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Chronic Activation of mTOR Complex 1 is Sufficient to Cause Hepatocellular Carcinoma

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Abstract

The mammalian target of rapamycin (mTOR) complex 1 (mTORC1) is a nutrient sensitive protein kinase that is aberrantly activated in many human cancers. However, whether dysregulation of mTORC1 signaling in normal tissues contributes to cancer risk is unknown. Here, we focused on hepatocellular carcinoma because it is a cancer with clear links to environmental factors that affect mTORC1, including dietary influences. Genetic ablation of the mTORC1 inhibitory component Tsc1 results in constitutively elevated mTORC1 signaling, an effect similar to that of obesity on this pathway. We found that mice with liver-specific knockout of *Tsc1* developed sporadic hepatocellular carcinoma with heterogeneous histological and biochemical features. The spontaneous development of hepatocellular carcinoma in this mouse model was preceded by a series of pathological changes known to accompany the primary etiologies of this cancer, including liver damage, inflammation, necrosis, and regeneration. Chronic mTORC1 signaling caused unresolved endoplasmic reticulum stress and defects in autophagy, which contributed to hepatocyte damage and hepatocellular carcinoma development. Therefore, we demonstrate a previously unrecognized role for mTORC1 in carcinogenesis, perhaps representing a key molecular link between cancer risk and environmental factors, such as diet.

Introduction

Liver cancer is the third leading cause of cancer-related deaths worldwide according to the World Health Organization (1, 2). Hepatocellular carcinoma (HCC) is the most frequent and aggressive primary tumor of the liver and has limited treatment options (3–5). Similar to other cancers, the risk of developing HCC is influenced by environmental factors, including HBV- or HCV-induced viral hepatitis, alcohol consumption, and obesity. The increasing incidence of HCC in the Western world has been linked epidemiologically to the increased rate of obesity (4, 6). The course of HCC development is a multistep process initiated by liver damage and followed by inflammation and cycles of necrosis and regeneration (7–9). This results in an environment that is permissive to genetic events leading to neoplastic

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transformation. Although the pathological features leading to HCC are shared amongst the common etiologies, the molecular events initiating this program and linking the environmental factors to HCC development are poorly understood.

Regardless of etiology, the excessive accumulation of triglycerides in the liver, or hepatic steatosis, has emerged as a potential risk factor in the development of human HCC (6, 10). The development of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis is thought to be the major link between obesity and increased risk of HCC (11–13). This idea has been supported by mouse models in which both dietary and genetic insults leading to HCC are often accompanied by hepatic steatosis (14–16). However, the molecular mechanisms linking this histopathological change to hepatocarcinogenesis, and whether hepatic steatosis itself is the true initiating event, are largely unknown. Here, we explore the potential role of the mammalian target of rapamycin (mTOR; also referred to as mechanistic target of rapamycin), which as part of mTOR complex 1 (mTORC1), is a key nutrient-sensing kinase that is aberrantly activated in the liver and other tissues under conditions of obesity (17, 18).

A network of oncogenic signaling pathways lie upstream of mTORC1, leading to its frequent activation in human cancers (19), including the majority of HCCs (20–24). The common activation of mTORC1 in human cancers is believed to reflect its role in promoting tumor growth, proliferation, and metabolism. Retrospective studies have found that HCC patients treated with the mTORC1 inhibitor rapamycin following liver transplant have substantially reduced incidence of recurrence (25). Based on such studies, there are currently ongoing trials with rapamycin and its analogs for the treatment of HCC (26). However, the contributions of mTORC1 signaling to HCC development and progression have not been rigorously explored. Distinct etiologies of HCC, including HCV infection and obesity, increase mTORC1 signaling in liver cells (Fig. S1A) (17, 18, 27–29), suggesting that aberrant activation of mTORC1 might underlie the risk of HCC attributed to these environmental inputs.

Various signaling pathways upstream of mTORC1 stimulate its activity through inhibition of the TSC1-TSC2 complex, the components of which are mutated in the genetic tumor syndrome tuberous sclerosis complex (TSC) (30). This complex is a key inhibitor of mTORC1 that functions as a GTPase-activating protein (GAP) for the small G-protein Rheb, which in its GTP-bound form is essential for the stimulation of mTORC1 activity. Disruption of this complex, through the loss of either TSC1 or TSC2, results in constitutive activation of mTORC1 that is largely independent of cellular growth conditions. Therefore, settings in which the TSC genes have been ablated offer genetic mTORC1 gain of function models and have been used in many settings to understand the cellular and tissue-specific functions of mTORC1 (31–39). Moreover, a high-resolution sequencing study of a primary HCC found a loss of function mutation in *TSC1* (40).

To explore the potential role of elevated mTORC1 signaling in the development of liver cancer, we used a genetic mouse model with liver-specific knockout of *Tsc1* (*LTsc1KO*) (38). We found that these mice, despite being protected from hepatic steatosis, develop spontaneous HCC with heterogeneous histological features and signaling. In this model, tumor development was preceded by all of the hallmarks of HCC and was initiated by hepatocyte damage resulting from chronic mTORC1 signaling. Sustained mTORC1 signaling in hepatocytes caused endoplasmic reticulum (ER) stress and defects in autophagy, which have been linked to the development of HCC (41–43). Therefore, we demonstrate that, in addition to its better-known function as a downstream effector of oncogenic signaling pathways controlling cell growth and proliferation in established tumors, chronic mTORC1 signaling in normal tissues can trigger the type of cellular damage that leads to

spontaneous transformation and cancer. We propose that mTORC1 represents a molecular link between environmental influences, such as dietary factors, and increased risk of certain types of sporadic cancers.

