Peng ZW et al. "Integrin αvβ6 critically regulates hepatic progenitor cell function and promotes ductular reaction, fibrosis and tumorigenesis"

SUPPLEMENTARY MATERIALS AND METHODS

Hepatic hydroxyproline, as a measure of collagen content was determined biochemically from two liver pieces from the median and left liver lobe (250-300mg in total, representing at least 10% of total liver volume) as described (1). Based on relative hepatic hydroxyproline (per 100mg of wet liver), total hepatic hydroxyproline was calculated (per liver).

Quantitative Real-Time RT-PCR (qRT-PCR) was performed on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) using TaqMan principle as described previously (1, 2). Validated TaqMan probes and primers (**Suppl Table 1)** were obtained commercially or designed using the Primer Express software (Perkin Elmer, Wellesley, USA).

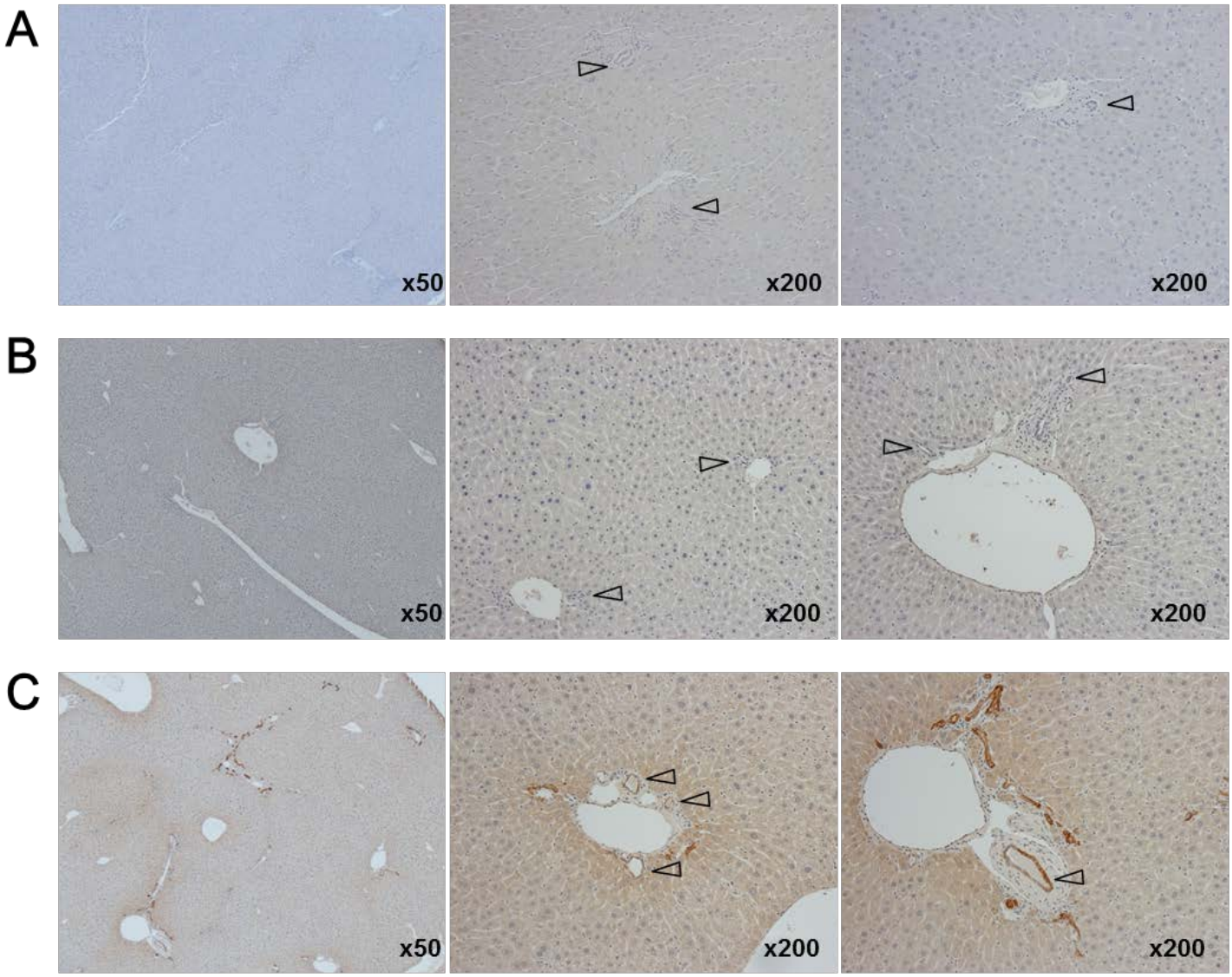
Serum levels of alanine aminotransferase (ALT) were measured on automated Catalyst Dx Chemistry Analyzer (IDEXX Laboratories, Inc., Westbrook, ME) as described (3).

