No Effect of Loss of E2F1 on Liver Regeneration or Hepatocarcinogenesis in C57BL/6J or C3H/HeJ Mice

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The E2F family of transcription factors regulates the expression of genes needed for DNA synthesis and cell-cycle control. However, the individual contributions of the different E2F family members in regulating proliferation in various tissues have not been well characterized. Mouse liver is an excellent system for investigating proliferation because its growth state can be experimentally manipulated. As observed in cell culture systems, E2F1 protein is present at low levels in the quiescent liver, with an increase in expression during proliferation. Therefore, we expected that E2F1 may play an important role in cell-growth control during periods of robust proliferation. Using *E2F1*-nullizygous mice, we performed partial hepatectomies to investigate the role of E2F1 in the synchronous proliferation of adult hepatocytes. We found that E2F1 deficiency resulted in only minor changes in gene expression and that the timing of liver regeneration was not altered in E2F1 nullizygous mice. E2F1 has displayed properties of both a tumor suppressor and an oncogene in different model systems. Therefore, we investigated the role of E2F1 in rapidly growing liver tumor cells in strains of mice that have high (C3H/HeJ) and low (C57BL/6J) rates of hepatocarcinogenesis. We observed no significant differences in the number of liver tumors that developed after diethylnitrosamine treatment of wild type versus *E2F1*-nullizygous mice. We suggest that abundant levels of E2F4 in the mouse liver compensate for loss of E2F1. *Mol. Carcinog. 25:295–303, 1999.* © 1999 Wiley-Liss, Inc.

Key words: cell cycle; liver; nullizygous mice; diethylnitrosamine

INTRODUCTION

From work in cell culture and studies of human tumors, it is clear that the retinoblastoma (Rb) pathway of proliferative control is a critical mutational target in the development of many tumors [1–3]. The focus of our studies is the E2F transcription factor family, a critical downstream mediator of the Rb pathway [4]. The E2F family, a group of helix-loop-helix leucine zipper transcription factors [5], is believed to regulate the expression of genes important for DNA synthesis and cell-cycle progression [6]. To date, six E2Fs have been identified, E2F1–6 [7–15]. Members of the E2F family function as heterodimers with the DP family of related proteins, including DP1 and DP2 [16–18]. Targets of the E2F family include genes such as *cdc2*, cyclin D1, cyclin E, *Rb, p107, E2F1, E2F2, B-myb*, cyclin A, and dihydrofolate reductase (*Dhfr*) [19–34]. A common characteristic of E2F-regulated genes is induction of mRNA expression at the G_1/S phase boundary of the proliferative cycle. In G_0 and G_1 cells, E2F is found in a complex with members of the Rb family, which includes pRb, p107, and p130 [35]. When an E2F–Rb family member complex occupies an E2F site in a target promoter, the Rb family member can actively repress transcription of that promoter. During G_1 progression, Rb family members become phosphorylated by G_1 cyclin–kinase complexes, causing dissociation from E2F. E2F in its "free" form remains bound to and transactivates certain genes such as *Dhfr*. For other E2F targets such as *B-myb*, dissociation of the E2F-Rb complex results in a loss of occupancy of the E2F site with a concomitant derepression of promoter activity. It is not yet clear whether different E2Fs regulate these two types of target genes.

E2F1 is the best-characterized member of the E2F family. E2F1 interacts exclusively with pRb [4], a member of the Rb family that is frequently lost or mutated in human tumors [1–3]. Overexpression of E2F1 has been shown to cause neoplastic transformation of cells in tissue-culture systems [12,36,37] and to enhance neoplasia in the skin of transgenic mice [38], suggesting that E2F1 promotes cell proliferation. *E2F1*-nullizygous mice exhibit exocrine tissue abnormalities and testicular atrophy [39], again suggesting that E2F1 activity is

positively correlated with the growth of certain cells in vivo. However, *E2F1*-nullizygous mice are also susceptible to tumor development in the reproductive tract, lung, and lymphatic system, providing evidence that E2F1 can also function as a negative regulator of cell growth [39]. These studies indicate that the role of E2F1 in either suppressing or promoting cell growth in an animal is tissue specific.

We previously examined the expression of E2F1 and the E2F target gene *Dhfr* in regenerating hepatocytes of C57BL/6J (B6) and C3H/HeJ (C3H) mice [40]. We found that although E2F1 is induced with different timing (relative to the onset of DNA synthesis) in B6 versus C3H mice, there is a striking correlation between the patterns of expression of E2F1 and Dhfr in both strains of mice. For example, we found that *E2F1* and *Dhfr* are induced at the G_1/S-phase boundary in C3H mice but that the mRNA levels of both *E2F1* and *Dhfr* peaked slightly after the first peak of DNA synthesis in B6 mice. These observations suggested that E2F1 may play an important role in regulating at least one E2F target gene (i.e., *Dhfr*). To further study the role of E2F1 in the liver, we used *E2F1*-nullizygous mice to study cell proliferation and gene expression in normal hepatocytes and after induction of hepatocellular carcinogenesis.

