Progression of HCC in Mice Is Associated With a Downregulation in the Expression of Hepatocyte Nuclear Factors

Natalia L. Lazarevich,1 Oksana A. Cheremnova,1,3 Ekaterina V. Varga,1 Dmitry A. Ovchinnikov,1,3 Elena I. Kudrjavtseva,1 Olga V. Morozova,2 Daria I. Fleishman,1,4 Natalia V. Engelhardt,1 and Stephen A. Duncan5

Hepatocyte nuclear factors (HNF) play a critical role in development of the liver. Their roles during liver tumorigenesis and progression of hepatocellular carcinomas (HCC) are, however, poorly understood. To address the role of HNFs in tumor progression, we generated a new experimental model in which a highly differentiated slow-growing transplantable mouse HCC (sgHCC) rapidly gives rise in vivo to a highly invasive fast-growing dedifferentiated variant (fgHCC). This in vivo model has allowed us to investigate the fundamental mechanisms underlying HCC progression. A complete loss of cell polarity, a decrease in cell-cell and cell-extracellular matrix (ECM) adhesion, elevation of telomerase activity, and extinction of liver-specific gene expression accompanies tumor progression. Moreover, cells isolated from fgHCCs acquired the ability to proliferate rapidly in culture. These alterations were coupled with a reduced expression of several liver transcription factors including HNF4, a factor essential for hepatocyte differentiation. Forced re-expression of HNF4α1 in cultured fgHCC cells reversed the progressive phenotype and induced fgHCC cells to re-establish an epithelium and reform cell-ECM contacts. Moreover, fgHCC cells that expressed HNF4α1 also re-established expression of the profile of liver transcription factors and hepatic genes that are associated with a differentiated hepatocyte phenotype. Importantly, re-expression of HNF4α1 in fgHCC reduced the proliferation rate in vitro and diminished tumor formation in congenic recipient mice. In conclusion, loss of HNF4 expression is an important determinant of HCC progression. Forced expression of this factor can promote reversion of tumors toward a less invasive highly differentiated slow-growing phenotype. (HEPATOLOGY 2004;39:1038–1047.)

Hepatocellular carcinoma (HCC) is one of the world’s most common cancers, with chronic hepatitis B and C infection and prolonged exposure to hepatocarcinogens being the major risk factors.1 The development of a malignant phenotype is considered a multi-step process that results from the accumulation of genetic alterations. The consequence of these alterations is the evolution of a tumor towards a more aggressive phenotype. HCC progression from a well differentiated to a less differentiated form is accompanied by striking changes in the morphological and genetic properties of the cells and is one of the most critical steps of liver tumorigenesis.2,3 Hepatic tumor progression is defined by a decrease in differentiation, an extinction of tissue-specific gene expression, acceleration of cell proliferation, loss of epithelial morphology, increased invasiveness, and ultimately metastasis. While a variety of inter- and intracellular signaling pathways essential for the control of liver function and proliferation have been described, the mo-
The molecular basis underlying HCC progression and its relationship to normal cell differentiation remains obscure.

Maintenance of hepatocyte differentiation and control of liver-specific gene expression is attributed in large part to hepatocyte nuclear factors (HNFs). This class of proteins includes five families of transcriptional regulators: HNF1, HNF3, C/EBP, HNF4, and HNF6 (as previously reviewed). In particular, the nuclear receptor HNF4 is a key regulator of both hepatocyte differentiation during embryonic development and maintenance of a differentiated phenotype in the adult. Differences in the biologic properties of experimental systems and tumor samples analyzed have led to conflicting reports concerning the role of HNFs in hepatocarcinogenesis. We therefore sought to elucidate the role of liver transcription factors in HCC progression by generating a new experimental model in which a minimally invasive slow-growing transplantable differentiated mouse HCC (sgHCC) rapidly gives rise in vivo to a highly invasive dedifferentiated fast-growing variant (fgHCC). Using this model, we demonstrate that HNF4 has a critical role in the progression of HCC toward an aggressive phenotype.

