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Alpha-fetoprotein-thymidine kinase–luciferase knockin mice: A novel model for dual modality longitudinal imaging of tumorigenesis in liver

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Background & Aims: Hepatocellular carcinoma (HCC) is frequently a lethal disease and one of the few malignancies that is still increasing in incidence around the world. Better animal models are highly desired to investigate the molecular basis of HCC and to develop novel therapeutic strategies. Alpha-fetoprotein (*Afp*) gene is expressed in fetal liver, silenced soon after birth, and highly re-expressed in hepatocellular carcinomas (HCC). We aimed to take advantage of the dramatic re-expression of the *Afp* gene in HCC to develop a hepatocarcinogenesis reporter (HCR) mouse model for dual-modality, longitudinal *in vivo* imaging of liver tumor development, and progression.

Methods: Knockin mice were established by placing a *thymidine kinase* (*tk*)*–luciferase (luc)* reporter gene cassette under the transcriptional control of the endogenous *Afp* promoter. DEN, a liver carcinogen, was used to induce liver tumors, which was monitored by both *luc*-based bioluminescent (BL) and *tk*-based positron emission tomography (PET) imaging.

Results: The expression profile of *luc* was identical to that of the endogenous *Afp* gene during development. As early as 2 months after the exposure to DEN, BLI revealed multifocal signals in the liver, long before the appearance of histologically apparent neoplastic lesions. By 6 months, BL and PET dual imaging showed strong signals in malignant HCC. By serendipity, a strong BL signal was also detected in adult testes, a previously unknown site of Afp expression.

Conclusions: The *HCR* model enables longitudinal monitoring of liver tumor development and progression, providing a powerful tool in developing chemoprevention and therapeutic strategies for HCC.

Keywords: Positron emission tomography; Bioluminescence imaging; Hepatocarcinogenesis; Hepatocellular carcinoma; Testes.
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Abbreviations: Afp, alpha-fetoprotein; HCC, hepatocellular carcinomas; *HCR*, hepatocarcinogenesis reporter; TK, thymidine kinase; LUC, luciferase; DEN, diethylnitrosamine; PET, positron emission tomography; BLI, bioluminescence imaging.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of human cancer and the third most common cause of cancer death worldwide. In North America, it is one of the few tumor types whose incidence is increasing, bucking the trend of continuing reduction for many other tumors. Typical HCC arises in a setting of chronic hepatitis with infection by hepatitis B and C viruses responsible for most HCC cases in humans [1,2]. Although the pathogenic causes are well known, no effective treatment is available for most HCC patients except for surgical resection [3].

Historically, studying naturally occurring HCC in model organisms, particularly mice, has been instrumental in advancing our understanding of the etiology of HCC. Genetically defined mouse models are increasingly used as powerful tools to evaluate genetic factors and therapeutic strategies for HCC [4–6]. However, because of the stochastic and non-visible nature of HCC occurrence, the detection and analysis of HCC from these models often requires the termination of the animals, precluding the real time assessment of the behavior of individual HCC tumors under a natural microenvironment and/or over a defined period of time. *In vivo* imaging in small laboratory animals is an emerging and promising technology that can be used to facilitate non-invasive,

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longitudinal monitoring of naturally occurring tumors. In mice, two types of reporter-based imaging modalities, the luciferase (Luc) reporter-based *in vivo* bioluminescence imaging (BLI) and the thymidine kinase (TK)-based Positron emission tomography (PET) imaging, respectively, are particularly useful. BLI has been widely used in cancer imaging often with multiple xenograft tumor-based systems where tumor cells express Luc. It provides a reliable, sensitive, and rapid modality for noninvasively tracking of tumor status in mice [7–9]. On the other hand, PET is a highly quantitative detection method that also provides three-dimensional information for the imaging target in the context of its surrounding [10–12].

In order to monitor HCC in mice by BLI and PET imaging, we decided to create a mouse model in which Luc and TK are expressed specifically in HCC by placing the coding sequences of Luc and TK under the control of the promoter of the Alpha-fetoprotein (*Afp*) encoding gene. Afp, a secreted polypeptide, is a serum tumor marker for HCC. It is highly expressed in fetal liver in both rodents and humans but is not expressed in normal adult liver. Interestingly, this protein is dramatically re-expressed in the vast majority of HCCs [13]. Thus, it is a unique HCC tumor specific marker in both mice and humans. Here we took advantage of the dynamic regulation of Afp expression and generated a novel reporter mouse model that enables dual modality imaging of the early and late stages of hepatocarcinogenesis *in vivo*.

