Transgenic mouse models generated by hydrodynamic transfection for genetic studies of liver cancer and preclinical testing of anti-cancer therapy

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Hepatocellular carcinoma (HCC) is one of the most lethal cancers worldwide; however, the genetic mechanisms underlying its pathogenesis are incompletely understood. Genetically engineered mouse (GEM) models of HCC have been developed to elucidate the role of individual cancer-related genes in hepatocarcinogenesis. However, the expensive and time-consuming processes related to generating a GEM model discourage the development of diverse genotype models. Recently, a simple and inexpensive liver-specific transgenic approach was developed, in which a hydrodynamics-based transfection (HT) method was coupled with the Sleeping Beauty transposase system. Various HT models in which different oncogenic pathways are activated and/or tumor-suppressing pathways inactivated have been developed in recent years. The applicability of HT models in liver cancer research is expected to broaden and ultimately elucidate the cooperation between oncogenic signaling pathways and aid in designing molecular therapy to target altered pathways.

Hepatocellular carcinoma (HCC) is one of the most lethal cancers worldwide, ranking third among all cancer-related mortalities and accounting for 500,000–600,000 deaths annually.1,2 Many of the treatment modalities developed for HCC offer limited success. Moreover, the 5-year survival rate remains considerably low.3,4 Surgical resection or local ablation therapy are preferred treatment options for early-stage HCC. However, tumors recur in approximately 70% of these patients within 5 years.2 Molecular target therapy has been investigated intensively in recent years as a promising treatment option for patients with advanced HCC.5,6

Epidemiological and molecular studies have shown that HCC development spans several decades and is associated with diverse factors and conditions.7–10 Patients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection are at a much higher risk of developing HCC than noninfected individuals. Other risk factors for HCC include alcohol abuse, diabetes, and obesity, which cause chronic liver injury, leading to liver fibrosis and cirrhosis.11,12 Recent years have seen an accumulation of knowledge regarding the genetic and epigenetic changes associated with HCC development. High-throughput oncogenomic studies have revealed deregulated signal transduction pathways in hepatocarcinogenesis.13–17 Hundreds of genes are known to undergo genetic alterations during HCC development.18,19 Hence, it is of critical importance to identify oncogenes that play a major role in hepatocarcinogenesis and those that are genetic modifiers or bystanders. Further, the identification of oncogenic partners that cooperatively induce HCC will lead to a greater understanding of the molecular mechanism of pathogenesis, as well as provide new insights into molecular target therapy design. In this sense, genetically engineered mouse (GEM) models for HCC with alterations in candidate oncogenes or tumor suppressor genes should serve as valuable tools for clarifying the contribution of any given gene to hepatocarcinogenesis as well as the development of new drugs targeting specific oncogenic signaling pathways.20–22

HCC: A multistep Carcinogenic Process Requiring Oncogenic Collaboration

The development of HCC is considered a complex multistep process; each stage requires an additional genetic or epigenetic alteration (Fig. 1). Thus, it is believed that cells of origin will acquire multiple genetic mutations during carcinogenesis to become malignant cancer cells.23,24 To better understand the roles of cancer-related genes in HCC development, various
GEM models for HCC have been developed.\textsuperscript{20–22} Consistent with the idea of multistep carcinogenesis, GEM models for liver cancer have shown that oncogenic mutations within a single gene are generally highly inefficient at inducing liver cancer (Table 1). This implies that oncogenic collaborations among multiple cancer-related genes are likely required for liver cells to become malignant.

The Myc protein is a transcription factor that activates various genes promoting cell proliferation and growth, and it plays a critical role in the carcinogenesis of various cancers.\textsuperscript{52,53} Genetic analyses have revealed that c-Myc overexpression, which is commonly caused by genomic amplification, is present in up to 70% of viral and alcohol-related HCCs.\textsuperscript{54} Transgenic mice overexpressing c-Myc alone inefficiently develop liver tumors with a long latency (65–90 weeks) and an incidence rate of 55%.\textsuperscript{27} However, coexpression of c-Myc with an additional oncogene, such as epidermal growth factor (EGF) or E2F transcription factor 1 (E2F1) greatly shortens the latency of HCC and induces liver cancer in 100% of mice.\textsuperscript{27,30,47} Thus, oncogenic collaborations with additional oncogenes and growth factors is likely critical in c-Myc-induced hepatocarcinogenesis.