Materials and Methods

Generation of HCC

Primary tumors were induced in male F1 hybrid (C57B1/DBA) mice by intraperitoneal injection of diethylnitrosamine (90 μg/g body weight) followed by promotion with phenobarbital (0.5% in drinking water for 14 months). The tumors were passaged by subcutaneous transplantation in F1 C57B1/DBA mice. All experimental procedures were performed according to ICCRC’s guidelines for laboratory animal use.

Cell Lines and Culture Conditions. All cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% of fetal calf serum (Gibco-BRL, Rockville, MD) and antibiotics. Where indicated, cells were grown on 0.1% gelatin-coated tissue culture dishes.

Transfection and Selection. Cells were co-transfected with either pLEN4S-HNF4 and pBabePuro, or pCMV-HNF4-Flag vector and pCMV-neo plasmids using DOTAP as directed (Roche). Stable transfected cells were selected for resistance to puromycin (5 μg/ml) or G418 (350 μg/ml).

RNA Isolation and Analyses

Total cellular RNA was purified using Qiagen RNeasy kit and reverse transcription polymerase chain reaction (RT-PCR) was performed as previously described. Primer sequences are available on request.

Proliferation Analyses

The proliferation rate was determined by BrdU incorporation in five independent experiments. The cells were incubated in BrdU-containing media for 2 hours and BrdU incorporation identified by immunohistochemistry (anti-BrdU Ab Sigma, 1:1,000). For proliferation kinetics analyses, 5 × 10^4 cells were cultured in triplicate and counted daily.

Tumor Growth Inhibition

Kinetics of tumor formation was estimated by subcutaneous injection of 5 × 10^6 cells into congenic mice (5 animals per each cell type). Tumor size was measured using a ruler to determine tumor lengths and converted to volume (diameter × diameter × length × π/6).

Immunohistochemistry

Cell cultures or tumor cryosections were fixed with cold methanol at –20°C and analyzed by an indirect peroxidase method as described previously. The primary antibodies used were rabbit polyclonal antibodies to collagen type IV 1:100 and rat monoclonal antibodies to ZO-1 1:100 (Chemicon, Temecula, CA).

Telomerase Activity

Telomerase activity was assayed by the telomeric repeat amplification protocol (TRAP) essentially as outlined by Kim and Wu.

Results

Comparison of Basic Biologic Properties of sg- and fg-HCC Variants

Several transplantable HCCs were established from primary mouse tumors that were induced using a diethylnitrosamine/phenobarbital protocol and propagated by subcutaneous transfer between mice. A fgHCC arose spontaneously in vivo from one of the sgHCC strains on the third passage. The parental sgHCC exhibited an in vivo passage-to-passage interval of 5–7 months, while the fgHCC strain required passage every 2 weeks (Fig. 1). While the overall development of HCC is a multistep process, the rapidity of the change from a sgHCC to a fgHCC suggested that this progression toward an invasive phenotype resulted from a single molecular event and implied that a comparison of sgHCC and fgHCC could elucidate fundamental mechanisms underlying HCC progression.

We had shown previously that the sgHCC is a partially solid carcinoma composed of large, moderately differentiated, octaploid cells. Trabeculae could be seen within the carcinoma that were several cell layers thick (Fig. 2A). In addition, mitotic cells were undetectable in the slow
growing tumor. In contrast to the sgHCC, the fgHCC was a small-cell anaplastic tumor that had lost its trabecular structure and contained cells with mainly diploid nuclei (Fig. 2A). Mitotic figures were commonly identified in fgHCC tumors, which is consistent with the observed rapid proliferation rate. In addition, Fig. 3 shows that we identified an increase in telomerase activity in fgHCC extracts compared to controls as defined by a typical ladder of 6 nucleotide base pair increments using a TRAP assay. Importantly, both sgHCC and fgHCC variants maintained their morphologic phenotype through multiple passages in vivo.