MATERIALS AND METHODS

Mouse Husbandry

The mice were housed in the McArdle Laboratory Animal Facilities in plastic cages on corncob bedding from Bed-O'Cobs (Anderson Cob Division, Maumee, OH) and fed Breeder Blox (Harlan, Madison, WI). Food and acidified water were available ad libitum. The mice used in experiments were bred in our mouse colony. Unless otherwise stated, the animals used in the experiments described are either progeny of the second-generation backcrosses of the *E2F1* null allele onto the B6 genetic background and thus have a genome 75% B6 and 25% 129SvPas (-+tyr-c+p) (129Sv) alleles or the progeny of an intercross of the progeny of the cross of third-generation mice carrying the *E2F1* null allele onto the C3H genetic background and thus have a genome approximately 50% C3H and 50% B6/129Sv (43.75% B6 and 6.25% 129Sv). Genotyping of mice to detect presence of the *E2F1* null allele was performed as described previously [39].

Seventy Percent Partial Hepatectomy

Partial hepatectomy was performed on male 6-wk-old *E2F1*-nullizygous and −wild-type mice by using ether anesthesia. The mice were shaved and scrubbed with 70% ethanol and Betadine before surgery. A lateral incision was made in each mouse's abdomen, and the left lateral and median lobes of the liver, which make up approximately 70% of the liver mass, were exteriorized, ligated with 0 silk cord from Roboz Surgical (Rockville, MD), and resected. The peritoneum and skin were sutured with 4-0 silk from Roboz Surgical, and wound clips from Fisher Scientific (Itasca, IL) were used to secure the incision. When required, the wound clips were removed, under ether anesthesia, 10 d after surgery. Mock hepatectomy was performed to control for changes in mouse weight, liver gene expression, and overall health that can occur as a result of the experimental procedure. Mock hepatectomy is identical to a 70% partial hepatectomy, except that no manipulations of the liver are performed and there is no regenerative response. Because of fluctuation that can occur as a result of the diurnal cycle, all animals were killed at the same time, and therefore, surgeries were performed at various times before the mice were killed.

Preparation of RNA

Total RNA was extracted from liver by the guanidine thiocyanate/CsCl method. Upon dissection, the livers were immediately frozen in liquid nitrogen and stored at −70°C. The livers were crushed while frozen and homogenized in a solution containing 4 M guanidine isothiocyanate; 26.5 mM sodium acetate, pH 6.0; and 125 mM 2-mercaptoethanol. The homogenate was layered over a solution containing 5.7 M cesium chloride and 25.5 mM sodium acetate, pH 6.0, in an SW.28 ultracentrifuge tube from Beckman Instruments Inc. (Palo Alto, CA.) and centrifuged overnight at 26°C at 96 000 x*g*. The RNA pellets were resuspended in double-distilled H_2O and precipitated with 0.1 vol of 3 M sodium acetate and 2.5 vol of ethanol on dry ice for 30 min. The precipitates were resuspended in double-distilled H_2O. The RNA samples were stored at −70°C. Poly(A)+ RNA was isolated by using the PolyA Tract kit from Promega Corp. (Madison, WI) to purify the mRNA from a total input of 5 mg of CsCl-purified total RNA.

Ribonuclease Protection Analysis

An RNA probe complementary to the 5′ end of mouse *Dhfr* was transcribed from pSP64-RT10 [41] linearized with EcoRI by using Sp6 RNA polymerase. RNase protection analysis was performed as described previously with the following modifications [32]. Probe (5×10^4 cpm) was incubated with 80 µg of cytoplasmic RNA in 16 µL of formamide, (pH 8.0, and 4 µL of hybridization buffer (200 mM PIPES, 2 M sodium chloride, and 5 mM EDTA) at 90°C for 15 min and then at 52°C for 3 h. The RNA was then digested with 10 µg of RNase A and 1 U of RNase T1 in 300 µL of RNase digestion buffer (10 mM Tris-Cl, pH 7.5; 5 mM EDTA; and 300 mM sodium chloride) for 30 min at 30°C. The reaction mixtures were treated with 0.65% (w/v) sodium dodecyl sulfate (SDS), and 0.167 µg/µL proteinase K for 15 min at 37°C and then extracted with phenol/chloroform. Samples were ethanol precipitated and resolved by gel electrophoresis. The products were visualized by autoradiography and phosphorimager analysis.

Northern Hybridization Analysis

Poly(A)+ RNA samples (1.5 µg) were precipitated and resuspended in sample buffer (50% formamide; 2.2 M formaldehyde; 1 mM MOPS, pH 7.0; 0.4 M sodium