Immunohistochemistry and immunofluorescence for progenitor (oval) cell markers were performed as previously reported(3, 4). Primary antibodies are summarized in Suppl. Table 2. Anti-αvβ6 (clone ch2A1, 1/200, Biogen Idec), A6 (1/50, kind gift of Dr. V. Factor, NIH, Bethesda, MD), and anti-pan-cytokeratin (antibodies were used as primary antibodies followed by HRP-conjugated secondary antibodies and DAB reagent. Morphometric analysis was performed by counting positive cells in at least 10 random portal tracts of at least four individual mice/group. Immunofluorescence was performed on acetone-fixed frozen liver sections or cells using primary antibodies against A6 (1/50), PCNA (1/50, Santa Cruz, #sc-7907), αvβ6 (clone ch2A1, 1/50, Biogen Idec), TROP2(1/50, AF1122, R&D Systems), pan-cytokeratin (1/50, Z0622, Dako, Glostrup, Denmark), Ki-67(1/200, TEC-3, Dako), K19 (1/200, TROMA-III, Developmental Studies Hybridoma Bank, University of Iowa), followed by secondary fluorophore-conjugated antibodies. DAPI was used as a nuclear counterstain. Images were captured using Zeiss Axioimager M1 (Carl Zeiss, Oberkochen, Germany).

Western blotting for α-SMA was performed and analyzed via band densitometry and normalized to β-actin as a loading control as described(5).

