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Impaired lipid accumulation in the liver of Tsc2-heterozygous mice during liver regeneration

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ABSTRACT

Tuberin is a negative regulator of mTOR pathway. To investigate the function of tuberin during liver regeneration, we performed 70% hepatectomy on wild-type and *Tsc2*+/− mice. We found the tuberin phosphorylation correlated with mTOR activation during early liver regeneration in wild-type mice. However, liver regeneration in the *Tsc2*+/− mice was not enhanced. Instead, the *Tsc2*+/− livers failed to accumulate lipid bodies, and this was accompanied by increased mortality.

These findings suggest that tuberin plays a critical role in liver energy balance by regulating hepatocellular lipid accumulation during early liver regeneration. These effects may influence the role of mTORC1 on cell growth and proliferation.

1. Introduction

Tuberin and hamartin, which are the products of genes TSC1 and TSC2, form a complex that negatively regulates mammalian target of rapamycin (mTOR). This complex works as a critical nutrient sensor, which regulates cell growth and proliferation via mTOR pathway. Mammalian target of rapamycin is activated by Ras-related small GTPase Rheb, which is a target of tuberin-hamartin complex. Tuberin is a GTPase-activating protein and stimulates the intrinsic GTPase activity of Rheb, thereby converting Rheb from its GTP-bound active state to GDP-bound inactive state. The insulin signaling pathway inactivates tuberin via the protein kinase, Akt. Inoki et al. reported that tuberin is directly phosphorylated by Akt at Thr1462, followed by an inactivation of tuberin, a disruption of its interaction with hamartin, and the activation of mTOR [1].

Under energy starvation (e.g., increased AMP:ATP ratio), AMPK becomes activated and phosphorylates tuberin to enhance its activity to inhibit mTOR signaling and cell growth/proliferation. Furthermore, phosphorylation of tuberin by AMPK protects cells from energy deprivation-induced apoptosis [2]. Taken together, these results indicate that tuberin inhibits cell growth/proliferation through the inactivation of mTOR under energy deprivation, and stimulates growth through the activation of mTOR when it receives growth-stimulating signals.

Jiang et al. reported that p70S6K, the downstream targets of mTORC1, was highly activated by phosphorylation at Thr389 in response to partial hepatectomy, and this activation was inhibited by rapamycin administration, leading to the attenuation of liver regeneration [3]. Accordingly, one would predict that enhanced mTORC1 activity could accelerate liver regeneration following partial hepatectomy by promoting cell growth and proliferation.

In the present study, we investigated the role of tuberin in liver regeneration by determining the time-dependent change of p70S6K phosphorylation at Thr389 and tuberin phosphorylation following 70% hepatectomy in C57BL/6 mice. We further performed 70% hepatectomy on *Tsc2*+/− mice, whose livers expressed half of the normal level of tuberin, and examined if liver regeneration is accelerated with hyperactivation of mTOR.

The results of these analyses indicate that tuberin phosphorylation strongly correlated with p70S6K activation during liver regeneration, but the process was not accelerated in the *Tsc2*+/− mice. Instead, we found impaired accumulation of lipid vesicles in the liver of these animals during the initial phase of liver regeneration, which serves as a primary energy source during early regeneration. These findings provide insights into the multiple functions of tuberin during liver regeneration.

Abbreviations: TSC, tuberous sclerosis; mTOR, mammalian target of rapamycin; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; p90 RSK, p90 ribosomal S6 kinase; ERK, extracellular signal-regulated kinase; P70S6K, p70 ribosomal S6 kinase; AMPK, 5′AMP-activated protein kinase; PH, partial hepatectomy; SH, sham operation; C57BL/6, c57 black 6; Rheb, ras homolog enriched in brain; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride.

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2. Materials and methods

2.1. Antibodies

Anti-tuberin (C-20) antibody (cat# sc-893) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin antibody (cat# A5441) was obtained from Sigma (St. Louis, MO). Anti-p70S6 kinase (cat# 9202), anti-p-p70S6 kinase (Thr389, 1A5)(cat# 9206), anti-phospho-Akt-substrate (RXRXXS*/T*, 110B7) (cat# 9614) were obtained from Cell Signaling (Beverly, MA). Anti-adipophilin antibody (cat# GP40) was obtained from Progen Biotechnik (Germany).