acetate; and 0.05 mM EDTA). The samples were heated for 5 min at 60°C and then placed on ice. A 0.2 vol of glycerol solution (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, and 0.4% xylene cyanol) was added to the samples, which were then separated on a 1% agarose formaldehyde gel by using MOPS electrophoresis buffer (20 mM MOPS, 8 mM sodium acetate, and 1 mM EDTA, pH 7.0). The gel was soaked in 20× standard saline citrate transfer buffer for 20 min before overnight transfer onto a Maximum Strength Nytran nylon hybridization membrane (Schleicher & Schuell, Kenne, NH). The membranes were baked for 1.5 h at 80°C in a vacuum oven after transfer. The blots were prehybridized in hybridization solution (50% formamide, 5× Denhardt's solution, 0.1% SDS, 100 μg of sonicated salmonsperm DNA, and 5× standard saline phosphate with EDTA (SSPE)) for 1 h. Probe fragments were labeled with ^{32}P by nick translation [42]. To make probes, pmcdc2 (a gift of Dr. Joe Nevins) was digested with EcoRI to release a 1.3-kb fragment that corresponds to the mouse *cdc2* sequence. pmAlb2 (a gift of Dr. Shirley Tilghman) was digested with HindIII to release a 700-bp fragment that corresponds to mouse albumin cDNA sequences. ^{32}P-labeled probe was added to hybridization mix, and hybridization was performed for 12–48 h. The blots were washed for 30–60 min in 1X SSPE and 0.5% SDS at 42°C, 30–60 min in 0.1X SSPE and 0.5% SDS at 42°C, and, if necessary, 30–60 min in 0.1X SSPE and 0.5% SDS at 60°C. Signals were visualized by autoradiography and phosphorimaging. The resulting signals were quantitated by phosphorimager analysis. For analysis, all signals were normalized to signals observed by using an albumin probe to control for sample loading. The values were plotted as the percentage of maximum experimental signal normalized to the albumin signal.

Chemical Carcinogenesis

B6 *E2F1*-heterozygous parents, the progeny of a third-generation backcross of the *E2F1* null allele onto the B6 background, were bred to produce *E2F1*–wild-type, *E2F1*-heterozygous, and *E2F1*-nullizygous progeny. C3H *E2F1*-heterozygous parents were bred to produce *E2F1*–wild-type, *E2F1*-heterozygous, and *E2F1*-nullizygous progeny. To control for any effects on liver tumor development that the 129Sv alleles passed on to the progeny, mating pairs were maintained throughout the experiment to produce families of progeny; e.g., the wild-type B6 family 1 mice and the nullizygous B6 family 1 mice were siblings from the same parents. As noted below, the mice were not genotyped until they were killed. This, in combination with the fact that the number of mice per litter varied, resulted in the analysis of a different number of mice of each genotype from the different families. Nine different wild-type and nullizygous families were created on the B6 background. However, due to death throughout the 32 wk of the experiment, the only wild-type B6 families that could be scored were families 1, 2, 3, and 9, and the only *E2F1*-nullizygous B6 families that could be scored were families 1, 2, 3, 4, 8, and 9. Five

different wild-type and nullizygous families were created on the C3H background; again, wild-type C3H family 1 mice and nullizygous C3H family 1 mice were siblings from the same parents. In this case, all families could be scored at the end of the experiment.

The relative tumor numbers of different genotypic classes were analyzed within each family. Male pups were injected intraperitoneally at 12 d of age with diethylnitrosamine (DEN) (Sigma Chemical Co., St. Louis, MO) at a dose of 0.1 μM/g body weight. The mice were killed at 32 wk of age. Their livers were dissected, and visible surface tumors greater than 1 mm in diameter were counted. The animals were then genotyped, and the relationship between genotype and tumor number was evaluated. The data were statistically analyzed by using the Wilcoxson rank sum test with Mstat software.

Nuclear Extract Preparation

After the mice were killed by cervical dislocation, their livers were dissected and either processed immediately or frozen in liquid nitrogen for storage at −70°C. The livers were placed in a 15 mL homogenizer with 11 mL of 0.25 M homogenization buffer (0.25 M sucrose; 10 mM HEPES, pH 7.6; 25 mM KCl; 1 mM EDTA; 0.1 mM spermine; 0.5 mM spermidine; 5 mM sodium fluoride; 1 mM dithiothreitol (DTT); 5 μg/mL aprotinin; 0.1 mg/mL phenylmethyl sulforyl fluoride (PMSF); 5 μg/mL leupeptin; 1 μg/mL pepstatin A; and 40 μL/mL Complete Inhibitor Mix (Boehringer Mannheim Corp., Indianapolis, IN). The livers were homogenized with a B pestle. Twenty-four milliliters of 2.3 M homogenization buffer (2.3 M sucrose; 10 mM HEPES, pH 7.6; 25 mM KCl; 1 mM EDTA; 0.1 mM spermine; 0.5 mM spermidine; 5 mM NaF; 1 mM DTT; 5 μg/mL aprotinin; 0.1 mg/mL PMSF; 5 μg/mL leupeptin; 1 μg/mL pepstatin A; and 40 μL/mL complete inhibitor mix (Boehringer Mannheim)) was added to the liver homogenate, and 32 mL of the diluted homogenate was added to an SW28 ultracentrifuge tube (Beckman Instruments, Palo Alto, CA). The homogenate was underlay with 7 mL of 2.3 M homogenization buffer and centrifuged at 4°C for 60 min at 24 000 x*g* in an SW28 rotor (Beckman Instruments). After centrifugation, the supernatant was aspirated, and the nuclear pellet was gently rinsed with 1 ml 0.1 M HEPES, pH 7.6. The pellet was resuspended in 150 μL of 5× 500 buffer (0.1 M HEPES, pH 7.6; 0.5 M KCl; 5 mM MgCl$_2$; 35% glycerol; 5 mM NaF; 1 mM DTT; 5 μg/mL aprotinin; 0.1 mg/mL PMSF; 5 μg/mL leupeptin; 1 μg/mL pepstatin A; and 40 μL/mL complete inhibitor mix (Boehringer Mannheim)) and pipetted gently. The resuspended nuclei were transferred to an Eppendorf tube and subjected to three repeats of a liquid-nitrogen freezing–37°C thawing cycle. The nuclear solution was incubated at 37°C for 10 min with nuclease stock solution (final concentrations 50 μg/mL RNase A, 100 μg/mL DNase I, 5 mM MgCl$_2$; and 10 mM Tris-Cl, pH 7.0). The nuclear solution was then placed on ice with a microstirbar on a stirring plate and stirred for 30 min. The nuclear homogenate was then microcentrifuged for 30 min at 4°C. The supernatant was