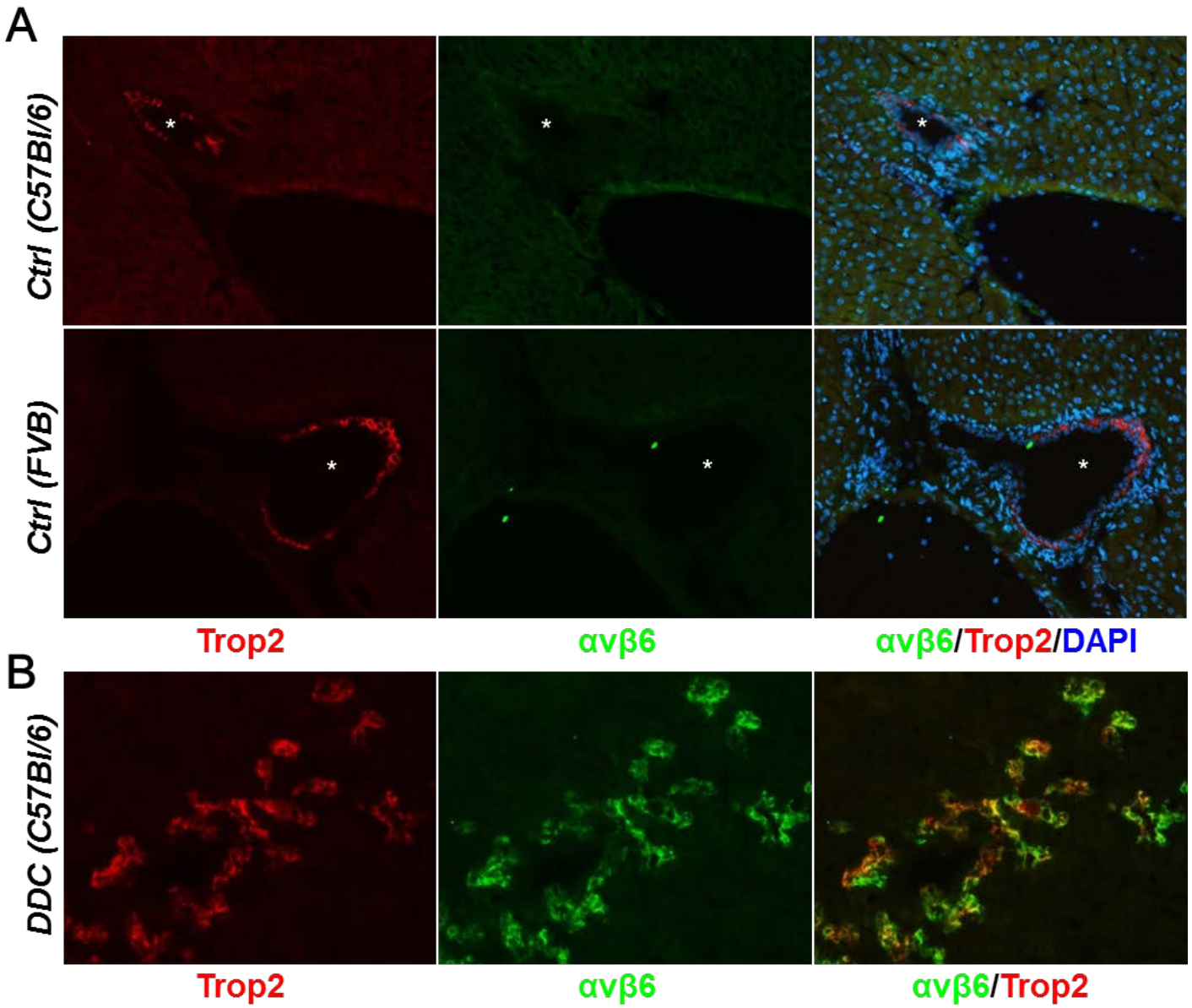
SUPPLEMENTARY RESULTS

Expression pattern of αvβ6 integrin in small ductular proliferations constituting ductular reaction and mature cholangiocytes lining larger intrahepatic bile ducts was investigated using immunohistochemistry in normal human and murine livers. No αvβ6 expression was detected in either human or murine normal liver (Suppl. Figure 1A and B, respectively). Normal cholangiocytes lining small or large intrahepatic bile ducts, as well as cells in the canals of Hering, were negative for αvβ6 (Suppl. Figure 1A and B). In contrast, in fibrotic livers of *Mdr2-/-* mice, intrahepatic bile ducts within fibrotic lesions demonstrated various degrees of αvβ6 expression, from strong immune-positivity in some large injured bile ducts to weak or absent expression in smaller and less scarred bile ducts (Suppl. Fig. 1C and Fig. 1A of main manuscript).



Supplementary Figure 1. **Integrin αvβ6 expression pattern in normal and diseased liver.** Immunohistochemistry for αvβ6 integrin in normal human liver (A), healthy murine liver (B, wildtype, FVB background) and fibrotic murine liver (C, Mdr2-/-, FVB background). Representative images at low (x50, left panel) and high (x200, middle and right panels) shown. Arrows indicate bile ducts.

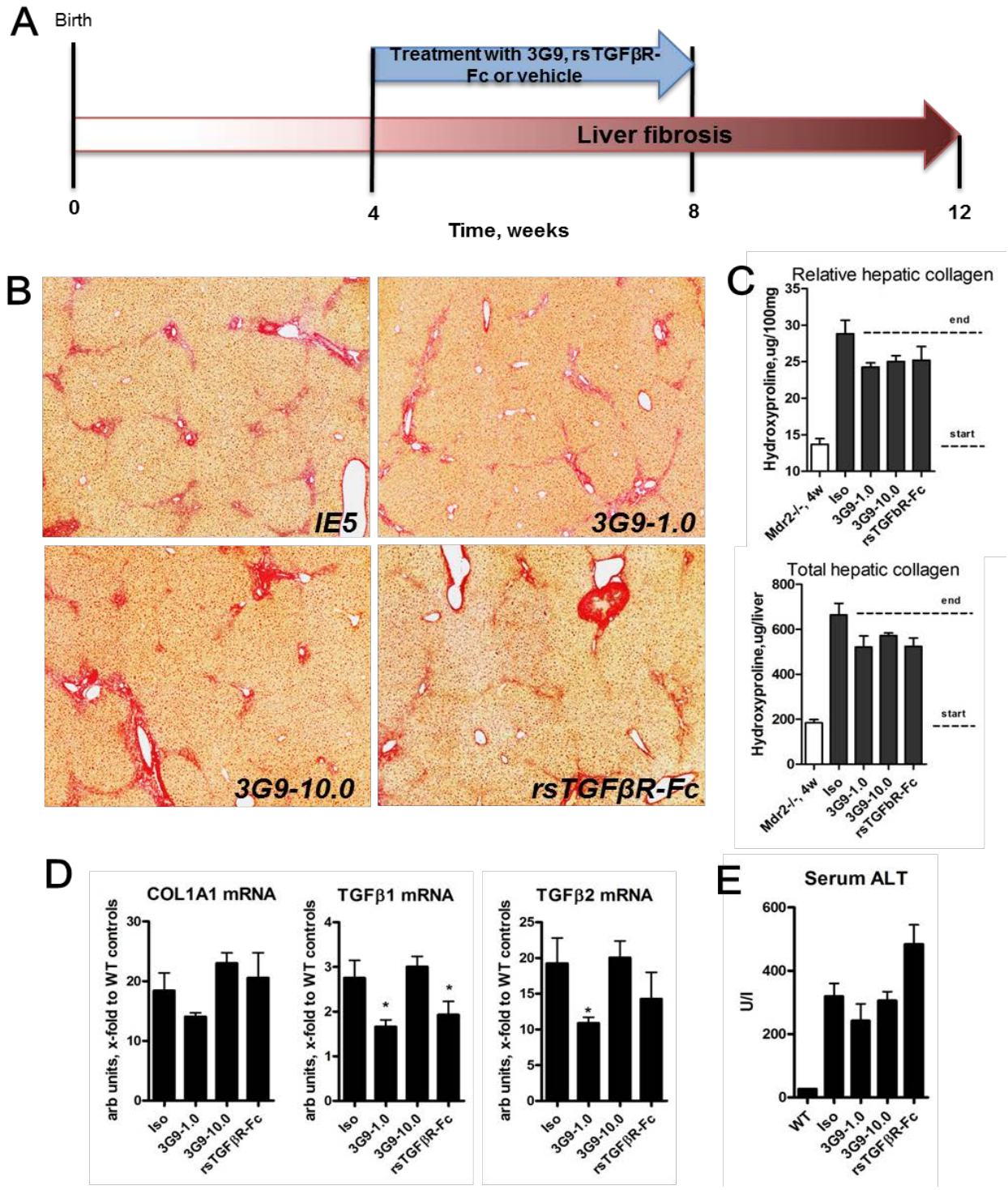
Lack of αvβ6 expression on mature normal cholangiocytes was further confirmed using immunofluorescence for αvβ6 and progenitor-specific marker Trop2. As expected, small bile ducts in livers of healthy C57Bl/6 and FVB mice were uniformly negative for both αvβ6 and Trop2 (not shown). Surprisingly, a few large intrahepatic bile ducts were negative for αvβ6 but demonstrated weak Trop2 expression (Suppl. Fig. 2A, exposure time twice as much as in DDC-fed mice images below). In contrast, proliferating cells comprising ductular reaction in chronically injured liver due to DDC feeding demonstrated robust Trop2 and αvβ6 expression with almost complete overlap (Suppl. Fig. 2B)