Materials and methods

Gene targeting in mouse embryonic stem (ES) cells and generation of knock-in mice

A targeting vector, *pAfp-tv1*, was constructed using the *BAC* modification system [14,15] as described [16]. This vector was designed to introduce a fusion gene cassette, designated *PuroTK-Luc*, consisting of the coding sequences for both the fruit fly luciferase (*Luc*) and the *PuroTK* [19], linked by an internal ribosomal entry site (*IRES*) along with a *loxP-PGKneo-loxP* cassette [20] in between the A and T of the translation start codon of the *Afp* gene (Detailed information for the plasmid construction will be made available upon request). This vector was electroporated into ES cells and targeted clones were identified by the standard mini-Southern approach. Two of these targeted clones were injected into blastocytes. Heterozygous mice carrying the targeted allele were obtained. Once heterozygous knock-in mice are obtained, they were mated with *Zp3/cre* transgenic mice which express the cre recombinase in the female oocytes and thus can facilitate the removal of *PGKpuro-tk* cassette flanked by a pair of *LoxP* sites [17]. This gave rise to mice in which the *PuroTK-Luc* cassette is placed right behind the *Afp* promoter. Such mice were identified by Southern blot analysis. Mice carrying this particular knock-in allele were identified by a PCR-based genotype using the following primers: 5′-ATG AAT TCG GCG CGC CGC CTG AAC TAC TGA AAC AAT-3′ and 5′-GAA GAG TTC TTG CAG CTC GGT-3′.

Animal care, liver carcinogenesis, and tissue analysis

Mice were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the USPHS "Policy on Human Care and Use of Laboratory Animals," and all studies were approved and supervised by The Case Western Reserve University Institutional Animal Care and Use Committee. Mice were generated on a C57 BL/6j-129 background originally and backcrossed to C3H and FVB/NJ mice for five generations by standard genetic crosses, which were then bred with each other to generate cohorts of *Afp-TK-ires-Luc* positive or negative mice that were used in subsequent studies. Knockin mice and wild type littermates were subjected to a single i.p. injection of 20 μg/g body weight of diethylnitrosamine (DEN) (Sigma) 14 days after birth. Mice were sacrificed at the indicated time points after injection. After imaging, the animals were euthanized by overdosing with pentobarbital and cut open for photography and tissue harvesting. Livers were excised, weighed, examined for macroscopic lesions, and photographed. When indicated, *ex vivo* imaging was performed. Individual liver lobes were fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA) or Z-FIX (ANATECH LTD, Battle Creek, MI). Paraffin-embedded sections were cut at 5 μm, and stained with H&E. In addition, a portion of the liver or testis was embedded in Tissue Freezing Media (Triangle Biomedical Sciences, Durham, NC), and submerged in cold 2-methylbutane for cryosectioning and stored at −80 °C.

Immunofluorescence

Frozen sections of liver and testis were fixed with 2% paraformaldehyde. After washing with PBS, the sections were blocked with 50 mmol/L NH_4Cl and permeabilized with 0.3% NP40 for 10 min. The sections were then incubated with goat anti-Afp (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 2 h followed by detection with donkey anti-goat IgG-Red X (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 30 min. Images were taken using a Leica microscope.

In vivo imaging with BLI & PET

BLI

For one-day-old pups, the animals were weighed precisely for calculating the amount of D-luciferin (Biosynth AG, Switzerland) to be carefully injected i.p. into the pups at 75 μg/kg body weight. Then, these one-day-old pups were placed in a home-made dark box with multiple light-absorbing cubicle chambers and subjected to imaging with a combined tri-imaging modality system developed by Thomas Jefferson National Lab (Newport News, VA) at either 3 or 6 min after injection of D-luciferin. For *BLI* of the adult mice, light signals were collected at 6 min after a single i.p. injection of 100 μg/kg body weight D-luciferin using a Xenogen IVIS 200 cooled-CCD camera system (Palo Alto, CA). During the period of signal collection, the animals were sedated continuously via inhalation of isoflurane gas (1.5% mixed with oxygen) through a nose corn device (EZAnesthesia, Palmer, PA). *Ex vivo* bioluminescence imaging of isolated organs was performed immediately after euthanasia of the animals. Dissected organs were placed on a sheet of black background and imaged with IVIS 200. The BLI images were then pseudo-colored and overlaid on top of a conventional photograph of the animals.

PET

Animals were imaged pair-wise with a presumptive control and a testing animal for each scan using a micro-PET scanner R4 (Siemens/Concord, Knoxville, TN). A 20 min transmission scan was conducted using a sealed point source in order to establish the attenuation map. During the period of image acquisition, the animals were sedated continuously via inhalation of isoflurane gas as in BLI. L-[F-18]-FMAU, the substrate of TK as well as the radiotracer used for PET imaging here was synthesized according to Mukhopadhyay et al. [18]. 250 μCi (9.25 MBq) of L-[F-18]-FMAU in 200 μl of saline solution was injected via tail vein into each animal. Forty minutes after injection, a 30 min emission scan was conducted in list mode acquisition. After the scan, the acquired data were binned into 5 min frames and reconstructed with attenuation correction using the ASIPRO package on the R4 scanner. The last frame of the emission images was colored in hot-metal for display.