Results

Mice with liver-specific knockout of *TSC1* (*LTsc1KO*) develop spontaneous hepatocellular carcinoma

As reported previously (38), the livers of *LTsc1KO* mice displayed constitutive mTORC1 signaling under fasting conditions at a magnitude comparable to that induced by feeding in control wild-type animals (Fig. S1B). By 9 to 10 months of age, the *LTsc1KO* mice spontaneously developed lower grade tumors, classified as dysplastic foci, nodules, or hepatomas (75%), and more aggressive and expansive hepatocellular carcinomas (50%; Fig. 1 A and B). These were detected at similar rates in both male (Fig. 1B) and female cohorts (Fig. S2). Liver tumors were not detected in the two control groups from these cohorts (*Tsc1^{fl/fl}* and *Albumin-Cre Tsc1^{fl/+}*). The *LTsc1KO* mice are protected from age-induced hepatic steatosis (38), and this was reflected in the non-tumor regions of their livers (Fig. S3). Therefore, these mice represent a new genetic model of spontaneous hepatocellular carcinoma that is independent of hepatic steatosis.

Histopathological and biochemical characterization of the HCCs arising in the *LTsc1KO* mice revealed heterogeneity between the tumors. In addition to the common trabecular histology, clear cell and lobular cell types were also observed in the HCCs (Fig. 1C), all of which are classical histological features of human HCC (44). Moreover, cholangiocarcinomas were not detected in the *LTsc1KO* mice, suggesting that the tumors were of hepatocyte origin, rather than arising from liver progenitor cells or cholangiocytes. Both the hepatomas and HCCs contained a large number of proliferating cells, as indicated by PCNA staining; furthermore, the hepatocytes within non-tumor regions showed a modest increase in proliferation relative to control livers (Fig. 1 D and E).

To begin to understand the molecular events driving tumorigenesis in this model, we examined effects on key oncogenic and inflammatory signaling pathways known to underlie some HCC development. As expected, mTORC1 signaling, as scored by staining for phosphorylated S6 (a ribosomal protein that is a downstream target of mTORC1 signaling), was detected in most hepatocytes in the *LTsc1KO* livers, but in only a small number of those in control livers (Fig. 2A). However, mTORC1 signaling was similar in tumors and adjacent non-tumor tissue from *LTsc1KO* livers (Fig. 2, A and B). Because Akt signaling is frequently increased in HCC (45, 46), we examined its activating phosphorylation on Ser⁴⁷³. As described previously (38), the *LTsc1KO* livers display attenuation of Akt signaling due to mTORC1-dependent feedback mechanisms. Surprisingly, Akt activation remained low in the tumors from these mice, with phosphorylation of Ser⁴⁷³ being similarly low in tumor and adjacent non-tumor regions of the *LTsc1KO* livers (Fig. 2B). We also evaluated the activation status of ERK signaling, another pathway commonly activated in HCC (47), and found that a subset of tumors showed robust activation of ERK (Fig. 2B and C). However, heterogeneity with respect to ERK signaling was evident when evaluating more than one tumor from the same mouse, with one tumor often being lower than normal tissue and the other being substantially higher (Fig. 2B and C). Moreover, the activation status of ERK was not dependent on tumor grade, with both low grade hepatomas and high grade HCCs exhibiting variable amounts of phosphorylated ERK (Fig. 2C). Various mouse models have established tumor-promoting (48–50) and suppressing (51–53) activities for the NF- κ B pathway in HCC development. In *LTsc1KO* tumors, we found that phosphorylation of I κ B was increased relative to non-tumor tissue (Fig. S4A), but only a small number of cells within any given tumor appeared to display activation of NF- κ B, as indicated by nuclear

accumulation of p65 (also known as RelA; Fig. S4B). This suggests that the canonical NF- κ B pathway is not a common driving force in HCC development in this model. Activation of STAT3 was found to be essential for tumor promotion in a carcinogen-induced HCC model lacking NF- κ B signaling (54), and activation of the JAK/STAT pathway is commonly observed in human HCC (54, 55). However, we did not find consistent activation of STAT3 in the tumor or non-tumor tissues of *LTsc1KO* livers (Fig. S4C). Genetic alterations leading to nuclear translocation of β -catenin and loss of p53 are frequent in HCC (9, 47). In *LTsc1KO* tumors and adjacent non-tumor tissue, β -catenin was localized largely at the cell-cell junctions (Fig. S4B), indicating that the Wnt- β -catenin pathway is unlikely to contribute to HCC development in this model. Finally, we found that p53 abundance was reduced to varying degrees in the *LTsc1KO* tumors (Fig. S4A). Taken together with the latency of tumor development, the histological heterogeneity, and the focal nature of the tumors, these findings suggest that, despite mTORC1 activation in all hepatocytes, sporadic HCC development in the *LTsc1KO* mice involves transforming events that are distinct between individual tumors, rather than arising from a common genetic alteration.