2.2. Partial hepatectomy

Eight to nine-week old C57 black 6 (C57BL/6) or *Tsc2*+/− (gift of D. Kwaitkowski, Harvard) male mice were fasted overnight. The next morning between 8:00 and 10:00, the mice were anesthetized with isoflurane (Abbott, cat# 05260-05) and the median and left lateral lobes of the liver were ligated at their stem and excised. Control mice were subjected to sham operation, which consisted of laparotomy and a brief manipulation of the liver with a cotton swab prior to wound closure. Food was re-introduced 6–8 h after surgery. The animals were sacrificed by cervical dislocation at the indicated time points following surgery. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Washington, which is certified by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3. Adipophilin immunostaining

Liver sections were fixed in 10% formalin, paraffin embedded, and stained with anti-adipophilin antibody using Vectastain Elite ABC Kit (Vector Laboratories, cat# PK-6101). Sections were reacted with 3,3′-diaminobenzizine (DAB) hydrochloride (Sigma, cat# D4293), followed by counterstaining with Harris hematoxylin.

2.4. Oil red O staining

Liver sections were fixed in 10% formalin for 2–3 h, equilibrated in 30% sucrose, and embedded in OCT. Frozen liver sections were stained with oil red O and counterstained with Harris hematoxylin.

2.5. Detection of phospho-tuberin recognized with anti-phospho-akt substrate (RXRXXS*/T*) antibody

Liver tissue was homogenized in NP-40 lysis buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2.5 mM ethylenediaminetetraacetate·$2H_2O$, 1% Nonidet P-40, 50 mM sodium fluoride, 0.5 mM AEBSF, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM sodium Orthovanadate, 1 mM dithiothreitol, 10 μg/ml leupeptin, 50 μg/ml SBTI, 200 nM Microcystin). Four micrograms anti-tuberin antibody was added to 5 mg protein and was rocked at 4 °C overnight for immunoprecipitation. Tuberin-antibody complex was absorbed with 40 μl Protein A Sepharose (1 g/4 ml, Sigma, cat# P3391, MO, USA), followed with washing by 1 ml NP-40 lysis buffer three times. The Protein A Sepharose was boiled with 25 μl of protein loading buffer at 1.5 time higher concentration for 5 min., and was subject to western blot analysis with anti-Phospho-Akt Substrate (RXRXXS*/T*) antibody as a primary antibody.

2.6. Western blot analysis

Liver tissue was homogenized in lysis buffer (50 mM Tris–HCl (pH7.4), 1% Triton X100, 150 mM NaCl, 50 mM β-glycerohosphate, 10% glycerol, 2 mM 2.5 mM ethylenediaminetetraacetate·$2H_2O$, 0.48 mM AEBSF, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/ml leupeptin, 50 μg/ml SBIT, 0.2 μM microcystin) and then centrifuged for 15 min at 4 °C. Protein concentration of the supernatant was quantified with BCA Protein Assay Kit (Pierce, cat#2161297A). An aliquot of protein was boiled with protein loading buffer for 5 min, and was loaded on SDS polyacrylamide gel. (7% for tuberin, 10% for p70S6K) After electrophoresis at constant voltage of 100 V, proteins were transferred onto PVDF membranes and blotted against primary antibodies. Membranes were washed with Tris-buffered saline with 0.1% Tween-20 and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies for 1 h. Protein bands were visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Pierce, cat#34080).

2.7. Expression of data and statistical analysis

The results are expressed as the mean ± SEM. The Student's *t* test was used for the comparison of data from two groups. The difference between groups was considered significant when *P* was less than 0.05.

3. Results and discussion

3.1. Hepatectomy induces P70S6K activation, downstream of mTOR signaling, during early liver regeneration

In order to investigate the time-dependent change of themTOR activation after 70% PH in C57BL/6 mice, we quantified the level of p-P70S6K (Thr389), using western blot analysis after PH.

We found that P70S6K was specifically phosphorylated in PH mice in contrast to sham-operated mice (Fig. 1A). The PH-specific phosphorylation already began at 0.5 h, peaked around 4–8 h, and gradually decreased by 24 h (Fig. 1A and B). These results indicate that mTOR signaling is activated at very early phase of liver regeneration.