Results

Generation of the Afp-TK-ires-Luc mouse model

We took advantage of dramatic re-expression of Afp in liver cancer and generated mice for visualizing the hepatocarcinogenesis processes *in vivo*. Toward this goal, we replaced the *Afp* coding sequence with those of imaging reporters, *thymidine kinase (TK)* and *luciferase (Luc);* two enzymes that are commonly used for facilitating PET and BLI, respectively. Fig. 1 shows the targeting strategy used to generate mutant alleles of the *Afp* gene. The *TK–Luc* dual-reporter fusion gene cassette (designated as Puro*TK-ires-Luc*) was introduced into the *Afp* locus by the standard knock-in gene targeting experiment in the mouse ES cells, giving rise to new *Afp.PGKneo.PuroTK-IRES-Luc* allele (Fig. 1A). Heterozygous mice carrying this allele were obtained. These mice were then mated with *Zp3-Cre* female mice to facilitate the removal of the PGKneo cassette from the original targeted allele, giving

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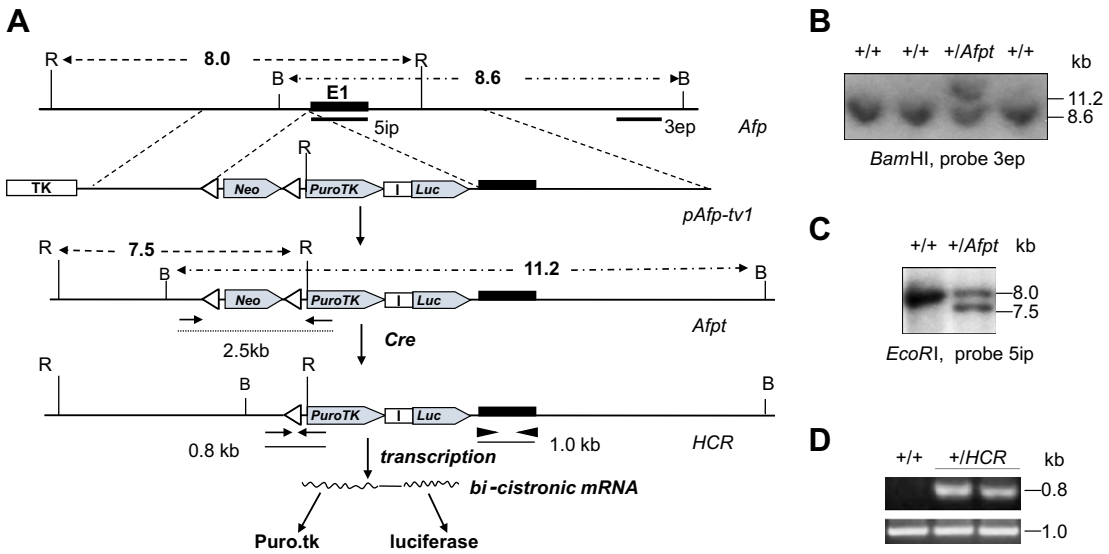

Fig. 1. Gene targeting at the *Afp* locus. (A) A schematic illustration of the gene targeting strategy. Targeting vector (*pAfp-tv1*) contains a *LoxP-Neo-LoxP-PuroTK-ires-Luc* cassette between the two arms of homology for gene targeting. By design, the cassette is inserted behind the A of the ATG translation start codon of the *Afp* gene, generating the primary knock-in allele (the *Afp-t* allele). *Cre*-mediated deletion of the *Neo* selection marker then gave rise to the final knock-in allele (the *HCR* allele). Transcription from this allele, driven by the *Afp* promoter, is expected to produce a bi-cistronic mRNA with which *Puro.TK* and luciferase can be produced upon translation, providing two potential reporters for imaging. Targeted clones were identified by Southern blot by the presence of a novel 11.2 Kb *BamHI* fragment in addition to the 8.6 Kb wild-type fragment using a 3′ external probe (3ep) and the presence of a 7.5 Kb *EcoRI* fragment in addition to the 8.0 wild-type fragment by 5′ internal probe (5ip). The *HCR* allele was detected by a PCR genotyping strategy using a pair of primers corresponding to the *Afp* promoter and the *Puro.TK* cassette, respectively. This PCR is designed to generate a 2.5 kb and 0.8 kb product from the *Afp-t* and *HCR* allele respective (the 2.5 kb product is not amplified under our experimental conditions). The sizes of the diagnostic *Bam HI* (B) and *Eco RI* (E) fragments of wild-type (*Afp*) and targeted (*Afpt*) allele, respectively, were indicated. The positions of the pair of primers used to identify the *HCR* allele are also shown. (B) Initial screening for putative clones containing the *Afp-t* allele by Southern blot. (C) Identification of correctly targeted clones by Southern blot from the putative targeted clones identified in B. (D) Identification of mice carrying the *HCR* allele by PCR genotyping. *HCR* mice were identified by detecting the 0.8 kb PCR fragment specific for the *HCR* allele (upper panel). As a control, another pair of primers was used to amplify a 1.0 kb fragment from the wild-type *Afp* gene (lower panel).

rise to a new modified *Afp* allele in which the *PuroTK-IRES-Luc* dual reporter cassette is placed right behind an endogenous *Afp* promoter as originally designed (Fig. 1). The imaging allele, designated as the *hepatocarcinogenesis-reporter* or *HCR*, is expected to produce the PuroTK fusion protein and luciferase with a profile identical to that of the unmodified endogenous *Afp* gene.