collected, and its protein content was determined by using the Bradford assay. The nuclear extract was flash-frozen in liquid nitrogen and stored at $-70°C$ until use.

Western Hybridization

Nuclear protein was resolved under reducing conditions on an 8% or 6% SDS-polyacrylamide gel by standard techniques [43]. The antibodies used were C-20 193X and KH95 sc251X (anti-E2F1) and C20 1082X (anti-E2F4) (Santa Cruz Biotechnology, Santa Cruz, CA). Antibody probing and washing were performed according to the manufacturers instructions. Signals were visualized by using secondary antibody conjugated to horseradish peroxidase and the ECL chemiluminescence kit (Amersham Corp., Arlington Heights, IL). The relative level of nuclear protein in each sample was assessed by analysis of the levels of TATA binding protein (TBP) by using an anti-TBP mouse monoclonal made by Dr. Nancy Thompson at the McArdle Laboratory.

RESULTS

No Deregulation of Liver Regeneration by Deficiency of E2F1 after 70% Partial Hepatectomy

A role for E2F1 in progression from a quiescent to a proliferating state has previously been suggested based on tissue-culture model systems [44]. However, the tissue-culture studies could not directly measure the effects of loss of E2F1 on proliferation. The liver of the *E2F1*-nullizygous mouse is a useful in vivo model for testing this hypothesis. In the adult mouse liver, only 0.1–1% of the cells are in S phase under normal conditions [45]. Surgical removal of the two largest lobes of the liver stimulates the remaining hepatocytes to synchronously proliferate, reaching an S-phase peak approximately 35 h after surgery in B6 male mice [40]. The remaining liver cells continue to proliferate until the liver mass is restored. At this point, approximately 14 d after surgery, proliferation ceases completely. Therefore, our first experiments to test the role of E2F1 in cell proliferation focused on regenerating liver.

We had previously demonstrated that *E2F1* mRNA levels are low in quiescent cells and high in S-phase cells after partial hepatectomy of B6 and C3H mice [40]. However, E2F1 protein levels had not been examined in liver samples. Therefore, we prepared nuclear extracts from the livers of newborn mice and from S-phase regenerating liver, as two different sources of proliferating hepatocytes. The levels of E2F1 in these samples were compared with the level in quiescent liver from 6-wk-old mice. In Figure 1, we demonstrate that E2F1 protein levels were greatly reduced in the quiescent adult livers relative to the newborn and S-phase livers. Thus, E2F1 protein levels correlated with active hepatocyte proliferation.

Studies have shown that expression of E2F1 can promote S-phase entry and that E2F1 can mediate the transcriptional activation of many E2F target genes in cultured cells [21, 46]. Because E2F1 may be important in
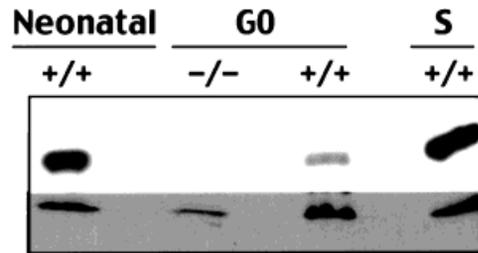

Figure 1. Liver growth state influenced E2F1 protein expression. Western hybribization using E2F1 antibody was performed with 200 µg of nuclear extract made from the livers of wild-type postnatal day 1 mice (Neonatal), 6-wk-old quiescent nullizygous and wild-type mice (G0), and 6-wk-old wild-type S-phase mice (S1). The relative level of nuclear protein in each sample was assessed by analysis of the level of TBP.

the activation of genes necessary for cell proliferation, a deficiency of E2F1 may result in an inhibition of liver regeneration. If this is true, then the livers of *E2F1*-nullizygous mice may not grow or reach their preoperative mass as quickly as the livers of wild-type mice do during the regenerative period. E2F1 may also be involved in the transcriptional repression of certain genes involved in DNA synthesis and cell-cycle progression by localizing Rb to their promoters [47]. If the role of E2F1 in liver cell growth control is to negatively regulate genes involved in proliferation, cells that lack E2F1 may be less responsive to antiproliferative signals than cells that are capable of forming active E2F1-pRb complexes. If the ability of hepatocytes to respond to antiproliferative signals is impaired because of deficiency of E2F1 in *E2F1*-nullizygous mice, liver regeneration in these animals may not cease at 14 d after surgery, as it does in wild-type mice.