Supplementary Figure 2. **Double-immunofluorescence for integrin αvβ6 and hepatic progenitor marker Trop2 in normal and diseased murine liver.** Immunofluorescence for αvβ6 integrin (green) and Trop2 (red) in healthy murine liver (A, wildtype, FVB and C57Bl/6 background) and fibrotic murine liver (B, DDC-fed, C57Bl/6 background). Representative images at x200 magnification shown. Asterisks indicate large intrahepatic bile ducts.

Early treatment with αvβ6-blocking antibody (3G9 mAB) fails to achieve significant antifibrotic effect in Mdr2-/- model of chronic biliary injury

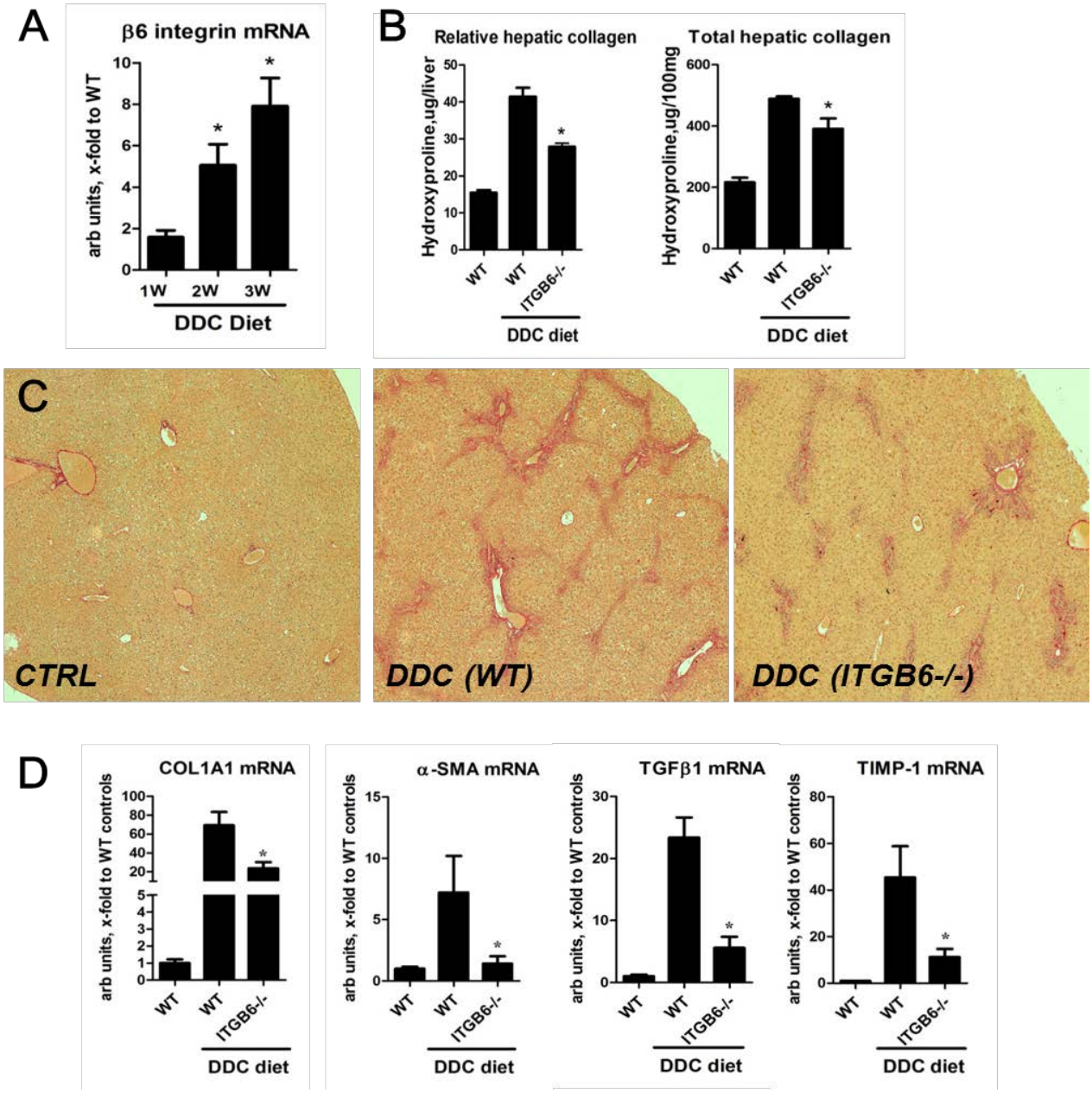
To test whether αvβ6 inhibition can prevent early-stage biliary fibrosis, we treated 4-week-old *Mdr2-/-* mice (with incipient fibrosis) with anti-αvβ6 antibody 3G9 (1 and 10mg/kg weekly i/p) for 4 weeks (early treatment protocol). At the end of treatment, 8-week-old Mdr2-/- mice that received isotype control antibody developed significant interstitial fibrosis with occasional bridging septae and significant increases in hepatic collagen levels (Suppl. Fig. 3B,C). Although mice treated with both doses of 3G9 mAB and systemic TGFβ inhibition (rsTGFβRII-Fc) showed signs of histological improvement (less ductular proliferation and bridging fibrosis, Suppl. Fig. 3B), hepatic collagen deposition showed a trend to lower values and failed to reach statistical significance (Suppl. Fig. 3). Analysis of fibrosis-related gene expression also did not reveal consistent changes due to αvβ6/TGFβ inhibition: procollagen 1 (COL1A1) mRNA did not change, while TGFβ1 decreased by 30-40% in low 3G9 dose and rsTGFβRII-Fc-treated groups, and TGFβ2 was 40% lower only in the low dose 3G9 group (Suppl. Fig. 3D). Serum ALT levels had a trend to higher values in rsTGFβRII-Fc-treated mice, but were not significantly affected by any of the treatments (Suppl. Fig. 3E).