Loss of cell-cell and cell-ECM contacts usually accompanies the progression of tumors toward a metastatic phenotype. We therefore compared changes in the adhesive properties of sgHCC and fgHCC. The sgHCC contained a basal membrane comprising of type I and IV collagens (Fig. 2A), laminin, entactin, and fibronectin (not shown). Moreover, each cell closely interacts with the matrix covering the basal space. In contrast to the sgHCC, ECM components are synthesized but not associated with the membranes, and cell-matrix interactions are disarrayed in the fgHCC (Fig. 2A). We also found that sgHCC cells strongly express E-cadherin (Cdh1), a cell adhesion molecule (Fig. 2C), and the gap junction protein connexin 32 (Gjb1) (Fig. 2B). The tight junction protein ZO-1 was identified close to the membranes of cells within the sgHCC by immunohistochemistry, indicating that these cells formed polarized epithelia. In contrast, fgHCC cells did not exhibit membrane staining of ZO-1 (Fig. 2A) and showed a vastly diminished expression of Gjb1 and Cdh1.

Fig. 1. HCC genealogical tree. Schematic depicts the in vivo transplantation schedule of sgHCC from a primary tumor established in 1995. Each box represents an individual animal injected by slow-growing (grey boxes) or fast-growing (black boxes) variants. Arrow indicates the animal used for cell culture (H33) isolation. Time scale in years and months is presented on the right.

Fig. 2. Epithelial morphology is lost during HCC progression. (A) Sections from sgHCC and fgHCC tumors were stained with hematoxylin-eosin (H&E) or by immunohistochemistry for collagen type IV (Col IV) or ZO-1 (magnification ×200). Large arrow denotes collagen IV staining around trabeculae and small arrow indicates ColIV staining around the cell perimeter in the sgHCC. (B) Northern-blot of total cellular RNA from adult normal adult liver (lane 1), two independently induced sgHCC tumors (lanes 2, 3), an fgHCC tumor (lane 4), and H33 cells in culture (lane 5). Equivalence of loading among all samples was verified by ethidium bromide staining (28S band). Connexin 32 (Gjb1) and α3 integrin (Itga3) were detected using radiolabeled DNA probes. (C) RT-PCR analysis of steady-state mRNA levels of E-cadherin (Cdh1), β-catenin (Catnb), Snail (Snai1), and Hprt in normal adult liver (lane 2), sgHCC (lane 3), fgHCC (lane 4), and H33 cell culture (lane 5). Amplification using Hprt primers confirmed that all samples started with similar amounts of RNA. RT-PCR reactions completed without reverse transcriptase confirmed the absence of genomic DNA (lane 1).
as well as an accompanying increase in Snai1 mRNA levels (Fig. 2B, C). This seems significant given that Snai1 encodes a transcriptional repressor of Cdh1 gene expression. We also observed an increase in expression of β-catenin (Catnb) and integrin subunit α3 (Itga3) (Fig. 2B, C). Integrin α3 is predominantly expressed in immature and transformed hepatocytes and is thought to affect tumor invasiveness and metastasis and β-catenin has been shown to modulate hepatic cell proliferation. From these data, we conclude that the transition from sgHCC to fgHCC is accompanied by a loss in cell-cell contact and in epithelial morphology.

fgHCCs Are Less Differentiated Than sgHCCs and Fail to Express Characteristic Hepatocyte Markers

