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Role of Myc in hepatocellular proliferation and hepatocarcinogenesis

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Abstract

Background & Aims—MYC is involved in cell growth, proliferation, apoptosis, energy metabolism, and differentiation. Whether it is essential for hepatocellular proliferation and carcinogenesis is unclear due to a lack of an efficient hepatocyte-specific *Myc* disruption model. This study used a novel genetic model to investigate the involvement of MYC in hepatocellular proliferation and hepatocarcinogenesis in mice.

Methods—Temporal hepatocyte-specific *Myc* disruption was achieved by use of the tamoxifen-inducible Cre-ERT² recombinase system under control of the serum albumin promoter. Hepatocyte proliferation was assessed by administering peroxisome proliferator-activated receptor α (PPAR α) agonist Wy-14,643. A diethylnitrosamine-induced liver cancer model was used to evaluate the role of *Myc* in hepatocarcinogenesis.

Results—Tamoxifen administration induced recombination of *Myc* specifically in hepatocytes of *Myc^{fl/fl},ERT2-Cre* mice. When treated with a known hepatocellular proliferative stimulus Wy-14,643, *Myc^{fl/fl},ERT2-Cre* mice showed a lower liver/body weight ratio and suppressed hepatocyte proliferation as compared to *Myc^{fl/fl}* mice. Hepatic expression of cell cycle control genes, DNA repair genes, and *Myc* target gene miRNAs were upregulated in Wy-14,643-treated *Myc^{fl/fl}* mouse livers, but not in Wy-14,643-treated *Myc^{fl/fl},ERT2-Cre* livers. However, no differences were observed in the lipid-lowering effect of Wy-14,643 between *Myc^{fl/fl},ERT2-Cre* and *Myc^{fl/fl}* mice, consistent with no differences in the expression of several PPAR α target genes involved in fatty acid β -oxidation. Moreover, when subjected to the diethylnitrosamine liver cancer bioassay, *Myc^{fl/fl},ERT2-Cre* mice exhibited a markedly lower incidence of tumor formation compared with *Myc^{fl/fl}* mice.

Conclusion—*Myc* plays an essential role in hepatocellular proliferation and liver tumorigenesis.

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Keywords

MYC; cell proliferation; tumorigenesis; BrdU; cell cycle control

INTRODUCTION

c-MYC, encoded by proto-oncogene *Myc*, is a pleiotropic transcription factor that belongs to the basic helix-loop-helix leucine zipper (bHLH) family of transcription factors [1, 2]. MYC binds to a 6-nucleotide DNA consensus sequence CACGTG (E-box) by forming a heterodimer with MAX, another bHLH protein to participate in the control of a wide variety of genes that are involved in the regulation of cell growth, cell proliferation, apoptosis, and differentiation by stimulating metabolism and protein synthesis [2].

MYC expression promotes the transition from G0/G1 to S phase of the cell cycle in multiple cell types by regulating cyclin/cyclin-dependent kinase (CDK) complexes [3, 4]. Rapid MYC induction is observed in hepatocytes of proliferating livers induced by peroxisome proliferator-activated receptor α (PPAR α) activation [5, 6], which is tightly correlated with G0/G1 to S-phase transition. Dysregulation of MYC is commonly found in several cancers [7–9]. In the liver, forced overexpression of MYC leads to spontaneous hepatocellular carcinomas [10]. In transgenic mice with inducible MYC, withdrawal of ectopic MYC expression in liver results in regression of tumors [11, 12]. However, a direct role for MYC in hepatocellular proliferation and carcinogenesis has not been established. Although most studies demonstrate that overexpression of MYC induces hepatocellular proliferation and tumorigenesis, direct evidence for an essential role of endogenous MYC in hepatocellular proliferation and tumorigenesis is lacking.

