Role of Cyclin D1 as a Mediator of c-Met– and β-Catenin–Induced Hepatocarcinogenesis

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Abstract

Activation of c-Met signaling and β-catenin mutations are frequent genetic events observed in liver cancer development. Recently, we demonstrated that activated β-catenin can cooperate with c-Met to induce liver cancer formation in a mouse model. Cyclin D1 (CCND1) is an important cell cycle regulator that is considered to be a downstream target of β-catenin. To determine the importance of CCND1 as a mediator of c-Met– and β-catenin–induced hepatocarcinogenesis, we investigated the genetic interactions between CCND1, β-catenin, and c-Met in liver cancer development using mouse models. We coexpressed CCND1 with c-Met in mice and found CCND1 to cooperate with c-Met to promote liver cancer formation. Tumors induced by CCND1/c-Met had a longer latency period, formed at a lower frequency, and seemed to be more benign compared with those induced by β-catenin/c-Met. In addition, when activated β-catenin and c-Met were coinjected into CCND1-null mice, liver tumors developed despite the absence of CCND1. Intriguingly, we observed a moderate accelerated tumor growth and increased tumor malignancy in these CCND1-null mice. Molecular analysis showed an up-regulation of cyclin D2 (CCND2) expression in CCND1-null tumor samples, indicating that CCND2 may replace CCND1 in hepatic tumorigenesis. Together, our results suggest that CCND1 functions as a mediator of β-catenin during HCC pathogenesis, although other molecules may be required to fully propagate β-catenin signaling. Moreover, our data suggest that CCND1 expression is not essential for liver tumor development induced by c-Met and β-catenin. [Cancer Res 2009;69(1):253–61]

Introduction

Human hepatocellular carcinoma (HCC) is the most widespread and grievous form of malignancies to be diagnosed in adults (1). HCC is becoming a concern in developed nations, particularly the United States, where at least 17,000 people are projected to die of liver cancer in 2008.[6] Patients with hepatitis B or C viral infections are at a higher risk of developing HCC, especially when the infection is accompanied by liver cirrhosis (2, 3). Liver transplantation and surgical resection are considered the most effective treatments of HCC. However, surgical treatments are only appropriate for a minority of patients. Therefore, there is an urgency to identify potential therapeutic targets for treatment of this deadly malignancy.

HCC progression is known to ensue a stepwise sequence of events (4). Separate genetic or epigenetic aberrations are thought to be involved in each step during hepatic carcinogenesis. These changes involve alterations in the expression or assembly of an oncogene or a tumor suppressor gene. Overexpression of the proto-oncogene c-Met is a common perturbation known to occur in HCC (5, 6). *c-Met* encodes a receptor tyrosine kinase, which becomes activated on binding to ligand hepatocyte growth factor or scatter factor. When stimulated, c-Met becomes phosphorylated and triggers mitogen-activated protein kinase (MAPK) signaling through the Ras-Raf-MAP/extracellular signal-regulated kinase (ERK) kinase pathway (7). It has been shown in mouse models that activation of c-Met can promote liver cancer development (8). Another pathway frequently mutated and activated is the Wnt/β-catenin signaling pathway. Wnt signals by binding to the frizzled family of receptors, which initiates a signaling cascade involving dishevelled, glycogen synthase kinase 3, Axin, and APC and regulates the nuclear localization and activation of β-catenin (9, 10). β-Catenin subsequently binds to T-cell factor (TCF)-4, a member of the TCF/lymphoid enhancer factor family of transcriptional factors, and induces downstream gene expression. Multiple targets have been identified for activated β-catenin, many of which seem to be tissue specific (9, 10). One of the well-characterized targets for activated β-catenin is cyclin D1 (CCND1; refs. 11, 12).

CCND1 belongs to the D-type cyclin family, which also includes cyclin D2 (CCND2) and cyclin D3 (CCND3). CCND1 interacts with cyclin-dependent kinase (Cdk) 4/6, which in turn phosphorylates the retinoblastoma (Rb) protein, thereby promoting the transition from the G_1 to S phase of the cell cycle (13, 14). CCND1 can cooperate with other oncogenes, including Ras, Src, and E1A, to transform cells (15–17). It has been shown that overexpression of CCND1 in liver can induce HCC in a transgenic mouse model (18). However, CCND1 transgenic mice develop HCC over a long period (17 months) and at a relatively low frequency (20–30%; refs. 18, 19). This observation suggests that overexpression of CCND1 alone may not be sufficient for HCC development and that a secondary mutation may be necessary to cooperate with CCND1 to induce HCC. In CCND1 knockout mouse models, ~75% of CCND1$^{-/-}$ mice survive past their first month (20, 21). The surviving mice though appear smaller than normal and have some developmental