We have recently described a series of metabolic changes induced by *Tsc* gene loss and mTORC1 activation that are driven by the transcription factors hypoxia-inducible factor 1 α (HIF-1 α) and sterol response element-binding protein 1 (SREBP1) (56). Consistent with these findings, the abundance of HIF1 α was basally increased in the non-tumor tissue of *LTsc1KO* mice and was further increased in the liver tumors (Fig. 2B). Furthermore, the abundance of the glucose transporter Glut1, which is encoded by a canonical HIF-1 α target gene, was increased in both non-tumor and tumor regions of the *LTsc1KO* mice (Fig. 2D). Consistent with the role of HIF-1 α in enhancing angiogenesis in tumors, the liver tumors arising in *LTsc1KO* mice were highly vascular, as indicated by staining of the endothelial marker endomucin (Fig. 2D). In our study characterizing the metabolic phenotype of the *LTsc1KO* mice (38), we found that chronic activation of mTORC1 in hepatocytes results in decreased activation of the transcription factor SREBP1c and de novo lipogenesis due to attenuation of Akt signaling. This resulted in lower amounts of SREBP1c targets, such as fatty acid synthase (FASN) and glucose-6-phosphate dehydrogenase (G6PD), as seen in the non-tumor tissue from these mice (Fig. 2D and Fig. S4D). However, the abundance of FASN and G6PD was increased in both low grade tumors (Fig. S4D) and HCCs (Fig. 2D) from the *LTsc1KO* mice, suggesting a restoration of SREBP1c activation in these tumors. These findings suggest that specific metabolic changes, commonly seen in human tumors (57), might contribute to tumor progression in this model.

The *LTsc1KO* livers display the pathological progression that commonly precedes HCC development

Regardless of underlying etiology, the course of HCC development in both mouse models and humans is generally a multistep process involving liver damage and hepatocyte death, inflammation, and cycles of necrosis and regeneration that precedes tumor formation (6, 8). Given our data suggesting that the tumors themselves are heterogeneous in nature, we sought to identify common tumor-initiating events in a cohort of younger *LTsc1KO* mice (6 months) without detectable tumors. Although there were no detectable abnormalities in the livers of control mice, the *LTsc1KO* livers showed various characteristics of liver damage, including the appearance of dysplastic hepatocytes (Fig. 3A), increased serum concentrations of the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fig. 3B) and hepatocyte death associated with cleaved-caspase 3 (Fig. 3C and D, Fig. S5A). In addition, we observed focal areas of necrosis and inflammation, with macrophage infiltration (as detected by F4/80 staining) within the *LTsc1KO* livers (Fig. 3E and F, Fig. S5B).

Hepatocyte death is generally accompanied by a regenerative response. Liver progenitor cells, sometimes referred to as oval cells, that can differentiate into both hepatocytes and cholangiocytes contribute to liver regeneration under conditions of hepatocyte damage. Expansion of this population is observed in both rodent models of HCC and chronic liver disease in humans, which can give rise to HCC (51, 58–62). In the *LTsc1KO* livers, oval cell expansion was observed in association with areas of immune infiltration, as detected with the oval-cell marker cytokeratin (59), whereas control livers displayed cytokeratin staining exclusively in the cholangiocytes comprising the bile ducts (Fig. 3G and Fig. S5B). In contrast to the normally quiescent hepatocytes of the adult liver (0.61% PCNA-positive hepatocytes in controls), *LTsc1KO* livers displayed a significant increase in proliferating hepatocytes (5.74%; Fig. 3H). Moreover, *LTsc1KO* livers contained more hepatocytes with γ -H2AX-positive nuclei (Fig. 3I and J), indicating that DNA damage was occurring at an age preceding tumor development. Consistent with this, mRNA and protein abundance of p53 and expression of its target *p21* were increased in *LTsc1KO* livers at this age, further suggesting the induction of a DNA damage response in the non-tumor tissue (Fig. S5A and C). This is in contrast to the tumors arising in older mice, which displayed reduced amounts of p53 (Fig. S4A). Collectively, these findings indicate that liver-specific deletion of *Tsc1* initiates a program of spontaneous hepatocyte death, followed by inflammatory and regenerative responses, and ultimately DNA damage that promote HCC development in a manner independent of hepatic steatosis.

Chronic mTORC1 activation is required for HCC development in *LTsc1KO* mice

Because constitutive activation of mTORC1 is the primary molecular defect caused by loss of function of the TSC1-TSC2 complex, we determined whether aberrant mTORC1 signaling was responsible for HCC development in the *LTsc1KO* model. A cohort of mice aged 5 months was treated with rapamycin or vehicle three times a week for 5 months. Rapamycin-treated *LTsc1KO* livers showed reduced mTORC1 signaling (Fig. S6A and B). Vehicle-treated *LTsc1KO* mice developed both hepatomas and hepatocellular carcinomas at a rate similar to our previous cohort (Fig. 4A and B). However, neither the vehicle-treated control mice nor the rapamycin-treated *LTsc1KO* mice developed liver tumors of any kind. Moreover, rapamycin treatment also blocked liver damage in these mice, as assessed by serum ALT concentrations (Fig. 4C) and hepatocyte apoptosis (Fig. 4D and Fig. S6B). Consistent with the lack of liver damage, and in contrast to vehicle-treated mice, the livers of rapamycin-treated *LTsc1KO* mice did not display hepatocyte proliferation, necroinflammatory regions, oval cell hyperplasia, or DNA damage (Fig. 4E and F, Fig. S6B). Therefore, chronic mTORC1 signaling is responsible for the tumor-initiating events leading to HCC development in the *LTsc1KO* mice.

