Activation of Inactive Hepatocytes through Histone Acetylation

A Mechanism for Functional Compensation after Massive Loss of Hepatocytes

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The mechanisms by which hepatic function is maintained after extensive parenchymal loss are unclear. In this study, we propose a novel concept of “functional heterogeneity” of hepatocytes based on their different expression of acetylated histones, the markers of active gene transcription, to explain the powerful compensatory capability of the liver. In the healthy human liver, only a fraction of the hepatocytes were marked by acetylated histones (ac-H2AK5, ac-H2BK5, ac-H3K9, ac-H3K14, ac-H3K27, and ac-H3K9/14). With the progression of cirrhosis, the ratio of the positive cells was gradually elevated, accompanied by the gradual exhaustion of the negative cells. By examining the global transcriptome of the mouse hepatocytes, we observed that the primed genes in the positive cells were much more numerous than those in negative cells. In a 70% hepatectomized mouse, the remnant hepatocytes were extensively activated, and the liver function was well maintained even when regeneration was severely inhibited. The functional compensation was absolutely dependent on the elevated expression of acetyl-histones. Additionally, when liver regeneration was blocked, the metabolism-related genes seemed to be preferentially transcribed. In conclusion, we demonstrate that normally, part of the active hepatocytes are competent for routine physiological requirements. The inactive hepatocytes, delicately regulated by acetyl-histones, act as a functional reservoir for future activation to restore the liver func-

tion after massive parenchymal loss. (*Am J Pathol* 2011, 179:1138–1147; DOI: 10.1016/j.ajpath.2011.05.029)

The liver has a unique regenerative capacity that restores its mass and function after diverse injuries, including a 70% to 95% partial hepatectomy (PH) in rodents and humans, by the proliferation of differentiated hepatocytes.^{1–3} Liver regeneration is commonly considered to be the adaptive process by which liver structure and function are recovered. However, in some cases, rodents can survive for an extended period of time after a 70% PH even when liver regeneration is chemically disrupted.^{4–6} Similarly, despite the on-going decrement of hepatocytes, patients suffering from chronic cirrhosis can keep their liver function normal for many years.^{7–9} This evidence suggests that the remnant liver can compensate for the initial loss of hepatic function independent of cell mass recovery. The powerful compensatory capability of the liver raises an interesting question: why does the body need so many hepatocytes when a minority of the liver mass seems competent? We hypothesize that only a subset of hepatocytes is functionally active at any given time, or each individual hepatocyte is only partially active. The subset of the inactive or the incompletely activated hepatocytes, therefore, acts as a functional reservoir that can be invoked for functional compensation. Molecular

Supported by the National Basic Research Program of China (2009CB522401) and by grants from the Natural Science Foundation of China (NO.30870983, NO.30971118, and NO.30972888).

Accepted for publication May 16, 2011.

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Supplemental material for this manuscript can be found at <http://ajp.amjpathol.org> or at doi:10.1016/j.ajpath.2011.05.029.

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studies of gene cascades have provided profound insights into the signaling pathways that are activated in the regenerating liver.^{10–13} However, just how the liver maintains its function during the process of liver regeneration remains unclear.^{11,14} Histone acetylation serves as a key modulator of chromatin structure and gene transcription.^{15,16} Histone acetylation, catalyzed by the histone acetyltransferases (HATs) to add an acetyl group to the lysine residuals (Lys or K), increases the accessibility of transcription factors to their target genes to prime transcription and elongation.^{17,18} Conversely, histone deacetylation, catalyzed by the histone deacetylases (HDACs) to remove the acetyl group from the lysine residuals, leads to chromatin compaction and results in transcriptional repression.¹⁵ HATs and HDACs coordinate to regulate gene expression and control numerous pathophysiological processes.^{19–21}

In the regenerating liver, histone acetylation is correlated with RNA synthesis and DNA replication.^{22,23} Enhanced histone acetylation promotes the transcription of many genes, whose products subsequently stimulate mitosis of the remnant hepatocytes.^{24,25} The role of histone acetylation in liver regeneration has been extensively investigated; however, its role in maintaining and compensating the liver function is still unclear.

As a marker of active transcription,¹⁵ the presence of acetylated histones might represent the functional status of hepatocytes. In this report, we examined the different expression levels of acetyl-histones in liver specimens from healthy humans and patients suffering from early to end-stage cirrhosis, to investigate the levels of histone acetylation during normal, compensatory, or decompensatory stages. We established a 70% PH mouse model, and the liver regeneration was severely inhibited by retrorsine pretreatment,^{5,6,26–29} to investigate the compensatory mechanisms of existing hepatocytes in the absence of proliferation.