The expression of the luciferase reporter in HCR mice recapitulates that of the endogenous Afp gene

Heterozygous *HCR* mice were fertile and did not display any phenotypic or histological abnormalities, showing that introduction of the *TK-ires-Luc* cassette into the *Afp* locus did not significantly affect either development or postnatal growth (Fig. 2A and C). Mating among these heterozygous mutant mice gave rise to progeny of all expected genotypes at a ratio consistent with Mendelian segregation. Homozygous *HCR* mice also were indistinguishable from their normal counterparts except that the females were sterile, consistent with a previous report [19].

Afp is known to be expressed in early developing liver or during hepatocarcinogenesis, but is largely absent in normal adult liver. Thus, we first examined whether *Luc* expression followed similar developmental regulation in *HCR* mice. The livers of mice between E16 and P30 were injected with D-luciferin, and livers were harvested and subject to a conventional biochemical enzymatic luciferase assay. We found that luciferase activity could be detected in the fetal liver from *HCR* mice but not in those from the wild-type control. The activity could be detected as early as E16.5, and the highest level was detected at postnatal day 1 (Fig. 2A). It rapidly dissipated and became completely undetectable by 3 weeks. The pattern is very similar to that of the endogenous *Afp* gene [20]. BLI confirmed the biochemical analyses and detected a strong signal in the neonatal liver (Fig. 2B), which disappeared from the adult liver (Fig. 2B and C).

Unexpectedly, strong BLI signals were also consistently detected in the adult male *HCR* mouse in areas around the testes (Fig. 2C). When the reproductive tract was dissected and imaged *ex vivo* (Fig. 2D and E), BLI signals were strongly detected in the testes and at lower levels in the seminal vesicles, suggesting that the *Afp* promoter is active in male reproductive cells. To verify Afp expression in the testes, immunofluorescence staining with an anti-Afp antibody was performed, as the heterozygous *HCR* mice still expressed Afp from the remaining wild type allele. Fig. 2G shows that Afp protein is indeed highly expressed in spermatids from the *HCR* mice that still retain one *Afp* allele, as did the wild type mice. It is possible that the lower BLI signals detected in seminal vesicles might have come from sperms stored in seminal vesicles. Consistent with the normal fertility of *HCR* mice, histological analysis did not reveal any defects in testes (Fig. 2F). To our knowledge, this is the first demonstration of Afp expression in an adult tissue.

In sum, these data suggest that the expression of the *PuroTK-IRES-Luc* transcript indeed recapitulates that of the endogenous *Afp* gene in the *HCR* mice.

HCR mice allow in vivo visualization of early pre-neoplastic lesions following DEN-induced hepatocarcinogenesis

We next asked whether the *PuroTK-IRES-Luc* dual reporter gene was specifically activated during hepatocarcinogenesis to allow

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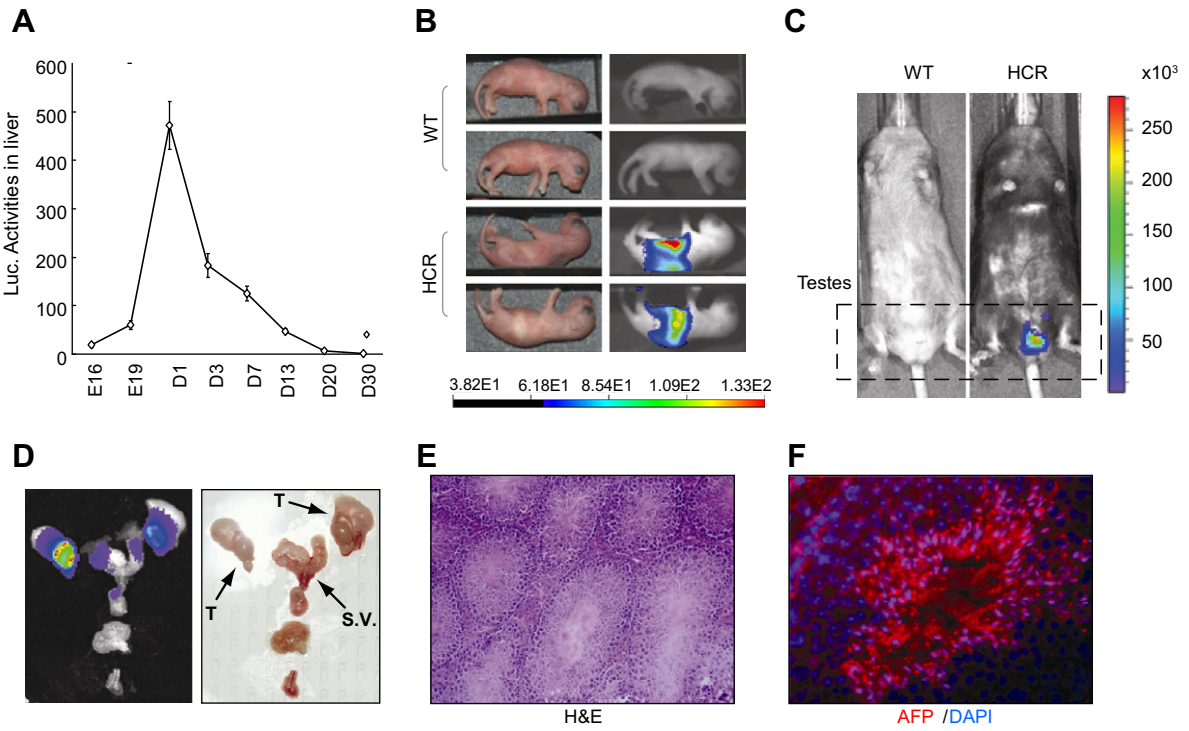