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[6] http://www.cancer.org

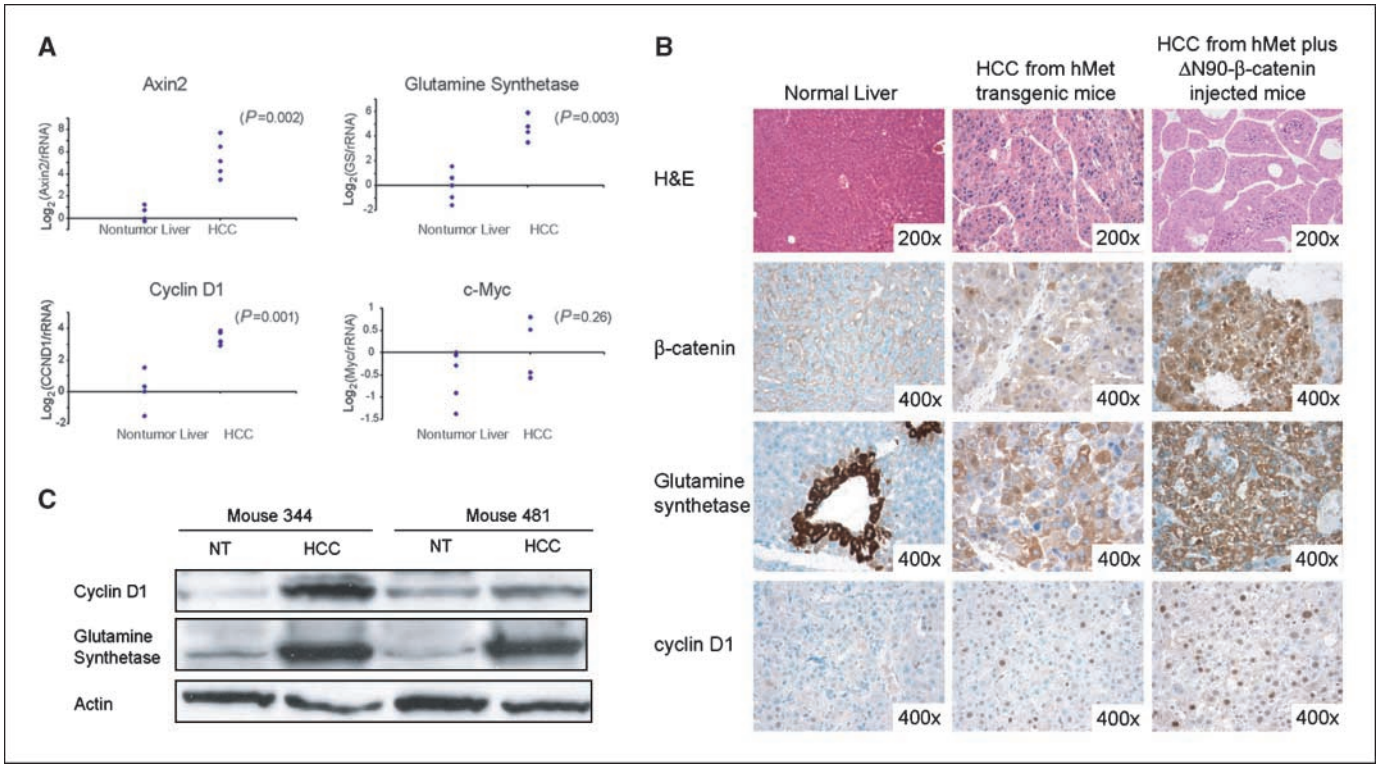

Figure 1. CCND1 is induced in hMet/β-catenin tumors. *A,* quantitative real-time RT-PCR analyses of β-catenin candidate target genes *Axin2, GS, CCND1*, and *c-Myc* in five paired HCC and nontumor liver tissues from hMet transgenic mice. Values are displayed as $\log_2$ ratio of tumor versus nontumor liver. *B,* immunohistochemical staining in normal (*left*), HCC from hMet transgenic (*middle*), or HCC from hydrodynamic cotransfection of hMet and ΔN90-β-catenin (*right*). *Row 1,* H&E staining; *row 2,* β-catenin; *row 3,* GS; *row 4,* CCND1. *C,* representative Western blots showing GS and CCND1 expression in two paired tumor and nontumor liver samples of hMet transgenic mice. Actin was used as the loading control.

defects in their retinas and mammary glands, are fertile, and have a similar life span as wild-type mice (20, 21). Using these CCND1-null mice, it has been found that CCND1 expression is required in certain tumor types in combination with different signaling pathways. For example, CCND1 is necessary for oncogenic Ras- and HER2-induced but not Wnt-1– or Myc-induced breast cancer (22). In colon cancer models induced by loss of APC, CCND1 is found to function as a tumor severity modifier and is required for efficient intestinal adenoma formation (23–25). Interestingly, in activated β-catenin–induced breast cancer, loss of CCND1 accelerates tumor development (26). Currently, how CCND1 contributes to and how it interacts with other oncogenic signals during liver cancer development remains unknown.

In a recent study, we conducted genetic analyses of tumors induced by human c-Met (hMet) in a mouse model (27). We found that β-catenin mutations are the second hit during malignant transformation, and these constitutively active β-catenin mutants are required to cooperate with hMet to promote hepatic carcinogenesis (27). In this report, we confirmed that CCND1 expression is up-regulated in liver tumor samples induced by hMet and β-catenin. We studied genetic interactions among CCND1, hMet, and β-catenin in hepatocarcinogenesis using murine models. Our results provide novel insight into how D-type cyclins function to promote liver cancer development *in vivo*.

Materials and Methods

Constructs and reagents. The pT3-EF1α-hMet and pT3-EF1α-ΔN90-β-catenin as well as pCMV/SB (the hyperactive sleeping beauty expression vector) constructs used for animal injections were previously described (27). Human CCND1 was cloned into pT3-EF1α via the Gateway PCR cloning strategy (Invitrogen). All plasmids were purified using the Endotoxin-Free Maxi Prep kit (Sigma) before being injected into the mice.

Mice breeding, genotyping, and hydrodynamic injections. Wild-type FVB/N mice were obtained from Charles River, and $CCND1^{+/-}$ mice (in FVB/N background) were obtained from The Jackson Laboratory. $CCND1^{+/-}$ mice were bred together to obtain $CCND1^{-/-}$ mice, and the genotyping procedure was as described (21). Hydrodynamic injection was performed as described (27). The injected mice were monitored weekly and sacrificed when appropriate or when they showed visibly enlarged livers or became moribund. All mice were housed, fed, and treated in accordance with protocols approved by the committee for animal research at the University of California at San Francisco (UCSF).

Hepatocyte isolation and transfection. Primary mouse hepatocyte isolation was performed using standard collagenase perfusion method as described (28). The hepatocytes were transfected with plasmids using Targefect-Hepatocyte (Targeting Systems) according to the manufacturer's instructions.

Histology. Animals were euthanized and their livers were removed and rinsed in PBS. Samples collected from the livers were either immediately frozen for RNA and protein extraction or fixed overnight in freshly prepared cold 4% paraformaldehyde. Fixed tissue samples were embedded in paraffin. Five-micron sections were placed on slides and stained with H&E to observe morphology of the cells.

Immunohistochemistry and immunofluorescence. Immunohistochemical staining was performed using avidin-biotin complex method kit (Vector Laboratories) as previously described (27). Immunofluorescence was performed in the similar manner, except that the appropriate Alexa-labeled secondary antibody (Invitrogen) was applied following incubation with the primary antibody. Antibody dilutions were as follows: anti-β-catenin (1:200), anti-E-cadherin (1:1,000), and anti-glutamine synthetase (GS; 1:500; BD Biosciences) and anti-Ki67 (1:150) and anti-CCND1 (SP4; 1:75; Lab Vision).