In this study, hepatocyte-specific temporal disruption of *Myc* was accomplished using the albumin promoter (SA)-expressing Cre-ER^{T2} system that yields hepatocyte-specific expression and activation Cre recombinase in the presence of tamoxifen [13]. Disruption of *Myc* suppressed hepatocellular proliferation induced by the PPAR α agonist Wy-14,643. Microarrays revealed that MYC regulates a large array of genes important for cellular proliferation and cell cycle progression that is not found in Wy-14,643-treated mice with hepatic *Myc* disruption. Disruption of MYC expression resulted in a lower incidence of diethylnitrosamine (DEN)-induced liver tumors. These data demonstrate that MYC is required for hepatocellular proliferation and liver tumorigenesis.

MATERIALS & METHODS

Animal and diets

Myc^{fl/fl} mice were provided by John M. Sedivy (Brown University) and described previously [14]. For temporal hepatocyte-specific disruption, *Myc*^{fl/fl} mice were crossed with mice harboring the Cre-ER^{T2} recombinase driven by the SA, designated SA-Cre-ER^{T2} [15], obtained from Pierre Chambon and Daniel Metzger (University of Strasbourg), to yield the *Myc*^{fl/fl},ERT2-Cre mouse line. *Myc*^{fl/fl},ERT2-Cre and *Myc*^{fl/fl} mice were maintained as littermates. For activation of the SA-Cre-ER^{T2} driven Cre recombinase, 6- to 8-week-old male mice were injected intraperitoneally with 2 mg tamoxifen and then fed a tamoxifen diet (1 mg/kg, Dyets Inc., Bethlehem, PA). For Wy-14,643 treatment, mice were fed for 3 days with tamoxifen diet before exposure to pelleted chow containing 0.1% Wy-14,643 (Bioserv, Frenchtown, NJ) and receiving 2 mg of tamoxifen injection every other day for the indicated time. All mice were housed in light and temperature-controlled rooms with free access to water and pelleted chow *ad libitum*. All animal experiments were performed in accordance

with the AAALAC international guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

DEN-induced hepatocarcinogenesis

Mye^{fl/fl} and *Mye^{fl/fl},ERT2-Cre* mice were injected intraperitoneally with 50 µg/g of tamoxifen at 10 days old and with 25µg/g of DEN (Sigma-Aldrich, St. Louis, MO) at 15 days old as described [16]. After weaning at 21 days old, mice were fed the tamoxifen diet for 5 days followed by a single dose of tamoxifen injection (2 mg/mice) every other week until killing and necropsy at 9 months. The livers were then separated into individual lobes, analyzed for the presence of tumors, and subjected to histological analysis.

Triglyceride and cholesterol analysis

Serum triglycerides and cholesterol levels were measured using Serum Triglyceride Determination Kit and Serum Cholesterol Determination Kit according to manufacturer's recommendation (Wako, Richmond, VA).

Quantitative real-time PCR

Total RNA extraction and quantitative real-time PCR (qPCR) were performed as previously described [15]. Primers were designed using qPrimerDepot and sequences were listed in Supplementary Table 1. Values were quantified using the comparative CT method, and normalized to β -actin mRNA. To determine the expression levels of microRNAs, Taqman microRNA assays (Invitrogen) directed to mmu-mir-17-5p, mmu-mir17-3p, mmu-mir-18a-5p, mmu-mir-19a, mmu-mir-290-5p, mmu-mir-291-3p, mmu-mir-293, mmu-mir-295, mmu-mir-100, mmu-let-7a-2 and an endogenous reference snoRNA55, were used following the manufacture's protocol (Applied Biosystems).

cDNA microarray analysis

Dye-coupled cDNAs were purified with MiniElute PCR purification kit (Qiagen) and hybridized to an Agilent 44K mouse 60-mer oligo microarray (Agilent Technologies, Santa Clara, CA). The data were processed and analyzed by Genespring GX software package (Agilent Technologies).

Western blot analysis

Liver whole-cell extracts were subjected to Western blot using primary monoclonal anti-MYC antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) as previously described [15], and the signals obtained normalized to GAPDH (Santa Cruz Biotechnology Inc).

Histological analysis

Hematoxylin and Eosin (H&E) staining were performed on formalin fixed paraffin embedded sections. Immunohistochemistry staining for BrdU was performed as previously described using antibodies against BrdU (AbDSerotec, Raleigh, NC) [17].

