Buitrago-Molina, L.E., Marhenke, S., Longerich, T., Sharma, A.D., Boukouris, A.E., Geffers, R., Guigas, B., Manns, M.P., Vogel, A.

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(2013) Hepatology, 58 (3), pp. 1143-1152.

The Degree of Liver Injury Determines the Role of p21 in Liver Regeneration and Hepatocarcinogenesis

Laura Elisa Buitrago-Molina[1§], Silke Marhenke[1§], Thomas Longerich[2], Amar Deep Sharma[1,3], Aristeidis E. Boukouris [1], Robert Geffers[3], Bruno Guigas[4,5], Michael P. Manns[1] and Arndt Vogel[1]

[1]Department of Gastroenterology, Hepatology and Endocrinology, Medical School Hannover, Hannover, Germany

[2]Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany

[3]Cluster of Excellence REBIRTH, Hannover Medical School, Hannover, Germany

[4]Department of Cell Biology, Helmholtz Centre for Infection Research, Braunschweig, Germany

[5]Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

[6]Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands

§ These authors contributed equally to this work.

Total word count: 5057

5 figures with no more than 6 panels

Title: 100 characters

Abstract: 209 words

Contact:

Arndt Vogel, MD

Medical School Hannover

Phone: +49 511 532 9590

Fax: +49 511 532 8392

email: vogel.arndt@mh-hannover.de

Summary

Hepatocellular carcinoma (HCC) frequently arises in the context of chronic injury that promotes DNA damage and chromosomal aberrations. The cyclin dependent kinase inhibitor p21 is an important transcriptional target of several tumor suppressors, which promotes cell cycle arrest in response to many stimuli. The aim of this study was to further delineate the role of p21 in the liver during moderate and severe injury and to specify its role in the initiation and progression of hepatocellular carcinoma. Deletion of p21 led to continuous hepatocyte proliferation in mice with severe injury allowing animal survival, but also facilitates rapid tumor development suggesting that control of compensatory proliferation by high levels of p21 is critical important to prevent tumor development. Unexpectedly however, liver regeneration and hepatocarcinogenesis was impaired in p21-deficient mice with moderate injury. Mechanistically, loss of p21 was compensated by activation of Sestrin2, which impaired mitogenic mTOR signaling and activated cytoprotective Nrf2 signaling. *Conclusion*: We conclude that the degree of liver injury and the strength of p21 activation determine its effects on liver regeneration and tumor development in the liver. Moreover, our data uncover a molecular link in the complex mTOR, Nrf2 and p53/p21-signaling network through activation of Sestrin2, which regulates hepatocyte proliferation and tumor development in mice with liver injury.

Introduction

Hepatocellular carcinoma is frequently associated with exposure to extrinsic factors that directly or indirectly induce DNA damage and chromosomal aberrations. Accumulation of DNA damage in hepatocytes ultimately leads to expanding foci of dysplastic hepatocytes, which progress to liver cancer if not rigorously controlled. ATM and ATR are serine/threonine kinases that sense DNA damage and coordinate DNA damage response pathways, most importantly p53. Activated p53 can inhibit proliferation to allow repair of DNA damage or trigger apoptosis if DNA damage is irreparable. p21 is one of the main effectors of p53 that induces cell cycle arrest and senescence in response to triggers such as DNA damage and telomere shortening by inhibiting the activity of cyclin-dependent kinase (CDK)–cyclin complexes and proliferating cell nuclear antigen (PCNA) (1). Due to its ability to induce growth arrest and as one of the main targets of several tumor suppressors, p21 was also considered as a potential tumor suppressor. Furthermore, several genetic studies in mice confirmed the importance of p21 for the regulation of liver regeneration and its ability to delay tumor development in the liver (2-5). The simple view on p21 as a tumor suppressor has however been complicated by findings that p21 can exhibit oncogenic activities in certain contexts. The first evidence for a pro-tumorigenic role of p21 came from observations that p21 suppresses apoptosis of thymic lymphoma cells thereby accelerating tumor growth (6). More recent data suggest that p21 may also induce proliferation of cancer cells by promoting the assembly of type-D cyclins with CDK4 and CDK6 (7).

The aim of this study is to further delineate the role of p21 in the liver during acute and chronic injury and to specify its role for the initiation and progression of hepatocellular carcinoma. For this aim, mice with a targeted genetic deletion of p21 were crossed into a mouse model of Hereditary Tyrosinemia Type 1 (HT1). HT1 is an autosomal-recessive human disease caused by a genetic inactivation of the enzyme fumarylacetoacetate hydrolase, FAH, which carries out the last step in the tyrosine catabolism pathway, that is mainly expressed in the liver and in the kidneys and accumulation of toxic metabolites, as fumarylacetoacetate (FAA), leads to acute or chronic liver failure (8). HT1 is characterized by an extremely high susceptibility for liver cancer. A murine model of Fah deficiency has been developed, which represent all phenotypic and biochemical manifestations of the human disease on an accelerated time scale (9-12).