3.2. Hepatectomy induces tuberin phosphorylation, which strongly correlates with P70S6K activation

Given that tuberin regulates mTORC1 and hence, p70S6K activity, we next investigated tuberin activity based on its phosphorylation state. Following partial hepatectomy, Hong et al. reported rapid activation of Akt, which in turn, can phosphorylates tuberin at multiple sites to suppress its function [4]. Based on the consensus sequence, RXRXX(S/T), Akt phosphorylation can be detected using a well-characterized anti-phospho PKB/Akt-substrate antibody. To determine such sites in tuberin following partial hepatectomy, we performed western blotting with this antibody following immunoprecipitation of tuberin from liver lysates. The western blot analysis at 2 h after surgery showed specific phosphorylation of tuberin in the PH group in contrast to minimally detectable phosphorylation in the sham group (Fig. 2A). In addition, there was strong correlation between the levels of phosphorylation of tuberin and that of P70S6K (Thr389) in the liver from multiple mice at 2 h. The temporal pattern of tuberin phosphorylation as detected by the Akt-substrate antibody paralled that of p70S6K phosphorylation beginning at 0.5 h, peaked around 2–6 h, and continued up to 12 h (Fig. 2C).

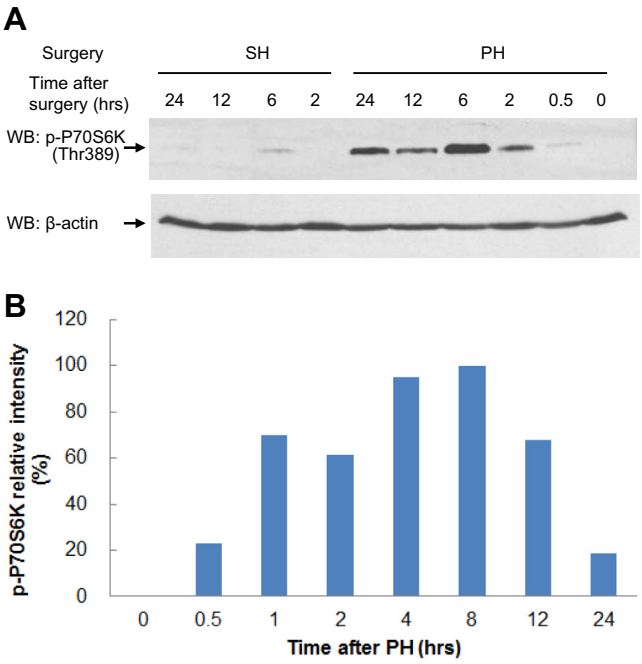

Fig. 1. Time course of phosphorylation of p70S6K during early liver regeneration. (A) Representative western blot of p-P70S6K (Thr389) from the liver of sham-operated and partially-hepatectomized mice at each time point. (B) Densitometric determination of the protein level of p-P70S6K. The protein level was determined by western blot from 2 mice at each time point. The average of density from 2 mice is expressed as the relative density to the highest one at 8 h after surgery.

