TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Pten Haplodeficiency Accelerates Liver Tumor Growth in miR-122a—Null Mice via Expansion of Periportal Hepatocyte-Like Cells

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Accepted for publication
July 26, 2018.

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This study aimed to shed light on the molecular and cellular mechanisms responsible for initiation and progression of liver malignancies by examining the role of phosphatase and tensin homolog on chromosome 10 (*Pten*) in liver tumor progression in miR-122a (*Mir122a*)—null mice. We generated and monitored liver tumor progression in *Mir122a*-null *Pten* heterozygous (*Mir122a*^{−/−};*Pten*^{+/-} and *Mir122a*^{−/−};*Alb-Cre*;*Pten*^{fx/+}) mice and compared the results with those in *Mir122a*^{−/−} mice. Both *Mir122a*^{−/−};*Pten*^{+/-} and *Mir122a*^{−/−};*Alb-Cre*;*Pten*^{fx/+} mice developed visible liver tumor nodules at 6 months of age. In premalignant livers of *Mir122a*^{−/−};*Pten*^{+/-} mice, decreased PTEN and increased phosphorylated AKT were specifically observed in periportal cells, associated with inflammatory and fibrotic microenvironments. Furthermore, IL-1β and tumor necrosis factor-α levels significantly increased in *Mir122a*^{−/−};*Pten*^{+/-} premalignant livers at 6 months of age. Oval cells expressing A6, epithelial cell adhesion molecule, keratin (K) 8, K19, and SRY (sex determining region Y)-box 9 (SOX9) were present in both *Mir122a*^{−/−} and *Mir122a*^{−/−};*Pten*^{+/-} livers. Interestingly, a hybrid hepatocyte-like population with intermediate levels of K8, HNF4α, and SOX9 was located proximally to the oval cells in *Mir122a*^{−/−};*Pten*^{+/-} livers. Lineage-tracing experiments revealed that these intermediate levels of K8 hepatocyte-like cells may be the cells of origin for *Mir122a*^{−/−};*Pten*^{+/-} liver tumors. These findings suggest that inflammatory microenvironments in the periportal area of *Mir122a*-null mice may locally cause *Pten* down-regulation and expand tumor-initiating cells, causing hepatocellular carcinoma. (*Am J Pathol* 2018, 188: 2688–2702; <https://doi.org/10.1016/j.ajpath.2018.07.019>)

Liver malignancies can be driven by a variety of factors, including extrinsic (eg, viral infection, diet, and toxic chemicals) and intrinsic (genetic and epigenetic changes) insults.^{1,2} Most liver malignancies are hepatocellular carcinoma (HCC) and cholangiocarcinomas that result in high morbidity and mortality rates in underdeveloped and developing countries and have elevated incidences in developed countries.^{3,4} Although multiple factors involved in hepatic tumorigenesis have been intensively studied, the molecular and cellular mechanisms responsible for initiation and progression of liver malignancies remain elusive to date.

miR-122 (official name *MIR122*) is the major miRNA of the liver.⁵ Expression of miR-122a in the mouse liver begins around mouse embryonic day 12.5 and reaches

maximal levels at birth and thereafter.^{6,7} Functionally, miR-122 regulates cholesterol and fatty acid metabolism.^{8,9} Expression of miR-122 is reduced in cases of HCC^{10–13} and is linked to poor prognosis and metastasis potential among patients with HCC.^{12,14,15} Recently, miR-122a—null mice have been shown to exhibit

Supported by the National Health Research Institutes grants NHRI-EX106-10515BI and NHRI-EX107-10515BI (C.-M.C.); the Ministry of Education Aim for the Top University Plan and the Featured Areas Research Center Program within the framework of the Higher Education Sprout Project; and the Ministry of Science and Technology grants MOST 104-2320-B-010-025, MOST 105-2320-B-010-003, and MOST 105-2320-B-010-028-MY3 (C.-M.C.).

Disclosures: None declared.

early-onset (at 2 months of age) liver inflammation, steatosis, and fibrosis and to subsequently develop HCC over 1 year.^{16,17}

The phosphatase and tensin homolog on chromosome 10 (*PTEN*) is a dual functional phosphatase and is a tumor-suppressor gene involved in a variety of malignancies.^{18–21} Decreased levels of *PTEN* in clinical HCC specimens have been reported.^{22–26} However, *PTEN* mutation appears to be a rare event in HCC.^{27–30} Hepatocyte-specific *Pten* ablation in mice results in the development of steatohepatitis, metabolic disorders, hepatomegaly at 6 months, hepatic adenomas at 10 to 11 months, and HCC at 18 to 19 months.^{31,32} Also, CD133⁺CD45[–] cells with stem/progenitor properties are increased at the premalignant stage and act as disease-initiating cells that cause liver cancer in the hepatocyte-specific *Pten*-ablated mice.³³ Thus, *PTEN* down-regulation may play a crucial role to drive or expand the disease-initiating cell populations to promote liver malignancies. Interestingly, decreased *PTEN* levels are observed in the *Mir122a*-null hepatic tumors that developed at 14 months of age.¹⁶ However, it remains unclear how *PTEN* down-regulation drives *Mir122a*-deficient hepatic tumorigenesis. Furthermore, the cellular origin for precancer or cancer lesions of *Mir122a*-null mice is largely undefined.

The cells of origin for liver malignancies are currently debatable. Previously, the ductular reactive cells in various human liver diseases and their equivalent oval cells in rodent liver injury models were postulated to have a progenitor property and were implicated as the cells of origin for HCC.^{34–37} However, the activated oval cells traced by the lineage-tracing approaches, which are conducted in diverse genetically and chemically induced HCC mouse models, do not cause HCC.^{38–40} A newly identified periportal hepatocyte subtype expressing a low level of *SRY* (sex determining region Y)-box 9 (*SOX9*), called hybrid hepatocytes, has greater regenerative potential than the oval cells. However, both hybrid hepatocytes and oval cells cannot cause diethylnitrosamine (*DEN*)-induced and chronic steatohepatitis-induced hepatic nodules and HCC.³⁹ Interestingly, in a mouse HCC model induced by hepatocyte-specific expression of human unconventional prefoldin RNA polymerase II subunit 5 interactor,⁴¹ the HCC can be generated from hepatocytes and expanded *SOX9*-expressing hepatic progenitor cells.⁴² Therefore, these lineage-tracing experiments in newly examined HCC mouse models suggest that HCC is predominantly developed from hepatocytes, but also possibly developed from oval progenitor cells in a context-dependent manner.

In this study, we monitored *Pten* haploinsufficiency in *Mir122a*-null liver tumor progression. We identified down-regulation of *Pten* in the periportal parenchyma and found aberrantly expanded keratin 8 (*K8*)/*SOX9*/hepatocyte nuclear factor 4 α (*HNF4 α*) hepatocyte-like cells that may be the cells of origin causing liver cancer.