Supplementary Figure 3. **Early treatment using function-blocking 3G9 antibody in Mdr2-/- mice.**
A. Scheme of experimental design. Integrin αvβ6-blocking antibody 3G9 mAB (1 and 10 mg/kg), 1E5 isotype control antibody (10 mg/kg) and rsTGFβRII-Fc protein (5 mg/kg) were administered four times (i.p. once a week) in *Mdr2-/-* mice with incipient biliary fibrosis starting at 4 weeks of age . **B.** Representative low-magnification images of connective tissue (Sirius Red) staining in *Mdr2-/-* mice (original magnification, x50). **C.** Relative (per 100mg of liver) and total (per whole liver) hepatic collagen levels in *Mdr2-/-* mice (biochemically via hydroxyproline). **D.** Hepatic transcript levels of COL1A1 and TGFβ1/2 as determined by real-time RT-PCR in total liver RNA (relative to β2-microglobulin, expressed as fold to healthy WT controls). **E.** Serum ALT levels in *Mdr2-/-* mice. Data expressed as mean±SEM (n=6-8), * $p \leq 0.05$ compared to isotype control group (ANOVA with Dunett's post-test)

Integrin αvβ6 deficiency confers protection from DDC-induced liver fibrosis

Wildtype (WT) C57Bl/6 and *Itgb6*-null mice (C57Bl/6 background) were fed DDC-supplemented diet for 3 weeks to induce oval cell reaction and progressive biliary fibrosis. Integrin beta 6 mRNA was up-regulated progressively up to 8-fold over baseline during 1-3 weeks in DDC model (Suppl. Fig. 4A). After 3 weeks of DDC feeding, WT mice developed sclerosing cholangitis with pronounced ductular reaction and advanced interstitial fibrosis, as expected. Fibrosis was significantly reduced in *Itgb6*-/- mice, with 50% and 30% reductions in relative and total collagen content, respectively, and no bridging fibrosis observed histologically (Suppl. Fig. 4B,C). Fibrosis-related gene expression was suppressed several fold compared to DDC-fed WT mice (Suppl. Fig. 2D). Progenitor responses in *Itgb6-/-* mice, as evaluated by progenitor cell marker A6 and Trop2 staining, were markedly attenuated (Fig. 7 in the main manuscript). Similar results were obtained when staining with pan-cytokeratin, an alternative progenitor cell marker (not shown).