To determine the role of E2F1 in the hepatocyte-regeneration cell cycle, we induced liver regeneration by performing 70% partial hepatectomies on 6-wk-old male wild-type and *E2F1*-nullizygous mice. The mice were killed 0, 3, 7, 11, 14, and 28 d after surgery, and the liver masses of the *E2F1*-nullizygous mice and *E2F1*–wild-type mice were determined as a measure of liver regeneration. As a control for the effects that the surgical procedure could have on liver weight, groups of *E2F1*-nullizygous and –wild-type mice were also subjected to mock hepatectomy. Because the mass of the liver is proportional to the body mass of the mouse, the liver weights of the experimental mice were normalized to this body weights for analysis. Three to 11 mice were used for each partial-hepatectomy and mock-hepatectomy data point.

Our results, shown in Figure 2, demonstrate that both the wild-type and *E2F1*-nullizygous mice that underwent mock hepatectomy displayed fairly constant liver–to–body weight ratios throughout the experimental period. The slight decrease in the liver–to–body weight ratio observed between 3 and 14 d was due to the normal increase in body weight of the mice over the experimental time period. The decreased liver–to–body weight ratios immediately after hepatectomy indicated that we successfully removed 70% of the liver. In both the *E2F1*-
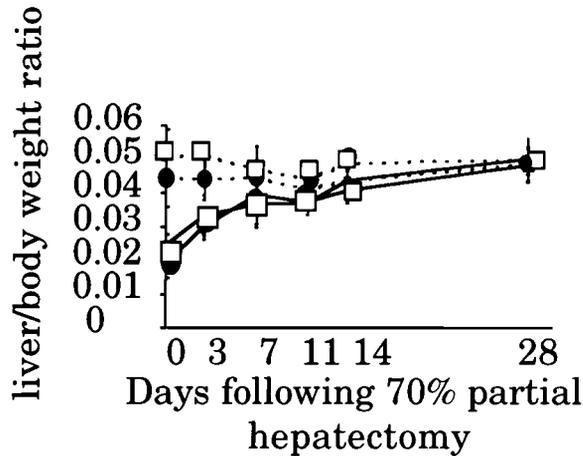
Figure 2. Deficiency of E2F1 did not deregulate liver regeneration after partial hepatectomy. Seventy percent partial hepatectomies and mock hepatectomies were performed on 6-wk-old male wild-type and *E2F1*-nullizygous mice. The mice were killed 0, 3, 7, 11, 14, and 28 d after surgery. To assess liver regeneration as a function of liver-mass restoration, liver–to–body weight ratios were determined. Three to 11 mice were used per partial-hepatectomy and mock-hepatectomy time point. Solid lines, animals receiving hepatectomies; dashed lines, animals receiving mock hepatectomies; white boxes, wild-type animals; black circles, nullizygotes.

nullizygous mice and the wild-type mice, the preoperative liver masses were restored during liver regeneration by 14 d after hepatectomy. At all experimental time points, the average liver weights of wild-type and nullizygous mice were not significantly different. These data suggest that deficiency of E2F1 does not impair liver regeneration. At 28 d after surgery, *E2F1*-nullizygous and *E2F1*–wild-type mice displayed similar liver masses, suggesting that the *E2F1*-nullizygous livers also respond properly to antiproliferative stimuli at the end or the 14-d regenerative period.

No Alteration by Deficiency of E2F1 of Regulation of *Dhfr* and *cdc2* mRNA after 70% Partial Hepatectomy

Even though no gross abnormalities were observed during liver regeneration in the *E2F1*-nullizygous mice, it remained possible that loss of E2F1 could influence the expression of particular E2F target genes. It is not yet known if different E2F family members regulate distinct subsets of cellular genes in vivo. However, we have previously shown that the pattern of *Dhfr* mRNA levels are very similar to the pattern of *E2F1* mRNA levels after partial hepatectomy in two different strains of mice [40]. To determine whether deficiency of E2F1 influences the expression of *Dhfr* mRNA during the proliferative cycle in mouse liver, we performed ribonuclease protection analysis on RNA samples taken at various times after 70% partial hepatectomy (Figure 3A). Although the overall levels of *Dhfr* mRNA slightly increased in *E2F1*-nullizygous mice relative to wild-type mice, the pattern of S-phase increase was not altered, suggesting that E2F1 does not influence the timing of S-phase activation of this gene in mouse liver. We have previously shown that the *Dhfr* gene has a G_1/S-phase induction of expression

primarily due to activation of transcription mediated through the E2F sites in its promoter [32,48]; mutation of the E2F site results in only a twofold increase in transcription in mouse fibroblasts [48]. Therefore, the twofold increase in *Dhfr* mRNA levels in the nullizygous mice could be due to loss of E2F1-mediated repression. Alternatively, preliminary data suggests that there are slightly more proliferating hepatocytes in this 6-wk-old *E2F1*-nullizygous mice, suggesting that the twofold increase in *Dhfr* mRNA could be due to an increased number of S-phase cells (Lukas, ER, unpublished data).