Materials and Methods

Mice

The *Mir122a*^{–/–} mice on a C57BL/6J;129 mixed background were described previously.¹⁶ To generate *Pten*^{+/-} mice, *Pten*^{fx/fx} mice⁴³ from The Jackson Laboratory (Bar Harbor, ME) were bred with *Prm-Cre* transgenic mice.⁴⁴ The *Pten*^{+/-} mice carrying the exon 5–deleted allele of *Pten* were obtained from the progeny of *Prm-Cre*;*Pten*^{fx/+} mice. The *Pten*^{+/-} mice were further crossed with C57BL/6J wild-type mice for 10 generations and then maintained on a C57BL/6J congenic background. *Mir122a*^{–/–};*Pten*^{+/-} mice were obtained by intercrossing *Mir122a*^{–/–} and *Mir122a*^{+/-};*Pten*^{+/-} mice. Albumin (*Alb*)–*Cre* mice⁴⁵ were obtained from The Jackson Laboratory and used to tissue specifically delete monoallelic *Pten* in *Mir122a*^{–/–};*Alb-Cre*;*Pten*^{fx/+} mice. The *K18-EGFP*;*K8-CreER*^{T2} transgenic mice^{46,47} and Cre reporter *R26R*^{lacZ/+} mice⁴⁸ were used for lineage-tracing experiments. All animals were housed in the specific pathogen-free areas on a 12:12-hour dark/light cycle in the Laboratory Animal Center of National Yang-Ming University. All animal studies were performed with the approval of the Institutional Animal Care and Use Committee at National Yang-Ming University.

Genotyping of Mice

Genomic DNA from 10-day-old mouse tails was isolated and subjected to PCR genotyping. *Mir122a* alleles were detected by the primers WT-1 (forward, 5'-GTGTTGGCCAGCTTATTC-CAAAGG-3'), Rev-2 (reverse, 5'-CTCACTGTAAAGGTCA-CAGGTGC-3'), and KO-1 (forward, 5'-CAACAATCCT-GTCAGCACCATAG-3'), yielding a 429-bp wild-type allele and an 825-bp null allele. The *Pten* alleles were detected by the primers oIMR1952 (forward, 5'-ACTCAAGGCAGGGAT-GAGC-3'), exon 5B (reverse, 5'-AATCTAGGGCCTCTT-GTGCC-3'), and pBS1 (reverse, 5'-GCTTGATATCGAATT-CCTGCAGC-3'), yielding a 1000-bp wild-type allele, a 1100-bp *Pten*^{fx} allele, and a 300-bp null allele. The primers for PCR genotyping of the Cre gene in *K18-EGFP*;*K8/K18-CreER*^T and *Alb-Cre* mice were Cre-1 (forward, 5'-GGACATGTTCA-GGGATCGCCAGGCG-3') and Cre- β (reverse, 5'-CGAC-GATGAAGCATGTTTGTAGCTG-3'), yielding a 230-bp product. The *R26R*^{lacZ/+} allele was detected by the primers Rosa-1 (reverse, 5'-GCGAAGAGTTTGTCTCTCAACC-3'), Rosa-2 (reverse, 5'-GGAGCGGGAGAAGATATG-3'), and Rosa-3 (forward, 5'-AAAGTCGCTCTGAGTTGTTAG-3'), yielding a 300-bp *R26R*^{lacZ/+} allele and a 550-bp wild-type allele.

Tamoxifen Administration

Tamoxifen (10 mg/mL; Sigma-Aldrich, St. Louis, MO) was dissolved in a 1:10 mixture of ethanol and sunflower seed oil (Sigma-Aldrich). Tamoxifen (50 mg/kg) was administered intraperitoneally for 1 (one dose) or 3 (triple dose) consecutive days to 6-week-old mice.

Histology, Sirius Red Staining, IHC, and IF Staining

Livers were fixed in 4% paraformaldehyde overnight and then embedded in paraffin or OCT compound (Tissue-Tek OCT; Sakura Finetek, Torrance, CA) for paraffin or frozen sectioning, respectively. The hematoxylin and eosin staining procedure was described previously.⁴⁷ Basophilic cells in periportal areas of hematoxylin and eosin-stained liver sections were manually counted under high-power views. Sirius red staining was performed by the Taiwan Mouse Clinic, National Phenotyping and Drug Testing Center (Academia Sinica, Taipei, Taiwan). The Sirius red positively stained areas were quantified by ImageJ software version 1.48 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). For the immunohistochemical (IHC) analysis, antibodies against HNF4 α (sc-8987; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), K19 (TROMA-III; 1:400; Developmental Studies Hybridoma Bank, Iowa City, IA), F4/80 (ab6640; 1:400; Abcam, Cambridge, MA), CD3 (ab5690; 1:100; Abcam), B220 (number 166501; 1:100; SouthernBiotech, Birmingham, AL), IL- β (ab9722; 1:100; Abcam), tumor necrosis factor (TNF)- α (ab6671; 1:100; Abcam), PTEN (number 9595; 1:100; Cell Signaling Technology, Danvers, MA), p-AKT (number 4058; 1:100; Cell Signaling Technology), Ki-67 (number 12202; 1:400; Cell Signaling Technology), epithelial cell adhesion molecule (EpCAM; G8.8; 1:400; Developmental Studies Hybridoma Bank), A6 (A6 BCM; 1:100; Developmental Studies Hybridoma Bank), K8/K18 (TROMA-1; 1:400; Developmental Studies Hybridoma Bank), and SOX9 (sc-20095; 1:400; Santa Cruz Biotechnology) were used. Positively stained cells in periportal areas detected by IHC were manually counted under high-power views. For immunofluorescence (IF) staining, liver sections were blocked by 10 mg/mL Sudan Black (Sigma-Aldrich) in 70% ethanol at room temperature overnight to reduce autofluorescence and then incubated with antibodies against β -galactosidase (55979; 1:1000; MP Biomedicals, Santa Ana, CA), EpCAM (Ab71916; 1:100; Abcam), or various antibodies described above. Quantification of co-IF data was manually counted under high-power views. Five tissue sections of each mouse (three to five mice from each genotype) were quantified.