Real-time reverse transcription-PCR. Total RNA was extracted from frozen liver tissues or primary hepatocytes using Trizol (Invitrogen) and digested with DNase I to remove genomic DNA contamination. Sybergreen-based real-time reverse transcription-PCR (RT-PCR) was carried out as described (29) and rRNA was used as an internal control. Transcript quantification was performed in triplicate for every sample and reported relative to rRNA. The primer pair sequences are as described previously (29).

Preparation of lysates and Western blotting. Liver tumors were frozen on dry ice on harvesting, and homogenates were sonicated in lysis buffer [150 mmol/L NaCl, 1.0% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8.0)] and centrifuged at 14,000 rpm at 4°C. Supernatant was boiled in Laemmli sample buffer for Western blot analysis. The antibodies used are as follows: anti-CCND1 (Ab3, 1:1,000, Lab Vision; C-20, 1:500, Santa Cruz Biotechnology), anti-CCND2 (M-20, 1:500, Santa Cruz Biotechnology), anti-CCND3 (C-16, 1:300, Santa Cruz Biotechnology), anti-Cdk4 (C-22, 1:500, Santa Cruz Biotechnology), anti-Cdk6 (1:300, Santa Cruz Biotechnology), anti-Cdk2 (M-2, 1:500, Santa Cruz Biotechnology), anti-GS (1:1,000), anti-actin (1:5,000, Sigma), anti-ERK (1:1,000), anti-phospho-ERK (1:1,000), anti-phospho-Met (1:1,000, Cell Signaling), and anti-V5 (1:5,000, Invitrogen). Western blots were quantified using the ImageJ software.[7]

Array-based comparative genomic hybridization. Mouse comparative genomic hybridization (CGH) arrays were obtained from the UCSF Cancer Center Array Core. The arrays contained 2,896 bacterial artificial chromosome clones spotted in triplicate, with an average spacing between clones of ~1 Mb. Array hybridization and data analyses were the same as previously described (30).

Results

CCND1 is induced in hMet/β-catenin tumors. In a recent study, we characterized a unique and efficient model of HCC in the mouse, driven by the inducible expression of hMet. Tumors arise sporadically in livers of such mice and all show mutation and activation of β-catenin (27). Using hydrodynamic transfection, we showed that whereas neither hMet nor activated β-catenin alone is able to promote HCC development, the cooperation between aberrant β-catenin signaling and activated Met is required for genesis of HCC in the mouse model (27).

To determine the molecular mechanisms of how activated β-catenin contributes to hepatic carcinogenesis, we searched for genes that are up-regulated by activated β-catenin in the mouse HCC samples. We determined the expression levels of candidate β-catenin targets, Axin2, GS, CCND1, and c-Myc, by quantitative real-time RT-PCR in five paired HCC and nontumor liver tissues from hMet transgenic mice (Fig. 1*A*). We chose to use the liver samples from hMet transgenic mice instead of those from hMet-injected and β-catenin–injected mice because in hMet transgenic mice, nontumor liver tissues also overexpress hMet. Therefore, the genes that are up-regulated in tumors versus nontumor tissues are most likely due to β-catenin activation. As expected, both Axin2 and GS expressions were approximately 23- and 38-fold higher in HCC samples than in surrounding nontumor liver tissue. CCND1 was also found to be expressed 7-fold higher in HCC than in nontumor liver tissue. However, c-Myc expression levels did not show significant differences (Fig. 1*A*).

To further validate our observations, we assayed the protein expression of CCND1 and GS. Immunohistochemical staining of liver tumor tissues revealed nuclear staining of β-catenin in HCC lesions, indicating the activation of β-catenin (Fig. 1*B*). In normal liver, GS was found to be only expressed in hepatocytes immediately adjacent to the central vein. In contrast, in HCC, GS expression was detected in virtually all malignant hepatocytes (Fig. 1*B*). Analysis of CCND1 expression revealed that there was little or weak expression of CCND1 in normal mouse liver cells. However, strong nuclear staining of CCND1 was apparent in the tumor samples (Fig. 1*B*). The expression patterns of these genes are similar for all tumors from hMet transgenic mice and hMet/β-catenin–injected mice (Fig. 1*B*). The up-regulation of CCND1 and GS in tumor tissues was also confirmed by Western blotting (Fig. 1*C*).

Our data suggest that CCND1 expression is induced by β-catenin during hepatic carcinogenesis. We next examined if this occurrence also takes place in normal hepatocytes. To access the induction of CCND1 by β-catenin, we expressed an activated form of β-catenin, ΔN90-β-catenin, into mice using hydrodynamic transfection, and their expression levels were analyzed using immunofluorescent staining. We found that whereas sporadic activated β-catenin staining could be visualized in normal hepatocytes, no CCND1 expression was detected in the same cells (Supplementary Fig. S1*A*). Consistent with our previous results, we observed colocalization of β-catenin and CCND1 in tumor cells induced by c-Met/ΔN90-β-catenin (Supplementary Fig. S1*A*). To confirm our findings, we transfected primary mouse hepatocytes with ΔN90-β-catenin (Supplementary Fig. S1*B*). Whereas we can detect strong expression of the β-catenin target gene *Axin2*, no up-regulation of CCND1 was observed (Supplementary Fig. S1*C*). These experiments suggest that CCND1 is not a direct target for β-catenin in normal hepatocytes, but rather CCND1 expression is induced during hepatic carcinogenesis.