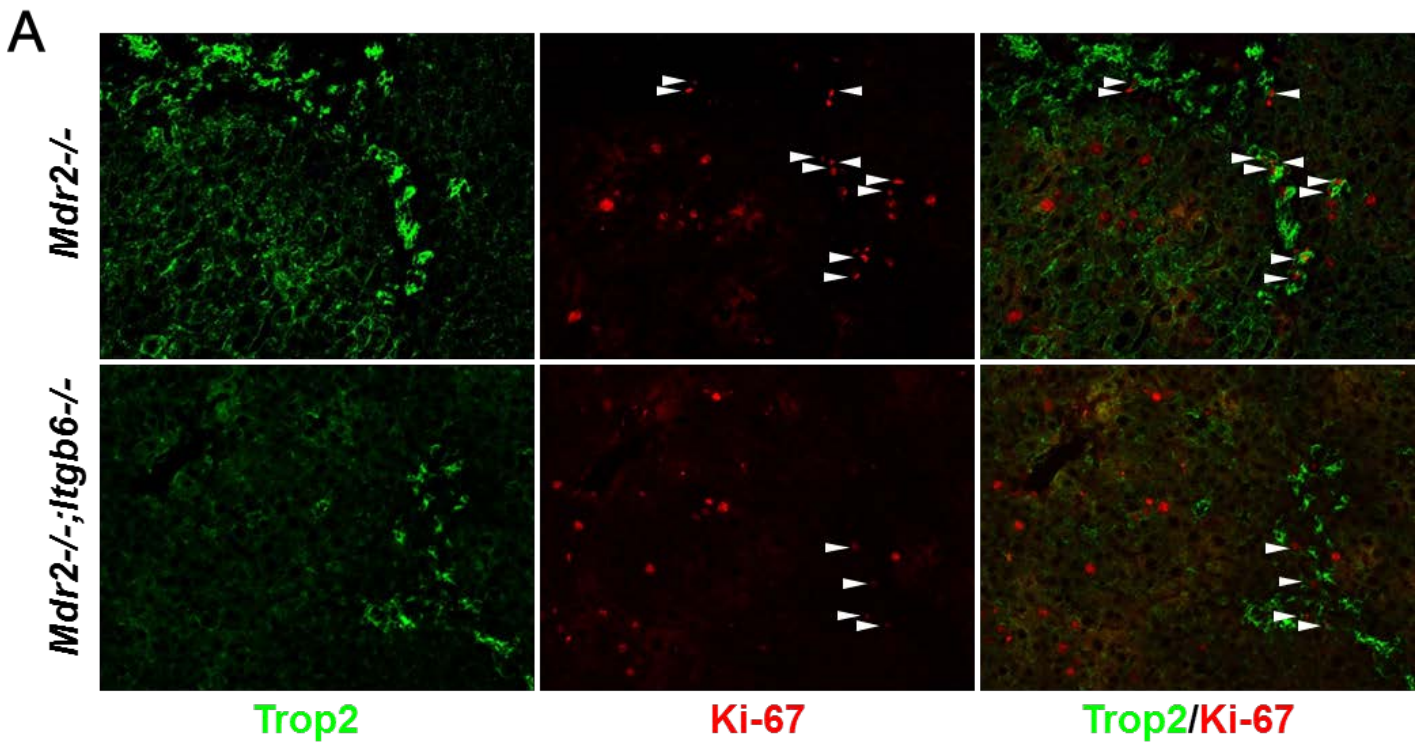


Supplementary Figure 4. **Integrin αvβ6 deficiency protects from liver fibrosis induced by DDC feeding.** **A.** Integrin β6 mRNA expression progressively increased after DDC diet from week 1 to week 3 in wildtype (WT) C57Bl/6 mice. **B.** Relative (per 100mg of liver) and total (per whole liver) hepatic collagen levels in WT and C57Bl/6.*Itgb6-/-* mice after 3 weeks on DDC diet (biochemically via hydroxyproline). **C.** Representative low-magnification images of connective tissue (Sirius Red) staining in WT and *Itgb6-/-* mice with DDC feeding (original magnification, x50). **D.** Hepatic transcript levels of COL1A1, αSMA, TGFβ1 and TIMP-1 as determined by real-time RT-PCR in total liver RNA (relative to β2-microglobulin, expressed as fold to healthy WT controls). Data expressed as mean±SEM (n=6-8), * p≤0.05 compared to WT on DDC diet (t-test).

Hepatic progenitor cell activation and fibrogenesis is markedly attenuated in the absence of integrin avβ6 in Mdr2-/- model of chronic biliary injury