RNA Isolation and Real-Time Quantitative RT-PCR

Total RNA content was extracted from frozen livers by TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) and reverse transcribed to synthesize cDNA using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). Real-time PCR was performed using the Kapa SYBR Fast qPCR kit (Kapa Biosystems, Wilmington, MA). The primer sequences used for real-time quantitative RT-PCR were *Pten* (forward, 5'-AGGCACAAGAGGCCCTAGAT-3'; reverse, 5'-CTGACTGGGAATTGTGACTCC-3'), *Epcam* (forward, 5'-CAGCTGGACACCGGCATT-3'; reverse, 5'-TGGACCTGCACCTATAAGACGTT-3'), *Il1b*

(forward, 5'-AGTTGACGGACCCCAAAAG-3'; reverse, 5'-AGCTGGATGCTCTCATCAGG-3'), *Tnf* (forward, 5'-CAGCCTCTTCTCATTCTGCTTGTTG-3'; reverse, 5'-CTGGAAGACTCCTCCCAGGTATAT-3'), *Il6* (forward, 5'-TAGTCCTTCTACCCCAATTTCC-3'; reverse, 5'-TTGGTCCTTAGCCACTCTTC-3'), and *Actb* (actin, β ; forward, 5'-CTAAGGCCAACCGTGAAAAG-3'; reverse, 5'-ACCAGAGGCATACAGGGACA-3').

Western Blot Analysis

Total protein extracted from frozen livers with lysis buffer [50 mmol/L Tris-HCl (pH 7.9), 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and phosphatase inhibitor cocktail (Sigma-Aldrich)] was subjected to Western blot analysis. Primary antibodies against PTEN (number 9595; 1:1000; Cell Signaling Technology), p-AKT^{S473} (number 4058; 1:1000; Cell Signaling Technology), β -actin (A5441; 1:5000; Sigma-Aldrich), pan-AKT (number 4691; 1:1000; Cell Signaling Technology), IL-1 β (ab9722; 1:1000; Abcam), and TNF- α (ab6671; 1:1000; Abcam) were used.

Fluorescence-Activated Cell Sorting

Livers were dissected and further digested with 0.012% type III collagenase (Worthington Biochemical Corp., Lakewood, CO) and DNaseI (1:1000; Sigma-Aldrich) at 37°C for 30 minutes. Enzymatic digestion was then blocked with collagenase blocking buffer (1% bovine serum albumin and 5 mmol/L EDTA/phosphate-buffered saline), and cells were washed, suspended in 1 $\times$ phosphate-buffered saline, and passed through cell strainers with 70- and 40- μ m pore sizes (BD Falcon, San Jose, CA). Dissociated single cells were incubated with PEcy7-conjugated anti-EpCAM (118215; 1:300; Biolegend, San Diego, CA), phosphatidylethanolamine-conjugated anti-CD133 (141203; 1:200; Biolegend), and APC-conjugated anti-CD45 (103112; 1:400; Biolegend) antibodies at 4°C for 30 minutes. Stained cells were analyzed and sorted with a BD FACSaria sorter (BD Biosciences, San Jose, CA).

Sphere-Forming Assay

A total of 10,000 isolated CD133⁺EpCAM⁺CD45⁻ cells were mixed with 50 μ L of Matrigel and plated in 12-well plates. Culture medium was added after polymerization of the Matrigel. The culture medium was composed of Dulbecco's modified Eagle's medium/F12 medium (Thermo Fisher Scientific) supplemented with 1 $\times$ B27 (Thermo Fisher Scientific), 1 $\times$ N2 (Thermo Fisher Scientific), 20 ng/mL epidermal growth factor (ScienCell, Carlsbad, CA), 50 ng/mL basic fibroblast growth factor (Peprotech, Rocky Hill, NJ), and 50 ng/mL human growth factor (Peprotech). Images of spheres under an inverted microscope (imt-2; Olympus, Tokyo, Japan) were taken on day 10 after plating.

X-Gal Staining

Whole-mount X-Gal staining of livers was conducted as previously described.⁴⁹ Post-fixed X-Gal-stained sections were counterstained with nuclear fast red (Muto Pure Chemicals, Tokyo, Japan), as described previously.⁵⁰

Statistical Analysis

Statistical analysis was performed using paired *t*-tests. Data are shown as means $\pm$ SD. Statistical significance was set at $P < 0.05$.

Results

Pten Haplodeficiency Accelerates *Mir122a*-Null Liver Tumorigenesis

To address whether *Pten* collaborates genetically with *Mir122a* in liver tumorigenesis, *Mir122a*^{-/-};*Pten*^{+/-} mice were generated to monitor their hepatic tumor acceleration in comparison with liver tumor development in *Mir122a*^{-/-} mice. At 6 to 9 months, the *Mir122a*^{+/-};*Pten*^{+/-} (nine males and eight females at 6 months of age; two males and two females at 9 months of age) and *Mir122a*^{-/-} (eight males and

Figure 1 Monoallelic *Pten* deletion accelerates *Mir122a*-deficient tumorigenesis. **A:** Liver gross views of *Mir122a*^{+/-};*Pten*^{+/-}, *Mir122a*^{-/-}, *Mir122a*^{-/-};*Pten*^{+/-}, and *Mir122a*^{-/-};*Alb-Cre*;*Pten*^{fx/+} mice at 6 and 9 months of age. **Yellow arrows** indicate macroscopic tumors observed in the *Mir122a*^{-/-};*Pten*^{+/-} livers and *Mir122a*^{-/-};*Alb-Cre*;*Pten*^{fx/+} livers. **B:** Immunohistochemistry reveals expression of the hepatocyte marker HNF4α and cholangiocyte marker keratin 19 (K19) in hepatic adenomas developed in *Mir122a*^{-/-};*Pten*^{+/-} and *Mir122a*^{-/-};*Alb-Cre*;*Pten*^{fx/+}, respectively, in 6-month-old mice. The **dashed curved lines** depict tumor boundaries. **Boxed areas** in images in the **first** and **third** columns are shown at higher magnification in the images in the **second** and **fourth** columns; **boxed areas** in images in the **top row** of the **second** and **fourth** columns are shown at higher magnification in the **insets**. Scale bars: 1 cm (**A**); 1 mm (**B**, **first** and **third** columns); 100 μm (**B**, **second** and **fourth** columns).

11 females at 6 months of age and two males and two females at 9 months of age) mice showed no sign of hepatic tumors. Interestingly, approximately 47% of 6-month-old (eight of 18 males and six of 12 females) and 80% of 9-month-old (two of three males and two of two females) *Mir122a*^{-/-}; *Pten*^{+/-} mice developed multiple liver tumors (Figure 1A; Table 1). In addition, *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} mice were generated, in which the monoallelic *Pten* was specifically deleted in the hepatocytes in the *Mir122a*-null background. Hepatic nodules and manifest liver tumors were consistently observed in all *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} mice at 6 and 9 months of age (two males and one female at each time point) (Figure 1A; Table 1). In these tumor-bearing mice, metastasis was not observed. IHC data revealed that most tumor cells expressed the hepatocyte marker HNF4 α in *Mir122a*^{-/-}; *Pten*^{+/-} and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} tumors (Figure 1B). A small proportion of tumor cells expressed K19, which formed ductal or acinar arrangements embedded within *Mir122a*^{-/-}; *Pten*^{+/-} and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} tumors (Figure 1B). Overall, our results demonstrate that monoallelic *Pten* loss facilitates hepatocellular adenoma and carcinoma development in *Mir122a*-null mice at 6 and 9 months of age.