Materials and Methods

Mice: C57Bl6-Fah^{Dexon5} and C57Bl6-Cdkn1a^{tm1Ty1}/J mice were crossed to generate *Fah$^{+/-}$/p21$^{+/-}$* breeders from which all *Fah$^{-/-}$* and *Fah$^{-/-}$/p21$^{-/-}$* animals used in these studies were derived. Drinking water was supplemented with NTBC at a concentration of 7.5 mg/ml. 2,5% percent of this normal dose was used for low-dose NTBC treatment.

Survival curves: Ten-week-old Fah-deficient mice were monitored for survival over the time after NTBC was reduced (2,5%) or withdrawn (0%). *Fah$^{-/-}$* and *Fah/p21$^{-/-}$* double knockout mice on 2,5% NTBC were followed for 400 days and *Fah/p21$^{-/-}$* 0% NTBC for 90 days.

Partial Hepatectomy: Briefly, mice were narcotized with an intraperitoneal injection of Ketanest/Rompun solution and subjected to a midline incision. Left and median lobes of the liver were ligated and resected. After closing the peritoneal and skin wounds, mice recovered from anesthesia on a warming pad. Thirty-eight hours or 1 week after PH, mice were sacrificed and livers were collected.

Immunoblotting: Frozen liver tissue was homogenized using an ultraturax (10 sec) in cell lysis buffer (50mM Hepes, 50mM KCl, 50mM NaF, 5mM NaPPi, 1mM EDTA, 1mM EGTA, 5mM ß-glycerophosphate, 1mM DTT, 1mM vanadate, 1% (v/v) NP40) containing Complete Protease Inhibitor mixture (Roche) and centrifuged for 10min at 16000xg. Protein concentration was measured using the Bio-Rad Protein Assay Dye Reagent and equal amounts of protein extracts were separated by SDS-PAGE and blotted to activated-PVDF membrane (Bio-Rad).

Transaminase and bilirubin levels: Mouse blood was collected from the orbital sinus in lithium heparin tubes (LH1.3, Sarstedt) and processed as per manufacturer instructions. Transaminase and bilirubin levels were measured using an Olympus AU 400 System.

RNA isolation and semi-quantitative RT-PCR: Total RNAs from liver tissue (n=4) were extracted by using the Qiagen RNAeasy kit. Transcriptor High Fidelity cDNA Synthesis kit (Roche) was utilized to synthetized cDNA. Sequences of PCR primers are provided by request.

Statistical analysis: Data are represented as mean ± SD. Data were analyzed by analysis of variance followed by Student's t-test to determine significance. p-values were considered statistically significant when $p < 0,05$ (*), 0,01 (**) or 0,001 (***).

Results

Loss of p21 Permits Survival of Fah-Deficient Mice with Severe Liver Injury

In order to determine the role of p21 in acute and chronic liver injury, *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice in the C57BL/6 background were generated. Body weight of healthy double knockout mice on 100% NTBC treatment was lower compared to *Fah*$^{-/-}$ mice, however liver/body weight ratio was not significantly different and there was no overt morphologic or biochemical phenotype. Next, NTBC treatment was completely stopped to induce severe liver injury. Following NTBC withdrawal, mean survival of *Fah*$^{-/-}$ mice was around 32 days (n=20) until they eventually died from liver failure accompanied by progressive weight loss. In agreement with our previous observation with *Fah/p21*$^{-/-}$ mice in the 129S background (2), double knockout mice survived the NTBC withdrawal for more than 4 months (n=20; p<0,0001) (Fig. 1A).

To further delineate the role of p21 in acute liver injury, mouse livers were collected 14 days after NTBC withdrawal. This time point was chosen because *Fah*$^{-/-}$ mice on 0% NTBC still had the same weight and overall health as mice on 100% NTBC despite hepatic dysfunction (10). As expected, histological examination revealed multiple small foci of necro-inflammation in *Fah*$^{-/-}$ mice on 0% NTBC (Fig. 1B). Furthermore, a few scattered TUNEL-positive hepatocytes were detectable in these livers (Fig. 1B,E). A similar picture was evident in the surviving double knockout mice suggesting that loss of p21 did not significantly modulate acute FAA-induced liver injury in this early phase.