Data analysis

Data are expressed as means $\pm$ standard deviations (SD). P values were calculated by independent t-test between two groups or one-way ANOVA analysis with Tukey correction between multiple groups using Prism 5. A $P < 0.05$ was considered significant.

RESULTS

Generation of a mouse model with temporal hepatocyte-specific disruption of *Myc*

Myc^{fl/fl} mice were crossed with SA-Cre-ERT² transgenic mice to generate a temporal and conditional disruption of *Myc* (*Myc^{fl/fl},ERT2-Cre*). To confirm hepatocyte-specific disruption of *Myc*, *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice were treated with one dose of vehicle or tamoxifen, and livers and extrahepatic tissues analyzed at 24 hours post-treatment. *Myc* recombination was only observed in livers from tamoxifen-treated *Myc^{fl/fl},ERT2-Cre* mice but not from *Myc^{fl/fl}* or vehicle-treated *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice (Figure 1A). A decrease in *Myc* mRNA level was also achieved in livers only from tamoxifen-treated *Myc^{fl/fl},ERT2-Cre* mice and not from *Myc^{fl/fl}* mice; *Myc* mRNA was not changed in extrahepatic tissues of tamoxifen-treated *Myc^{fl/fl},ERT2-Cre* mice (Figure 1B). Western blot analysis of liver extracts revealed diminished MYC protein levels in tamoxifen-treated *Myc^{fl/fl},ERT2-Cre* mice (Figure 1C). These data indicate that an efficient inducible liver-specific disruption of *Myc* was achieved using the SA-Cre-ERT² system.

Myc disruption represses hepatocellular proliferation and hepatomegaly but not β -oxidation induced by PPAR α agonist Wy-14,643

Administration of PPAR α agonists results in hepatocyte proliferation and hepatomegaly in mice and rats [18]. Previous studies demonstrated that PPAR α agonists induce *Myc* mRNA in rats and mice and that increased MYC protein levels correlated with hepatocellular proliferation [6]. When treated with Wy-14,643 for two weeks, *Myc^{fl/fl},ERT2-Cre* mice showed a lower number of BrdU-positive hepatocytes compared to *Myc^{fl/fl}* mice (Figure 2A). The labeling index was $21 \pm 4.7\%$ for hepatocytes in Wy-14,643-treated *Myc^{fl/fl},ERT2-Cre* mice for hepatocytes compared to $67 \pm 7.1\%$ in Wy-14,643-treated *Myc^{fl/fl}* mice (Figure 2B), demonstrating that *Myc* disruption markedly suppresses Wy-14,643-induced hepatocyte proliferation. It was also noteworthy that the BrdU labeling index of non-parenchymal cells was comparable between Wy-14,643-treated *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice (Figure 2B), indicating that proliferation of non-parenchymal cells is not affected by disruption of *Myc* in hepatocytes. The decrease in hepatocellular proliferation was further revealed by a lower liver/body weight ratio in the *Myc^{fl/fl},ERT2-Cre* mice as compared to the *Myc^{fl/fl}* mice (Figure 2C). However, the serum triglyceride-lowering extent was similar in the mouse lines (Figure 2D), and the induction of well-characterized PPAR α target genes, acyl-CoA oxidase (*Acox1*), cytochromes P450 4a10 and 4a14 (*Cyp4a10* and *Cyp4a14*) did not differ between *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice (Figure 2E), indicating that MYC is not required for PPAR α -dependent fatty acid β -oxidation.

Myc controls cellular growth and proliferation networks in hepatocytes

To further explore the expression profile of genes controlled by MYC, microarray analysis was conducted on liver RNA from *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice on chow diet or Wy-14,643-containing diet. The data revealed that 1247 genes were significantly decreased and 825 genes increased in *Myc^{fl/fl},ERT2-Cre* mice compared with *Myc^{fl/fl}* mice (Supplementary Fig. 1, microarray data in <http://www.ncbi.nlm.nih.gov/geo/>, GEO # GSE43842). Thirty-three of the decreased genes are known to be involved in cell cycle control and 13 are involved in DNA replication, recombination and repair (Table 1). Pathway analysis revealed that the top down-regulated network was associated with cellular growth and proliferation (Table 2). These data reinforce a role for MYC in driving hepatocellular growth and proliferation by controlling the cell cycle, and DNA replication, recombination and repair.