Inflammation and Fibrosis in *Mir122a*^{-/-}, *Mir122a*^{-/-}; *Pten*^{+/-}, and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} Premalignant Livers

Hematoxylin and eosin staining revealed expanding basophilic cells residing in the periportal areas of *Mir122a*^{-/-} and *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers at 6 months of age (Figure 2A). More prominent basophilic lesions, including larger oval nuclear cells and small round immune cells, were identified in the premalignant livers of *Mir122a*^{-/-}; *Pten*^{+/-} mice than in the *Mir122a*^{-/-} mice at 6 months of age (Figure 2A). Quantitative results showed that the larger basophilic cells were significantly increased in the periportal areas of *Mir122a*^{-/-}; *Pten*^{+/-} livers and, to a lesser extent, in *Mir122a*^{-/-} livers, compared with those of *Mir122a*^{+/-}; *Pten*^{+/-} livers (Figure 2A). Macrophages, T cells, and B cells residing at the basophilic lesions were identified by the macrophage marker F4/80, the T-cell marker CD3, and the pan B-cell marker B220, respectively (Figure 2B). Many F4/80-stained macrophages surrounded the ductular reactive cells in *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers and were also scattered at the parenchyma of *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers (Figure 2B). More CD3- and B220-positive cells were detected in the periportal lesions of *Mir122a*^{-/-}; *Pten*^{+/-} than in those of *Mir122a*^{+/-}; *Pten*^{+/-} and *Mir122a*^{-/-} livers (Figure 2B), indicating higher levels of reactive immune cells in premalignant lesions in *Mir122a*^{-/-}; *Pten*^{+/-} livers. Consistently, infiltrating macrophages and lymphocytes were also increased in premalignant livers of *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} mice compared with those in their littermate controls (Supplemental Figure S1). mRNA

Table 1 Liver Tumor Incidence of *Mir122a*^{-/-}; *Pten*^{+/-} and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} Mice

Genotype	Sex	Tumor incidence by age, months	
		6	9
<i>Mir122</i> ^{+/-} ; <i>Pten</i> ^{+/-}	M	0/9 (0)	0/2 (0)
	F	0/8 (0)	0/2 (0)
<i>Mir122a</i> ^{-/-}	M	0/8 (0)	0/2 (0)
	F	0/11 (0)	0/2 (0)
<i>Mir122a</i> ^{-/-} ; <i>Pten</i> ^{+/-}	M	8*/18 (44)	2 [†] /3 (67)
	F	6*/12 (50)	2 [†] /2 (100)
<i>Mir122</i> ^{+/-} ; <i>Alb-Cre</i> ; <i>Pten</i> ^{fx/+}	M	0/3 (0)	0/2 (0)
	F	0/2 (0)	0/2 (0)
<i>Mir122a</i> ^{-/-} ; <i>Alb-Cre</i>	M	0/3 (0)	0/3 (0)
	F	0/2 (0)	0/2 (0)
<i>Mir122a</i> ^{-/-} ; <i>Alb-Cre</i> ; <i>Pten</i> ^{fx/+}	M	2*/2 (100)	2 [†] /2 (100)
	F	1*/1 (100)	1 [†] /1 (100)

Data are given as number/total (percentage).

*Hepatic adenoma-bearing mice.

[†]HCC-bearing mice.

F, female; M, male.

levels of proinflammatory cytokines were examined, and it was found that *Il1b* and *Tnf*, but not *Il6*, were significantly increased in both *Mir122a*^{-/-}; *Pten*^{+/-} and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} livers compared with those in other groups (*Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, *Mir122a*^{+/-}; *Alb-Cre*; *Pten*^{fx/+}, and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+}) (Figure 2, C and D). Using IHC to assess IL-1 β and TNF- α positivity *in situ*, both IL-1 β - and TNF- α -positive cells could be detected in *Mir122a*^{-/-} livers and were highly increased in *Mir122a*^{-/-}; *Pten*^{+/-} periportal areas (Supplemental Figure S2, A–D). IL-1 β was found to be slightly increased in *Mir122a*^{-/-} livers and further up-regulated in *Mir122a*^{-/-}; *Pten*^{+/-} whole-liver lysates by Western blot analysis (Supplemental Figure S2, E and F). However, using Western blot analysis, the protein levels of TNF- α were unable to be detected, which might be attributable to a low percentage of TNF- α -positive cells in whole-liver lysates (Supplemental Figure S2E). In parallel, the inflammatory phenotypes, including the percentages of IL-1 β - and TNF- α -positive cells, as well as the protein levels in *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} livers, were also significantly increased compared with those of *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} and *Mir122a*^{+/-}; *Alb-Cre*; *Pten*^{fx/+} livers (Supplemental Figure S3). In addition to the inflammatory phenotypes, prominent Sirius red positive-stained areas were observed and quantified in *Mir122a*^{-/-}; *Pten*^{+/-} livers compared with the *Mir122a*^{+/-}; *Pten*^{+/-} and *Mir122a*^{-/-} livers (Figure 2, E and F), indicating more fibrotic lesions. Similarly, Sirius red-positive areas were also greater in the *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} livers than their controls (Supplemental Figure S4). These data indicate that inflammation and fibrosis occurred earlier in *Mir122a*^{-/-}; *Pten*^{+/-} and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fx/+} livers and promoted liver tumor progression.

PTEN Down-Regulation, AKT Phosphorylation, and Cellular Proliferation in *Mir122a*^{-/-}; *Pten*^{+/-} Premalignant Livers and Tumors