As expected, a strong activation of p21 and almost no Ki67 positive hepatocytes were seen in *Fah*$^{-/-}$ mice on 0% NTBC despite a clear induction of cyclin D (Fig. 1B,E). Loss of p21 caused continuous hepatocyte proliferation in mice on 0% NTBC, thereby allowing survival of these mice in line with our previous observation (Fig. 1A,B,E) (2).

Loss of p21 Causes Rapid Tumor Development in Fah-Deficient Mice with Severe Liver Injury

To study the role of the p21 signaling in severe FAA-induced liver injury at later time points, livers were analyzed 2 months after NTBC withdrawal. Histologic examination of the surviving mice revealed moderate to severe acinar inflammation and numerous ballooned and dysplastic hepatocytes (Fig. 1D). Biochemically liver injury measured by transaminase and bilirubin levels accordingly increased over time (Fig. 1C). Almost no TUNEL positive hepatocytes were detectable in any mouse on 0% NTBC (Fig. 1D,E).

In the absence of p21, proliferation of hepatocytes with DNA damage further increased

compared to the earlier time point (Ki67 labeling index of 47% at 2 months compared to 14% at 14 days, p=0,005). In contrast, proliferation of hepatocytes was still markedly inhibited in the few surviving *Fah*$^{-/-}$ mice and almost no Ki67-labeled hepatocytes were detectable (n=4 out of 41 mice) (Fig. 1D,E). Similar results were obtained with BrdU as a DNA-synthesis marker and with phosphorylated histone H3 as a mitosis-specific marker (data not shown). In agreement with the proliferation assays, liver weight was significantly reduced in *Fah*$^{-/-}$ compared to *Fah/p21*$^{-/-}$ mice (p=0,01) (Fig. 1F). Interestingly however, the average hepatocyte cross-sectional area measured by β-catenin staining increased by 55% in *Fah*$^{-/-}$ mice suggesting a switch from proliferation-based liver regeneration to a regenerative process mediated by cell hypertrophy to at least partially compensate the strong p21-induced cell cycle arrest (Fig. 1E).

Due to the ongoing proliferation of hepatocytes with DNA damage, 85% of *Fah/p21*$^{-/-}$ mice (n=17) developed macroscopic detectable HCCs within 2-3 months. Interestingly, 25% of the few surviving *Fah*$^{-/-}$ mice (one out of four) also developed liver tumors despite the profound cell cycle arrest induced by p21 (Fig. 1F). Overall however, tumor incidence was significantly higher in the double knockout mice (p=0,006).

p21 is Required for Proliferation of Hepatocytes in Fah-Deficient Mice with Moderate Chronic Liver Injury

To analyze the role of p21 in chronic liver injury and their potential involvement in cancer formation under moderate hepatocellular damage, mice were exposed to a reduced treatment regimen of NTBC (2,5%) for up to 12 months. This suboptimal treatment closely mimics the human liver disease leading to HCC formation in HT1 patients (10, 13). *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice survived the low dose NTBC treatment (Fig. 2A).

Three months following NTBC reduction, histological examination revealed only mild acinar inflammation (Fig. 2B). Transaminase and bilirubin levels were accordingly not significantly increased in both groups (Fig. 2C). In contrast to Fah-deficient mice on 0% NTBC, multiple proliferating hepatocytes were found in livers of *Fah*$^{-/-}$ mice on 2,5% NTBC treatment. In agreement with the Ki67 staining, cyclin D levels were elevated and p21 was only slightly induced (Fig. 2B,D,E). TUNEL staining did not reveal any apoptotic hepatocytes (Fig. 1B,D). Surprisingly, the number of Ki67-labeled hepatocytes was significantly reduced in livers of *Fah/p21*$^{-/-}$ mice under 2,5% NTBC treatment compared to *Fah*$^{-/-}$ mice (Fig. 2B,D). Similar results were obtained with BrdU as a DNA-synthesis marker and with phosphorylated histone H3 as a mitosis-specific marker (data not shown). Thus, proliferation-based liver regeneration was

unexpectedly impaired in p21-deficient livers suggesting loss of p21 may actually impair hepatocyte proliferation during chronic liver injury. Similarly to mice on 0% NTBC, hepatocyte cross-sectional area measured by β-catenin staining increased in *Fah*$^{-/-}$ mice (p=0,05).