In conclusion, we found that CCND1 expression is up-regulated in mouse liver tumors induced by hMet and β-catenin. Consistent with our observation, overexpression of CCND1, but not c-Myc, has been reported in two HCC mouse models involving the activation of β-catenin: conditional APC knockout mice and RasV12 and β-catenin double conditional transgenic mice (31, 32). Because CCND1 is an important factor in the regulation of cell

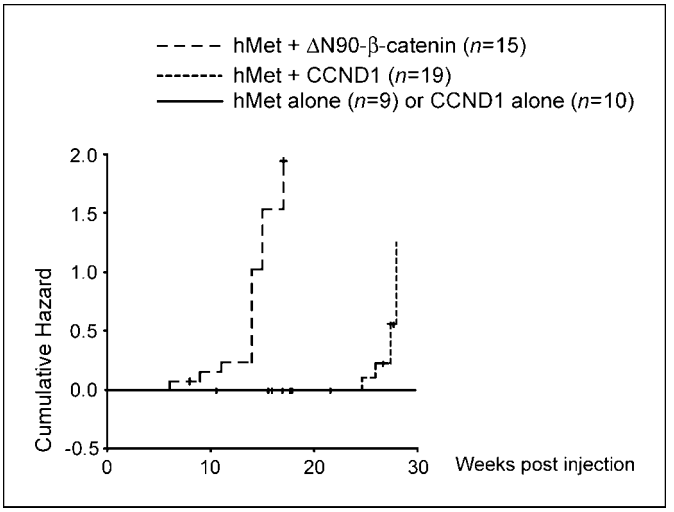

Figure 2. CCND1 cooperates with hMet to induce HCC in mice. Cumulative hazard curve comparing the latency and frequency of tumor development in hMet alone, CCND1 alone, hMet/CCND1-injected, and hMet/ΔN90-β-catenin–injected mice. The cumulative hazard represents the relative probability of tumor development in each condition.

[7] http://rsbweb.nih.gov/ij/

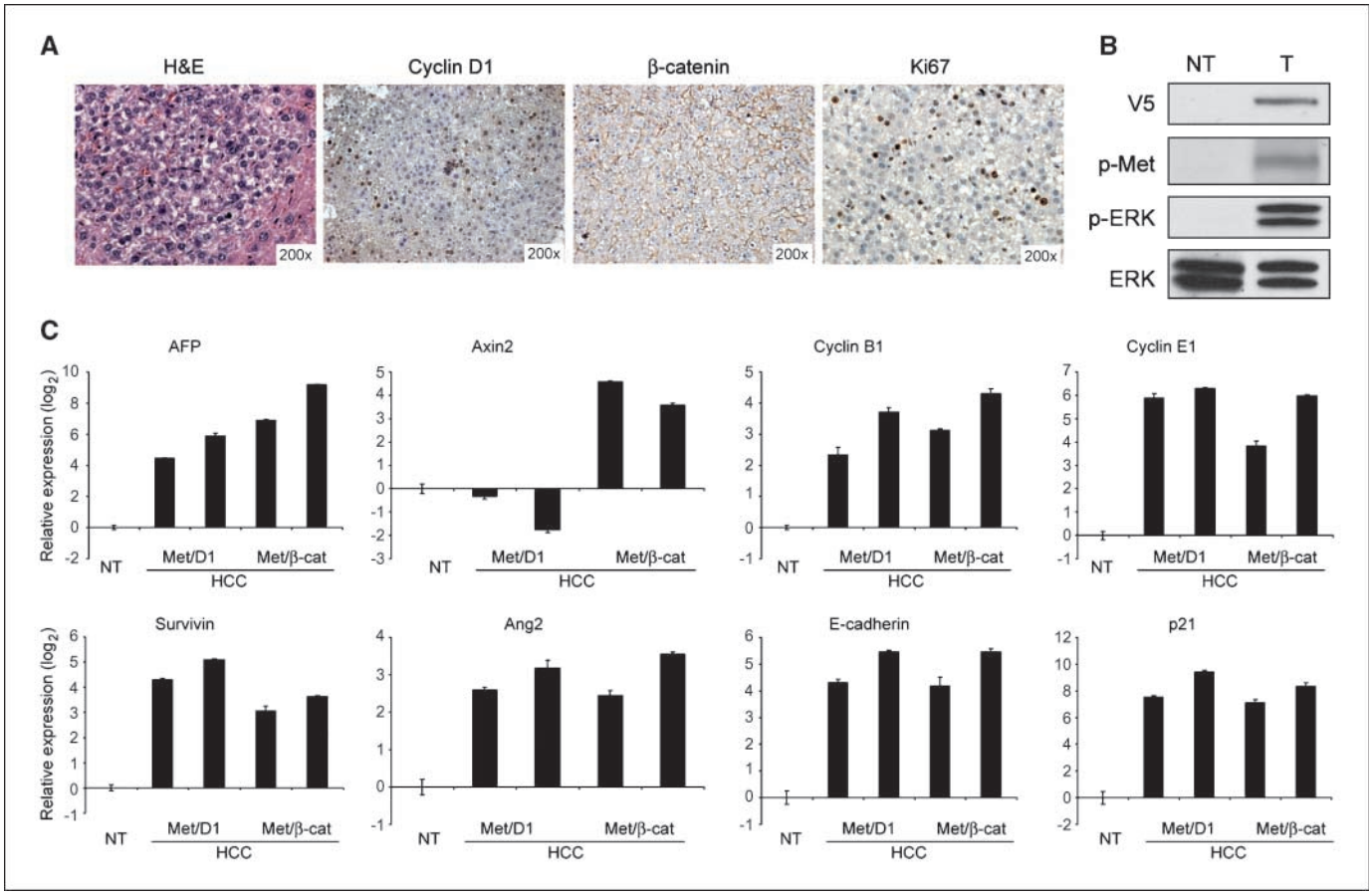

Figure 3. Molecular features of liver tumors induced by hMet/CCND1. *A,* immunohistochemical staining of liver tumor tissues from hMet/CCND1 mice: H&E, CCND1, β-catenin, and Ki67 staining (*left* to *right*). *B,* Western blot analyses showing the expression of V5-tagged hMet, activation of hMet [phospho-Met (*p-Met*)], and elevated MAPK signaling [phospho-ERK (*p-ERK*)] in hMet/CCND1 tumor cells. *C,* quantitative real-time PCR analyses of liver tumor markers in normal liver (*NT*), hMet/CCND1 tumors, and hMet/ΔN90-β-catenin tumor samples. In all cases, the expression in normal liver was set to 1 and used to normalize all the other samples.

cycle progression, we hypothesized that CCND1 may be a key mediator of β-catenin in promoting hepatic carcinogenesis.