To evaluate the efficacy of rapamycin treatment on established liver tumors in *LTsc1KO* mice, we treated an aged cohort (12 months) of *LTsc1KO* mice with vehicle or rapamycin for 1 month. Rapamycin-treated *LTsc1KO* livers showed robust inhibition of mTORC1 signaling, as scored by staining for phosphorylated S6, both in the tumors and adjacent non-tumor tissue (Fig. 5A). In contrast to the protective effects of rapamycin on liver damage and HCC development in the preventative trial described above, treatment with rapamycin at an age following tumor development did not affect tumor burden, with both tumor number and grade being similar between rapamycin and vehicle-treated mice (Fig. 5B). However, rapamycin treatment decreased the number of proliferating cells in the majority of tumors (Fig. 5C and D). Consistent with the fact that rapamycin generally elicits a cytostatic rather than a cytotoxic response, no increase in the number of apoptotic cells was apparent in the liver tumors of rapamycin-treated mice compared to vehicle-treated mice (Fig. S7A). Despite loss of mTORC1 signaling, a subset of hepatomas and focal regions within HCCs displayed large numbers of proliferating cells after rapamycin treatment (Fig. S7B),

suggesting that some of these liver tumors are resistant to the cytostatic effects of rapamycin. Taken together, these findings are consistent with increased mTORC1 signaling being carcinogenic in the *LTsc1KO* mice but playing a more limited role in the established liver tumors.

Chronic mTORC1 signaling triggers liver damage and HCC development subsequent to endoplasmic reticulum stress and defective autophagy

Having established a role for mTORC1 in promoting the type of liver damage that triggers cancer-initiating events, we sought to reveal the underlying downstream processes. We focused on two adaptive responses that are affected by mTORC1 signaling that have also been implicated in the development of HCC: the unfolded protein response and autophagy. The unfolded protein response (UPR) is an adaptive stress response activated upon accumulation of misfolded proteins in the endoplasmic reticulum (ER stress). The UPR induces apoptosis during prolonged ER stress. Two independent branches of the UPR comprise the IRE1 (inositol-requiring enzyme 1) pathway, which induces splicing of the *Xbp1* mRNA leading to translation of an active XBP1 transcription factor, and the PERK (pancreatic eukaryotic initiation factor 2 α (eIF2 α) kinase; double stranded RNA-activated protein kinase-like endoplasmic reticulum kinase) pathway, which phosphorylates eIF2 α to attenuate cap-dependent translation and increase translation of ATF4 (activating transcription factor 4) (41). Activation of the UPR is observed in many liver diseases, including viral hepatitis and obesity-induced hepatic steatosis (41, 63, 64), and chronic mTORC1 activation can cause ER stress in other settings (65, 66). Indeed, preceding signs of liver damage, aberrant mTORC1 signaling induces ER stress in the livers of young (3 months) *LTsc1KO* mice, as demonstrated by activating phosphorylation events for the IRE1 and PERK pathways (Fig. 6A and Fig. S8A). The activation of both of these pathways is blocked by short-term (4 days) rapamycin treatment of the *LTsc1KO* mice. Additional UPR markers downstream of PERK (phosphorylation of eIF2 α at Ser⁵¹ and ATF4, Fig. S8A) and IRE1 (*Xbp1* splicing, Fig. S8B), and transcriptional targets of the UPR (*Ero1* and *Chop*, Fig. 6B) were similarly affected.

mTORC1 signaling is a major regulatory link between nutrient status and macroautophagy, the process by which cellular constituents, including long-lived proteins and damaged organelles, are recycled through targeted lysosomal degradation (67, 68). Autophagy is decreased in the liver under conditions of obesity (69, 70) and is suppressed by mTORC1 signaling (67, 71–74). Several studies have found that defects in autophagy can give rise to liver tumors, and liver-specific deletion of genes encoding autophagy factors results in the accumulation of protein aggregates and damaged organelles followed by hepatocyte death, thereby initiating the classic path to liver tumor development also seen in the *LTsc1KO* mice (42, 43, 75–78). We found that livers from young *LTsc1KO* mice displayed accumulation of p62 (also known as Sequestosome1 or SQSTM1; Fig. 6A and C), which is believed to target ubiquitinated proteins and organelles to autophagosomes and which is selectively degraded by autophagy (79, 80). Furthermore, *LTsc1KO* livers show increased abundance of the non-lipidated form of LC3B (LC3B–I), which is lipidated to form LC3B–II as one of the initiating steps in autophagosome formation. Like markers of ER stress, these indications of defective autophagy in the *LTsc1KO* livers were reversed by short-term rapamycin treatment (Fig. 6A). However, there was no significant difference in the transcript abundance of *p62* (*Sqstm1*) or *LC3B* (*Map1lc3b*) between the control and knockout livers (fig. S8C). We noted that LC3B–II abundance was basally higher in the *LTsc1KO* livers compared to controls, suggesting that the autophagy defect does not lie in LC3B–I lipidation per se. Because LC3B–II is degraded by autophagy, like p62, we hypothesized that there was a defect in autophagic flux in *LTsc1KO* livers. To test this, we analyzed relative flux through autophagy by treating control and *LTsc1KO* mice with

chloroquine, which inhibits autophagosome degradation by neutralizing the lysosome. Relative to vehicle-treated animals, the chloroquine-treated control mice showed accumulation of p62 in their livers, indicative of active flux through autophagy (Fig. 6D). In contrast, although p62 abundance in *LTsc1KO* livers was increased relative to control mice, it remained unchanged following chloroquine treatment, suggesting that there is little to no flux through autophagy in these livers.