Loss of p21 Impairs Tumor Development in Fah-Deficient Mice with Moderate Liver Injury

To examine tumor onset and progression in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice under moderate chronic liver injury, livers of Fah-deficient mice were examined after 6, 9 and 12 months on 2,5% NTBC treatment. At 6 months, liver tumors were evident by macroscopic and histological examination in 50% of *Fah*$^{-/-}$ mice (n=10); tumor incidence increased over time reaching 76% after 9 months (n=20) and 100% after 12 months (n=20; Fig. 3A,B). Surprisingly, loss of p21 significantly delayed tumor development; no tumors were detectable after 6 (n=15) and 9 months (n=15) and only 50% of *Fah/p21*$^{-/-}$ mice developed liver tumors 12 months on 2,5% NTBC treatment (n=10, p=1,7E-2). Furthermore, *Fah*$^{-/-}$ livers displayed a significant greater number and size of tumors than *Fah/p21*$^{-/-}$ livers (Fig. 3C,D). In contrast to the findings described here, *Fah/p21*$^{-/-}$ mice in the 129S background still displayed a higher tumor incidence on 5% NTBC (2). The background specific differences are most likely due to a higher sensitivity of *Fah*$^{-/-}$ mice in the 129S background to NTBC reduction. Additionally, we cannot rule out that the higher tumor incidence in the 129S background might also be related to a general higher tumor susceptibility of these mice, epigenetic adaptations, which might occur in the backcrossed mice and/or cleanliness of the mouse facilities, which has been shown to significantly modulate hepatocarcinogenesis (14).

Together these data indicate that loss of p21 dramatically accelerates tumor development in *Fah*$^{-/-}$ mice with severe liver injury, but surprisingly delays tumor development in mice with moderate liver injury.

Differential Regulation of Cell Cycle Related Genes in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ Mice

FAA is a highly electrophilic compound, which induces DNA damage, mitotic abnormalities, chromosomal instability and ER stress *in vitro* and *in vivo* (15, 16). To better understand how loss of p21 modulates the cellular stress response in Fah-deficient mice, microarray analysis was performed with mice on 0% and 2,5% NTBC before visible tumor nodules development and compared with their respective controls on 100% NTBC. First, transcriptional profiles from tumor prone mice (*Fah*$^{-/-}$ mice on 2,5% NTBC and *Fah/p21*$^{-/-}$ mice on 0% NTBC) and from *Fah*$^{-/-}$ mice were compared to profiles from healthy mice (*Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice on 100% NTBC) and *Fah/p21*$^{-/-}$ mice on 2,5% NTBC. KEEG Pathway analysis identified 334 genes significantly regulated. The most significant category modified in tumor prone mice was related to “cell cycle”

(p=9,55E-5) followed by “DNA repair” (p=1,1E-3; Fig. 4A). Interestingly, direct comparison of gene expression from *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice revealed a similar profile in tumor prone *Fah*$^{-/-}$ mice on 2,5% NTBC, *Fah*$^{-/-}$ tumors and *Fah/p21*$^{-/-}$ mice on 0% NTBC mice. In contrast, the expression profiles of *Fah/p21*$^{-/-}$ mice with moderate liver injury (2,5% NTBC), in which liver regeneration was impaired and tumor development delayed, clustered with expression profiles from healthy mice (Fig. 4A). Together, the pathway analysis identified cell cycle related genes as modified by p21 and as most significantly associated with tumor development.

The Role of p21 for Hepatocyte Proliferation After Partial Hepatectomy Depends on Pre-existing Liver Injury

The above data strongly suggest that p21 differently modulates liver regeneration and hepatocarcinogenesis in mice with moderate and severe liver injury. To further analyze the role of p21 for hepatocyte proliferation, partial hepatectomies (PH) were performed.

First, the role of p21 was analyzed in *p21*$^{+/+}$ and *p21*$^{-/-}$ mice. Multiple Ki67 positive cells were clearly visible in *p21*$^{+/+}$ and in *p21*$^{-/-}$ mice 38 hours after PH and there was now significant difference between both groups (Fig. 4B). Liver mass recovery monitored by body/liver weight ratio was slightly accelerated in *p21*$^{-/-}$ mice one week after PH (Fig. 4C). At this time point, almost no Ki67 positive cells were detectable in either group. Overall, there were only minor differences between knockout and WT hepatocytes suggesting that p21 does not play a major role for the initiation and termination of hepatocyte proliferation in healthy mice. Next, partial hepatectomies were performed with *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice with preexisting liver injury. We have previously shown that *Fah*$^{-/-}$ mice on 0% NTBC do not survive PH due to the complete p21-mediated block of hepatocyte proliferation (2). Here, Fah-deficient mice 3 months on 2,5% NTBC with moderate liver injury were used. Surprisingly, hepatocyte proliferation following PH was markedly inhibited in *Fah*$^{-/-}$ mice in which basal liver regeneration before PH was not impaired (Fig. 4E). Importantly, the profound cell cycle arrest was associated with a strong induction of p21 (Fig. 4F). In contrast to *Fah*$^{-/-}$ mice, multiple Ki67 positive cells were clearly visible in *Fah/p21*$^{-/-}$ mice on 2,5% NTBC 38h after PH (Fig. 4E). Together, these data indicate that p21 has no lasting effect on liver regeneration in healthy mice after PH. In contrast, PH in mice with preexisting liver injury leads to a strong induction of p21, which subsequently impairs liver regeneration.