In contrast to *Dhfr*, other E2F target genes such as *cdc2* are thought to be mainly regulated by G_0-phase mediated repression of transcription. Therefore, we also examined the pattern of expression of *cdc2* mRNA in the regenerating livers of wild-type and nullizygous mice. If E2F1 is the major E2F responsible for the repression of cdc2, we would expect to see higher levels of *cdc2* mRNA in quiescent hepatocytes (i.e., at the 0-h time point). As shown in Figure 3B, *cdc2* mRNA levels increased after
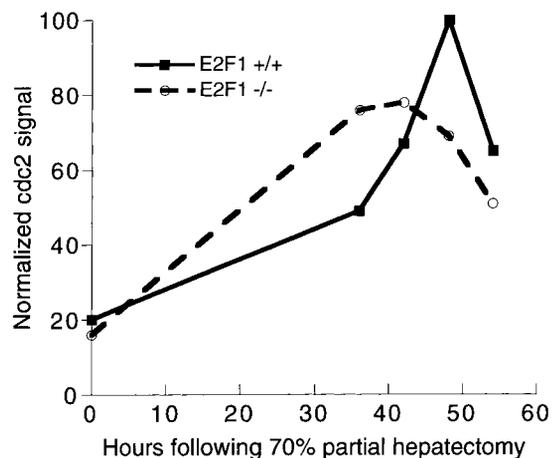
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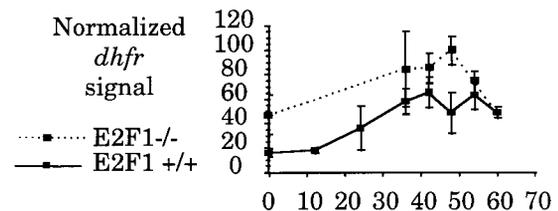
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Figure 3. The growth regulation of *Dhfr* and *cdc2* mRNA was not altered in *E2F1* nullizygous mouse liver. (A) Ribonuclease protection assays were performed with a ^{32}P-labeled antisense probe to the *Dhfr* mRNA and 1.5 μg of poly(A)+ RNA isolated from the livers of 6-wk-old male mice at the indicated time after 70% partial hepatectomy. The data shown are averages of several experiments; the error bars represent the standard deviation. (B) Northern hybridization was performed with a ^{32}P-labeled *cdc2* cDNA probe and 1.5 mg of poly(A)+ RNA isolated from the livers of 6-wk-old male *E2F1*-nullizygous and wild-type mice at the indicated times after 70% partial hepatectomy.
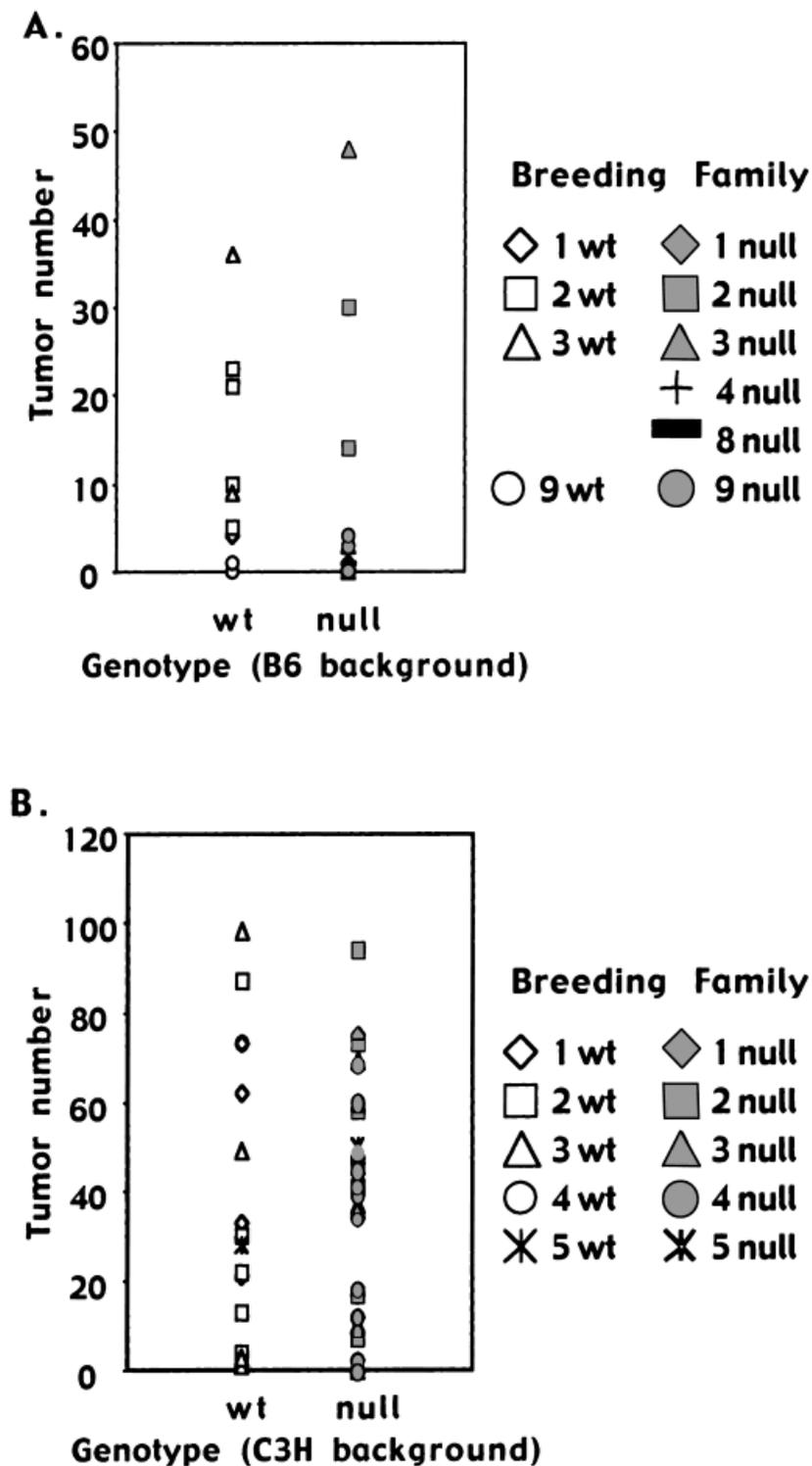
Figure 4. Hepatocarcinogen susceptibility was not different in *E2F1* wild-type and nullizygous animals. *E2F1* heterozygous parents of B6 (A) and C3H (B) mice were bred to produce *E2F1* wild-type and *E2F1* nullizygous progeny. Male pups were injected with DEN at 12 d of age and killed 30 wk later. Then, surface tumors >1 mm in diameter were counted. Tumor number and genotype were then analyzed, and the results are reported for each of the different breeding families. Each data point indicates the number of tumors in an individual animal. The family to which the animal belonged is indicated by the symbol, with open symbols indicating wild-type animals and filled symbols indicating nullizygous animals.