The observed morphologic changes that occurred within the tumor cells as the sgHCC transformed to a fgHCC implied that they were reverting to a less-differentiated state. Hepatocyte de-differentiation is accompanied by loss of expression of genes that impart mature hepatocyte function, as well as the ectopic expression of genes, such as alpha-fetoprotein (Afp), that are normally restricted to fetal hepatoblasts. We therefore compared steady-state levels of messenger RNAs (mRNAs), which are normally found in differentiated hepatocytes, in sg- and fgHCCs, cultured H33 cells (see below), and normal adult liver using RT-PCR (Fig. 4). Figure 4 shows that Afp mRNA levels were undetectable in the differentiated hepatocytes of the adult liver; however, Afp mRNAs were clearly detected in sgHCC cells. This expression of the fetal hepatoblast marker Afp indicates that sgHCC cells are less differentiated than adult hepatocytes. Surprisingly, Afp mRNA could not be detected in fgHCC samples; however, when fgHCC cells were grown in culture, Afp mRNA was then readily identified. We considered two possible explanations for this result. One was that the fgHCC tumors had lost their ability to express any hepatic marker genes and were, therefore, severely de-differ-
entiated, but in response to culture began to re-establish a hepatic phenotype. Alternatively, the fgHCC tumors contained cells that were, in fact, fully differentiated and so had repressed levels of Afp expression and upon culture began a de-differentiation program that led to expression of Afp mRNA. To distinguish between these possibilities, we examined the expression of an array mRNAs that are characteristically expressed in mature differentiated hepatocytes. Figure 4 shows that sgHCC tumors have a gene expression profile that closely resembles that of adult hepatocytes with the exception of Afp whose expression was increased. However, when we examined mRNA levels in fgHCCs, we found that most hepatic mRNAs were undetectable or severely repressed. These included mRNAs encoding albumin; aldolase b; α1-antitrypsin (SERPIN1A); transthyretin; phenylalanine hydroxylase (Pah); cytochrome p450 subfamily IIIa (CYP3A); cholesterol 7α-hydroxylase (CYP7A); L-type pyruvate kinase; glucokinase; L-type fatty acid binding protein; hepatocyte growth factor-like protein; apolipoproteins (APO) A1, A2, A4, B, C2, and C3; and retinol binding protein. We also found that expression of a small number of hepatic mRNAs was retained in the fgHCC cells, such as those encoding ApoC1 and E, transferrin, and IGF-1. We believe these findings—that expression of a wide array of hepatic genes is lost in fgHCC cells—support the proposal that Afp expression is absent from fgHCC cells because they are poorly differentiated compared to either adult liver cells or even those of sgHCCs. It is worth noting, however, that the fgHCC tumors are clearly of hepatic origin because they express a small subset of liver mRNAs.

**Tumor Progression Is Accompanied by Changes in Expression of Liver Transcription Factors**

Hepatocyte differentiation is controlled by the combinatorial action of multiple liver transcription factors, including HNF1, HNF3, HNF4, C/EBP, and HNF6.\(^{28}\) These factors establish a regulatory network that provides prompt regulation of diverse liver functions at the level of gene expression. We therefore sought to test whether the cellular de-differentiation that occurred during progression of sgHCC to fgHCC was accompanied by changes in expression of specific liver transcription factors. Figure 5 shows the result of comparing levels of steady-state mRNAs encoding liver transcription factors in adult liver, sgHCCs, and fgHCCs using RT-PCR. While the majority of the mRNAs encoding liver transcription factors were detected at comparable levels in adult liver and in sgHCCs, several were undetectable or reduced in fgHCCs. These included Hnf6 (both α1 and α7 isoforms), Hnf1α (Tcf1) and –1β (Tcf2), Foxa3, Hnf6 (One-

| Gene | sgHCC1 | sgHCC2 | fgHCC1 | fgHCC2 | liver | H33 |
|------|--------|--------|--------|--------|-------|-----|
| Hprt |        |        |        |        |       |     |
| Hnf4α1 |   |        |        |        |       |     |
| Hnf4α7 |   |        |        |        |       |     |
| Hnf1α |   |        |        |        |       |     |
| Hnf3γ |   |        |        |        |       |     |
| Hnf6 |   |        |        |        |       |     |
| Ftf |   |        |        |        |       |     |
| Pxr |   |        |        |        |       |     |
| Cebpα |   |        |        |        |       |     |
| Cebpβ |   |        |        |        |       |     |
| Cebpd |   |        |        |        |       |     |
| Coup-tfl |   |        |        |        |       |     |

Fig. 5. Changes in transcription factor gene expression accompany HCC progression. RT-PCR analysis of steady-state levels of mRNAs encoding Hnf3 (α1 and α7 isoforms), Hnf1α (Tcf1), Hnf1β (Tcf2), Hnf3α (Foxa1), Hnf3β (Foxa2), Hnf3γ (Foxa3), Hnf6 (One cut1), Cebpα (Cebpα), fetoprotein transcription factor (Nr5a1/Fit), and the pregnane-x-receptor (Nr112/Per). We also observed a considerable up-regulation in Coup-tfl (Nr2f1) mRNA levels in fgHCC compared to adult liver and sgHCC. These data may be functionally significant because Coup-tfl and Coup-tfl (Nr2f1) are nuclear receptors that can act as antagonists of HNF4 in regulating expression of several liver genes.\(^{29}\) We also found that expression of Hnf4α7 mRNA is induced in sgHCCs (Fig. 5). HNF4α7 has been described as the predominant fetal isoform of HNF4 and is consistent with our proposal that the sgHCC cells are partially de-differentiated.\(^{30}\)
HNF4 Re-expression in Cultured fgHCC Cells Increases Hepatic Gene Expression, Induces a Transition to an Epithelial Phenotype and Suppresses Proliferation