Interaction of the mTOR and p21 Pathways Facilitates Tumor Development in the Liver

Several molecular pathways, in particular MAPK and mTOR, have been implicated in hepatocarcinogenesis in previous clinical and experimental studies (3, 17, 18). Interestingly, most of these pathways are also important for liver regeneration suggesting that they are likely candidates contributing to the “cell cycle” gene expression profile in tumor prone Fah-deficient mice. To determine the role of these pathways in Fah-deficient mice, activation of JNK/c-jun, ERK, p38 and mTOR was analyzed 14 days after NTBC withdrawal and three months on 2,5% NTBC. The activation of the JNK/c-jun, ERK and p38 stress kinases did not correlate to the phenotype of Fah-deficient mice (Fig. 5A). A strong activation of the mTOR pathway, as monitored by immunblot analysis of phosphorylated S6, was evident in *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice on 0% NTBC. Similarly, a moderate phosphorylation/activation of S6 was seen in *Fah*$^{-/-}$ mice with moderate liver injury (2,5% NTBC). Interestingly however, S6 phosphorylation was significantly reduced by 50% in *Fah/p21*$^{-/-}$ mice on 2,5% NTBC, in which hepatocyte proliferation was reduced (n=6, $p<0,05$) (Fig. 5A,B). Moreover, reduction of NTBC induced an increase of 4-EPB1 protein levels in *Fah*-deficient mice albeit without significantly changing the ratio between the phosphorylated and non-phosphorylated protein.

Very recently, it has been shown that genotoxic and ER stress can inhibit mTOR activity in the liver through induction of Sestrin-2 (19, 20). Here, a significant stronger induction of Sestrin-2 was evident in *Fah/p21*$^{-/-}$ mice 3 months after NTBC reduction (increase of 50%) (Fig. 5C) suggesting that loss of p21 leads to a compensatory activation of Sestrin-2, which subsequently inhibits mTOR activity. Moreover, Sestrin-2 has been shown to activate Nrf2 signaling in mouse livers by promoting p62-dependent autophagic degradation of Keap1 (20). Accordingly, microarray and RT-PCR analysis revealed a significant stronger activation of several known downstream targets genes of Nrf2 including HO-1, Nqo1 and GSTm4 in livers of *Fah/p21*$^{-/-}$ mice compared to *Fah*$^{-/-}$ mice (Fig. 5D,E).

Discussion

Liver injury is often accompanied by severe DNA damage of hepatocytes, which leads to an activation of DNA repair pathways, including p53 and p21. Subsequent development of pre-neoplastic lesions and their progression to HCCs reflects the convergence of genetic and epigenetic defects that provoke dysregulation of pathways controlling cell cycle progression. Several previous studies have shown that p21 regulates liver regeneration and hepatocarcinogenesis. JNK1-dependent down-regulation of p21, for example, is required for proliferation of hepatocytes and tumor progression in chemically induced carcinogenesis (3). Similarly, we confirmed here our previous findings in Fah-deficient mice that loss of p21 permits proliferation of hepatocytes with severe DNA damage, which rapidly progress to dysplastic hepatocytes and HCCs (2). These studies established p21 as a negative regulator of hepatocyte proliferation and as a tumor suppressor. Paradoxically however, we report here that hepatocyte proliferation was significantly reduced and, more importantly, tumor development was profoundly delayed in p21-deficient mice with moderate liver injury, which provides further insight into the complex regulation of cellular processes required for liver regeneration and tumor development. The late spontaneous tumor onset in p21-deficient mice and the rarity of p21 loss of function mutations in cancer already provided some evidence that p21 is not a classical tumor suppressor. Here, we provide evidence that loss of p21 may actually promote or delay tumor development in the same disease and the same organ depending on the degree of preexisting injury.

Previous studies and our own observation suggest that the ability of p21 to modulate liver tumor development is closely linked to its ability to control cell cycle progression of hepatocytes. Interestingly however, the role of p21 for liver regeneration appears to depend on the degree of liver injury and the strength of subsequent induction of p21. In healthy mice, p21 is not required for normal liver development and does not significantly affect initiation and termination of liver regeneration following PH. On the other hand, abundantly expressed transgenic p21 dramatically reduced hepatocyte cell cycle progression in an otherwise healthy and normal environment. Moreover, this function even overrides the powerful mitogenic signals induced by 70% PH (21). Similarly, high levels of p21 in WT mice following extended PH or in Fah-deficient mice on 0% NTBC following 70% PH almost completely inhibit liver regeneration resulting in a dramatically increased mortality (2, 4). Here, we provide evidence that 70% PH induces to a strong and robust induction of p21 in mice with preexisting liver injury subsequently impairing liver regeneration. Together, these data indicate that the degree of overall (acute and chronic)

liver injury determines the strength of p21 induction in the liver and subsequently its effect on hepatocyte proliferation.