To confirm these pathways, qPCR analysis of mRNAs encoded by cell cycle control genes was performed in *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice treated with Wy-14,643 for 48 hours.

Ablation of MYC expression significantly down-regulated a series of cyclins, including cyclin mRNAs, *Ccnb1*, *Ccne1* and *Ccne2*, and cyclin-dependent kinase 1 (*Cdk1*) that are critical for control of the G1/S phase in hepatocytes (Figure 3A). Loss of MYC in hepatocytes also suppressed genes that are crucial for the G2/M DNA damage checkpoint, such as checkpoint kinase 1 homologue (*Chk1*) and RAD51 homolog (*S. cerevisiae*) (*Rad51*). Moreover, a family of minichromosome maintenance (*Mcm*) genes required for the initiation and elongation of DNA replication, were dramatically reduced by liver-specific *Myc* disruption (Figure 3B). These data demonstrate that disruption of *Myc* suppresses Wy-14,643-induced hepatocellular proliferation via inhibition of genes responsible for G1/S cell cycle, checkpoint and DNA damage repair.

MYC regulates miR17-92 cluster in hepatocytes

PPAR α activation in mice leads to an increase in mir-17-92 polycistronic cluster [6]. A critical mechanism by which MYC induces proliferation and tumorigenesis is direct regulation of the mir17-92 miRNA cluster [19]. To test whether PPAR α -dependent induction of mir-17-92 cluster is MYC dependent, qPCR analysis was performed. Mir-17-5p was rapidly induced by over 5-fold after two days of Wy-14,643 exposure in *Myc^{fl/fl}* mice, but not in *Myc^{fl/fl},ERT2-Cre* mice (Figure 3C). Disruption of hepatic *Myc* could also inhibit the induction of other mir-17-92 cluster family members such as mir-17-3p, mir-18a-5p, and mir-19a. However, no significant changes were observed in expression of the mir-290-295 cluster (homolog of human miR-371-3 cluster in mouse) or mir-100/let-7a2 (Figure 3C). This is consistent with previous studies that MYC activates this miRNA cluster [6].

Myc^{fl/fl},ERT2-Cre mice are resistant to DEN-induced hepatocarcinogenesis

To investigate the direct role of MYC in liver tumorigenesis, the susceptibility of *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice to DEN-induced liver cancer was determined. Injection of 25 μ g/g DEN resulted in 100% incidence of visible tumors in *Myc^{fl/fl}* mice whereas only 33% incidence in *Myc^{fl/fl},ERT2-Cre* mice (Figure 4A); the number and size of tumors were also decreased in *Myc^{fl/fl},ERT2-Cre* mice (Figure 4B and 4C). Histological analysis demonstrated that *Myc^{fl/fl},ERT2-Cre* mice had a 55% incidence of preneoplastic foci and 33% adenomas but no carcinomas, whereas 100% of *Myc^{fl/fl}* mice had both preneoplastic foci and adenomas, with 67% of them developing carcinomas (Figure 4D and 4E). Ki67 immunostaining showed a significant increase in of hepatocyte proliferation in DEN-treated *Myc^{fl/fl}* that was markedly reduced in *Myc^{fl/fl},ERT2-Cre* livers (Figure 4F). qPCR analysis further demonstrated a significant increase of mir-290-295 RNA in DEN-induced liver tumors from the DEN-treated *Myc^{fl/fl}* mice as compared to but not in DEN-treated *Myc^{fl/fl},ERT2-Cre* mice; normal liver tissues from saline-treated *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice were used as controls (Figure 4G), which is consistent with a previous study showing that miR-371-3 (homolog of mir-290-295 in humans) could be a direct target of MYC in HCC [20]. To investigate whether the 55% incidence of preneoplastic foci and 33% adenomas developed in *Myc^{fl/fl},ERT2-Cre* mice was due to the residual *Myc*-floxed allele, *Myc* recombination was assessed in a few tumors. PCR analysis showed that a slight band for *Myc*-floxed allele was detected in adenomas from *Myc^{fl/fl},ERT2-Cre* mice suggesting that a small amount of MYC could be produced in these tumors, but likely less than that in tumors from *Myc^{fl/fl}* mice (Supplementary Fig. 2). This indicates that DEN-induced tumor hepatocytes from *Myc^{fl/fl},ERT2-Cre* mice could escape *Myc* disruption through the transformation of residual hepatocytes or stem cells that did not undergo recombination of the *Myc*-floxed allele.