Studies in mice have demonstrated that HNF4 is a central regulator of a differentiated hepatocyte phenotype. This suggested that loss of HNF4 may be the predominant mechanism through which sgHCCs progress to the less differentiated fgHCC phenotype. If this were true, we predicted that expression of HNF4 within fgHCC cells may cause them to re-differentiate and re-establish hepatocyte characteristics.

To test this, we attempted to generate cultures of cells, called H33, from fgHCC that could be transfected with an HNF4 expression plasmid. We found that such cultures were easily obtained by simple mechanical dispersion of the fgHCC after short-term trypsin treatment. The success in this approach presumably reflected the decrease in cell-cell and cell-matrix anchorage within the fgHCC. H33 cells exhibited a weak anchorage to substrates and a loss of contact inhibition that resulted in a tendency toward three-dimensional growth. Cultivation of H33 cells in Matrigel, serum-free media, or in the presence of differentiation/promoting agents and growth factors such as dimethyl sulfoxide, dexamethasone, fibroblast growth factor 1, epidermal growth factor, or hepatocyte growth factor (HGF) failed to induce differentiation of fgHCC cells in culture (not shown). H33 cells, like fgHCC, failed to express the majority of genes that are normally expressed by hepatocytes including Hnf4 (Fig. 4, 5). In contrast to fgHCC tumors, the sgHCCs possessed strong cell-cell and cell-matrix contacts and attempts to obtain cell suspensions using EDTA/collagenase perfusion were unsuccessful. Stable G418-resistant cell lines were established from fgHCC-H33 cells that had been co-transfected with plasmids that either expressed a FLAG (Sigma-Aldrich Co.) epitope-tagged version of HNF4α1 from the cytomegalovirus (CMV) promoter or a cassette that conferred resistance to neomycin. A control cell line H33-neo that did not express HNF4α1 was generated by transfecting solely with the neomycin-resistance expression plasmid. Three cell lines were selected for study that were judged by immunoblot analysis using anti-FLAG antibodies (not shown) and by RT-PCR to abundantly express HNF4 (Fig. 6). Figure 4 shows that, in contrast to fgHCC-H33 cells, expression of several liver genes was re-established in H33 cells expressing HNF4. These genes included ApoA1, -A4, -B, α1-antitrypsin, Cyp7a, and Gjb1. Moreover, Fig. 6 shows that when HNF4 was expressed in fgHCC cells they re-established expression of several other liver transcription factor mRNAs including Hnf1α, Hnf6, Nr2f1, and Foxa3 and a downregulation of Coup-tfl.

Immunocytochemical staining using anti-FLAG antibodies found that there was a decrease in the number of HNF4-FLAG expressing cells during the first weeks of cultivation (not shown) possibly due to a reduction in cell proliferation after HNF4 re-expression. To overcome this problem, we generated stable cell lines from the fgHCC-H33 cells that expressed low levels of HNF4 by transfecting with the pLEN4S-HNF4 plasmid. In this construct, transcription of an Hnf4α1 cDNA is controlled by the metallothionein promoter, which in the absence of heavy metals regulates expression at low levels. As was the case for cell lines expressing HNF4 from the CMV promoter, pLEN4S transfected H33 cells again expressed Hnf4 target genes including Hnf1α (Fig. 7B). After approximately one month in culture, fgHCC cells expressing HNF4 began to form epithelial sheets (Fig. 7A). We were able to confirm that this was a true epithelial conversion because ZO-1 staining was clearly detected at the plasma membranes, indicating that these cells had formed tight junctions (Fig. 7A). In addition, fibrils of collagen type IV present along cell-cell interfaces, which are a characteristic of epithelial formation, could be identified by immunohistochemistry in HNF4 transformed H33 cells. Importantly, no formation of an epithelial morphology or localization of junction proteins was observed in the control H33 cells (Fig. 7A). If HNF4 expression reversed tumor progression, as was suggested by the previous experiments, we predicted that forced expression of HNF4 would reduce the rate of cell proliferation. We therefore determined the rate of DNA synthesis in HNF4 transformed H33 cells by a BrdU incorporation assay. Figure 8A shows...
that there was approximately a 1.5-fold lower incorporation of BrdU in HNF4 expressing H33 cells compared to control H33 cells. This reduction in DNA synthesis translated into considerably diminished proliferation kinetics of cells expressing HNF4 (Fig. 8B). These data implied that HNF4 expression should reduce the aggressiveness of HCC toward a malignant character by inducing re-differentiation toward a hepatocyte phenotype and by suppressing tumor cell proliferation.