It is likely that expression of monoallelic *Pten* may be down-regulated in the *Mir122a*^{-/-}; *Pten*^{+/-} hepatocytes, resulting in the subsequent loss of tumor-suppressive function that could lead to HCC initiation. Thus, PTEN and p-AKT^{S473} levels were examined in the liver samples of controls, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} mice, and liver tumors of *Mir122a*^{-/-}; *Pten*^{+/-} mice at 6 months of age. Using Western blot analysis, PTEN protein levels were found to be comparable in *Pten*^{+/-} and *Mir122a*^{-/-}; *Pten*^{+/-} liver samples (Figure 3, A and B). Down-regulated PTEN and increased p-AKT^{S473} were only specifically detected in *Mir122a*^{-/-}; *Pten*^{+/-} liver tumors (Figure 3, A and B). We hypothesized that down-regulated PTEN and a lack of detectable level of p-AKT^{S473} in the *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers may occur locally in a subpopulation, so the changes of PTEN and p-AKT^{S473} cannot be detected in the whole-liver lysates used in Western blot analysis. Therefore, PTEN and p-AKT^{S473} were examined using IHC, and it was found that the cells with PTEN down-regulation and AKT phosphorylation were specifically detected in the periportal reactive areas (Figure 3C). Cellular proliferation was further examined in *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers. The Ki-67-stained sections exhibited a highest level of proliferative cells in periportal areas of *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers than that in *Mir122a*^{-/-} and *Mir122a*^{+/-}; *Pten*^{+/-} livers (Figure 3, D and E). Because the infiltrating immune cells were identified in periportal areas and associated with higher levels of IL-1 β and TNF- α in *Mir122a*^{-/-}; *Pten*^{+/-} livers (Supplemental Figure S2, A–D), the possibility of IL-1 β - and/or TNF- α -mediated NF- κ B activation (nuclear localization) was examined in control, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers. Higher nuclear staining of NF- κ B was observed in the periportal areas of *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers than in the periportal areas of *Mir122a*^{-/-} premalignant livers (Supplemental Figure S5). As shown by the early reports,^{51–53} activation of NF- κ B could exhibit antagonistic effects to down-regulate PTEN levels. Thus, our findings imply that the down-regulated PTEN in periportal areas of *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers is likely to be associated with the inflammatory microenvironments, as shown by the elevated levels of IL-1 β /TNF- α and local activation of NF- κ B.

Expansion of Oval Cells in *Mir122a*^{-/-} and *Mir122a*^{-/-}; *Pten*^{+/-} Premalignant Livers

To examine the characteristics of periportal lesions in *Mir122a*^{-/-}; *Pten*^{+/-} livers, ductular reaction was examined using biliary or oval cell markers (A6, EpCAM, and K19) in immunostaining, and these markers were found to be expressed in the periportal basophilic cells in *Mir122a*^{-/-} and *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers (Figure 4A). The

ductular reaction was more prominent in *Mir122a*^{-/-}; *Pten*^{+/-} than in *Mir122a*^{-/-} premalignant livers (Figure 4A). Similar biliary/oval cell markers A6 and K19 were also expressed in the periportal basophilic cells in *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{fl/+} livers (Supplemental Figure S6). Coimmunofluorescence staining was performed, and it was found that EpCAM levels with K19 and A6 were coexpressed in cholangiocytes of the control *Mir122a*^{+/-}; *Pten*^{+/-} livers and in oval cells of *Mir122a*^{-/-} and *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers, respectively (Supplemental Figure S7, A and B). Real-time quantitative RT-PCR data confirmed that the expression of *Epcam* was significantly up-regulated in *Mir122a*^{-/-} livers and more profoundly up-regulated in *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers (Figure 4B). Flow cytometry analysis to detect EpCAM protein on the cell surface also revealed increased EpCAM-positive cells in *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers (Supplemental Figure S7, C and D). A previous study has identified expanded CD133⁺CD45⁻ oval cells as liver bipotent progenitors and cancer-initiating cells in the hepatocyte-specific *Pten* deletion mouse model.³³ In this study, CD133⁺ populations were further quantified by flow cytometry using CD133⁺EpCAM⁺ in CD45 negatively gated cells of control, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers. A trend toward increased numbers of CD133⁺EpCAM⁺CD45⁻ cells was found in *Mir122a*^{-/-}; *Pten*^{+/-} livers compared with that in *Mir122a*^{-/-} livers (Supplemental Figure S8). Next, it was examined whether CD133⁺EpCAM⁺CD45⁻ cells in *Mir122a*^{-/-}; *Pten*^{+/-} livers exhibited anchorage-independent sphere-forming ability. Indeed, it was found that the sphere-forming ability of CD133⁺EpCAM⁺CD45⁻ cells was higher in *Mir122a*^{-/-}; *Pten*^{+/-} livers compared with *Mir122a*^{-/-} livers (Figure 4, C and D). The size of spheres, however, was comparable among different genotypes (Figure 4E). Our data indicate that the expansion of CD133⁺EpCAM⁺CD45⁻ progenitor cells in the *Mir122a*^{-/-}; *Pten*^{+/-} livers was correlated with PTEN down-regulation and exhibited anchorage-independent sphere-forming ability.

Aberrant K8-Stained Patterns Associated in Periportal Areas of *Mir122a*^{-/-}; *Pten*^{+/-} Premalignant Livers

K8 and K18 have been used to identify ductular reactive cells in human liver diseases.³⁶ Using IHC, K8 expression was detected by TROMA-I monoclonal antibody in sections of *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers. K8 was highly expressed in the biliary ducts in the *Mir122a*^{+/-}; *Pten*^{+/-} and ductular reactive cells in *Mir122a*^{-/-} and *Mir122a*^{-/-}; *Pten*^{+/-} livers (Figure 5A). Strikingly, the hepatic parenchyma of *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers revealed heterogeneous patterns with high, intermediate, and low K8-staining intensities (Figure 5A). The intermediate-stained K8 cells, referred to as K8^{int} cells, were round and larger hepatocyte-like cells, which were apparently unlike the highly intense K8-stained ductular cells (hereafter referred to as K8^{hi} cells) in *Mir122a*^{-/-};

Pten^{+/-} premalignant livers (Figure 5A). Co-IF staining of p-AKT^{S473} and K8 was performed, and p-AKT^{S473} was detected in both K8^{hi} and K8^{int} cells in the periportal area of *Mir122a*^{-/-}; *Pten*^{+/-} livers (Figure 5B). To characterize the cellular identity of K8^{int} cells, hepatocyte differentiation marker HNF4 α was costained with K8 in the sections of *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}; and *Mir122a*^{-/-}; *Pten*^{+/-} livers. Only K8^{int} cells were HNF4 α positively stained hepatocytes in the periportal area of *Mir122a*^{-/-}; *Pten*^{+/-} livers, whereas K8^{hi} cells were HNF4 α negatively stained ductular cells in all samples (Figure 5C). Quantification data showed slightly increased K8^{int} and HNF4 α double-positive cells in the *Mir122a*^{-/-} premalignant livers and greatly increased *Mir122a*^{-/-}; *Pten*^{+/-} in premalignant livers (Figure 5D). Interestingly, the aberrant K8^{int} cells were significantly increased and located next to K8^{hi} cells, which is indicative of a cell-lineage relationship between K8^{hi} and K8^{int} cells in the periportal area of *Mir122a*^{-/-}; *Pten*^{+/-} livers.

SOX9 and K8 expression was examined using dual-colored IHC on the sections of *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers. SOX9 was coexpressed with some K8^{hi} cells in the periportal areas of *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers (Figure 5E). Interestingly, SOX9 was also present in hepatocyte-like K8^{int} cells in *Mir122a*^{-/-}; *Pten*^{+/-} periportal areas (Figure 5E). It was further confirmed that SOX9 was coexpressed with HNF4 α specifically in *Mir122a*^{-/-}; *Pten*^{+/-} livers, but not in *Mir122a*^{-/-} livers (Figure 5F). These data indicate that the expanded K8^{int} hepatocyte-like cells in the periportal areas of *Mir122a*^{-/-}; *Pten*^{+/-} livers included SOX9⁺HNF4 α ⁺ hybrid hepatocytes.