Fig. 2. Detection of luciferase activity in *HCR* mice. (A) Detection of luciferase activity in the liver of *HCR* mice at various ages by chemoluminescent assay. (B) Detection of luciferase activity in newborn *HCR* mice by bioluminescent imaging. (C) Detection of luciferase activity in adult *HCR* mice. Note the pattern of luciferase activity in testes. (D) BLI of a male reproductive tract. Abbreviations used are: T, testes; S.V., seminal vesicles. (E) Photographic image of reproductive tract. (F) Histology of testes (magnification 20×). (G) Detection of Afp in testes by immunofluorescence (magnification 40×).

Cancer

longitudinal monitoring of the hepatocarcinogenesis process by BLI, PET, or both. Liver tumors can be induced with DEN, a classical hepatocarcinogen, in young mice on susceptible genetic backgrounds. To facilitate induction of liver tumors, we initially transferred the *HCR* allele from the original liver tumor-resistant C57BL/6j-129Sv mixed genetic background into C3H genetic backgrounds that is highly susceptible to DEN-induced hepatocarcinogenesis. After four rounds of backcrossing, two week-old C3H male mice were given a single intraperitoneal injection of DEN. Longitudinal imaging was commenced twice a month later. Remarkably, significant levels of BLI signals were detected in *HCR* mice but not the control mice as early as 2 months after the DEN injection (Fig. 3A). To confirm this observation, livers from a subgroup of mice were dissected and subject to BLI *ex vivo*. Fig. 3C shows that multiple Luc-positive foci were present on every lobe. However, gross inspection under a dissection microscope and detailed histological examination failed to identify any clear neoplastic lesions at this early stage (Fig. 3B, D and E). Indeed no clear cell or basophilic foci were detected. PET imaging is known to be less sensitive than BLI imaging. As a result, PET imaging of the same mice at this early stage did not detect any consistent signals. Because luciferase and thymidine kinase were expected to be translated from the same bi-cistronic transcript (Fig. 1), it is possible that PuroTK was expressed but at a level that was too low to be visualized by PET imaging.

Since these heterozygous *HCR* mice also carry a normal copy of the endogenous *Afp* gene, we examined whether Afp was also expressed in these livers. Consistent with the multifocal BLI signals, numerous microscopic Afp-positive foci were readily detected across the liver sections (Fig. 3F and G). Although the cellular origin of Afp-positive cells is yet to be defined, hepatocytes appeared to constitute a significant fraction where Afp displayed a cytosolic staining pattern (Fig. 3H). These results indicate that Afp expression takes place during the early stages of hepatocarcinogenesis, before the appearance of histologically evident transformation. Importantly, these early events of hepatocarcinogenesis can be detected by BLI in *HCR* mice.

Longitudinal monitoring of liver tumor progression by both BLI and PET imaging

Next, we determined whether *HCR* mice will permit the long-term real time monitoring of the natural progression of liver tumor formation. Because DEN induces a large number of fast-growing liver tumors that rapidly occupy most of the liver parenchymal space on C3H genetic background, we backcrossed *HCR* mice with FVB/NJ mice that are much less susceptible to DEN-induced hepatocarcinogenesis. Upon DEN exposure FVB mice develop a small number (less than 10) of discrete liver tumors, a fraction of which progress to malignant HCC. As such, the FVB/NJ genetic background is more suitable for imaging later stage individual liver tumors. DEN-treated FVB/NJ mice also displayed BLI signal at early stage, albeit somewhat delayed compared with C3H mice. BLI signals became stronger over time. An example at 6 months after DEN treatment is shown in Fig. 4A, revealing strong BLI signals. Note the difference in signal intensities between Fig. 3A and Fig. 4A on the scale bars. The distribution patterns of Luc signals were suggestive of the presence of