**Discussion**

In this work, we have characterized a new experimental model of tumorigenesis in which a differentiated slow-growing transplantable mouse HCC rapidly gave rise to a fast-growing de-differentiated variant. We are confident that the fgHCC tumor is a direct descendant of the sgHCC for a number of reasons. First, fgHCC tumor or cultured cells express unaltered levels of some liver transcription factor genes including *Hnf3α*, *β*, *Cebpβ*, and *δ* and, in addition, maintain diminished but detectable expression of a subset of hepatoblast marker genes such as *Transferrin*, *Apoc1*, -c2, -b, -e; and *Hgfl*. We did observe some relatively subtle differences in the levels of marker gene expression between independent fgHCCs and cultured cell lines and we believe this reflects slight differences in the extent of tumor progression in these isolates. Moreover, our finding that forced expression of HNF4 causes re-differentiation of the fgHCC cells toward a hepatocyte phenotype also suggests that the cells are originally of hepatic origin.

The observed change from a slow-growing differentiated to a fast-growing undifferentiated phenotype appeared to occur rapidly, implying that the transformation was a consequence of dysfunction of a limited set of genes or perhaps even a single event. Moreover, it seemed likely that HCC progression might result from alterations in expression of transcription factors because the observed tumor progression was accompanied by significant changes in gene expression. An involvement for liver transcription factors in HCC progression is supported by analyses of the role of HNFs in controlling the differentiation and epithelial transition of hepatoma cells in culture. These studies have identified a correlation between expression of HNF1α and HNF4α with the differentiated state of hepatoma cells. HNF1α expression has also been examined in HCCs and found to be expressed at lower levels in poorly differentiated compared to cells that are well-differentiated. Similarly, expression of C/EBPα, whose activity is associated with a quiescent, differentiated state of hepatocytes, is downregulated in rat liver nodules and HCCs and forced expression of C/EBPα was found to impair proliferation and suppress tumorigenicity of human hepatoma.
cells in culture. Although these findings are intriguing, and imply that hepatocyte transcription factors may inhibit HCC progression, the exact role played by HNFs remained unclear, primarily due to the absence of a reproducible in vivo experimental model. In our current one-step progression model, we have shown that HCC de-differentiation closely correlates with massive alterations in liver transcription factor gene expression. This includes down-regulation in expression of HNF4, HNF6, FTF, PXR, HNF1α, HNF1β, HNF3γ, and C/EBPα and up-regulation of COUP-TFI.

The observation that expression of multiple transcription factors was affected in the fgHCC was surprising given that the progression of sgHCC to fgHCC was so rapid. This suggests the existence of a limited number of factors that have global control of the hepatic gene expression program. One factor that exhibits transcriptional control over a large number of genes expressed in hepatocytes is HNF4. Studies in knockout mice have shown that HNF4 is crucial for expression of a large array of hepatic genes. We found that expression of HNF4 is indeed repressed as the HCC progresses from slow-growing to fast-growing and, importantly, that forced expression of HNF4 in fgHCC cells significantly suppresses tumor cell proliferation both in vivo and in vitro demonstrating that HNF4 can exert a strong anti-tumor effect on HCC progression. Consistent with a role as a tumor suppressor, we found that forced expression of HNF4α1 in cultured fgHCC cells re-established many characteristics of differentiated hepatocytes including the expression of several hepatocyte markers and transcription factors. In addition, fgHCC cells forced to express HNF4 re-established epi-

