideal for cell migration, which may explain why PBGs are distributed evenly throughout the fetal EHBD but are in collagen-encircled clusters in the adult EHBD. This may also explain the formation of outpouchings in the collagen/HA-filled bile duct-on-a-chip – PBGs can easily increase in number in an HA-rich environment, as demonstrated by the remarkable PBG hyperplasia observed in fetal EHBDs.

The increased PBG volume, including higher mucus production leading to increased bile viscosity and expanded HA layer during epithelial regeneration make it likely that fetal EHBDs can swell and are particularly susceptible to obstruction, potentially even during normal development. It is also possible that in the setting of EHBD injury during gestation, some ducts progress to total obstruction and BA but others undergo successful repair, potentially accompanied by transient luminal narrowing and bilirubin elevations. A recently published large clinical trial that examined the efficacy of serum bilirubin measurements as diagnostic tools for BA reported that, of 123,279 total newborns tested, all seven babies who went on to develop BA had elevated bilirubin levels at 60 hours and 2 weeks after birth, but so did a significant number of newborns who never developed cholestatic symptoms or disease.[1] We speculate that these newborns experienced transient EHBD swelling causing elevated bilirubin levels either as part of a healing injury – a forme fruste of BA – or even as part of normal growth.

Steroid treatment of certain tumors leads to a reduction in HA synthesis and stromal edema.[36,37] Interestingly, the administration of steroids after a Kasai procedure did not influence post-operative survival or the number of transplantation-free months, but it did lead to decreases in serum bilirubin levels.[38] It is possible that steroids decreased HA synthesis and/or swelling in the large intrahepatic bile ducts of these patients, effectively widening the lumen. Notably, this effect was most prominent in the younger cohort, the group that would be most likely to have high levels of HA. It is conceivable that as BA progresses, more collagen is deposited around the ducts and other epithelial structures, limiting the effect of steroids on reducing EHBD wall volume.

We observed numerous goblet cells in the EHBDs of the hypoxic animals, although intestinal metaplasia was found in some control fetal sheep EHBDs as well. The occurrence of goblet cells in the surface epithelium of the EHBD has been described previously in autopsy samples from human newborns between 28-41 weeks of gestation,[27] although it is not a general feature of BA. We noted higher overall viscosity of fetal compared to adult bile in sheep and it is possible that the same is true in humans; we hypothesize that the combination of swelling and luminal narrowing from HA and viscous bile contributes to luminal obstruction and BA pathophysiology, although this would be difficult to test in human fetuses and neonates.

In summary, the damage-repair response of the fetal EHBD is an example of fetal wound healing. The expansion of the HA layer in the EHBD wall potentially leads to obstruction of the EHBD lumen, which can be detected by elevated serum

bilirubin levels. The initial luminal narrowing could lead to progression of fibrosis/scarring when accompanied by the transition to adult-type wound healing in late gestation, including increased degradation of HA. Viewing the damage-repair response of fetal EHBDs in the context of fetal wound healing will be crucial to understanding the early pathogenesis of BA, including rapid progression to biliary fibrosis after birth, and to the development of potential treatments.

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Abbreviations

AE2, anion exchange protein 2; BA, biliary atresia; EHBD, extrahepatic bile duct; ECM, extracellular matrix; HA, hyaluronic acid; HAS, hyaluronic acid synthase; HMW, high molecular weight; HYAL, hyaluronidase; LMW, low molecular weight; PBG, peribiliary gland; PSC, primary sclerosing cholangitis.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

IEMdJ and RGW designed the study, interpreted the results and wrote the manuscript. IEMdJ performed the image analyses. RGW obtained funding for the experiments. IEMdJ, DC, YD, JL, DL, KG, DE, FR, and AD performed the experiments. MLH performed the fetal sheep surgeries under the supervision of JWG, AWF, and MGD. RGW, ARH, JB, RJP, and PAR supervised the experiments. EEF, AN, and CL were instrumental in obtaining access to human tissue. All authors critically reviewed and approved the manuscript.

Data availability statement

Data presented in this study are available upon reasonable request.

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Supplementary data

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