Autophagy is a homeostatic response that limits cellular damage by clearing defective proteins and organelles. Persistence of p62 aggregates due to impaired autophagy is associated with various human liver diseases, including non-alcoholic fatty liver disease and HCC (81), and is believed to cause accumulation of defective mitochondria. Indeed, electron micrographs of hepatocytes from *LTsc1KO* livers revealed enlarged, morphologically abnormal mitochondria with disorganized cristae, which were not observed in littermate control mice (Fig. S8D). These mitochondria are reminiscent of those in hepatocytes lacking essential autophagy genes *Atg5* or *Atg7* (43, 78). Consistent with the presence of dysfunctional mitochondria, we found increased concentrations of mitochondrial-derived reactive oxygen species (ROS) in hepatocytes isolated from *LTsc1KO* livers compared to controls (Fig. S8E). Finally, as seen in the tumors arising in other autophagy-deficient liver models (75–78, 82), the accumulation of p62 protein and aggregates was more pronounced in the tumors compared to adjacent normal tissue in 9 month-old *LTsc1KO* livers (Fig. 6E and F). As seen in the 3-month cohort (Fig. 6A), the accumulation of p62 was reversed by rapamycin treatment in these mice (Fig. 6E). Taken together, these data suggest that chronic activation of mTORC1 signaling in the liver causes defects in autophagic flux and accumulation of damaged organelles.

Discussion

This study demonstrates that chronic activation of mTORC1 signaling is sufficient to initiate a pathological program of liver damage, inflammation, and regeneration that triggers sporadic development of HCC. Aberrant mTORC1 signaling in the liver causes early onset ER stress and defects in autophagy that precede signs of liver damage. We propose that the resulting proteotoxic stress and organelle damage, perhaps manifesting itself in oxidative stress, creates a tumorigenic environment that is shared by the major etiological factors underlying the development of human HCC (41, 83, 84). Therefore, in addition to the established role of mTORC1 activation in promoting anabolic growth and proliferation downstream of oncogenic signaling pathways in tumors (19, 57), we reveal a previously unappreciated role for dysregulated mTORC1 signaling in promoting cancer-initiating events. These findings suggest that chronically increased mTORC1 signaling could be a key molecular link between genetic or environmental factors and the type of cell and tissue damage that contributes to the development of HCC and perhaps other cancers (Fig. 6G).

The *LTsc1KO* mice represent a unique and mechanistically informative genetic model of HCC driven by the PI3K–Akt–mTOR pathway. Inactivating mutations in PTEN, another tumor suppressor in this pathway, are frequently observed in human HCCs and are associated with advanced disease stage and decreased overall survival (85–89). Like the *LTsc1KO* model described here, mice with liver-specific knockout of PTEN also exhibit constitutive activation of mTORC1 and HCC development (90–93). However, PTEN loss in hepatocytes results in increased activation of Akt leading to the development of hepatic steatosis, which has been proposed to underlie HCC development in this model. In contrast, the *LTsc1KO* mice display reduced Akt signaling in the liver and are protected from hepatic steatosis (38). Therefore, the *LTsc1KO* mice demonstrate that mTORC1 activation, independent of Akt and hepatic steatosis, is sufficient to initiate the pathological progression to HCC.

The liver has a remarkable capacity to regenerate in response to toxin-induced damage or physical injury (94). Differentiated hepatocytes are quiescent, but can be induced to proliferate during regenerative responses. In addition, the proliferation of hepatic progenitor cells (for example, oval cells), which can give rise to both new hepatocytes and cholangiocytes, is stimulated by liver damage. However, the role of progenitor cell hyperplasia in human HCC is unknown, and the contribution of this cell population to the development of liver tumors varies between different mouse models (58). Genetic mouse models resulting in pronounced and pervasive expansion of progenitor cells give rise to hepatocellular carcinomas, cholangiocarcinomas, and mixed lineage tumors originating from this population (59, 91). The liver tumors arising in *LTsc1KO* mice originate from hepatocytes. Although increased numbers of progenitor cells (oval cell hyperplasia) are observed in the *LTsc1KO* livers, these areas of expansion are generally restricted to inflammatory and necrotic foci within these livers. The findings suggest that progenitor cell expansion is secondary to hepatocyte damage and is not the driving force underlying sporadic hepatoma and HCC development in this particular model.