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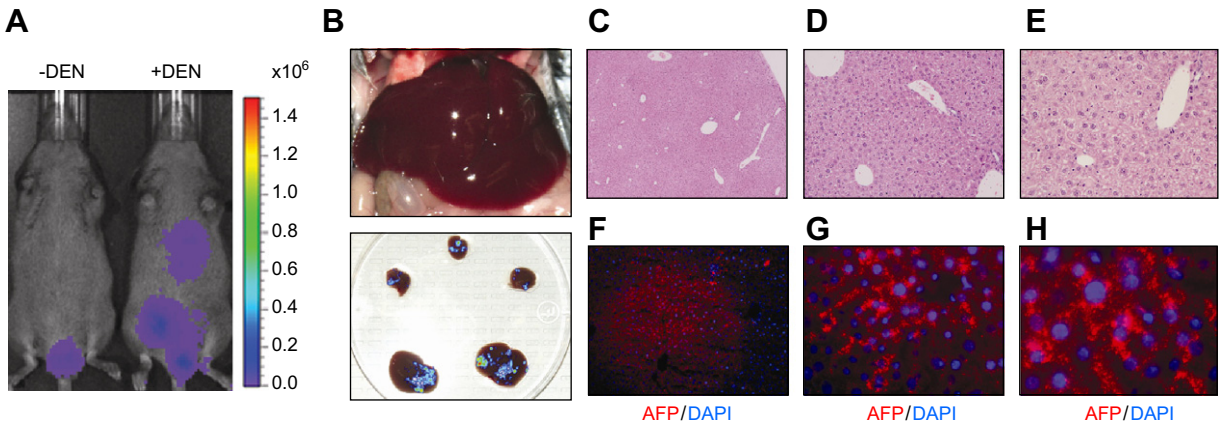

Fig. 3. Visualization of early pre-neoplastic lesions in *HCR* mice by BLI. (A) BLI on a pair of *HCR* mice. The animal on the right was injected with a single dose of DEN at 2 weeks of age and imaged 2 months later. The animal on the left was injected with vehicle control. Note the detection of hepatic BLI signal only in the DEN-injected animal. The testicular signals varied from animal to animal and were not correlated with DEN treatment (not shown). (B) A photograph of the liver showing normal gross morphology. (C) *Ex vivo* BLI of dissected liver lobes from DEN injected mouse superimposed on a digital photograph. (D and E) Hemotoxylin and eosin staining of liver sections from DEN injected mouse (magnification, D, 5×; E, 20×). (F and G). Expression of Afp in DEN-treated mouse liver detected by immunofluorescence (red, Afp. blue, DAPI. Magnification, F, 10×; G, 40×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

multiple tumors. However, the two-dimensional nature of BLI made it hard to ascertain whether they represent individual tumors.

More importantly, at 6 months, the DEN-induced tumors could be readily detected by PET imaging based on *Afp* promoter-driven thymidine kinase activities (Fig. 4B). Interestingly, coronal ventral to dorsal serial imaging showed the presence of multiple PET-positive nodules at different depths, illustrating the power of three-D imaging by PET to detect distinct internal tumor masses. Indeed, autopsy and histological analyses revealed that the livers of these animals harbored multiple liver tumors (Fig. 4C and E). *Ex vivo* BLI of the liver was performed with the excised liver (Fig. 4D), which was correlated with visible surface tumor (arrows in Fig. 4C and D), although the internal tumors revealed by BLI and PET imaging could not be seen by visual inspection.

Effective dual modality imaging of malignant HCC

Most liver tumors induced by DEN at 6 months were benign hepatomas characterized by uniform nuclear morphology and clear tumor margins (Fig. 4E). To determine if malignant HCC can be effectively imaged by BLI and PET, tumors were allowed to progress for three more months and the mice were imaged again. In one of these mice, dramatically strong signals were detected by both BLI and PET imaging (Fig. 5A and B). Upon autopsy, a single large tumor was found that showed strong BLI signal by *ex vivo* imaging (Fig. 5C and D). Histological analyses revealed the tumor was a malignant HCC that had invaded into surrounding normal liver parenchyma (Fig. 5E and F). Similar observations were made in two other malignant mice, suggesting that the *HCR* model may allow effective imaging of early pre-neoplastic lesions, hepatomas, as well as late stage malignant HCCs.

Discussion

In this study, we developed a novel hepatocarcinogenesis reporter (*HCR*) mouse model that allows non-invasive and longitudinal imaging of the entire natural process of liver tumor development and malignant progression. Our results demonstrate that the *HCR* model faithfully recapitulated dynamic regulation of endogenous Afp gene expression. More importantly, the *HCR* mouse enabled simple BLI detection of early stage neoplastic lesions before they became histologically apparent, while the later stages of tumor progression can be monitored by dual BLI and PET imaging. Unexpectedly, the *HCR* model also led to the characterization of testes as the only organ that strongly express Afp proteins in normal adult mice.