DISCUSSION

MYC, a transcription factor encoded by proto-oncogene *c-myc*, is thought to contribute to hepatocyte proliferation, liver regeneration and tumorigenesis in response to hepatic injury

and carcinogens exposure. Given the potential key role of *Myc*, it would be of value to determine whether MYC is essential for these processes using a genetic model *in vivo*. In the current study, mice with a temporal hepatocyte-specific *Myc* disruption were developed using SA-Cre-ERT² system [13]. Continuous treatment with tamoxifen led to maintenance of *Myc* disruption in hepatocytes. Disruption of *Myc* in hepatocytes represses hepatocellular proliferation induced by PPAR α agonist Wy-14,643. Mice with hepatocyte-specific *Myc* disruption were also largely resistant to DEN-induced hepatocarcinogenesis, thus demonstrating that MYC is essential in hepatocellular proliferation and tumorigenesis.

Since embryonic *Myc* knockout mice are lethal [21], liver-specific *Myc*-deficient mice were used to investigate the role of MYC in liver physiology and pathology. Previously, other groups targeted *Myc* disruption in liver [22, 23]. *Myc* alleles were disrupted by injecting newborn *Myc*^{fl/fl} homozygous mice with pIpC to activate the α/β interferon-inducible *Mx*-promoter driving Cre recombinase (*Myc*^{fl/fl} Mx-Cre) revealing that lower MYC in newborn mice had decreased hepatocyte size and polyploidization as the liver develops [23]. A model of hepatocyte-specific *Myc* disruption was also generated by crossing *Myc*^{fl/fl} mice to SA-Cre-harboring mice [22, 24]. However, the efficiency of *Myc* disruption was low, with *Myc* mRNA expression remaining higher than 30% in 10-week-old mice [22]. A similar result was obtained when the *Myc*^{fl/fl} mouse was crossed with the SA-Cre line [25], where efficient disruption of *Myc* could not be achieved (unpublished data). This lack of efficient MYC loss in the *Alb*-Cre mice is likely due to the rapid proliferation of MYC-expressing hepatocytes during liver growth and the regenerative capacity of the liver with non-recombined *Myc* alleles. In the present model, recombination of the *Myc*^{flxed} alleles was achieved by the hepatocyte-specific Cre-ERT² system, which temporally activates the Cre recombinase expression under the control of serum albumin promoter in the presence of tamoxifen [13, 15]. This mouse line achieved stable and efficient disruption of MYC protein specifically in hepatocytes, yielding a more suitable model for assessing its physiological and pathological roles in hepatocytes compared with the previous mouse models.