CCND1 cooperates with hMet to induce HCC in mice. To test the hypothesis that CCND1 is a mediator of activated β-catenin signaling during hepatic carcinogenesis, we investigated whether overexpression of CCND1 can substitute for activated β-catenin and cooperate with hMet to induce HCC in mice. We used hydrodynamic transfection to coexpress CCND1 and hMet in mouse liver. Histologic examination revealed that none of the nine mice that were injected with hMet/CCND1 showed any signs of tumor development between 11 and 22 weeks after injection (Fig. 2). Tumor formation was first observed in these mice at 25 weeks after injection. In total, 6 of the 19 mice injected with CCND1 and hMet developed liver tumors within 29 weeks after injection (Fig. 2). Gross examination of livers of these mice also showed multiple small lesions scattered throughout the surface of the liver (Supplementary Fig. S2). In contrast, mice expressing only hMet (n = 9) or CCND1 (n = 10) failed to develop liver cancer during this time period (Fig. 2). The fact that tumors were only observed when CCND1 and hMet are coexpressed suggests that overexpression of CCND1 cooperates with hMet to promote liver cancer formation.

We found that coexpression of hMet and ΔN90-β-catenin induces HCC in 12 of 15 mice within 17 weeks after injection (Fig. 2), much earlier than mice injected with CCND1 and hMet. We also observed that the frequency of tumor development in hMet and ΔN90-β-catenin (80%) was higher than that of hMet- and CCND1-injected mice (32%; Fig. 2). Together, the data suggest that tumor initiation is likely to be much later in hMet/CCND1-injected mice compared with hMet/β-catenin–injected mice.

Molecular features of liver tumors induced by CCND1/hMet. To gain further insight into the molecular features of liver tumors induced by hMet/CCND1, we examined tumor samples using histologic analysis and quantitative RT-PCR. We found up-regulation of liver tumor–specific marker α-fetoprotein (AFP) in hMet/CCND1 tumor samples (Fig. 3*C*), confirming the neoplastic nature of these cells. Histologic examination of liver tissue from hMet/CCND1-injected mice revealed that the majority of the tumors seemed to be adenoma that compressed the surrounding nontumorous liver parenchyma (Fig. 3*A*). Increased plate thickness and trabecular disorganization were rarely observed in these tumors (Fig. 3*A*). This is distinct from tumors found in hMet transgenic mice or mice injected with hMet/ΔN90-β-catenin, where a majority of the tumors display features consistent with malignant HCC (Fig. 1*B*). Nuclear staining of CCND1 was observed, revealing the presence of CCND1 in neoplastic hepatocytes, but rarely in nontumor liver tissues (Fig. 3*A*). The level of CCND1 expression in hMet/CCND1 tumor cells is similar to what we observed in tumors from hMet/ΔN90-β-catenin–injected mice.

Overexpression of hMet (with COOH-terminal V5 tag) was confirmed by Western blotting using the V5 antibody (Fig. 3*B*), and the activation of hMet as illustrated by the high levels of phospho-Met and phospho-ERK in hMet/CCND1 tumor cells (Fig. 3*B*). To rule out the possibility that the tumors induced by hMet/CCND1 were due to endogenous mutations of β-catenin, we analyzed β-catenin and its target gene expression in tumor samples. We found no evidence of activation of β-catenin signaling in tumor cells, as only membrane β-catenin staining was observed (Fig. 3*A*), and no up-regulation of β-catenin target genes *Axin2* and *GS* was detected by real-time RT-PCR (Fig. 3*C*; data not shown). Altogether, these analyses support our hypothesis that hMet and CCND1 together promote liver adenoma formation in mice.

Next, we examined the genes involved in cell cycle, apoptosis, and cell adhesion in CCND1/hMet tumor samples. We observed frequent cell proliferation in tumor cells as indicated by positive staining for the proliferative marker Ki67 (Fig. 3*A*). This observation is also confirmed by the high expression of cell cycle regulatory genes, *cyclins B1* and *E1*, as well as Cdk inhibitor p21Cip1 in the tumors (Fig. 3*C*). In addition, tumor cells expressed high levels of the antiapoptotic protein survivin as well as the angiogenic gene *Ang2* (Fig. 3*C*). Immunostaining revealed that tumor cells are positive for the cell-cell adhesion molecule E-cadherin (data not shown). This observation is confirmed by up-regulation of E-cadherin as indicated by real-time PCR analysis (Fig. 3*C*). Although there are striking differences in tumor latency and incidence between the hMet/ΔN90-β-catenin–injected and hMet/CCND1-injected mice, molecular features of both tumors are similar to a certain extent. For example, elevated expressions of cyclins, survivin, Ang2, E-cadherin, and p21Cip1 were observed in all tumor samples examined (Fig. 3*C*).

In conclusion, our study shows that overexpression of CCND1 can cooperate with hMet to promote liver cancer formation, supporting the hypothesis that CCND1 is a critical downstream signaling molecule of activated β-catenin. However, the differences between tumors induced by hMet/CCND1 and hMet/ΔN90-β-catenin indicate that other factors, in addition to CCND1, may be required to fully transduce the signaling generated by aberrant β-catenin.

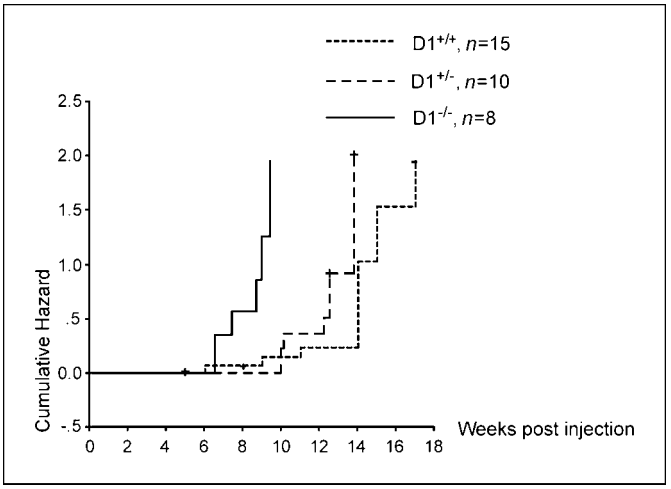

Figure 4. Accelerated tumor development induced by hMet/ΔN90-β-catenin in CCND1 knockout mice. Cumulative hazard curve comparing the latency and frequency of tumor development induced by hMet/ΔN90-β-catenin in wild-type, CCND1$^{+/-}$, or CCND1$^{-/-}$ mice. The cumulative hazard represents the relative probability of tumor development in each condition.