β-catenin, one of the key downstream effectors of the Wnt signaling pathway, plays an important role in liver development and regeneration.\textsuperscript{55,56} Activation of the Wnt/β-catenin pathway is found in 30% of human hepatic tumors, occurring through either an activating mutation within β-catenin itself or reduced expression of APC, a negative regulator of β-catenin.\textsuperscript{57} GEM models expressing an activated form of β-catenin or liver-specific APC knockout induce hepatomegaly or HCC with a long latency, while coexpression of activated β-catenin with an additional oncogene, such

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**Table 1. Genetically engineered mouse models for HCC**

| Genes       | Incidence rate (%) | Latency (weeks) | Refs. |
|-------------|--------------------|-----------------|-------|
| AAT         | 100                | 52–90           | 25    |
| APC\textsuperscript{+/−} | 67            | 38              | 26    |
| c-myc       | 55                 | 65–90           | 27    |
| E2F-1       | 33                 | 52              | 28–30 |
| EGF         | 100                | 24–36           | 31,32 |
| HBx         | 84                 | 52–104          | 33–35 |
| HCV core    | 32                 | 80–105          | 36,37 |
| NEMO\textsuperscript{+/−} | 100          | 52              | 38–40 |
| P53\textsuperscript{+/−} | 10.9           | 60              | 41    |
| PTEN\textsuperscript{+/−} | 66           | 44              | 42    |
| SV40 T-antigen | 100         | 20              | 43    |
| TAK1\textsuperscript{+/−} | 80            | 39              | 44    |
| TGF-α       | 50                 | >52             | 45,46 |
| c-myc + E2F1| 100                | 26–39           | 30,47 |
| c-myc + EGF | 100                | 12–18           | 32    |
| β-catenin(Δex3) + HRAS\textsuperscript{12V} | 100       | 8              | 48    |
| KRAS\textsuperscript{12D} + HBx | 62.5     | 34              | 49    |
| P53\textsuperscript{+/−} + c-myc | 75      | 21              | 50    |
| PTEN\textsuperscript{+/−} + GRP94\textsuperscript{−/−} | 80 | 25              | 51    |
as HRAS$^{G12V}$, leads to HCC as early as 8 weeks following oncogenic expression.\textsuperscript{26,48,58,59}

Phosphatase and tensin homolog (PTEN) is a tumor suppressor that negatively regulates the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway, which regulates cell survival, proliferation, and energy metabolism.\textsuperscript{60} Absence or reduced expression of PTEN was found in approximately 40% of HCC cases.\textsuperscript{61} GEM models with knockout of PTEN in the liver resulted in HCC in 66% of mice at 44 weeks.\textsuperscript{42} A concomitant knockout of Grp94, a major endoplasmic reticulum (ER) chaperone protein, highly accelerated the development of HCC in PTEN-null livers; no tumors were found in the livers of PTEN-null mice, while 100% of mice with PTEN and Grp94 double knockout developed HCC by 8 months of age.\textsuperscript{51}

In humans, P53 inactivation is a common event in a variety of cancers including HCC.\textsuperscript{62,63} Mutations within P53 are found in aflatoxin-induced HCC at a frequency of 50%, and in HCC not induced by aflatoxin at 28–42%.\textsuperscript{64–66} GEM models have revealed that HCC development induced by p53 inactivation is greatly accelerated when additional oncogenes are expressed.\textsuperscript{41,50}

### Hydrodynamics-Based Transfection (HT) Model for HCC

Although traditional GEM models have significantly contributed to our understanding of the molecular mechanisms underlying hepatocarcinogenesis, development of a GEM model usually involves expensive and time-consuming processes, such as microinjection of transgenes into pronuclei (for transgenic models), transfection and manipulation of embryonic stem cells (for knockout models), as well as implantation and subsequent breeding. Additionally, proto-oncogenes or tumor suppressor genes are often critical for embryonic or fetal development, and thus genetic manipulations can cause unwanted developmental side effects.

To overcome these issues, a very elegant and simple method for liver transgenesis was recently developed in which naked DNA plasmids encoding a gene of interest were directly delivered into the liver via HT.\textsuperscript{67–70} The HT method uses the physical force generated by rapid injection of a large volume of DNA solution into the lateral tail vein. This creates increased pressure in the vena cava, pushing the DNA solution into the large hepatic vein and subsequently hepatic tissue. The fenestrae of liver sinusoids are enlarged by the hydrostatic pressure, allowing plasmids to pass through capillary walls and reach the hepatocytes.\textsuperscript{71} HT is highly specific for hepatocytes, thus enhancing the versatility of this method in liver-specific transgenesis.\textsuperscript{68}

One major limitation of HT is that prolonged expression of a transgene is difficult to achieve. Plasmids delivered into hepatocytes are eventually degraded without chromosomal integration, and furthermore, gene expression from the episomal DNA is rapidly turned off within several days.\textsuperscript{68,72,73} This problem can be resolved by use of the Sleeping Beauty (SB) transposon system.\textsuperscript{74–76} The SB system consists of a transposon made up of a gene expression cassette flanked by a specific DNA sequence of inverted repeats and a source of transposase enzyme (SB transposase) that recognizes the DNA sequence. During SB-mediated transposition, the SB transposase excises the transposon from the delivered plasmid DNA and integrates into any one of approximately $10^8$ TA dinucleotide base pairs scattered randomly throughout the genome, guaranteeing random chromosomal insertion of the transgene.\textsuperscript{73}