partial hepatectomy in both wild-type and nullizygous mice. Importantly, the levels of *cdc2* mRNA in quiescent hepatocytes did not increase in mice lacking E2F1. In this experiment, it appears that there may be a slightly different pattern of *cdc2* mRNA expression in the regenerating hepatocytes of *E2F1*-nullizygous mice versus wild-type mice. However, based on the results of several other hepatectomy time-courses, we believe that the slight differences were due to mouse-to-mouse variation (data not shown). Therefore, we conclude that the absence of E2F1 does not greatly affect the growth-regulated expression of E2F target genes that are regulated by activation (*Dhfr*) or repression (*cdc2*).

Liver Tumor Development in E2F1-Deficient Mice

We did not observe significant alterations in cell-cycle progression after partial hepatectomy, a model of transient hepatocyte proliferation that includes one or two cell-cycles. However, we also wished to determine if deficiency of E2F1 influences the growth properties of neoplastic hepatocytes undergoing continuous proliferation. Inbred strains of mice can vary significantly in their liver cell growth properties and susceptibility to liver tumor development [49]. The genetic background of the *E2F1*-nullizygous mice consists of B6 and a 129Sv substrain [50], strains that have been shown to be resistant to liver tumor development [49,51]. Thus, the original studies of *E2F1*-knockout mice were not conclusive concerning the role of E2F1 in liver tumor development. We therefore turned to chemically induced hepatocarcinogenesis as a model system to examine the effects of E2F1 deficiency in hepatocytes that have undergone neoplastic transformation. Also, because E2F1 has been shown to have both tumor-promoting and tumor-suppressing capabilities, we transfered the *E2F1*-knockout allele to a strain of mice that develops a low number of tumors (B6) and a strain of mice that develops a high number of tumors (C3H).

To determine the relative susceptibility of *E2F1*−wild-type and -nullizygous mice to liver tumor development, we treated 12-d-old male mice with DEN. To control for any effects on liver tumor development that the 129Sv alleles passed on to the progeny from the parents, mating-pairs were maintained throughout the experiment to produce families of progeny. Each data point in Figure 4 represents the number of tumors in an individual animal; the family to which the animal belonged is indicated by the symbol, with the open symbols indicating wild-type and the filled symbols indicating nullizygous animals. Analysis of the tumor number and genotype was performed within each family by using the Wilcoxson rank-sum statistical test. The obtained Wilcoxson rank sum scores were then combined by using the method of Lehman to produce a *P* value of 0.68 for the B6 mice (Figure 4A) and a *P* value of 0.35 for the C3H mice (Figure 4B). Based on this data, we concluded that there is no significant correlation between liver tumor susceptibility and characteristics and genotype at the *E2F1* locus in either strain of mice. We also observed that the sizes and
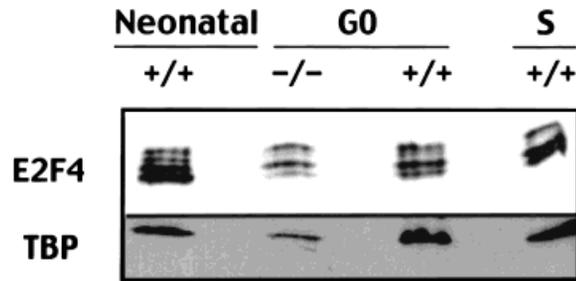