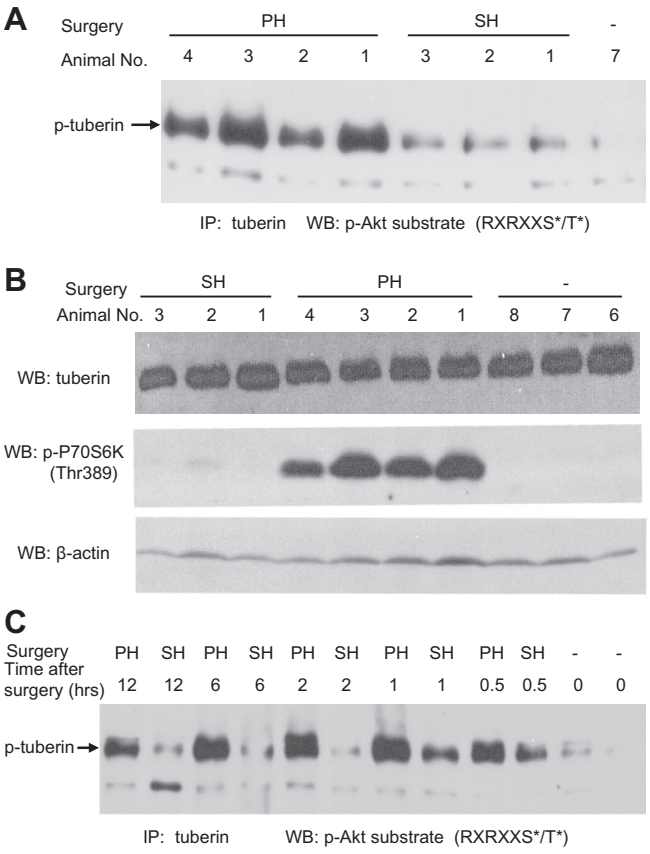

Fig. 2. Specific phosphorylation of tuberin, which correlates with the activation of mTORC1 pathway after PH. (A) Specific phophosrylation of tuberin in the liver after PH. Total protein extracts from the remnant livers of three sham-operated and four partially-hepatectomized animals at 2 h after surgery were subjected to immunoprecipitation with anti-tuberin antibody followed by western blot analysis with anti-p-Akt substrate (RXRXXS*/T*) antibody. (B) Western blot of tuberin and p-P70S6 K in the liver after PH. The same protein extracts with (A) was subject to western blot analysis. (C) Time course of phosphorylation of tuberin during early liver regeneration. Total protein extracts from the remnant livers of sham-operated and partially-hepatectomized animals at each time point were subject to immunoprecipitation with anti-tuberin antibody followed by western blot analysis with anti-p-Akt substrate (RXRXXS*/T*) antibody. Representative results from each time points are shown.

To further determine which Akt sites were phosphorylated in tuberin, we used a phospho-specific antibody to detect phosphorylation of TSC2 (Thr1462). However, the antibody failed to detect significant expression of tuberin throughout the first 24 h after PH (data not shown) suggesting that Thr1462 was not significantly phosphorylated in this setting. Others have also reported that the anti-Akt substrate antibody did not detect the TSC2 (Thr1462) site [5]. Besides Akt, phosphorylation of the RXRXX(S/T) motif is catalyzed by other AGC-family kinases such as cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), protein kinase C and $p90^{RSK}$. Therefore, we predict that the phosphorylation of tuberin during early liver regeneration may be triggered by AGC-family kinase besides Akt/PKB.

Collectively, we found that tuberin in wild-type liver is specifically phosphorylated immediately following PH at a site independent of Thr1462. This phosphorylation is recognized by the anti-phospho PKB/Akt-substrate antibody and strongly correlates with P70S6K phosphorylation (Thr389), which is induced by mTOR signaling.

3.3. The effect of tuberin deficiency on liver regeneration

It is known that tuberin deficiency induces constitutive hyperactivation of mTOR signaling [6,7]. We postulate that liver may regenerate faster under condition of mTORC1 hyperactivity. To test this hypothesis, we performed 70% hepatectomy in *Tsc2*+/− mice; these animals have been bred in the C57BL/6J background.

First, we determined the expression level of tuberin in the livers of *Tsc2*+/− mice before and after partial hepatechtomy. Fig. 3A and B shows that tuberin expression in the heterozygous liver was approximately 50% of the wild-type controls, and the amount did not significantly change following partial hepatectomy (Fig. 3B). However, this did not translate to significantly higher levels of p70S6K phosphorylation in the *Tsc2*+/− compared to *Tsc2*+/+ livers (data not shown).

Nonetheless, we observed several deaths among the *Tsc2*+/− mice between 48 and 72 h in contrast to no mortality in the *Tsc2*+/+ mice. Liver:body weight ratios were not significantly different between the two groups (Fig. 3C). Therefore, despite lower expression levels of tuberin in the *Tsc2*+/− livers, hepatic mTORC1 activity was not enhanced, and liver regeneration was not accelerated in the mutant mice. Hence, it was surprising to find a higher mortality rate occurring between 48 and 72 h after PH in the *Tsc2*+/− mice. These results indicate that tuberin may have other functions during liver regeneration, in addition to negative regulation of mTORC1.

3.4. Hepatocellular lipid body formation after PH was severely Impaired in Tsc2+/−mice

It has been reported that lipid accumulation during early (first 24 h) liver regeneration plays a critical role on liver regeneration [8]. Shtyer et al. showed the suppression of lipid accumulation in the liver by leptin administration impaired liver regeneration [9].