This time and cost-effective methodology has allowed for diverse HCC models expressing various oncogenes to be developed rapidly. The first report of an HCC model generated by HT was published in 2005 by Dr. Largaespada’s research group.\textsuperscript{77} In that study, HT of transposons expressing activated NRAS (NRAS$^{G12V}$) with plasmids encoding SB transposase induced multiple tumors in livers of p19Arf-null mice approximately 1 month post-HT, demonstrating the applicability of HT technology coupled with the SB transposon system for generating HCC transgenic models. Soon after publication, Tward et al. used the simple liver-specific transgenic approach and reported that HT of transposons encoding Met and activated β-catenin gave rise to HCC in 74% of mice within 1 month.\textsuperscript{78} Since then, dozens of HCC models expressing a variety of oncogenes have been developed.

### HT Models for Oncogenic Collaboration Studies in HCC

Considering the diverse genetic factors and multistep carcinogenic process in HCC, the relationships between tumor-related genes are of high importance for understanding the genetic mechanisms involved in HCC pathogenesis. To investigate potential oncogenic collaborations between two cancer-related genes using traditional GEM models, breeding experiments between two GEM strains carrying individual oncogenic mutations should be performed to acquire genetic alterations in the two target genes. Furthermore, mutations within tumor suppressor genes are, in general, only effective when both alleles are mutated, requiring a well-designed mating strategy. Such multiple time-consuming breeding methods limit the generation of diverse mouse models with alternations in various combinations of proto-oncogenes and tumor suppressor genes. However, this problem can easily be circumvented in HT models; coexpression of two oncogenes in hepatocytes can be achieved by simply mixing transposons encoding individual oncogenes and performing HT using the transposon mixture. Thus, transgenic livers carrying various combinations of oncogenic mutations can be developed efficiently using this approach, suggesting the versatility of HT models in elucidating potential oncogenic collaborations in HCC development.

HT models have shown increased tumor development when specific combinations of oncogenic mutations were introduced in the liver. For example, mice expressing activated AKT developed HCC approximately 6 months post-HT.\textsuperscript{79} The latency was significantly shortened when activated AKT was coexpressed with NRAS$^{G12V}$ or an activated form
of β-catenin. In addition, HT models expressing short hairpin RNA downregulating p53 (shp53) alone failed to induce tumors. However, coexpression of shp53 with gene X of HBV (HBx) induced HCC 139 days post-HT. Lee et al. successfully used HT to develop double transgenic mouse models expressing an activated form of β-catenin together with NRAS<sub>G12V</sub> or a dominant-negative Spry2 (SPRY2<sup>Y55F</sup>), revealing that an oncogenic collaboration between Wnt and RAS signaling pathways is critical in hepatocarcinogenesis. Further, Xu et al. investigated oncogenic cooperation between Bmi1 and activated RAS during hepatocarcinogenesis using an HT model. They concluded that neither Bmi1 nor activated RAS alone was sufficient to develop HCC, while coexpression induced HCC in 78.6% of mice between 15 and 30 weeks post-HT. Patil et al. also reported that coexpression of cyclinD1 and c-Met resulted in HCC, while expression of either gene individually failed to induce tumors, revealing an oncogenic collaboration between these two genes in HCC.

To investigate a potential oncogenic collaboration among RAS, c-Myc, Hedgehog, and P53 signaling pathways in HCC development, Ju et al. used an HT-mediated transgenic approach. Transposons encoding HRAS<sub>G12V</sub> (for activation of RAS signaling), a constitutively active form of Smo (SmoM2, for activation of Hedgehog signaling), c-Myc (for activation of Myc-mediated signaling), and shp53 (for suppressing P53 signaling) were developed first, and then HT was performed using various combinations of oncogenes (Fig. 2a). Mice transfected with HRAS<sub>G12V</sub> and shp53 showed numerous nodules as early as 4 weeks post-HT (Fig. 2b and Table 2). Tumors were found in the livers of c-Myc plus HRAS<sub>G12V</sub> mice 2 months post-HT, while c-Myc plus shp53 mice showed tumors with a low frequency around 7 months post-HT.
post-HT (Fig. 2b and Table 2). No tumors were observed in livers coexpressing SmoM2 and c-Myc, HRASG12V or shp53. Of note, histopathologic examination revealed that livers of HRASG12V and shp53 mice showed highly malignant and poorly differentiated HCCs, while moderately differentiated and well-differentiated HCCs were observed in HRASG12V plus c-Myc and c-Myc plus shp53 mice, respectively. Thus, depending on the oncogenic combination, it was determined that the incidence rate, tumor latency and histopathologic features of tumors were highly variable, revealing that collaborations among different oncogenic signaling pathways greatly affect HCC pathogenesis.