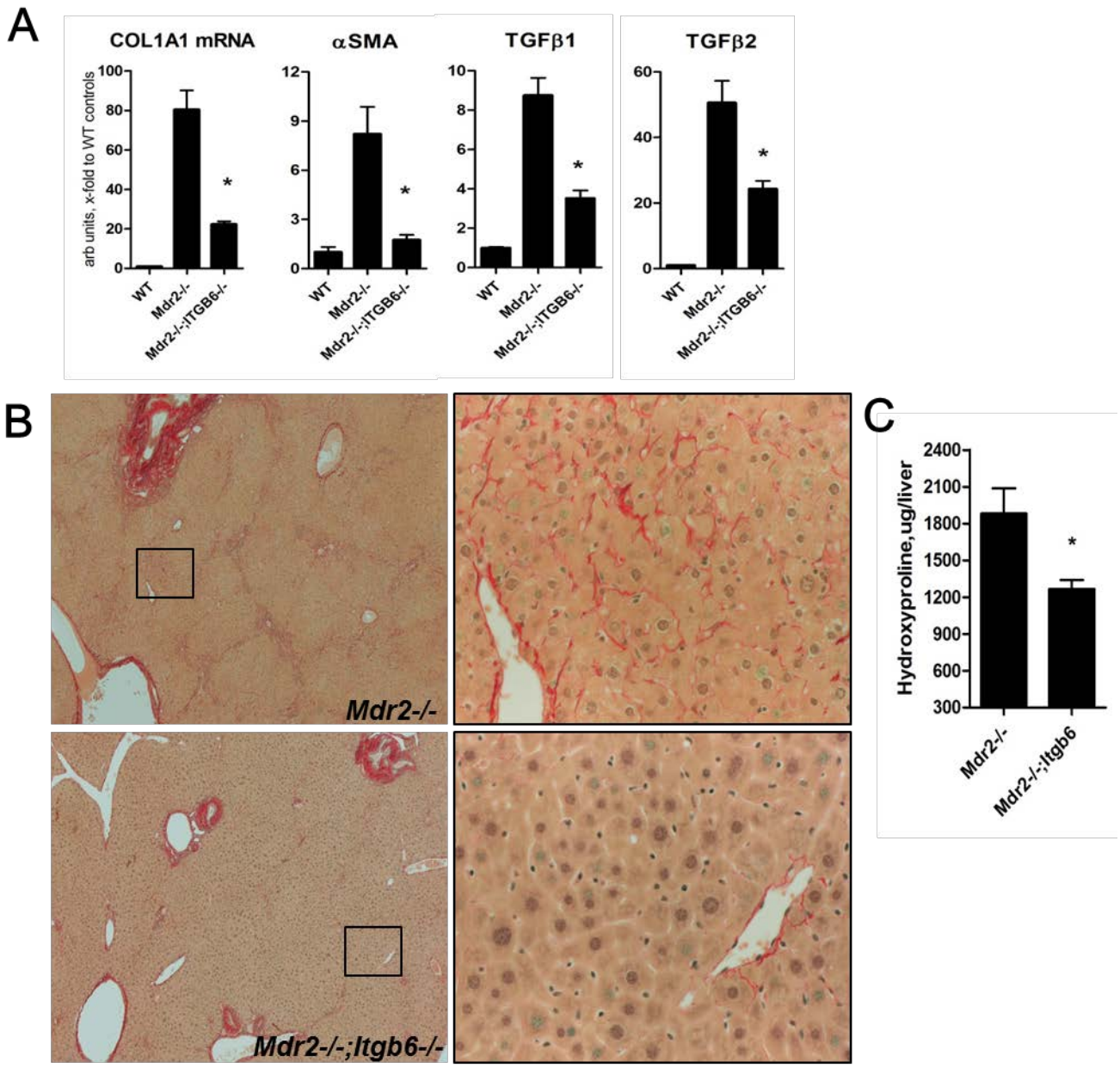
To assess the role of integrin αvβ6 in the progenitor response, hepatic fibrosis, and hepatocellular carcinoma, we generated *Mdr2-/-;Itgb6-/-* double knockouts (as described in M&M) and analyzed them in comparison to integrin αvβ6-sufficient *Mdr2-/-* mice. Analysis of F2 progeny (8 weeks of age) revealed higher hepatic collagen levels in *Mdr2-/-;Itgb6-/-* mice compared to *Mdr2-/-* littermates (not shown). This was unexpected in the context of significant protection from fibrosis in the DDC model in adult *Itgb6-/-* mice (Suppl. Fig. 4), and the antifibrotic effect of anti-αvβ6 antibody treatment at advanced disease stages (Fig. 5 of the main manuscript). Because of the well-documented expression of αvβ6 in developing, but not healthy adult liver, we hypothesized that the absence of αvβ6 during liver development along with congenital liver disease in Mdr2-/- may produce artifacts related to early developmental events rather than fibrogenesis.

To test this, we used a low-dose cholic acid challenge (which aggravates liver injury and fibrosis in Mdr2-/- mice (6)) in adult (5week-old) male *Mdr2-/-;Itgb6-/-* and *Mdr2-/-* mice for 4 weeks, and analyzed the progenitor (oval) cell compartment, liver injury and fibrogenesis. Cholic-acid feeding led to a 3-fold increase in serum ALT levels, which did not differ between *Mdr2-/-;Itgb6-/-* and *Mdr2-/-* mice (not shown). Histological examination showed that *Mdr2-/-* mice developed severe ductular reaction with expansion of A6+ progenitors around portal tracts and along fibrotic septae. However, ductular reaction and progenitor expansion were notably reduced in *Mdr2-/-;Itgb6-/-* mice, with A6+ cell counts reduced by 65% compared to *Mdr2-/-* littermates (See Fig. 6C,D of main manuscript). Double-immunofluorescence for progenitor marker Trop2 and Ki-67 (nuclear cell proliferation antigen) revealed that replication of progenitors appears to be reduced in the absence of integrin αvβ6 (Suppl. Fig. 5). The fibrogenic response, as measured by fibrosis-related hepatic mRNA levels, was indeed dramatically attenuated in *Mdr2-/-;Itgb6-/-* mice (Suppl. Fig. 6A). Accordingly, fibrosis induced by cholic acid challenge (5-9 weeks of age) was significantly inhibited in *Mdr2-/-;Itgb6-/-*, which deposited less than half of hepatic collagen compared to their Mdr2-/- littermates (average increase in hydroxyproline 301.8ug/liver versus 627ug/liver in *Mdr2-/-*, not shown).