Fibrate drugs used to lower plasma triglycerides [26], are agonist for PPAR α , a ligand-activated nuclear receptor that controls genes involved in fatty acid transport and catabolism, primarily in liver, but also in kidney, heart and skeletal muscle [27, 28]. In rodents, short-term administration of PPAR α agonists, such as fibrate drugs and the experimental compound Wy-14,643, initiates a pleiotropic response of hepatomegaly, peroxisome proliferation, and increased fatty acid transport and oxidation in liver, whereas long-term exposure leads to tumorigenesis in a PPAR α -dependent manner [5, 29]. Clues to the molecular link between PPAR α and hepatocellular proliferation was recently uncovered. Given the importance of MYC in regulating cellular growth, proliferation and metabolism, MYC might serve as a critical link between PPAR α and hepatocellular proliferation. Indeed, *Myc* mRNA and MYC protein levels were robustly induced as early as 24 hours upon Wy-14,643 treatment in mice as a result of suppression of expression of the let-7C miRNA-encoding gene [6]. Let-7C destabilizes *Myc* mRNA and the lower let-7C expression leads to increased *Myc* mRNA and MYC protein and the resultant hepatocellular proliferation. Wy-14,643 treatment for two weeks caused a significant increase of liver weight in *Myc*^{fl/fl} mice which was mainly due to hepatocellular proliferation, as revealed by the BrdU labeling index, consistent with earlier studies [30]. However, this response was markedly blunted by *Myc* disruption in hepatocytes of *Myc*^{fl/fl,ERT2-Cre} mice, as the BrdU labeling index dropped from about 68% to 21% in hepatocytes. The difference of liver/body weight ratio between *Myc*^{fl/fl} and *Myc*^{fl/fl,ERT2-Cre} mice occurred as early as two days post Wy-14,643 treatment. Mechanistically, several critical MYC target genes involved in cell cycle control, including *Ccna2*, *Ccnb1*, *Ccne1*, *Ccne2* and *Cdk1*, were suppressed in *Myc*^{fl/fl,ERT2-Cre} mice upon Wy-14,643 treatment. In addition to cell cycle control genes, minichromosome maintenance (*Mcm*) 2–7 genes, identified as direct MYC target genes in neuroblastoma [31], were down-

regulated by *Myc* disruption. MCM2-7, a family of six related proteins required for the initiation and elongation of DNA replication, form heterohexameric MCM complexes and act as a replicative helicase at the DNA replication fork, a key component of the pre-replication complex [32–34]. The decreased MCM2-7 indicates that *Myc* disruption reduces DNA replication in hepatocytes upon Wy-14,643 challenge. Moreover, two checkpoint genes, *Chek1* and *Rad51*, were repressed by *Myc* inactivation. CHEK1 kinase acts downstream of ATM/ATR kinase and plays an important role in DNA damage checkpoint control, embryonic development and tumor suppression [35, 36]. RAD51 was also reported to be involved in DNA damage signaling, recombination repair, and tumorigenesis [37]. Many of the above genes were previously found regulated and critical for PPAR α -induced hepatocellular proliferation in PPAR α ligand-independent constitutively-active mice [17]. This further confirms that MYC drives the proliferative response to PPAR α activation in mice.

A mechanism by which PPAR α regulates gene expression and hepatocellular proliferation was revealed in which MYC is the central player [6]. As noted above, activated PPAR α indirectly induced *Myc* mRNA and protein expression via suppression of let-7C. In addition, a series of miRNAs were up-regulated upon PPAR α activation, including the mir-17–92 polycistron cluster, and ChIP assays demonstrated that MYC could bind to the promoter of the gene encoding mir-17-5p. The mir-17-92 cluster is directly regulated by MYC leading to enhanced cell cycle progression and a blockade of tumor cell apoptosis [38, 39]. Consistent with this data, hepatic disruption of MYC in the current study inhibited Wy-14,643-induced expression of mir-17-5p, mir-17-3p, mir-18a-5p, and mir-19a, indicating that the mir-17-92 cluster is a direct MYC target and might be partially responsible for PPAR α activation-induced hepatocellular proliferation and tumorigenesis.

Numerous studies suggest that MYC plays a role in glucose metabolism and ribosomal biogenesis, [40] and that overexpression of MYC improved glucose homeostasis in diabetic mice [41]. However, the present data suggest that *Myc* disruption in *Myc^{fl/fl},ERT2-Cre* mice did not alter the expression of genes involved in glucose metabolism, nor the genes responsible for glutamine metabolism under normal conditions (data not shown). In addition, *Myc* inactivation does not alter the induction of fatty acid-catabolizing genes induced by PPAR α activation, such as *Cyp4a10*, *Cyp4a14* and *Acox1*. These data indicate that MYC may not be essential for glucose, fatty acid and glutamine metabolism under these conditions. *Myc* disruption may increase the β -oxidation-independent carbohydrate and lipid metabolism via an indirect regulatory pathway. Whether MYC is crucial for glucose and glutamine metabolism during tumorigenesis and other hepatic pathological processes needs to be elucidated by additional experimentation.