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Fig. 8. Forced expression of Hnf4α1 in fgHCC cells diminishes cell proliferation in vitro and suppresses tumor formation. (A) Percent of BrdU incorporation into H33-Puro (black bar) and H33 cells expressing HNF4 (gray bar). (B) Proliferation kinetics of control H33-Puro (black triangles) and H33 cells expressing HNF4 (gray squares). Cells (5 × 10⁴) were plated on 6-cm Petri dishes and counted daily. (C) Volumes of tumors formed by H33-Puro (black triangles) or H33-HNF4 cells (gray squares) after subcutaneous injection into congenic mice.

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thelia and increase expression of junction proteins. Consistent with this, recent studies using mouse embryos, in which the Hnf4α gene is ablated specifically from fetal hepatocytes, have shown that HNF4 is essential for the generation of a hepatic epithelium. Furthermore, in the same study, it was demonstrated that HNF4 could induce a mesenchymal to epithelial transition when expressed in naive NIH 3T3 fibroblasts. Based on these studies and the current work, we propose that loss of HNF4 expression is a critical event during HCC progression that mediates de-differentiation, loss of cell-cell contacts, and, as a consequence, the cells of the HCC become highly proliferative and invasive.

Although our data support the proposal that HNF4 is a key regulator of HCC progression, it seemed unlikely that mutation of the Hnf4α gene itself was responsible. If progression were a direct consequence of Hnf4α mutation then we would have expected to identify a recapitulation of the phenotype presented by hepatocyte-specific Hnf4α knockout mice. However, although the fgHCC cells and Hnf4α−/− hepatocytes share many characteristics, some important differences are evident. For example, while Hnf4α−/− hepatocytes show a massive disruption in expression of hepatic mRNAs, the expression of liver transcription factors, including HNF1α, HNF1β, HNF3γ, and FTF, is only moderately affected. In contrast, we have shown that the levels of Hnf1α, Hnf1β, Hnf3γ, C/EBPα, and Fif mRNAs are all significantly reduced or undetectable in fgHCCs compared to the sgHCCs. Furthermore, 7 kb of the Hnf4α1 5′-regulatory region was unable to direct expression of a reporter gene in fgHCC cells, indicating that loss of HNF4α1 expression in fgHCC cells is determined at the level of transcription (data not shown). We therefore conclude that in our model HCC progression results from mutations that affect factor(s) that act upstream of HNF4 and likely regulate its expression.

The change in the profile of genes expressed as the sgHCC progresses to a fgHCC is reminiscent of the extinction phenotype observed in somatic cell hybrids. In this case, the presence of tissue specific extinguisher loci results in widespread repression of hepatic gene expression. One of these loci, Tse1, has been identified and shown to encode the regulatory subunit RIα of protein kinase A (PKA). We find that the PKA R1a subunit is expressed in both sgHCC and fgHCC cultures suggesting that the mechanism(s) that control sgHCC to fgHCC progression is distinct from TSE-1 (not shown). This idea is also supported by the finding that the presence of the Tse1 locus results in repression of Foxa2 (Hnf3β) expression in somatic cell hybrids and Foxa2 expression is unaffected in fgHCC cells.

In conclusion, we described a new in vivo model of HCC progression. In this model, the tumor progression of chemically-induced mouse HCCs is accompanied by profound changes in the differentiation state and in cell proliferation that closely mimics that found in naturally occurring HCCs. We have shown that these alterations are coupled with a deregulation of a block of transcription factors that are essential for hepatocyte differentiation. Multiple phenotypic changes associated with tumor progression in vivo are at least partially defined by HNF4α1 suppression and can be reversed by restoration of Hnf4α1 gene expression. This data defines an important role for HNF4 in in vivo hepatocarcinogenesis and implies the possibility of reversing HCC invasiveness by expression or activation of HNF4. We believe this animal model will not only facilitate further elucidation of the molecular mechanisms controlling tumor progression but could also facilitate testing of potential therapeutics.

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