Research on liver cancer has been facilitated by mouse model systems, which share morphologic, histological, and molecular features with human HCC [21,22]. These include chemically-induced liver cancers using several carcinogens and genetically engineered mouse models. The advent of the *HCR* model described in this paper will significantly increase the power and utility of the mouse HCC models. Our data have demonstrated the successful application of *HCR* mice to monitor the DEN-induced liver tumor development and progression. By simply breeding of the *HCR* mice to other genetically engineered mouse models for HCC, the natural process of hepatocarcinogenesis by the defined molecular pathways can be imaged *in vivo*. Remarkably, as early as 2 months after DEN treatment, BL imaging detected significant *Afp* promoter-driven luciferase expression in the liver, long before the appearance of clear cell and basophilic foci that are characteristic of early neoplastic lesions. Thus HCR mice may enable much earlier detection of cellular changes associated with carcinogenic insults. Immunofluorescence analysis confirmed the multifocal expression of Afp in the liver, in what appeared to be hepatocytes. A significant body of evidence exists that hepatocarcinogen exposure leads to rapid proliferation of oval cells and transitory hepatocyte-like cells, both of which express Afp (Sell, 2008, and Abelev and Eraiser, 1999). Whether or not the Afp-positive cells constitute oval cells, transitory hepatocyte-like cells or mature hepatocytes, or a mixture of all these cell types is yet to be determined.

It should be noted that not all HCC developed in mice are Afp positive (Jalanko and Ruoslahti, 1979). A similar situation also exists in human HCC as well as HCC progenitor/stem cells.

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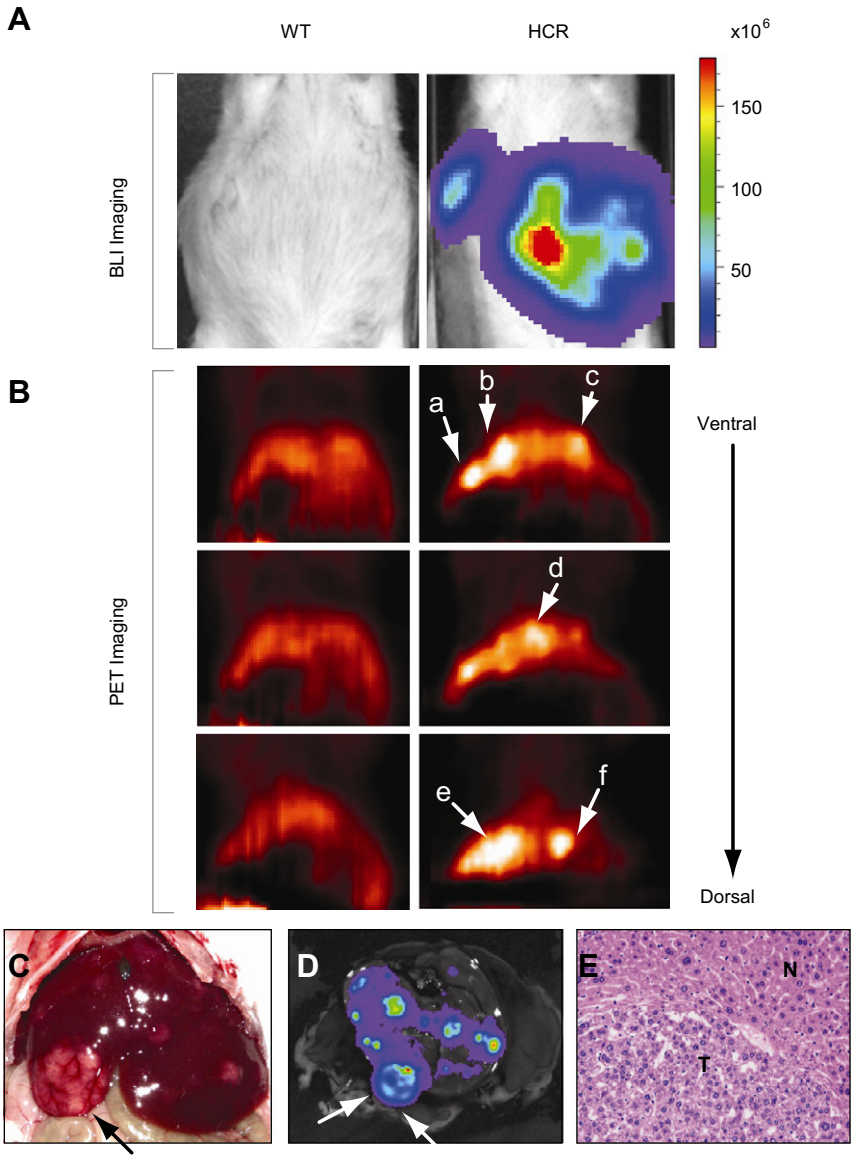