To investigate the role of MYC in liver carcinogenesis, the DEN-induced hepatocarcinogenesis model was used. *Myc^{fl/fl},ERT2-Cre* mice were found to be resistant to development of liver adenomas and carcinomas as compared to the *Myc^{fl/fl}* mice. The mechanism leading to a decrease in hepatocellular cancer is likely due to a lower proliferative capacity of hepatocytes during development resulting in less DEN-induced DNA damage being fixed as gene mutations. Dysregulation of *Myc* is a hallmark of many human malignancies, including liver cancers. In liver, constitutive overexpression of MYC induces tumorigenesis, whereas inhibition of MYC expression resulted in regression of established tumors *in vivo* [11, 12], indicating that targeting MYC is a potential approach for therapeutics of liver tumors. Further studies are required to determine how MYC contributes to multiple carcinogenic steps, such as initiation and promotion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Myc^{fl/fl}	floxed Myc allele
Cre-ER^{T2}	cre recombinase:tamoxifen-inducible estrogen receptor ligand-binding domain fusion protein
Myc^{fl/fl},ERT2-Cre	inducible liver-specific <i>Myc</i> knockout mouse
bHLH	basic helix-loop-helix leucine zipper
CDK	cyclin-dependent kinase
PPARα	peroxisome proliferator-activated receptor α
DEN	diethylnitrosamine
SA	serum albumin promoter
BrdU	bromodeoxyuridine
Acox1	acyl-CoA oxidase
Cyp4a10 and Cyp4a14	cytochromes P450 4a10 and 4a14
Chk1	checkpoint kinase 1 homologue
Rad51	RAD51 homolog (<i>S. cerevisiae</i>)
Mcm	minichromosome maintenance
Myc^{fl/fl}Mx-Cre	α/β interferon-inducible <i>Mx</i> -promoter driving Cre recombinase

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Fig. 1. Conditional and temporal disruption of *Myc* in hepatocytes

(A) PCR diagnostic for activated ER^{T2}-Cre-mediated recombination of *Myc* allele in genomic DNA isolated from kidney (lanes 1) and liver (lanes 2) of *Myc^{fl/fl}* or *Myc^{fl/fl,ERT2-Cre}* mice treated with vehicle (VEH) or tamoxifen (TM) for 24 hours. (B) qPCR analysis measuring *Myc* mRNA level in liver, lung, heart, kidney and skeletal muscle from *Myc^{fl/fl}* or *Myc^{fl/fl,ERT2-Cre}* mice treated with tamoxifen and killed 24 hours post-treatment. n=6–8. ***p<0.001 versus *Myc^{fl/fl}* group. (C) Western blot of MYC for whole liver extracts from *Myc^{fl/fl}* and *Myc^{fl/fl,ERT2-Cre}* mice treated with TM and killed 24 hours post treatment.

Fig. 2. *Myc* disruption suppresses hepatocyte proliferation but not the lipid-lowering effect of Wy-14,643

(A) Representative BrdU staining of liver sections from vehicle or Wy-14,643-treated *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice. (B) BrdU labeling index for hepatocytes and non-parenchymal cells. n=6–8. **p<0.01 versus *Myc^{fl/fl}*+Wy. (C) Liver/body weight ratios at two weeks post-treatment. n=6–8. **p<0.01 versus *Myc^{fl/fl}*+Wy. (D) Serum triglyceride levels in vehicle or Wy-14,643-treated *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice. (E) mRNA levels of PPARα target genes *Acox1*, *Cyp4a10* and *Cyp4a14* in *Myc^{fl/fl}* and *Myc^{fl/fl},ERT2-Cre* mice determined by qPCR.