CCND1 expression is not required for tumor development induced by activated β-catenin plus hMet. Because CCND1 expression is induced by hMet and β-catenin in liver tumors, we next determined whether the expression of CCND1 is required for activated β-catenin and hMet to promote HCC development *in vivo*. Toward this aim, we generated CCND1 knockout mice in the FVB/N background. These CCND1-null mice show similar phenotypes as described in the C57/BL6 background (20, 21). Liver tissues seemed to be normal in these mice. ΔN90-β-catenin and hMet were coinjected into CCND1$^{+/-}$ and CCND1$^{-/-}$ mice, and tumor development was monitored.

We found that hMet and ΔN90-β-catenin could induce liver cancer development independent of the CCND1 genotype. In particular, we observed 7 of 8 CCND1$^{-/-}$ mice developed tumors within 11 weeks after injection, 8 of 10 CCND1$^{+/-}$ mice developed tumors 14 weeks after injection, and 12 of 15 wild-type mice developed tumors 17 weeks after injection (Fig. 4). Intriguingly, we noticed that the CCND1 gene dosage affected the onset and progress of hepatocarcinogenesis: tumors progressed earlier with lower CCND1 gene doses (i.e., CCND1$^{-/-}$ mice showed the fastest tumor progression), whereas wild-type mice showed the longest latency time for tumor development (Fig. 4). Tumors from CCND1$^{-/-}$ or CCND1$^{+/-}$ mice are multifocal and scattered around the liver, similar to what has been observed in wild-type FVB/N mice (Supplementary Fig. S2).

Histologic analyses revealed that tumor lesions from CCND1$^{+/-}$ and CCND1$^{-/-}$ mice emerge as HCC with cytologic atypia and frequent trabecular disorganization (Fig. 5*A*; data not shown). This is further verified by high expression levels of AFP in these tumor samples (Fig. 5*D*). Ectopic expression of β-catenin is validated by nuclear and cytoplasmic staining in tumor cells (Fig. 5*A*). Expression of hMet in tumor samples is indicated by the presence of the V5 marker. The activation of hMet signaling is confirmed by an increase in the expression of phospho-Met and phospho-ERK (Fig. 5*C*). CCND1 expression was not detected in tumors from CCND1$^{-/-}$ mice by immunohistochemical staining or Western blotting (Figs. 5*A* and 6*A*).

We further assayed the molecular signatures of tumors from different CCND1 genetic backgrounds. Increased cell proliferation was detected in tumors from CCND1$^{-/-}$ mice, as indicated by positive Ki67 staining (Fig. 5*A*). The overexpression of cyclin B1, E1, and Cdk inhibitor p21Cip1 in tumors from CCND1$^{-/-}$ as well as CCND1$^{+/-}$ and wild-type mice further verifies this observation (Fig. 5*D*). The antiapoptotic gene *survivin* is also found to be overexpressed in all tumor samples (Fig. 5*D*).

We next examined the expression of the cell-cell adhesion molecule E-cadherin. In the normal liver, hepatocytes show weak staining of E-cadherin around the periportal area (Fig. 5*B*; ref. 33). Tumors induced by hMet and β-catenin in wild-type mice displayed ubiquitous expression of E-cadherin in all tumor cells (Fig. 5*B*). In contrast, there seemed to be a heterogeneous pattern of E-cadherin expression in tumors from CCND1$^{-/-}$ mice: whereas some tumor nodules retained E-cadherin staining, others showed no expression (Fig. 5*B*). Because loss of E-cadherin has been linked with malignant phenotype, our data indicate that tumors from CCND1-null mice show moderate accelerated tumor growth and increased malignancy.

A study by Calvisi and colleagues (34) showed that mouse liver tumors with β-catenin activation have a stable genome. Consistent