Supplementary Figure 5. **Integrin αvβ6 deficiency attenuates progenitor cell proliferation in Mdr2-/- mice.** **A.** Double-immunofluorescence for cell proliferation marker Ki-67 (red, arrows) and progenitor marker Trop2 (green) suggest diminished progenitor cell replication (double-positive cells indicated by arrows) in *Mdr2-/-;Itgb6-/-* mice (lower panel) compared to *Mdr2-/-* (upper panel). Note similar numbers of proliferating hepatocytes (large Ki-67(+) nuclei within hepatic lobule) between mice of both genotypes. Representative images shown (original magnification, x200).

We kept a parallel groups of *Mdr2-/-* and *Mdr2-/-;Itgb6-/-* mice on regular chow for an extended period of time (1 year) for primary liver tumor assessment (see main manuscript), which were also analyzed for fibrotic/cirrhotic outcomes. Liver fibrosis in *Mdr2-/-;Itgb6-/-* developed less progressively throughout their life-time compared to *Mdr2-/-*, as evidenced by lack of characteristic cirrhotic changes(5) in aged FVB.*Mdr2-/-* mice such as significant sinusoidal fibrosis (Suppl. Fig. 6B), as well as 33% decrease in hepatic collagen content at 12 months of age (Suppl. Fig. 6C).



Supplementary Figure 6. Fibrogenesis is markedly attenuated in the absence of integrin avβ6 in double-mutant Mdr2-/-;Itgb6-/- mice.

A. Profibrogenic genes COL1A1, TGFβ1/2 and αSMA expression is strongly diminished in cholic acid-fed *Mdr2-/-;Itgb6-/-* mice. Hepatic transcript levels of target genes were determined by real-time RT-PCR in total liver RNA (relative to β2-microglobulin, expressed as fold to healthy WT controls). Data are mean±SEM (n=4-5 per bar), **B.** Connective tissue staining in aged mice kept on regular chow (12 months old). Note severe sinusoidal fibrosis in *Mdr2-/-* that is absent in *Mdr2-/-;Itgb6-/-*mice. Representative Sirius Red images (magnification: left panel, 50x; right panel, 400x). **C**. Hepatic collagen content in 12 months-old *Mdr2-/-* and *Mdr2-/-;Itgb6-/-* mice on a regular chow. Data are mean±SEM (n=12 per bar)*, p≤0.05 compared to *Itgb6*-sufficient *Mdr2-/-* mice (t-test).

Supplementary Table 1. **Primers and probes used in quantitative RT-PCR.**

Target gene	5´-Primer	TaqMan probe	3´-Primer
β_2MG	CTGATACATACGCCTGCAGAGTTAA	GACCGTCTACTGGGATCGAGACATGTG	ATGAATCTTCAGAGCATCATGAT
COL1A1	TCCGGCTCCTGCTCCTCTTA	TTCTTGGCCATGCGTCAGGAGGG	GTATGCAGCTGACTTCAGGGATGT
TGFβ1	AGAGGTCACCCGCGTGCTAA	ACCGCAACAACGCCATCTATGAGAAAACCA	TCCCGAATGTCTGACGTATTGA
α-SMA	ACAGCCCTCGCACCCA	CAAGATCATTGCCCCTCCAGAACGC	GCCACCGATCCAGACAGAGT
TGFβ2	GTCCAGCCGGCGGAA	CGCTTTGGATGCTGCCTACTGCTTTAGAAAT	GCGAAGGCAGCAATTATCCT
Itgb6	GCAGAACGCTCTAAGGCCAA	TGGCAAACGGGAACCAATCCTCTGT	AAAGTGCTGGTGGAACCTCG
Fn14	TGCCGCCGGAGAGAAAA	CTCTCCACCAGTCTCCTCTATGGGGGTAGTAAA	GCCACACCTGGGCAGC
Albumin	GCTACGGCACAGTGCTTG	-	CAGGATTGCAGACAGATAGTC
TAT	ACGCACAGACGCACGCACTCAC	-	CTTTCCGGTCCGCAGCAATGT
K19	GCTGCAGATGACTTCAGAACC	-	CATGGTTCTTCTTCAGGTAGGC
EpCAM	TTGCAGACTGCGCTTCAAGA	-	TACGATCGGTAATGCCGGTG
CD133	ACCGGAGAGGGATGGTACTT	-	GGCAATCTTCAGAGCCAAGAC
HNF1β	TaqMan assay ID: Mm00447459_m1 (Life Technologies)		
K19	TaqMan assay ID: Mm00492980_m1 (Life Technologies)		
HNF4α	TaqMan assay ID: Mm01247712_m1 (Life Technologies)		
Albumin	TaqMan assay ID: Mm00802090_m1 (Life Technologies)		
Trop2	TaqMan assay ID: Mm00498401_s1 (Life Technologies)		
EpCAM	TaqMan assay ID: Mm000493214_m1 (Life Technologies)		
SOX9	TaqMan assay ID: Mm00448840_m1 (Life Technologies)		
α-Fetoprotein	TaqMan assay ID: Mm00431715_m1 (Life Technologies)		
Foxl1	TaqMan assay ID: Mm00514937_s1 (Life Technologies)		
Lgr5	TaqMan assay ID: Mm00438890_m1 (Life Technologies)		

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