Chronic activation of mTORC1 causes cellular stresses leading to liver damage that are shared by the common etiologies of HCC. Chronic HBV and HCV infection (95, 96) and non-alcoholic steatohepatitis (41, 63, 64) are associated with unresolved ER stress and apoptotic responses in hepatocytes. Previous studies have indicated that cells, tissues, and tumors with aberrant activation of mTORC1 signaling display sustained ER stress and activation of the UPR (65, 66), responses that are also detected in the livers of young *LTsc1KO* mice before liver damage and tumor development. Non-alcoholic steatohepatitis associated with obesity, in addition to leading to chronic mTORC1 activation (17, 18) and ER stress (41, 63, 64) in the liver, causes defects in hepatic autophagy (69, 70). As a highly integrated sensor of cellular nutrient and energy status, mTORC1 is a key regulator of autophagy, playing an evolutionarily conserved role in inhibiting this process (67, 68, 71, 72). We find that, like other genetic settings with loss of function of the TSC1-TSC2 complex (73, 74), the *LTsc1KO* livers display defective flux through autophagy resulting from sustained mTORC1 signaling. Moreover, defects in autophagy have been known to contribute to tumorigenesis since the discovery of *Beclin1* (also known as *Atg6*) as a tumor suppressor gene (97), and *Beclin1* heterozygous mice develop hepatocellular carcinoma at advanced ages (76, 77). The susceptibility of the liver, in particular, to defects in autophagy leading to tumorigenesis has been supported by mosaic and liver-specific knockouts of essential autophagy genes (43, 78). Impaired autophagy can exacerbate ER stress, as observed under conditions of obesity (69, 70), and this can result in oxidative stress, further deterioration of cellular organelles and genomic instability (78, 82, 98, 99). Therefore, it is likely that the combination of unresolved ER stress and defective autophagy resulting from chronic mTORC1 signaling promotes organelle dysfunction, proteotoxic and oxidative stress, and a similar form of liver damage caused by the major environmental etiologies of HCC.

The growing incidence of HCC in both developing and developed countries underlies the critical importance of defining the molecular links between the etiologies associated with HCC and the initiating events in tumor development. As a downstream effector of oncogenes (such as *PIK3CA*) and tumor suppressors (such as *PTEN*) that are commonly mutated in HCCs, mTORC1 signaling is likely to promote tumor progression in the liver. However, the data presented here suggest that mTORC1, as a central node for sensing cellular growth conditions, might be a pivotal link between environmental risk factors, such as viral infection and obesity, and the cellular damage that initiates the inflammatory and regenerative responses leading to HCC (Fig. 6G). Aberrant mTORC1 signaling results in loss of control over two key homeostatic responses in liver cells, the UPR and autophagy, and dysregulation of these adaptive responses contribute to chronic liver disease and the

development of HCC. The sporadic and heterogeneous nature of the liver tumors developing in the *LTsc1KO* model provides an opportunity for future investigation into the molecular and genetic events underlying spontaneous tumorigenesis arising from these common causes of liver damage. Furthermore, this genetic model will be powerful for testing novel therapeutic avenues aimed at either preventing the pathological sequence to tumor development shared amongst the etiologies of HCC or targeting established tumors at different stages of progression.

MATERIALS AND METHODS

Mouse Studies

Mice used in this study were described previously (38). Study cohorts were generated by crossing *Tsc1^{fl/fl}* mice with *Tsc1^{fl/+} Albumin-Cre* mice (C57BL/6J background), and PCR genotyping was performed as described (100). For rapamycin treatment, a 50 mg/ml stock solution was diluted into vehicle (5% Tween-80, 5% PEG-400 in 1X phosphate buffered saline (PBS)) for long- (2 mg/kg on Mondays, Wednesdays, and Fridays) or short- (5 mg/kg, 4 consecutive days) term treatments through intraperitoneal (i.p.) injections. For chloroquine treatment, mice were injected with 10 mg/kg or vehicle (PBS) through i.p. injections for 2 consecutive days and were fasted overnight before harvesting the livers.

Histology and Immunohistochemistry

Histological preparation and analyses were performed in the Dana-Farber/Harvard Cancer Center Rodent Histopathology Core, directed by R.T.B. Freshly dissected tissues were fixed in formalin and paraffin-embedded. 4- μ m sections were stained with hematoxylin and eosin (H&E) or processed further for immunohistochemistry (IHC). For IHC, slides were deparaffinized and antigen retrieval was done by autoclaving for 20 min in 1X Target Retrieval Solution, pH 6.0 (DAKO, S1699). For PCNA, PanCK and γ -H2AX detection, an additional incubation with 20 μ g/ml Proteinase K (Sigma) in 10 mM Tris-HCl buffer, pH 7.4 (20 min at 37°C) was done. Sections were stained by the immunoperoxidase technique using AEC (HRP-AEC Cell & Tissue Staining Kit, R&D Systems) and counterstained with hematoxylin. Primary antibodies: PCNA (Abcam); F4/80 (AbD Serotec); PanCK (DAKO); p62 (Enzo Life Sciences); Glut1 (Alpha Diagnostic); Endomucin, G6PD, and NF κ B (p65) (Santa Cruz); phospho-S6 (S240/244), phospho-ERK1/2 (Thr202/Tyr204), cleaved-caspase 3, γ -H2AX, FASN, and β -catenin (Cell Signaling Technologies (CST)).

Immunoblotting

Freshly dissected tissues, snap frozen in liquid nitrogen, were homogenized in SDS lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM MgCl₂, 1% SDS, 10% glycerol, 1 mM DTT, 50 mM β -glycerophosphate, 50 mM NaF, 1X protease inhibitor cocktail (Sigma) [pH 7.4]), and remaining debris was cleared by subsequent 10 and 30 min centrifugations at 16,000 rpm. Normalized protein lysates were resolved by SDS-PAGE and immunoblots were performed using antibodies from CST, except p62 (Abnova), HIF1 α (Cayman), phospho-IRE1 (Abcam), p53 (Vector labs), and actin and tubulin (Sigma).