Fig. 4. BLI and micro-PET dual modality imaging of liver tumor mice 6 months after DEN treatment. (A) BLI of an *HCR* mouse (right) together with a wild-type control (left). Mice were treated as described in the Fig. 3 legend. Note the focal nature of BLI signals. (B) Micro-PET imaging of the same mice. Serial coronal sections from ventral to dorsal were collected, and three sections are presented. Note distinct patterns of PET signals detected at different depths from the ventral surface of the liver. (C) A photograph showing multiple visible liver tumors upon autopsy (top), most of which were Luc-positive based on *ex vivo* BLI as shown in (D). Arrows point to a large tumor visible by both visual inspection and BLI. (E) H&E analysis of a typical tumor. Most tumors were classified as benign hepatomas at this stage.

Intriguingly, AFP-positive, but not AFP-negative human HCC exhibit features of hepatoblasts or mature hepatocytes (Yamashita et al. 2008; 2009; Mishra et al. 2009), suggesting that AFP positive HCCs are derived from hepatocyte lineages. Thus, our model could be incorporated into the studies that model these specific types of AFP positive HCCs.

Between the dual imaging modalities, BLI provides a sensitive approach for detecting early neoplastic lesions, which represents an unprecedented tool in studying chemoprevention at a very early stage. On the other hand, both BLI and micro-PET could be used to detect larger hepatomas and malignant HCC at later stages during hepatocarcinogenesis. Although PET is less sensitive in detecting liver tumors at early stages, it does provide a unique opportunity for acquiring quantitative and 3-D information regarding both individual tumors as well as its relationship with the surrounding hepatic tissues.

Serendipitously, we have found that in adult mice, the knockin reporter was highly expressed in the male reproductive tract, independent of their expression in the HCC. The function of Afp in testis is still not clear, although Afp is known to be elevated in testicular cancer [23]. Nevertheless, the testicular BLI signal provides an appealing and convenient internal control for BLI signal quantitation.

In addition to the basic studies on the molecular basis of hepatocarcinogenesis, the *HCR* model will prove valuable in the development and evaluation of chemopreventive and therapeutic strategies for HCC. Moreover, recent studies have indicated that human AFP represents a marker for hepatoblasts and its

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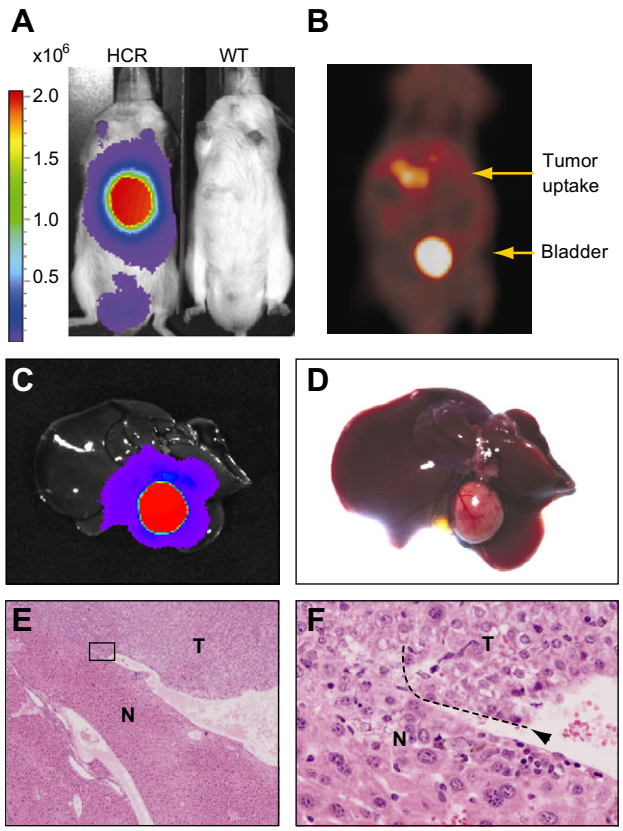

Fig. 5. BLI and micro-Pet imaging of malignant HCC. Mice were treated with DEN and imaged nine months later to allow sufficient time for tumor progression. (A) BLI of wild type and *HCR* mice *in vivo* as described in Fig. 3A. (B) The *HCR* mouse was also subject to micro-PET imaging. (C and D) A single tumor was identified upon autopsy that was highly positive by *ex vivo* BLI. (E and F) H&E staining of tumor sections. Note the highly heterogeneous nuclear morphology and extensive invasion of tumor cells into surrounding normal liver tissues.

immediate derivatives as well as the stem/progenitor-like cells of HCC [24,25]. Thus, the *HCR* mice reported here may also be used to monitor the activity of hepatocyte progenitor cells, particularly during the course of hepatocarcinogenesis.

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