Tracing of K8^{hi} and K8^{int} Cells in *Mir122a*^{-/-}; *Pten*^{+/-} Livers

Lineage-tracing experiments were performed to examine whether K8^{hi} or K8^{int} cells in *Mir122a*^{-/-}; *Pten*^{+/-} livers are the cells of origin for liver tumorigenesis. Previously, we generated a transgenic mouse *Tg(Krt18-EGFP, Krt8-CreER^{T2})* (hereafter referred to as *K8-CreER*) carrying *EGFP* and *CreER^T* transgenes driven by K18 and K8 promoters, respectively.^{46,47} *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} mice, which carried *K8-CreER* and

Rosa26 Cre LacZ reporter (*R26R^{LacZ/+}*) transgenes in *Mir122a*^{-/-}; *Pten*^{+/-} backgrounds, were generated. Two different induction protocols (administration of either a single dose or a triple dose of tamoxifen) were examined, and *LacZ* reporter expression was analyzed in *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers at 1, 8, and 30 weeks after tamoxifen administration (Figure 6A). We hypothesized that a one-dose tamoxifen administration would induce Cre activity only in K8^{hi} cells, whereas a triple-dose tamoxifen administration would be able to induce Cre activity in both K8^{hi} and K8^{int} cells, for tracing their derivatives in *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers and tumors (Figure 6A). Compared with the X-Gal-stained livers 1 week after tamoxifen treatments, the result showed that a higher X-Gal-stained intensity was observed in the 8-week trace for *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} livers after one- or triple-dose tamoxifen treatments (Figure 6B). Co-IF staining using antibodies against K8 and β -galactosidase showed that β -galactosidase was only expressed in K8^{hi} cells in *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} periportal areas at 1 week after the one-dose tamoxifen administration (Figure 6C). At 8 weeks after the one-dose tamoxifen treatment, β -galactosidase expression could be traced in the K8^{int} cells, in addition to K8^{hi} cells, in *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} periportal areas (Figure 6C). For the triple dose of tamoxifen treatment, β -galactosidase expression was detected in both K8^{hi} and K8^{int} cells of *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} periportal areas (Figure 6D). The β -galactosidase labeling efficiency in K8^{hi} and K8^{int} cells was quantified and revealed that the β -galactosidase-labeled K8^{int} cells were significantly increased in the 8-week tracing (13.2% $\pm$ 6.5% in a one-dose treatment; 64.2% $\pm$ 6.2% in a triple-dose treatment) compared with that in the 1-week tracing experiment (0.8% $\pm$ 1.8% in a one-dose treatment; 43.9% $\pm$ 6.9% in a triple-dose treatment) (Figure 6E). Co-IF staining was also performed using antibodies against K19 (oval cell marker) and β -galactosidase. Under both one- and triple-dose tamoxifen treatment protocols, K19/ β -galactosidase expression exhibited similar staining patterns (Supplemental Figure S9, A and B) and labeling efficiency (Supplemental Figure S9C) in *K8-CreER*; *R26R^{LacZ/+}*; *Mir122a*^{-/-}; *Pten*^{+/-} periportal areas. In a 30-week trace experiment, liver tumors

Figure 2 Inflammation, fibrosis, and putative tumor-initiating lesions in *Mir122a*^{-/-}; *Pten*^{+/-} premalignant livers. **A:** Hematoxylin and eosin (H&E)-stained sections reveal periportal areas of *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers from 6-month-old mice. Basophilic lesions in *Mir122a*^{-/-} and *Mir122a*^{-/-}; *Pten*^{+/-} periportal areas are shown at higher magnification in the insets; yellow arrowheads indicate oval cells. Cholangiocytes of *Mir122a*^{+/-}; *Pten*^{+/-} and oval cells of *Mir122a*^{-/-} and *Mir122a*^{-/-}; *Pten*^{+/-} liver sections (five sections per mouse; five mice per group) were manually counted and quantified in the bar graph. **B:** Immunohistochemistry using F4/80 antibody for detecting macrophages, anti-CD3 for detecting T cells, and anti-B220 for detecting B cells reveals inflammatory cell infiltration in *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers at 6 months of age. Boxed areas depict representative positive staining of F4/80, CD3, and B220 and are shown at higher magnification in the insets. Quantification of F4/80-, CD3-, and B220-expressing cells in the basophilic lesions is shown in the bar graphs. **C:** Real-time quantitative RT-PCR (RT-qPCR) was used to detect *Il1b*, *Tnf*, and *Il6* expression in *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers. **D:** RT-qPCR was used to detect *Il1b*, *Tnf*, and *Il6* expression in *Mir122a*^{+/-}; *Alb-Cre*; *Pten*^{+/+}, *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{+/+}, and *Mir122a*^{-/-}; *Alb-Cre*; *Pten*^{+/+} livers. Relative levels of indicated genes were normalized with *Actb* expression. **E:** Sirius red-staining sections reveal collagen-deposit areas in *Mir122a*^{+/-}; *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-}; *Pten*^{+/-} livers. **F:** Percentage of fibrotic areas was quantified by calculating Sirius red-stained collagen-deposited areas and dividing by the yellow-stained hepatic parenchyma. Data are expressed as means $\pm$ SD. *n* = 5 mice per group (D and F). **P* < 0.05, ***P* < 0.01, and ****P* < 0.005. Scale bar = 100 μ m (A, B, and E). Original magnification, $\times$ 800 (A, insets). PV, portal vein.

Figure 3 Phosphatase and tensin homolog on chromosome 10 (PTEN) down-regulation, AKT activation, and cellular proliferation detected in periportal areas of *Mir122a*^{-/-};*Pten*^{+/-} premalignant livers. **A:** Total lysates of wild-type (WT), *Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/-} livers and *Mir122a*^{-/-};*Pten*^{+/-} hepatic tumor were used for detecting levels of PTEN and AKT^{S473} phosphorylation (p-AKT) by Western blot analysis. β-Actin and pan-AKT levels were used as internal controls. **B:** Relative PTEN levels were quantified from multiple experiments of Western blot analysis, as shown in **A**. **C:** Immunohistochemical analysis of PTEN and p-AKT in periportal regions in 6-month-old *Mir122a*^{+/-};*Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/-} livers and *Mir122a*^{-/-};*Pten*^{+/-} tumor. Dashed lines depict down-regulated PTEN and p-AKT in periportal areas. **D:** Liver sections from 6-month-old *Mir122a*^{+/-};*Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/-} mice were immunostained with anti-Ki-67 antibody. Boxed areas in the top panels are shown at higher magnification in the bottom panels. **E:** Quantification of the percentages of cells stained for Ki-67 by immunohistochemical analysis. Data are expressed as means ± SD. *n* = 5 mice per group (E). ****P* < 0.005. Scale bar = 100 μm (C and D). PV, portal vein.