Interestingly, gene set enrichment analysis revealed that proliferation-related genes were most significantly, differently regulated between tumor prone Fah-deficient mice and *Fah/p21*$^{-/-}$ mice on 2,5% NTBC suggesting that other mitogens might be affected by loss of p21. Factors that drive proliferation of hepatocytes and hepatocarcinogenesis in chronic liver injury are not yet completely understood. The mTOR pathway is increasingly recognized to regulate growth and proliferation of hepatocytes and tumor cells (11, 22-24). In contrast to 4E-BP1, which appears to play only a minor role in mediating the effects of mTOR on mitogen-stimulated hepatocyte proliferation (23), pharmacological and genetic studies revealed that specifically S6k1 promotes hepatocyte proliferation by regulating cyclin D1 promoter activity and mRNA levels in hepatocytes. Moreover, the biological importance of S6 ribosomal-mediated translation has been shown in adult mouse livers that have a conditionally deleted S6 gene and which fail to proliferate due to a block in cyclin E mRNA expression. Here, we observed a striking correlation between mTOR activation/S6 phosphorylation and hepatocyte proliferation/tumor development. Importantly, we have previously shown that activation of the mTOR pathway is required for proliferation of hepatocytes during FAA-induced liver injury. Moreover, pharmacological inhibition of mTOR signaling and specifically S6 phosphorylation impaired cell cycle progression of *Fah*$^{-/-}$ hepatocytes following NTBC withdrawal and markedly suppressed liver regeneration and tumor development in *Fah/p21*$^{-/-}$ mice (11). mTOR activity can be inhibited by multiple mechanism including nutrient limitations and DNA damage. Very recently, Sestrin-2 has been identified to suppress mTOR activity in the liver following genotoxic and ER stress (19, 20). Here, the strong compensatory induction of Sestrin-2 significantly inhibited mTOR activity thereby impairing baseline liver regeneration in *Fah/p21*$^{-/-}$ mice with moderate liver injury. Moreover, Sestrin-2 has also been to shown to activate Nrf2 in the liver (20). Accordingly, a stronger activation of Nrf2 target genes was evident in livers of *Fah/p21*$^{-/-}$ mice. Nrf2 is a transcription factor, which regulates a battery of anti-oxidants and other cyto-protective genes in many tissues (25). Importantly, we have previously shown a high mortality and accelerated tumor development in mice with a targeted deletion of Nrf2 in Fah-deficient mice (12). Thus, our data suggest that the compensatory induction of Sestrin-2 does not only inhibit mTOR mediated hepatocyte proliferation, but also enhanced the Nrf2-regulated oxidative stress response thereby protecting mice against subsequent injury and tumor development.

In conclusion, we provide evidence that the degree of liver injury and the strength of p21 activation determine its effects on hepatocyte proliferation and hepatocarcinogenesis. Moreover, our data uncover a molecular link in the complex mTOR, Nrf2 and p53/p21-signaling network through activation of Sestrin-2, which can compensate for the loss of p21 in the liver during chronic injury.

References

1. Choudhury AR, Ju Z, Djojosubroto MW, Schienke A, Lechel A, Schaetzlein S, Jiang H, et al. Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. Nat Genet 2007;39:99-105.

2. Willenbring H, Sharma AD, Vogel A, Lee AY, Rothfuss A, Wang Z, Finegold M, et al. Loss of p21 permits carcinogenesis from chronically damaged liver and kidney epithelial cells despite unchecked apoptosis. Cancer Cell 2008;14:59-67.

3. Hui L, Zatloukal K, Scheuch H, Stepniak E, Wagner EF. Proliferation of human HCC cells and chemically induced mouse liver cancers requires JNK1-dependent p21 downregulation. The Journal of clinical investigation 2008;118:3943-3953.

4. Lehmann K, Tschuor C, Rickenbacher A, Jang JH, Oberkofler CE, Tschopp O, Schultze SM, et al. Liver Failure After Extended Hepatectomy in Mice Is Mediated by a p21-Dependent Barrier to Liver Regeneration. Gastroenterology 2012;143:1609-1619 e1604.

5. Albrecht JH, Poon RY, Ahonen CL, Rieland BM, Deng C, Crary GS. Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver. Oncogene 1998;16:2141-2150.