The studies described above demonstrate that HT models can be applied efficiently to investigate oncogenic collaborations among various signaling pathways during hepatocarcinogenesis. The application of HT transgenic models to study HCC genetically will likely increase due to the simplicity and reduced time and resources of the method compared with traditional transgenic models.

**Application of the HT Model in Preclinical Testing of HCC Anti-Cancer Therapy**

Xenograft models for HCC have been preferred over transgenic models in preclinical studies, mainly due to the rapid generation of human cancers in vivo. However, criticism has been raised in applying xenograft models for preclinical research from the microenvironmental perspective. To develop xenograft models for HCC, established human cancer cells are transplanted into immunocompromised mice. Considering the important roles of immune cells and inflammatory responses in HCC development, the impaired immune system in xenograft models provides an unnatural microenvironment in which tumor development can go awry. Furthermore, cancer cells are often transplanted ectopically, mainly in subcutaneous locations, which hardly recapitulates the liver microenvironment and thus enhances the problem of disparate microenvironments. Lastly, the neovascularature that develops in xenografts often shows histologically distinct features when compared with human tumors, possibly explaining why many anti-angiogenic agents effective in xenograft models have failed in clinical trials.

In HT models for HCC, however, tumors develop under the natural microenvironment of the liver. Mice with functional immune systems are used to generate HT models and tumors develop in the orthotopic location. Furthermore, the HT model for HCC is easily generated and tumors can be efficiently induced in the liver within a short period of time. It has been reported that HCC development can be achieved within 1 month post-HT, depending on the oncogene(s) expressed from transposon vectors. Thus, this fast and simple method for the induction of autochthonous tumors would greatly benefit preclinical studies of anti-cancer therapy for HCC.

Rudalska et al. showed that treatment with sorafenib in HCC models generated by HT improved the median survival of HCC-bearing mice by 20–30%. The survival advantage in these mice highly resembled that in HCC patients treated with sorafenib (35% median survival advantage compared with those given placebo). However, sorafenib treatment was highly effective in HCC xenograft models, exhibiting a median survival advantage of 50–100%. Tumor growth regression was even observed in xenograft mouse models treated with sorafenib at a higher dose. Thus, it is believed that HT models for HCC can complement xenograft models for predicting the treatment response in human patients.

The HT methodology can easily provide suitable HCC models for preclinical testing of drugs targeting specific signaling pathways. For example, to investigate an anti-tumor effect by targeting the AKT-mTOR signaling pathway, Wang et al. developed a murine HCC model induced by an activated form of AKT via hydrodynamic transfection and administered rapamycin, an mTORC1 inhibitor, to the mice following HT. They found that 100% of the rapamycin-treated mice survived, whereas none survived in the vehicle-treated group at 6 weeks post-HT. Similarly, to investigate whether an inhibition of the Notch signaling affects tumor development in the liver, Huntzicker et al. used an HT model of liver cancer induced by activated forms of RAS and AKT, which exhibited an active Notch signaling. They treated mice with antagonistic antibodies specific to Notch2 or the Notch ligand, jagged1, for 5 weeks following hydrodynamic transfection and found that the inhibition of the Notch signaling pathway significantly reduced tumor burden in the liver. Another study using an HT model of HCC for preclinical testing of molecular target therapy was reported by Dr. Scott Lowe’s research group. Using a liver cancer model with YAP overexpression and p53 suppression generated by hydrodynamic transfection, they showed that inhibition of Nestin using a transposon-mediated knockdown strategy completely suppressed tumor formation in the liver.

In addition to the application to molecular target therapy, an HT model of HCC has been applied to investigate chemoprevention of HCC by dietary carbohydrate restriction. Malignant cells
heavily rely on glucose for increased energy production and metabolic processes required for rapid cellular proliferation. Studies have shown that dietary carbohydrate restriction is a promising anti-cancer therapy. To test a tumor prevention effect of dietary carbohydrate restriction in hepatocarcinogenesis, Lee et al. used an HT model of HCC induced by an activated RAS and p53 suppression. Mice were fed an isocaloric carbohydrate-restriction diet beginning 2 weeks prior to hydrodynamic transfection and throughout the 6-week experiment. Liver cancer development in the HT mouse model was significantly suppressed by the dietary carbohydrate restriction. This suggests that HT models can be applied for preclinical testing of various HCC anti-cancer strategies.

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