Fig. 3. Altered expression of cell cycle control, DNA repair genes and miRNAs in mice lacking hepatic expression of MYC

(A) qPCR analysis of cell cycle control genes-encoded mRNAs. (B) qPCR analysis for mRNAs encoding proteins involved in DNA damage repair and checkpoints. n=6–8.

*p<0.05 and **p<0.01. (C) qPCR analysis of the mir-17–92 cluster, mir-290–295 cluster and mir-100/let-7a-2 cluster in livers from vehicle or Wy-14,643-treated $Myc^{fl/fl}$ and $Myc^{fl/fl,ERT2-Cre}$ mice. Expression was normalized to endogenous reference snoRNA55. n=6–8. **p<0.01 and ***p<0.001 compared to all the other three groups.

Fig. 4. *Myc^{fl/fl,ERT2-Cre}* mice are resistant to DEN-induced liver tumorigenesis

(A) Incidence of visible tumors from in *Myc^{fl/fl}* and *Myc^{fl/fl,ERT2-Cre}* mice treated with 25 μ g/g of DEN at age of 15 days and killed at 9 months later. (B) The number of tumors counted from the surface of liver lobes. n=6–9 per group. (C) Tumor size measured on the surface of liver lobes. n=9 per group. (D) Incidence of preneoplastic foci, hepatocellular adenoma, and hepatocellular carcinoma in DEN-treated *Myc^{fl/fl}* and *Myc^{fl/fl,ERT2-Cre}* mice. Data are expressed as percentage of total number of mice (n=9). (E) Representative images of H.E. staining from DEN-treated *Myc^{fl/fl}* and *Myc^{fl/fl,ERT2-Cre}* mice. “T” denotes tumor area. (F) Representative images of Ki67 staining from DEN-induced tumors in *Myc^{fl/fl}* and *Myc^{fl/fl,ERT2-Cre}* mice. (G) qPCR analysis of mir-290–295 cluster and mir-100/let-7a-2

cluster in saline-treated livers and DEN-induced tumors in *Mye^{fl/fl}* and *Mye^{fl/fl,ERT2-Cre}* mice. Expression was normalized to endogenous reference snoRNA55. n=6 for saline treatment, n=9 for *Mye^{fl/fl}*+DEN and n=3 for *Mye^{fl/fl,ERT2-Cre}*+DEN. *p<0.05 and **p<0.01, ***P<0.001.

Table 1Top Biological Functions Regulated in Liver by *Myc* in Hepatocytes

Name	p-value	# Molecules
Downregulated		
Cell cycle	5.57E-05 – 3.83E-02	27
DNA replication, recombination, and repair	5.57E-05 -4.53E-02	13
Protein trafficking	5.26E-04 -3.83E-02	6
Cell death	6.84E-04 -3.83E-02	18
Cell-to-cell signaling and interaction	1.40E-03 -4.99E-02	33
Upregulated		
Carbohydrate metabolism	7.81E-04 -4.25E-02	11
Cell morphology	7.81E-04 -4.97E-02	21
Cell-to-cell signaling and interaction	7.81E-04 -4.95E-02	19
Lipid metabolism	7.81E-04 -4.28E-02	16
Molecular transport	7.81E-04 -4.97E-02	19

Table 2Associated Networks Regulated in Liver by *Myc* in Hepatocytes

Down-regulated		
ID	Associated network functions	Score
1	Cellular development, cell death, cellular growth and proliferation	31
2	Cellular growth and proliferation, hematological system development and function, cell-to cell signaling and interaction	29
3	Tissue development, embryonic development, organ development	18
4	Cellular growth and proliferation, cell death, post-translational modification	15
5	Lipid metabolism, small molecule biochemistry, vitamin and mineral metabolism	15
Up-regulated		
1	Cellular movement, cell-to-cell signaling and interaction, nervous system development and function	36
2	Cell death, cell-mediated immune response, cellular development	30
3	Inflammatory response, amino acid metabolism, molecular transport	15
4	Cell cycle, DNA replication, recombination, and repair, cell death	15
5	Tissue development, cellular development, reproductive system disease	13

The score was calculated by Ingenuity Pathway Analysis software. Details are given in the Supplementary Information file.