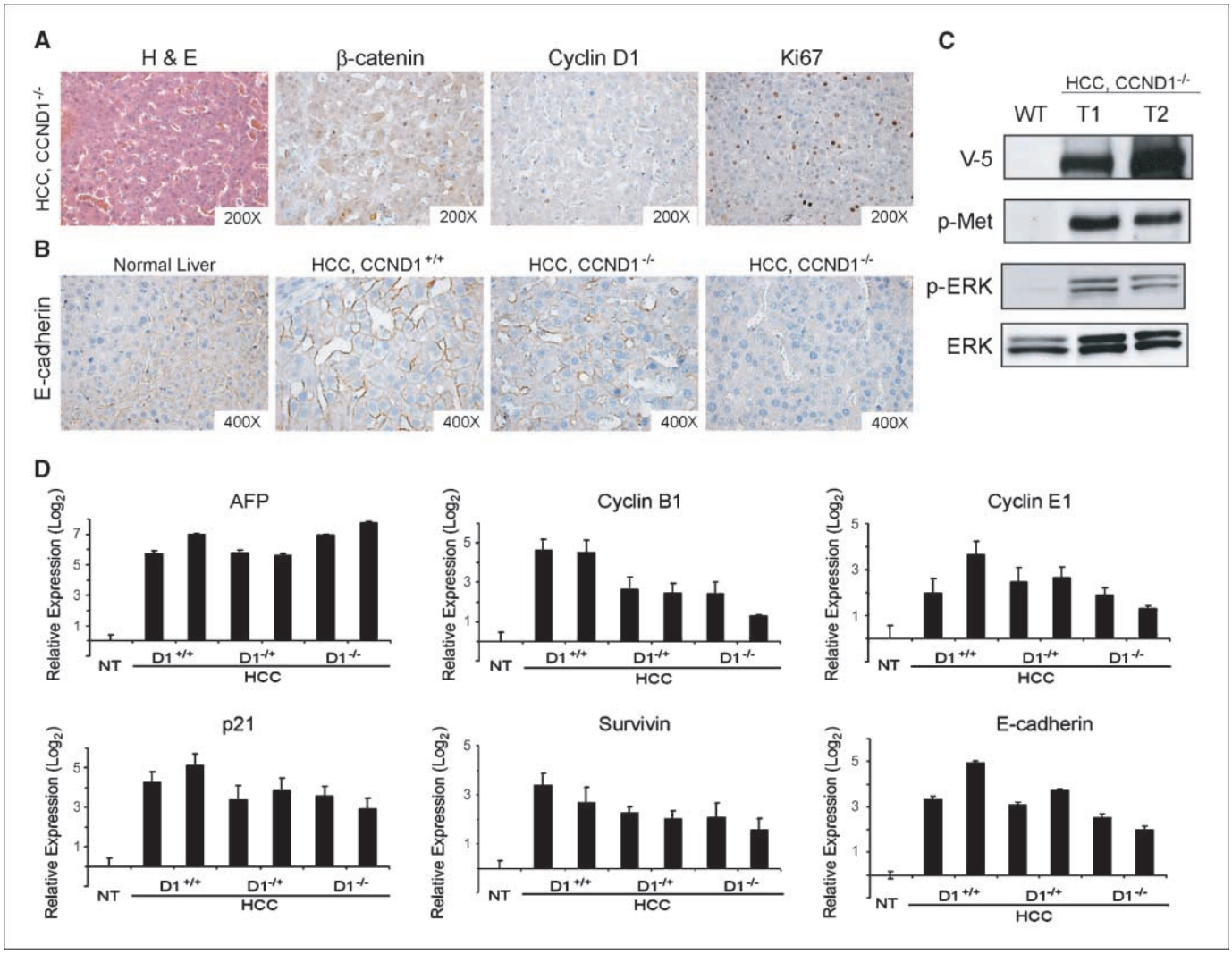

Figure 5. Molecular features of liver tumors induced by hMet/ΔN90-β-catenin in different CCND1 genetic backgrounds. *A,* immunohistochemical staining of liver tumor tissues from hMet/ΔN90-β-catenin–injected CCND1$^{-/-}$ mice: H&E, β-catenin, CCND1, and Ki67 staining (*left* to *right*). *B,* immunohistochemical staining of E-cadherin expression in normal liver and liver tumors from wild-type or CCND1$^{-/-}$ mice. *C,* Western blot analyses showing the expression of V5-tagged hMet, activation of hMet (phospho-Met), and elevated MAPK signaling (phospho-ERK) in hMet/ΔN90-β-catenin;CCND1$^{-/-}$ tumor cells. *D,* quantitative real-time PCR analyses of tumor markers in normal liver and liver tumors from wild-type, CCND1$^{+/-}$, or CCND1$^{-/-}$ mice. In all cases, the expression in normal liver was set to 1 and used to normalize all the other samples.

with this observation, we found that tumors induced by hMet and ΔN90-β-catenin also have a stable genome, with no abnormal chromosomal gains or losses (Supplementary Fig. S4). We examined genomic instability in tumor samples from CCND1$^{-/-}$ mice using array-based CGH. Similar to what we observed in wild-type mice, liver tumors induced by hMet and ΔN90-β-catenin in CCND1$^{-/-}$ mice have no genomic instabilities (Supplementary Fig. S4). The study therefore suggests that the accelerated tumor growth in CCND1$^{-/-}$ mice is not due to the increased genomic instability of these tumor samples.

Up-regulation of CCND2 in CCND1-null liver and liver tumor samples. Our preliminary studies show that mRNA of all three members of the D-type cyclin family is expressed in the mouse liver (data not shown). To determine whether the loss of CCND1 was compensated by the other two D-type cyclins, we assayed for the protein expression of CCND2 and CCND3 in tumor tissues from wild-type, CCND1$^{+/-}$, and CCND1$^{-/-}$ mice. Strikingly, we observed that although CCND2 is expressed at very low levels in wild-type tumor samples, its expression is significantly up-regulated in tumors from CCND1$^{+/-}$ and CCND1$^{-/-}$ mice (Fig. 6*A*). The expression of CCND3 remains the same in all tumor samples. We further examined the expression of the D-type cyclin partners, Cdk4 and Cdk6, as well as Cdk2, which binds to cyclin E and A. We found the expression of Cdk2 and Cdk6 to be independent of tumor genotypes. However, the expression of Cdk4, a major signaling partner of CCND1, in CCND1$^{-/-}$ mice tumors is significantly decreased by 60% in comparison with tumors from wild-type mice (Fig. 6*A*; Supplementary Fig. S3).

We next determined whether the up-regulation of CCND2 also compensates for the loss of CCND1 during normal liver development or occurs only during tumorigenesis. We assayed the expression of CCND1 and CCND2 in normal wild-type liver and in the liver of CCND1-null mice. We found that CCND2 expression is decreased in normal liver samples compared with CCND1$^{+/-}$ and CCND1$^{-/-}$ mice (Fig. 6*B*). The results suggest that CCND2 replaces CCND1 function when the *CCND1* gene is deleted in the liver and

the CCND2/Cdk6 complex replaces the CCND1/Cdk4 complex during hepatic carcinogenesis.

Discussion

In this article, we showed that CCND1 expression is up-regulated in liver tumors induced by hMet/β-catenin. Consistent with the studies by Cadoret and colleagues (35), we found that CCND1 is not a direct target of activated β-catenin in normal mouse hepatocytes. On the other hand, overexpression of CCND1 has been found to be correlated with β-catenin activation in multiple mouse liver tumor models (31, 32, 36). Recently, Zeng and colleagues (37) reported that RNA-mediated β-catenin knockdown in human HCC cell lines with activated β-catenin mutations leads to decreased expression of CCND1. Together, all the data suggest that CCND1 is likely to be induced by activated β-catenin during hepatic carcinogenesis. Our experiments show that CCND1 can partially substitute activated β-catenin and cooperates with hMet to induce liver cancer formation *in vivo*, thus providing additional evidence of CCND1 as a target of β-catenin during malignant transformation.