6. De la Cueva E, Garcia-Cao I, Herranz M, Lopez P, Garcia-Palencia P, Flores JM, Serrano M, et al. Tumorigenic activity of p21Waf1/Cip1 in thymic lymphoma. Oncogene 2006;25:4128-4132.

7. Liu Y, Yeh N, Zhu XH, Leversha M, Cordon-Cardo C, Ghossein R, Singh B, et al. Somatic cell type specific gene transfer reveals a tumor-promoting function for p21(Waf1/Cip1). The EMBO journal 2007;26:4683-4693.

8. Grompe M. The pathophysiology and treatment of hereditary tyrosinemia type 1. Semin Liver Dis 2001;21:563-571.

9. Grompe M, Al-Dhalimy M, Finegold M, Ou CN, Burlingame T, Kennaway NG, Soriano P. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. Genes Dev 1993;7:2298-2307.

10. Grompe M, Lindstedt S, al-Dhalimy M, Kennaway NG, Papaconstantinou J, Torres-Ramos CA, Ou CN, et al. Pharmacological correction of neonatal lethal hepatic dysfunction in a murine model of hereditary tyrosinaemia type I. Nat Genet 1995;10:453-460.

11. Buitrago-Molina LE, Pothiraju D, Lamle J, Marhenke S, Kossatz U, Breuhahn K, Manns MP, et al. Rapamycin delays tumor development in murine livers by inhibiting proliferation of hepatocytes with DNA damage. Hepatology 2009;50:500-509.

12. Marhenke S, Lamle J, Buitrago-Molina LE, Canon JM, Geffers R, Finegold M, Sporn M, et al. Activation of nuclear factor E2-related factor 2 in hereditary tyrosinemia type 1 and its role in survival and tumor development. Hepatology 2008;48:487-496.

13. Al-Dhalimy M, Overturf K, Finegold M, Grompe M. Long-term therapy with NTBC and tyrosine-restricted diet in a murine model of hereditary tyrosinemia type I. Mol Genet Metab 2002;75:38-45.

14. Dapito DH, Mencin A, Gwak GY, Pradere JP, Jang MK, Mederacke I, Caviglia JM, et al. Promotion of hepatocellular carcinoma by the intestinal microbiota and TLR4. Cancer Cell 2012;21:504-516.

15. Jorquera R, Tanguay RM. Fumarylacetoacetate, the metabolite accumulating in hereditary tyrosinemia, activates the ERK pathway and induces mitotic abnormalities and genomic instability. Hum Mol Genet 2001;10:1741-1752.

16. Zerbini C, Weinberg DS, Hollister KA, Perez AAR. DNA ploidy abnormalities in the liver of children with hereditary tyrosinemia type I. Correlation with histopathologic features. Am J Pathol 1992;140:1111-1119.

17. Hui L, Bakiri L, Mairhorfer A, Schweifer N, Haslinger C, Kenner L, Komnenovic V, et al. p38alpha suppresses normal and cancer cell proliferation by antagonizing the JNK-c-Jun pathway. Nature genetics 2007;39:741-749.

18. Sakurai T, He G, Matsuzawa A, Yu GY, Maeda S, Hardiman G, Karin M. Hepatocyte necrosis induced by oxidative stress and IL-1 alpha release mediate carcinogen-induced compensatory proliferation and liver tumorigenesis. Cancer Cell 2008;14:156-165.

19. Budanov AV, Karin M. p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell 2008;134:451-460.

20. Bae SH, Sung SH, Oh SY, Lim JM, Lee SK, Park YN, Lee HE, et al. Sestrins Activate Nrf2 by Promoting p62-Dependent Autophagic Degradation of Keap1 and Prevent Oxidative Liver Damage. Cell Metabolism 2013;17:73-84.

21. Wu H, Wade M, Krall L, Grisham J, Xiong Y, Van Dyke T. Targeted in vivo expression of the cyclin-dependent kinase inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development and regeneration. Surg Oncol Clin N Am 1996;5:215-229.

22. Espeillac C, Mitchell C, Celton-Morizur S, Chauvin C, Koka V, Gillet C, Albrecht JH, et al. S6 kinase 1 is required for rapamycin-sensitive liver proliferation after mouse hepatectomy. The Journal of clinical investigation 2011;121:2821-2832.

23. Jiang YP, Ballou LM, Lin RZ. Rapamycin-insensitive regulation of 4e-BP1 in regenerating rat liver. J Biol Chem 2001;276:10943-10951.

24. Volarevic S, Stewart MJ, Ledermann B, Zilberman F, Terracciano L, Montini E, Grompe M, et al. Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. Science 2000;288:2045-2047.

25. Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A 1994;91:9926-9930.

Acknowledgment

This work was supported by the Wilhelm-Sander-Stiftung (to A.V.), the Deutsche Krebshilfe (Max-Eder Grant to A.V.) and the Deutsche Forschungsgemeinschaft (TRR-77 (Liver Cancer – From Molecular Pathogenesis to Targeted Therapies) to A.V. and the Cluster of Excellence REBIRTH II (Regenerative Biology and Reconstructive Therapies) (to A.V.)

Figure 1

Loss of p21 Allows Survival of Fah-Deficient Mice with Severe Liver Damage, but Accelerates Hepatocarcinogenesis

A Kaplan-Meyer survival curves of *Fah*$^{-/-}$ (n=20) and *Fah/p21*$^{-/-}$ (n=20) mice on 0% NTBC. **B-F** Eight-week old *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice were on 0% NTBC for 14d or 2 months and compared with their 100% NTBC counterparts. **B** Representative H&E, TUNEL, Ki67 and p21 immunohistochemistry. **C** GOT and bilirubin levels were significantly increased after NTBC withdrawal, especially in *Fah/p21*$^{-/-}$ mice. **D** Representative photographs of livers and the indicated immunohistostainings from mice on 0% NTBC for 2 months. **E** Quantification of TUNEL and Ki67 positive hepatocytes. Western blots of total liver lysates from pooled samples (n=4) and hepatocyte size. **F** Tumor incidence and liver weight from *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice on 0% NTBC.

Figure 2

p21 is Required for Proliferation of Hepatocytes with DNA Damage

A Kaplan-Meyer survival curves of *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice on 2,5% NTBC treatment. **B-F** *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice were 3 months on 2,5% NTBC and compared with their 100% NTBC counterparts. **B** Representative pictures of indicated immunohistostainings. **C** Blood serum GOT and bilirubin levels. **D** Quantification of TUNEL and Ki67 positive hepatocytes. **E** Western blots of total liver lysates from pooled samples (n=4). **F** Hepatocyte size.

Figure 3

Loss of p21 Delays Tumor Formation in Fah-Deficient Mice with Moderate Liver Damage

A-D *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice were treated with either 100% or 2,5% NTBC for 6, 9 and 12 months. **A** Representative photographs of livers at indicated time points. Surprisingly, loss of p21 delayed tumor development in Fah-deficient livers. **B** Graphs representing tumor incidence and liver weight of Fah-deficient mice at different time points. Interestingly, only 50% of *Fah/p21*$^{-/-}$ mice exhibited tumors at 12 months. **C-D** Scatter plots displaying size of tumors and tumor numbers in Fah-deficient livers at indicated time points.

Figure 4

The Role of p21 for Liver Regeneration After Partial Hepatectomy Depends on Overall Liver Injury

A Hierarchical clustering of mouse cohorts, where cohorts with similar expression profiles were grouped together. Each row represents a value of four pooled RNA samples of each cohort. First heat map correspond to cell cycle-related genes identified from subtracting disease-associated genes from tumor-associated genes; and second, to expression profiles related to the Gene Ontology Term “DNA repair” extracted from cancer-related genes (see Supplementary Materials and Methods). **B-D** *p21*$^{+/+}$ and *p21*$^{-/-}$ mice underwent 70% PH. Livers were collected 38h and 1 week after PH. **B** Ki67 immunostaining and quantification of positive hepatocytes. **C** Liver/body weight ratio of *p21*$^{+/+}$ and *p21*$^{-/-}$ mice 1 week after PH. **D** Western blots of total liver lysates from pooled samples. **E-F** *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice were 3 months on 2,5% NTBC, underwent 70% PH. Livers were collected 38h after. **E** Ki67 immunostaining and quantification of positive hepatocytes. **F** Western blots of total liver lysates from pooled samples.

Figure 5

Crosstalk Between mTOR and p21 Signaling Pathways During Tumor Formation in the Liver

A-E *Fah*$^{-/-}$ and *Fah/p21*$^{-/-}$ mice were 14d on 0% NTBC (**A**) or 3 months on 2,5% NTBC (**A-E**) and compared with their 100% NTBC counterparts (n=4). **B** Quantification of S6 phosphorylation. **C** Expression of Sestrin2 at RNA and protein level. (**D**) RNA levels from microarrays analysis and semi-quantitative RT-PCR (**E**) of different Nrf2 target genes (F: *Fah*$^{-/-}$, Fp21: *Fah/p21*$^{-/-}$). **F** Schematic representation of how hepatocyte proliferation and tumor formation are reduced by inhibition of mTOR and activation of Nfr2 pathways via sestrin-2 activation in p21-deficient livers with moderate injury.