Figure 4 Activation of oval cells occurs in periportal areas in *Mir122a*^{-/-};*Pten*^{+/+} premalignant livers. **A:** Immunohistochemical analysis of oval cell markers [A6, EpCAM, and keratin 19 (K19)] in periportal areas in 6-month-old *Mir122a*^{+/+};*Pten*^{+/+}, *Mir122a*^{-/-};*Pten*^{+/+}, and *Mir122a*^{-/-};*Pten*^{+/+} livers. **B:** Real-time quantitative RT-PCR analysis of *Epcam* expression in 6-month-old *Mir122a*^{+/+};*Pten*^{+/+}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/+} livers. **C:** Phase-contrast images of spheres generated from fluorescence-activated cell-sorted (FACS) ductal progenitor cells (CD45⁻CD133⁺EpCAM⁺) of 6-month-old *Mir122a*^{+/+};*Pten*^{+/+}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/+} livers. **Arrows** show spheres grown in Matrigel. **Top panels** show representative images of sphere-forming abilities. **Bottom panels** show the size and morphology of *Mir122a*^{+/+};*Pten*^{+/+}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/+} hepatic spheres. **D and E:** Sphere-forming ability (**D**) and sphere size (**E**) of FACS ductal progenitor cells in 6-month-old *Mir122a*^{+/+};*Pten*^{+/+}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/+} livers. Data are expressed as means $\pm$ SD. $n = 5$ mice per group (**B**, **D**, and **E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$. Scale bars: 100 μ m (**A**); 0.5 cm (**C**).

were collected from *K8-CreER*;*R26R*^{LacZ/+};*Mir122a*^{-/-};*Pten*^{+/+} mice and whole-mount X-Gal staining was performed. Notably, tamoxifen seemed to suppress tumorigenesis in *K8-CreER*;*R26R*^{LacZ/+};*Mir122a*^{-/-};*Pten*^{+/+} livers compared with age-matched *Mir122a*^{-/-};*Pten*^{+/+} livers, in which multiple tumors were developed within the same time period. Nonetheless, four tumors were assessed from one-dose and three tumors were assessed from triple-dose tamoxifen-treated *K8-CreER*;*R26R*^{LacZ/+};*Mir122a*^{-/-};*Pten*^{+/+} mice. X-Gal positively stained tumors (two of three) were found in triple-dose, but none (zero of four) were found in one-dose, tamoxifen

administered to *K8-CreER*;*R26R*^{LacZ/+};*Mir122a*^{-/-};*Pten*^{+/+} mice (Figure 6F). Under the one-dose tamoxifen treatment, poorly β -galactosidase-labeled K8^{int} cells after a 1-week trace (Figure 6E) were correlated with X-Gal negatively stained tumors after a 30-week trace (Figure 6F). Taken together, the lineage-tracing experiments revealed that the K8^{int} cells were likely generated from K8^{hi}- or K19-expressing oval cells in *K8-CreER*;*R26R*^{LacZ/+};*Mir122a*^{-/-};*Pten*^{+/+} periportal areas. The expanded K8^{int} hepatocyte-like cells in *Mir122a*^{-/-};*Pten*^{+/+} premalignant livers could further act as the cells of origin causing liver tumors.

Figure 5 Expanded hepatocyte-like cells express p-AKT, intermediate levels of keratin 8 (K8^{int}), HNF4α, and SOX9 in periportal areas of *Mir122a*^{-/-}*Pten*^{+/-} premalignant livers. **A:** Immunohistochemical analysis of K8 in periportal areas in 6-month-old livers of indicated genotypes. **B:** Immunofluorescently stained liver sections of 6-month-old *Mir122a*^{-/-}*Pten*^{+/-} mice reveal the expression of K8 (red) and p-AKT (green). Nuclei were stained by DAPI (blue). **C:** Immunofluorescence (IF) analysis of K8 (green) and HNF4α (red) in the periportal areas in 6-month-old *Mir122a*^{+/-};*Pten*^{+/-}, *Mir122a*^{-/-}, and *Mir122a*^{-/-};*Pten*^{+/-} livers. **D:** Quantification of the percentages of cells stained for K8^{int} and HNF4α assessed by IF analysis. **E:** Dual-colored immunohistochemistry reveals K8 (blue cytoplasm) and SOX9 (red nuclei) expression in the periportal areas in 6-month-old livers of indicated genotypes. **A–C** and **E:** Boxed areas in top panels are shown at higher magnification in the bottom panels. Arrowheads and arrows indicate highly intense K8-stained ductular cells and K8^{int} cells, respectively. **F:** IF-stained liver sections reveal SOX9 (green) and HNF4α (red) expression in the periportal areas in 6-month-old *Mir122a*^{-/-} and *Mir122a*^{-/-};*Pten*^{+/-} livers. Arrows indicate the cells with double positivity for SOX9 and HNF4α (yellow). Arrowheads indicate the cells with single positivity for SOX9 (green). Boxed areas are shown at higher magnification in the insets. Data are expressed as means ± SD. *n* = 5 mice per group (D). ****P* < 0.005. Scale bar = 100 μm (A–C, E, and F). PV, portal vein.

Discussion

Our study provides novel genetic evidence to show that *Pten* is haploinsufficient for *Mir122*-null HCC initiation and

progression in mouse liver. Infiltrating inflammatory cells in the periportal area are associated with locally down-regulated PTEN and up-regulated p-AKT. Also, the ductular cells and K8^{int} hepatocyte-like cells of