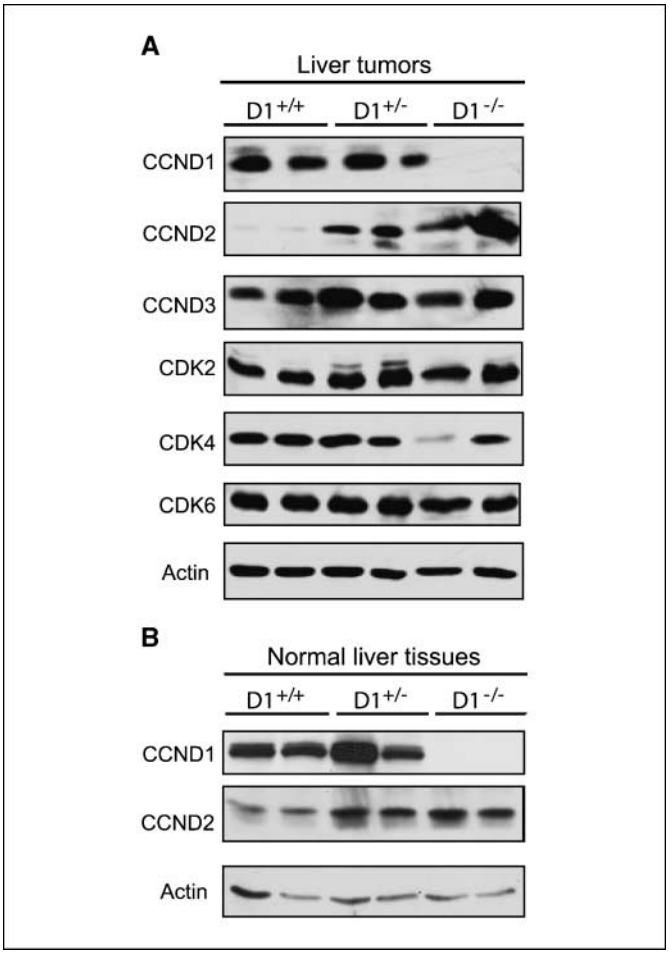

Figure 6. Expression in D-type cyclins and Cdks in mouse liver tissues. *A,* Western blotting analysis of the expression of CCND1, CCND2, CCND3, Cdk2, Cdk4, and Cdk6 in liver tumor samples with different CCND1 genetic backgrounds. *B,* expression of CCND1 and CCND2 in normal liver tissues from mice with different CCND1 genetic backgrounds. Actin was used as the loading control.

Although coexpression of CCND1 and hMet can induce liver cancer formation in mice, we found that the tumors induced by CCND1/hMet require longer latency, form at a lower frequency, and seem to be more benign compared with tumors induced by β-catenin/hMet. These observations indicate that CCND1 is only part of the signaling triggered by β-catenin activation, and other molecules may be required to fully transduce the aberrant β-catenin signaling. Other targets of β-catenin include Tbx3 (38) and Gpr49 (39), both found to be up-regulated in liver tumors induced by hMet/β-catenin.[8] Tbx3, a member of the T-box transcriptional repressor family, has been found be overexpressed in melanoma, breast cancer, and ovarian cancer (40–42). In addition, Tbx3 is a potent inhibitor of p19Arf and, hence, a regulator of the p19Arf-MDM2-p53 pathway (43). Thus, Tbx3 may provide a novel link between activated β-catenin and p19Arf tumor suppressor pathways. Gpr49, also known as Lgr5, is an orphan G protein–coupled receptor. Gpr49 has been found to be overexpressed in human colon and ovarian tumors and is proven to be a marker for intestinal stem cells (44, 45). Altogether, it would be of great importance to elucidate how Tbx3, Gpr49, and CCND1 function together and mimic the activity of β-catenin in cooperation with c-Met to induce liver cancer.

In our study, we have shown that expression of CCND1 is not required in murine HCC pathogenesis induced by activated β-catenin and hMet. Therefore, whereas CCND1 expression is up-regulated in liver cancer cells, it seems to be dispensable for the activity of c-Met and β-catenin in liver tumor development. Unexpectedly, we observed that hMet- and ΔN90-β-catenin–induced tumor development was accelerated by the loss of CCND1. Tumor cells seemed to be more aggressive, with frequent E-cadherin–negative tumors present in the HCCs of CCND1$^{-/-}$ mice. Interestingly, increased breast tumorigenesis has also been observed in CCND1$^{-/-}$ mice when they were crossed with mice expressing activated β-catenin targeted to the mammary gland (26). Our study provides additional evidence that CCND1 plays divergent roles under different oncogenic signals and in diverse cell types. For each specific oncogenic signal and each cell type, one has to assay for the tumorigenic activity of the oncogene in a CCND1-null background to elucidate the requirements of CCND1 in the specific circumstance.

What are the molecular mechanisms for the accelerated and more aggressive phenotype observed in CCND1 knockout mice? One possible clue comes from our investigation of the expression of other D-type cyclins and Cdks in the CCND1$^{-/-}$ tumor samples. Increased expression of CCND2 in CCND1$^{+/-}$ and CCND1$^{-/-}$ tumors strongly suggests that this member of D-type cyclin family can replace CCND1. In addition, lack of CCND1 seems to decrease the Cdk4 protein level, likely through reduced stability of free Cdk4. Whether CCND2/Cdk6 is more efficient in the phosphorylation of Rb than CCND1/Cdk4 during liver tumor development warrants further investigation and may provide functional roles for CCND2 in hepatic carcinogenesis. A recent study supports a positive role for CCND2 in tumorigenesis, where CCND2 transgenic mice are more susceptible to developing skin tumors, a characteristic that is not shared by CCND1 and CCND3 transgenic mice generated under the same promoter (46, 47). In addition, D-type cyclins play Cdk-independent roles in certain cell types. For example, D-type

[8] M.A. Patil et al., unpublished results.

cyclins bind to nuclear receptors such as androgen, estrogen, and vitamin D receptors to regulate the expression of several genes in prostate, mammary gland, and skin keratinocytes (48, 49). Although the interaction of D-type cyclins with nuclear receptors has not been described in liver, whether CCND1 acts through this pathway during liver tumorigenesis clearly needs to be evaluated. A third possibility is that D-type cyclins may modulate tumorigenesis via regulating cell types other than hepatocytes. For example, D-type cyclins may regulate tumor immunity or angiogenesis, and the more rapid tumor growth in CCND1-null mice may be due to the reduced immune response or more robust angiogenesis because CCND1 is deleted in all cell types in mice. This hypothesis can be tested by generating hepatocyte-specific deleted CCND1 mice using the albumin Cre system. If we fail to observe this accelerated tumor growth phenotype in these mice, the result will support an additional nonhepatocyte role of D-type cyclins during HCC pathogenesis.

It has been speculated that small molecules targeted against CCND1/Cdk4 may be useful as therapeutic reagents against human tumors. However, our study and the study by Rowlands and colleagues suggest that we need to be cautious about such treatments because it could lead to unfavorable consequences under certain conditions. For example, CCND1/Cdk4 inhibitors may not be suitable for patients who have chronic HBV or HCV infection, as these patients are at a greater risk of developing HCC, and loss of CCND1/CDK4 activity may accelerate the progression of this malignancy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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