Grading of liver damage and inflammation

Serum ALT and AST were determined using Infinity™ Reagents (Thermo Scientific). Sera were obtained by tail bleeding (Fig. 3), or collected directly from the heart after sacrifice (Fig. 4). Inflammation grade was scored by examination of H&E-stained liver sections, with the following scale: 0, no immune infiltration; 1, one or two foci of immune infiltration in at least two lobes; 2, greater than two foci in at least two; 3, large areas of immune infiltration in more than three lobes.

Gene Expression Analysis

RNA was isolated from mouse tissue using TRIzol (Invitrogen) and was reverse transcribed into cDNA using the Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). SYBR Green-based qRT-PCR was performed using an Applied Biosystems 7300 RT-PCR System. Triplicate runs of each sample were normalized to Rplp0 (m36b4) mRNA to determine relative expression. Primer pair sequences are listed in Table S1.

Electron Microscopy

Anesthetized mice were subjected to sequential portal vein perfusions of 10 ml NaCl (0.9%) and fixative (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)). 1–2 mm cubes of liver tissue were incubated for 2 hours in fixative, washed in 0.1 M cacodylate buffer and postfixed with 1% osmium tetroxide/1.5% potassium ferrocyanide for 1 hour, washed in water 3 times and incubated in 1% aqueous uranyl acetate for 1 hour followed by 2 washes in water and subsequent dehydration in increasing concentrations of ethanol (10 min each; 50%, 70%, 90%, 2×10 min 100%). Samples were put in propylene oxide for 1 hour and infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Marivac Canada Inc.), followed by embedding in TAAB Epon, polymerized at 60°C for 48 hours. Ultrathin sections (approximately 60 nm) were cut on a Reichert Ultracut-S microtome, transferred to copper grids stained with lead citrate and examined in a JEOL 1200EX transmission electron microscope, with images recorded using an AMT 2k CCD camera.

Primary hepatocyte isolation and FACS analysis

Primary hepatocytes were isolated from 10-week-old male mice following portal vein collagenase perfusion (Blenzyme 3; Roche) and Percoll gradient purification. The cells were cultured in medium containing 5% fetal bovine serum overnight and then incubated with 5- μ M MitoSOX Red (Invitrogen) for 15 min at 37°C. The cells were washed twice and collected by trypsinization in phenol red-free medium. FACS analysis was performed using BD FACSCalibur (BD Biosciences). Gating on live cells was performed before the collection of data.

Statistical Analysis

Data are reported as mean $\pm$ SEM. Following a normality test of the data (Kolmogorov-Smirnov), comparisons between two groups were performed with either Student's t-test (2-tailed) (Figs. 1E, 3B,D,F,J, 6B, S5C, and S8B,C) or Mann-Whitney test (Figs. 4C,F and 5D) as appropriate. *P* values $\leq$ 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Spontaneous Development of Hepatocellular Carcinoma (HCC) in *LTsc1KO* mice. (A) Macroscopic appearance of representative livers from *Tsc1^{fl/fl}* and *Tsc1^{fl/fl} Albumin-Cre* (*LTsc1KO*) mice at 9–10 months. (B) Percentage of *Tsc1^{fl/fl}* (n=8), *Tsc1^{fl/+} Albumin-Cre* (n=6), and *LTsc1KO* (n=12) mice with hepatomas (or nodular foci) and HCC at 9–10 months of age. Two 4- μ m H&E-stained sections per liver were scored. (C) Representative H&E-stained liver sections showing normal histological architecture in *Tsc1^{fl/fl}* mice and histological subtypes of HCCs observed in *LTsc1KO* mice. (D,E) Detection and quantification of proliferating hepatocytes by PCNA immunostaining of *Tsc1^{fl/fl}* and

LTsc1KO liver sections. **(E)** Five 200X fields (approx 200 hepatocytes each) were counted for each liver section, and data are presented as the mean $\pm$ SEM (n=3). *P<0.01 compared to *Tsc1^{fl/fl}* normal liver, **P<0.05 and ***P<0.0005 compared to *LTsc1KO* non-tumor region. Scale bars, **A**, 1 cm; **C**, 100 μ m (100X), **D**, 50 μ m (200X).

Fig. 2. Status of signaling and metabolic pathways in the liver tumors of *LTsc1KO* mice. **(A)** Representative images of phospho-S6 (Ser^{240/244}) immunostaining of liver sections from *Tsc1^{fl/fl}* and *LTsc1KO* mice. T, tumor; N, non-tumor. **(B)** Immunoblot analysis of liver lysates from non-tumor (N) or tumor (T) regions of *Tsc1^{fl/fl}* (2 mice) and *LTsc1KO* mice (4 mice with 1 or 2 tumors each) with the indicated antibodies. Phospho-specific antibodies: Akt (Ser⁴⁷³), S6 (Ser^{240/244}), ERK (Thr²⁰²/Tyr²⁰⁴). **(C)** Phospho-ERK immunostaining of liver sections from *LTsc1KO* mice showing heterogeneous distribution in hepatomas and regions of HCC. **(D)** Immunostaining of liver sections from *Tsc1^{fl/fl}* and *LTsc1KO* mice with antibodies to the given proteins. Scale bar, **A and C**, 50 μ m (200X), **D**, 100 μ m (100X).