Figure 5. E2F4 was abundant in mouse liver. Western hybridization using E2F4 antibody was performed with 200 μg of nuclear extract made from the livers of wild-type postnatal day 1 mice (Neonatal), 6-wk-old quiescent nullizygous and wild-type mice (G0), and 6-wk-old wild-type S-phase mice (S). The relative level of nuclear protein in each sample was assessed by analysis of the levels of TBP.

stages of the tumors, as established by histological analysis, were similar in the wild-type and nullizygous mice. The tumors were mainly type A and type B hepatomas, with no difference in distribution observed between genotypes (data not shown).

Abundant E2F4 in Mouse Hepatocytes

Our results suggest that E2F1 activity is not required for proliferation of normal or tumorigenic hepatocytes. It is possible that other E2F family members are critical for liver cell growth and are present in *E2F1* nullizygous mouse liver. Therefore, we performed western hybridization analysis of other E2F family members by using nuclear extracts from both quiescent and proliferating livers. The most abundant E2F in all cell lines examined to date is E2F4. In tissue-culture systems, E2F4 protein is expressed constitutively in all stages of the cell cycle; E2F4 activity is regulated by interaction with p107, not by protein levels. As shown in Figure 5, E2F4 was very abundant in postnatal, adult quiescent, and adult S-phase regenerating livers. We have not been able to detect other E2F family members by using western analysis, suggesting that the levels of the other E2Fs must be extremely low in liver tissue. These data demonstrate that E2F1 is not the only E2F in wild-type mice. Therefore, the loss of E2F1 may not dramatically alter the overall amount of E2F activity in the liver.

DISCUSSION

We used the *E2F1*-nullizygous mouse to characterize the effects of E2F1 deficiency on cell growth, E2F target gene expression, and tumorigenesis. We had previously shown that both *E2F1* and *Dhfr* show a similar pattern of expression in regenerating hepatocytes; the levels of both mRNAs peak at the G_1/S-phase boundary in C3H mice, but the peak of both mRNAs is in late S-phase in B6 mice [40]. These observations demonstrating that E2F1 levels correlate with the expression pattern of at least one E2F-regulated gene in two different strains of mice suggested that E2F1 may be a critical regulator of hepatocyte proliferation. However, our studies using the *E2F1*-nullizygous mice demonstrated that liver regeneration proceeded similarly in them and wild-type animals. We

also found that *Dhfr* mRNA was maintained at a high level in S phase in the absence of E2F1 and that the *cdc2* promoter activity was not derepressed in quiescent hepatocytes in *E2F1*-nullizygous mice. Our results are surprising in light of a recent study published while our work was in progress. Wang et al. [52] showed that quiescent embryonic fibroblasts derived from *E2F1*-nullizygous mice have a defect in cell-cycle reentry after stimulation with high concentrations of serum. Wang and colleagues suggested that E2F1 plays a unique role in the transition of resting fibroblasts from G_0 to a proliferating state. Our examination of the role of E2F1 in the transition of resting hepatocytes to a proliferating state indicated that the role of E2F1 in regulating cell proliferation is probably different in different tissues.

Loss of E2F1 can both reduce and promote tumorigenesis, depending upon the tissue studied. For example, loss of E2F1 results in increased tumor development in the lung, reproductive tract, and lymphatic system [39]. In contrast, loss of E2F1 can reduce the frequency of pituitary and thyroid tumors that occur in $Rb1^{(+/-)}$ mice [50]. Therefore, we used two different strains of mice, one with low tumor susceptibility (B6) and one with high tumor susceptibility (C3H) to determine the influence of E2F1 on hepatocarcinogenesis. However, we found that the loss of E2F1 was neither a tumor promoter nor a tumor suppressor in the liver, again emphasizing the conclusion that the role of E2F1 in particular tissue cannot be assumed based on data collected by using a different system.

In conclusion, our results suggest that E2F1 does not influence liver growth or tumorigenicity. It is important to note that the inactivation of the *E2F1* locus has been shown to result in the loss of functional E2F1 protein in embryonic fibroblasts [39]; the western blot shown in Figure 1 confirmed the absence of E2F1 in the liver of the nullizygous animals. E2F1 is a member of a multigene family, and thus functional redundancy may prevent the loss of E2F1 from having a major effect in the liver. Accordingly, we showed that E2F4 was very abundant in the liver. We also recently showed using a formaldehyde cross-linking protocol, that E2F4 was bound in vivo to all E2F target genes tested (Wells J, Farnham PJ, unpublished data). In contrast, E2F1 was bound to only a subset of those target genes. Thus, we suggest that E2F4 may be the major E2F activity that controls proliferation of normal and neoplastic hepatocytes. Therefore, in our future analyses of the role of the E2F family in liver growth and tumorigenicity, we will use either *E2F4*-nullizygous mice or transgenic mice expressing a dominant negative *E2F4*.

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