Figure 6 Lineage tracing of highly intense K8-stained ductular cells (K8^{hi}) and intermediate levels of K8 (K8^{int}) cells in pre-malignant livers and hepatic tumors in *Mir122a*^{-/-}; *Pten*^{+/-} mice. **A:** Hypothetical illustration of β-Gal–labeled K8^{hi} and K8^{int} cells (blue cells) causing hepatocytic tumor cells in *K8-CreER*; *R26R*^{LacZ/+}; *Mir122a*^{-/-}; *Pten*^{+/-} mice. The experimental time lines are shown at the bottom. One- and triple-dose tamoxifen administration protocols were used to trace K8^{hi} cells and to trace both K8^{hi} and K8^{int} cells, respectively. After tamoxifen injection for 1, 8, and 30 weeks (W), the *K8-CreER*; *R26R*^{LacZ/+}; *Mir122a*^{-/-}; *Pten*^{+/-} liver samples were harvested for assessing *LacZ* reporter expression. **B:** Gross views of X-Gal–stained livers show overall *LacZ* reporter expression in *K8-CreER*; *R26R*^{LacZ/+}; *Mir122a*^{-/-}; *Pten*^{+/-} mice at 1 and 8 weeks after one- and triple-dose tamoxifen injections. **C** and **D:** Immunofluorescence staining of K8 (green) and β-Gal (red) in *K8-CreER*; *R26R*^{LacZ/+}; *Mir122a*^{-/-}; *Pten*^{+/-} livers at 1 and 8 weeks after one-dose (**C**) or triple-dose (**D**) tamoxifen injection. Boxed areas in the top panels are shown at higher magnification in the bottom panels. Arrowheads and arrows indicate K8^{hi} and K8^{int} cells, respectively. **E:** Quantification of the percentages of K8^{hi} and K8^{int} cells stained by β-Gal in *K8-CreER*; *R26R*^{LacZ/+}; *Mir122a*^{-/-}; *Pten*^{+/-} livers at 1 and 8 weeks after one- and triple-dose tamoxifen injections. **F:** Gross (left panels) and histologic (right panels) views of X-Gal–stained *K8-CreER*; *R26R*^{LacZ/+}; *Mir122a*^{-/-}; *Pten*^{+/-} liver tumors 30 weeks after one- and triple-dose tamoxifen injection. Nuclei fast red was used for counterstaining in the right panels. Data are expressed as means ± SD (**E**). *n* = 3 mice per group (**E**). ***P* < 0.01, ****P* < 0.005. Scale bars: 0.5 cm (**B**); 100 μm (**C** and **D**); 0.1 cm (**F**).

Mir122a^{-/-};*Pten*^{+/-} premalignant livers are expanded. Subsequently, down-regulated PTEN and activated AKT promote liver tumor initiation and progression in the *Mir122a*-null background. Lineage-tracing experiments under different tamoxifen-induced LacZ reporter tracing among K8-expressing cells in *Mir122a*^{-/-}*Pten*^{+/-} premalignant livers enabled us to demonstrate that K8^{int} hepatocyte-like cells are derived from K8^{hi} cells and can be the cells of origin for liver tumor initiation and progression. However, the lower β-galactosidase-labeling efficiency in K8^{int} hepatocyte-like cells (approximately 1% in 1-week trace; approximately 13% in 8-week trace) after a one-dose tamoxifen induction appears to be inefficient for long-term (30-week) lineage tracing, which limits our finding to identify X-Gal-positive tumors.

Interestingly, the K8^{int} cells identified in the periportal area in this study express SOX9 and hepatocyte marker HNF4α that resemble the hybrid hepatocytes in a previous report.³⁹ However, the hybrid hepatocyte cells, previously described by Font-Burgada et al,³⁹ cannot cause a chemical- or diet-induced liver tumor. In this study, down-regulation of PTEN or activation of AKT appears to be the crucial step for generating K8^{int} hepatocyte-like cells. Immunohistologic analysis and lineage-tracing experiments revealed that the K8^{int} hepatocyte-like cells are often juxtaposed with the K8^{hi} oval cells and are derived from K8^{hi} oval cells in *Mir122a*^{-/-}*Pten*^{+/-} premalignant livers. Thus, these findings suggest that PTEN down-regulation in *Mir122a*-null oval cells and their derivatives in periportal areas can drive HCC initiation and progression. Although the previous reports demonstrate that the oval cells generated from various mouse liver disease models fail to cause HCC,^{38–40} our results suggest that periportal inflammatory and fibrotic microenvironments can induce certain oncogenic insults (ie, PTEN down-regulation or AKT activation) that promote periportal oval-to-hepatocyte-like cell expansion and tumorigenesis.

DEN is a potent carcinogen driving hepatic tumorigenesis in rodents. Bioactive DEN requires cytochrome p450 enzymes, including Cyp2E1, in the differentiated hepatocytes located at the pericentrilobular area.^{54,55} On DEN administration, the *Mir122a*-null mice are susceptible to biliary cystogenesis and hepatocellular carcinogenesis, suggesting that *Mir122a* has a protective role against the hepatotoxic consequences of DEN.⁵⁶ In this study, hepatocellular tumors, but not biliary cyst formation, were observed in *Mir122a*^{-/-}*Pten*^{+/-} mice. This finding suggests that PTEN down-regulation and AKT activation in *Mir122*-null livers are crucial for driving hepatocellular tumorigenesis, but not required for biliary cystogenesis.

In the current study, PTEN down-regulation specifically occurred in periportal parenchyma and was associated with infiltrating inflammatory cells. The inflammatory phenomena and local PTEN down-regulation appeared to be independent of the sex; thereby, no significant sex-related differences were observed in liver tumor development in

both *Mir122a*^{-/-}*Pten*^{+/-} and *Mir122a*^{-/-};*Alb-Cre*;*Pten*^{fl/+} mice. According to the previous reports,^{51–53} IL-1β/TNF-α-mediated NF-κB signaling might be one of the potential antagonistic mechanisms to down-regulate PTEN levels. However, other mechanisms causing PTEN down-regulation in hepatic periportal microenvironments cannot be ruled out. A recent study shows that microenvironment-induced PTEN down-regulation by exosomal miRNAs promotes brain metastasis outgrowth.⁵⁷ In the *Mir122a*-null liver, aberrantly produced exosomal miRNAs that target *Pten* mRNA in periportal parenchyma remain unclear and require further investigation.

In summary, this study shows that PTEN levels are crucial to control the initiation and expansion of hepatocyte-like tumor cells under periportal inflammatory and fibrotic microenvironments in *Mir122a*-null background. By generating compound mutants in mice, this approach facilitates our understanding in the cooperatively suppressive mechanism of *Mir122a* and *Pten* in mouse hepatocellular carcinogenesis that may explain the consequences of down-regulation of both tumor suppressors in patients with HCC.

Acknowledgments

We thank Drs. Hsin-Fang Yang-Yen and Jeou-Yuan Chen for comments on this study; the Taiwan Mouse Clinic, National Phenotyping and Drug Testing Center (Academia Sinica, Taipei, Taiwan) for technical support; and the Developmental Studies Hybridoma Bank, which was started by the Eunice Kennedy Shriver National Institute of Child Health and Human Development of the NIH, at the University of Iowa for maintaining the A6 (A6 BCM, distributed by Valentina M. Factor), EpCAM (G8.8, distributed by Andrew G. Farr), keratin 8 (TROMA-I, distributed by Philippe Brulet and Rolf Kemler), and keratin 19 (TROMA-III, distributed by Rolf Kemler) monoclonal antibodies.

W.-L.T. and C.-M.C. designed the experiments, analyzed the data, and wrote the article; W.-L.T. performed the experiments; and W.-L.T., L.-R.Y., A.-P.T., and C.-M.C. interpreted data and provided critical input.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.07.019>.

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