Fig. 3. HCC development in *LTsc1KO* mice is preceded by liver damage, hepatocyte death, inflammation and regeneration. Analysis of male mice at 6 months. **(A)** Representative H&E-stained sections from *Tsc1^{fl/fl}* and *LTsc1KO* livers. **(B)** Serum concentrations of ALT and AST are presented as the mean $\pm$ SEM (n=8–9). *P<0.05 (ALT) and *P<0.005 (AST). **(C,D)** Immunostaining and quantification of cleaved caspase-3. Data from ten 200X fields per liver are presented as the mean $\pm$ SEM (n=3), *P<0.05. **(E)** A representative *LTsc1KO* liver stained with H&E or the macrophage marker F4/80. **(F)** Inflammation grade in *Tsc1^{fl/fl}* and *LTsc1KO* livers. Data are presented as the mean $\pm$ SEM (n=10–12), *P<0.5E–04. **(G)**

Representative H&E and anti-PanCK staining of cholangiocytes in *Tsc1^{fl/fl}* livers and expanded progenitor cells (arrows) in *LTsc1KO* livers. **(H)** Detection and quantification of proliferating hepatocytes by PCNA immunostaining. Data from five 200X fields per liver are presented as the mean $\pm$ SEM (n=3), *P<0.05. **(I and J)** Detection and quantification of hepatocytes with DNA damage by γ -H2AX immunostaining. Data from ten 200X fields per liver are shown as the mean $\pm$ SEM (n=3), *P<0.05. Scale bars, **A,C,E** and **H** 100 μ m (100X); **G** and **I**, 50 μ m (200X).

Fig. 4.

Chronic mTORC1 activation is required for HCC development in *LTsc1KO* mice. (A) Macroscopic appearance and H&E-stained sections of representative livers from *Tsc1^{fl/fl}* and *LTsc1KO* mice at 10 months of age, following 5-months treatment with vehicle or rapamycin. (B) Percentage of mice from (A) with hepatomas (or nodular foci) and HCC (n=6 per condition). Tumors were scored in five 4- μ m H&E-stained section, each separated by 0.5 mm, for each liver. (C) Serum levels of ALT in the mice described in (A). Data are presented as the mean $\pm$ SEM (n=6). *P<0.05 compared to vehicle treated *Tsc1^{fl/fl}* mice, **P<0.01 compared to vehicle treated *LTsc1KO* mice. (D) Immunoblot analysis of non-tumor liver lysates from the mice described in (A), showing relative levels of cleaved caspase-3 (n=3). (E) Representative H&E-stained sections of the non-tumor regions of liver

from *LTsc1KO* mice treated, as in (A), with vehicle or rapamycin, showing reduced immune infiltration after rapamycin treatment. (F) Inflammation grade in the livers of mice described in (A). Data are presented as the mean $\pm$ SEM (n=6). *P<0.05 compared to vehicle treated *Tsc1^{fl/fl}* mice, **P<0.01 compared to vehicle treated *LTsc1KO* mice. Scale bars, A, 400 μ m (40X); E, 50 μ m (200X).

Fig. 5. Effect of rapamycin treatment on the liver tumors of *LTsc1KO* mice at 12 months of age. Mice were treated with vehicle or rapamycin from 12 to 13 months of age (n=4). Tumors were scored in five 4- μ m H&E-stained section, each separated by 0.5 mm, for each liver. (A) Representative H&E- and phospho-S6 stained liver sections from vehicle- and rapamycin-treated *LTsc1KO* mice showing inhibition of mTORC1 signaling by rapamycin. T, tumor and N, non-tumor. (B) Number of hepatomas and HCCs in the vehicle and rapamycin treated mice. (C,D) Detection and quantification of proliferating hepatocytes by PCNA immunostaining of liver sections from vehicle- and rapamycin-treated *LTsc1KO* mice. (D) Approximately 200 hepatocytes were counted for each tumor, and data are presented as the mean $\pm$ SEM (n=5 hepatomas and 5 HCCs per treatment). *P<0.05

compared to hepatomas in vehicle treated mice, $**P<0.01$ compared to HCCs in vehicle treated mice. Scale bars, **A**, 100 μm (100X), **C**, 50 μm (200X).

Fig. 6.

Chronic mTORC1 signaling in the liver causes ER stress and defects in autophagy. (A, B) Mice aged 3 months were treated with vehicle or rapamycin for 4 consecutive days and then fasted overnight (n=3). Liver protein and RNA were isolated for (A) immunoblot and (B) qRT-PCR analyses. Phospho-specific antibodies: IRE1 (Ser⁷²⁴), PERK (Thr⁹⁸⁰), S6 (Ser^{240/244}). qRT-PCR data are presented as the mean $\pm$ SEM relative to vehicle-treated controls. *P<0.05 compared to vehicle-treated *Tsc1^{fl/fl}* mice, **P<0.05 compared to vehicle-treated *LTsc1KO* mice. (C) Representative immunostaining of p62 in liver sections from 6 month-old mice. (D) Mice aged 3 months were treated with vehicle or chloroquine (CQ) for

2 days and then fasted overnight before harvesting the liver for immunoblot analysis. **(E)** Immunoblot analysis of liver lysates from mice, aged 10 months, treated with vehicle or rapamycin for the preceding 5 months. For the vehicle-treated *LTsc1KO* group, non-tumor (N) and tumor (T) lysates from each liver are shown. **(F)** Representative immunostaining of p62 in the non-tumor and tumor tissues of liver sections from 9 month-old *LTsc1KO* mice. Scale bars, **C** and **F**, 50 μ m (200X). **(G)** Model of the role of chronic mTORC1 signaling in HCC development. See discussion for details.