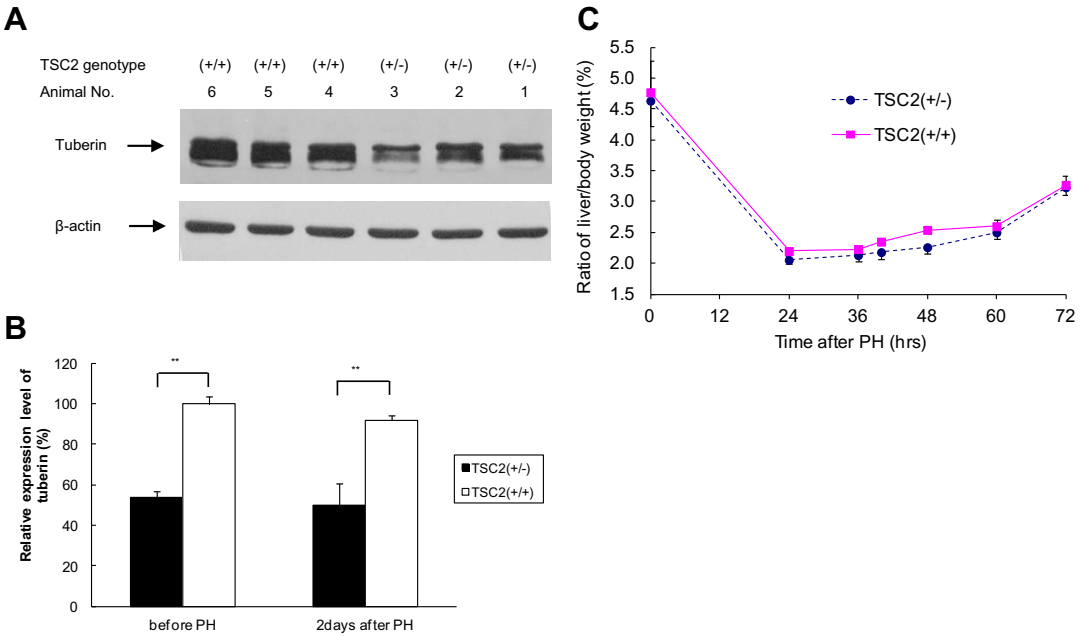

Fig. 3. The protein expression level of tuberin in the liver and liver:body weight ratio of *Tsc2*(+/−) and *Tsc2*(+/+) mice before and after PH. (A) Western blot of tuberin in the liver of *Tsc2*(+/+) and *Tsc2*(+/−) mice before surgery. Protein extracts from the liver was subject to western blot analysis with anti-tuberin antibody. (B) Densitometric determination of the hepatic protein level of tuberin in *Tsc2*(+/+) and *Tsc2*(+/−) mice before and 2 days after PH. The average of density from 6–7 mice is expressed as the relative density to that of the *Tsc2*(+/+) mice before PH. Results are mean ± SEM. $^{**}P < 0.01$ vs. *Tsc2*(+/+) mice. (C) Liver:body weight ratio of *Tsc2*(+/−) (solid line) and *Tsc2*(+/+) (dotted line) mice after PH. The average of 4–6 mice at each time point was shown. Results are mean ± SEM.

These results suggest the importance of lipid as a primary energy source for hepatocyte proliferation during early liver regeneration.

We examined hepatic lipid accumulation after hepatectomy in the *Tsc2*+/− mice using oil red O staining. At 24 h following surgery, we found a striking difference in the amount of hepatocyte lipid accumulation between the wild-type and mutant livers (Fig. 4A and B). As expected, there was considerable oil red O staining in the wild-type livers, but very little was found in the *Tsc2*+/− livers. To confirm this finding, we performed immunohistochemical analyses using formalin-fixed paraffin-embedded liver sections with anti-adipophilin antibody, which recognizes a surface marker of lipid droplets. We found that the average size of the adipophilin-stained hepatocellular lipid bodies of *Tsc2*+/− mice at 24 h was markedly smaller than that of the *Tsc2*+/+ mice. Furthermore, the shape of the lipid bodies was severely distorted (Fig. 4D). In contrast, wild-type mice exhibited considerable accumulation of adipophilin+lipid droplets with large, round vesicles at the same time point (Fig. 4C). These results suggest that even a 50% reduction in tuberin expression may cause a disruption in hepatocellular lipid body formation during early liver regeneration.

Surgical injury induces the release of a variety of stress hormones such as catecholamines, glucocorticoid, glucagon, which trigger a cascade of metabolic adjustments to promote catabolism and substrate mobilization in the postoperative period. It is reported that catabolism of systemic adipose stores is essential for hepatic lipid accumulation after PH and normal liver regeneration [10]. Walldorf et al. reported that beta-adrenergic blocker, propranolol, impaired liver regeneration, accompanied by a lower hepatic triglyceride content after PH [11]. These studies suggest that hepatic lipid accumulation after PH is triggered by catecholamines to mobilize peripheral adipose tissue and provides lipid substrates to the liver. The observation that glucose supplementation after PH exhibits inhibitory effect on the regeneration [12] and that systemic lipolysis followed by hypoglycemia is essential for normal liver regeneration [10] support the notion that lipid accumulation in the liver after PH provides the necessary energy substrates during

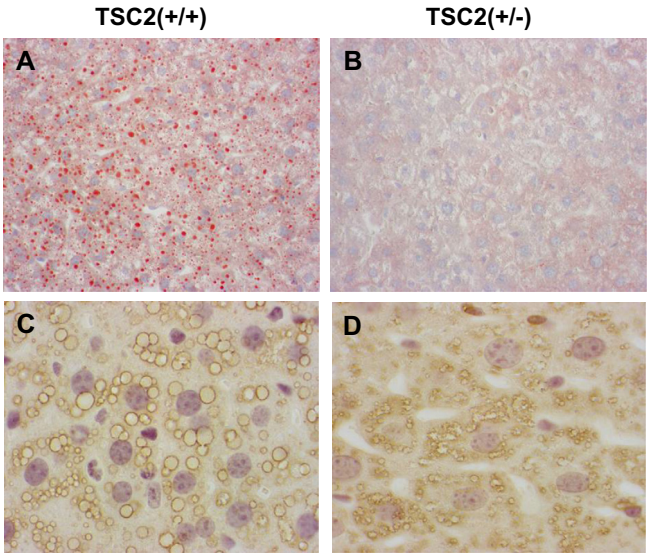

Fig. 4. Hepatic lipid accumulation at 24 h after PH. (A and B) oil red O staining of Frozen liver sections of *Tsc2*(+/+) (A) and *Tsc2*(+/−) mice (B). (C and D) Hepatic lipid body formation in *Tsc2*(+/+) (C) and *Tsc2*(+/−) mice (D). Immunohistochemical staining of adipophilin (marker of lipid body) was performed on paraffin-embedded liver sections, followed by Harris hematoxylin staining.

the initial phase of liver regeneration. The higher mortality rate in the hepatectomized *Tsc2*+/− mice may be associated with a relative deficiency of lipid-based energy source.

3.5. What is the mechanism of impaired hepatocellular lipid body formation during early liver regeneration in Tsc2+/− mice?

Tuberin was reported to reside in multiple subcellular compartments, servingmultiple functions. Jones et al. reported that tuberin

was cofractionated with caveolin-1 in lipid raft fraction and regulated its localization. In cells lacking tuberin, most of the endogenous caveolin-1 was displaced from plasma membrane to a Berefeldin-A-sensitive post-Golgi compartment, resulting in a disruption in caveolae formation. These results suggest that tuberin plays a role in the subcellular localization of caveolin-1 and consequently caveolae formation during post-Golgi transport [13]. We further showed that the trafficking of caveolin-1 was under the influence of microtubule-associated protein, CLIP-170, which acts as a substrate for mTORC1 [14]. Interestingly, caveolin-1 deficient mice exhibit impaired liver regeneration and low survival rate after PH [15]. Livers of these mice show dramatically reduced lipid droplet accumulation, and their survival was rescued by glucose supplementation. While this work supports our findings, the role of caveolin-1 in liver regeneration remains controversial [16]. In a recent study comparing multiple strains of Cav1−/− mice, Fernandez-Rojo et al. reported that mice of certain genetic background can compensate for caveolin-1 deficiency through the activation of anabolic metabolism, thus explaining the discrepant findings [17]. They concluded that caveolin-1 is required for efficient hepatic lipid storage during liver regeneration. Taken together, these results suggest that the impaired lipid droplet accumulation in the *Tsc2*+/− livers may be secondary to dysregulated caveolin-1 trafficking.

In summary, we found that tuberin phosphorylation is induced by partial hepatectomy, and this correlated with P70S6K activation during the early phase of regeneration. However, contrary to our expectation, liver regeneration of *Tsc2*+/− mice was not enhanced, and we attribute this to the reduction in lipid body formation following hepatectomy, which serves as an important energy source. This is consistent with the effects of tuberin on caveolin-1 trafficking and the role of caveolin-1 on regulating lipid-dependent energy metabolism in the liver. It remains to be determined whether the complete absence of Tsc2 in the liver accelerates or retards hepatic regeneration. It is known that mice with liver-specific deletion of Tsc1 are resistant to steatosis even when challenged with high-fat diet [18]. But whether hyperactivation of mTORC1 can compensate for the diminished lipid pool to promote hepatocyte proliferation and regeneration remains to be investigated.

Acknowledgments

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