We demonstrate for the first time that in healthy liver, only part of hepatocytes are positively stained by acetyl-histones; however, in developing cirrhotic liver, the ratio of the positive hepatocytes is gradually elevated, accompanied by the gradual exhaustion of the negative cells. In intact mouse liver, the hepatocytes could also be divided into the acetyl-histone-positive and -negative subgroups. The positive cells possess much more active transcription than the negative cells. Therefore, our data indicate a novel concept of “functional heterogeneity” of the hepatocytes based on their different expression of acetyl-histones. As an active marker, histone acetylation also participates in the regulation of functional compensation after massive parenchymal loss.

Materials and Methods

Immunohistochemistry of Human Livers

Human liver tissues were obtained from three healthy living liver transplant donors (two males and one female; the median age was 36 years and ranged from 29 to 44 years), whose livers were partial hepatectomized for liv-

ing donor liver transplantation, and the tissue specimens were immediately subjected to quality examination, 10 patients (seven males and three females; the median age was 56 years and ranged from 33 to 72 years) suffering from early-stage cirrhosis (as determined by ultrasonography and biopsy) with a Child-Pugh score of A, and 10 patients (nine males and one female; the median age was 53 years and ranged from 28 to 66 years) with end-stage cirrhosis with a Child-Pugh score of C, whose livers were removed before liver transplantation. The liver sections were stained with antibodies against ac-H2AK5 (acetylation of the fifth lysine residual at the N terminal) (ab45152), ac-H2BK5 (ab1759), and ac-H3K27 (ab4729), from Abcam, Cambridge, UK; ac-H3K9 (#1328-1) and ac-H3K14 (#1921-1), from Epitomics, Burlingame, CA, and ac-H3K9/14 (#06-599) from Millipore, Billerica, MA. Three pathologists independently reviewed all of the slides. For each of the patients, 10 high-power fields ($\times 200$) were randomly selected; the positive and negative hepatocytes were counted, respectively, and their ratio was calculated. All of the patients were treated at the West China Hospital, Sichuan University, and their medical records were reviewed to determine their clinical presentation.

Animals and Treatments

Six-week-old C57BL/6 male mice were used for this study. The mice were maintained in alternating 12-hour light/dark cycles with regular chow. Animal procedures and care were conducted in accordance with the institutional guidelines in compliance with national and international laws and policies.

The mice were randomized into retrorsine-treated (Sigma Chemical Co., St. Louis, MO) and vehicle-treated groups at the onset of the experiment. The mice received two treatments of retrorsine [30 mg/kg intraperitoneal injection (i.p.)] 2 weeks apart, as previously described.²⁷ Five weeks after the second injection, the mice were randomized to receive hepatectomy, anacardic acid^{30,31} [AA, a product of Merck (Whitehouse Station, NJ), diluted in glycerol at 1 mg/mL, 10 mg/kg i.p.] injection, or both hepatectomy and acid injection ($n = 13$ in each group). The mice were fasted overnight but allowed free access to water before treatment. Administration of isoflurane inhalation anesthesia and surgical PH (70%) were performed as previously described.³²

Three mice in each group were sacrificed to harvest liver tissue and to isolate hepatocytes 48 hours after treatment. A single dose of 5-bromo-2'-deoxyuridine (BrdU) (Sigma) was administered (50 mg/kg i.p.) 30 minutes before sacrifice. The body weight and liver weight were recorded. The liver tissues were subjected to DNA quantification, paraffin-embedded sections for pathological examination, and BrdU immunohistochemistry. The serum was used for alanine transaminase (ALT) and glutamic-oxaloacetic transaminase (AST) measurement. Hepatocytes were isolated by a two-step perfusion method as previously described.³³

Another three mice in each group were subjected to the indocyanine green (ICG) 15-minute retention (R15)

test at 48 hours. Briefly, ICG (0.5 mg/kg) was administered via the femoral vein. Samples of venous blood (0.1 mL) were taken before and 15 minutes after injection via the contralateral femoral vein. Each sample was diluted to one-fourth of the concentration with saline, and the specimens were analyzed for ICG concentration in a spectrophotometer at 805 nm.³⁴ The remaining seven mice in each group were analyzed for survival rates, which were expressed using Kaplan-Meier curves. In addition, all of the surviving animals were subjected to the ICG-R15 test at the end of experiment.

Hepatocyte Sorting

Hepatocytes isolated from mice were suspended in phosphate buffered saline (PBS) and fixed by the addition of paraformaldehyde at a final concentration of 0.5% for 30 minutes. The cells were then washed and resuspended in PBS with 0.1% Triton X-100 (Sigma) and incubated for 3 minutes. The cell suspension was then centrifuged and resuspended in 0.2 mL of PBS with 2 μ g of anti-ac-H2AK5 antibody, and the mixture was incubated for 1 hour at room temperature. Following incubation, the cells were centrifuged, resuspended in 0.2 mL of PBS with 0.1% Triton X-100 and 2 μ g of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, incubated for 30 minutes in the dark, and again washed in 1 mL of 0.1% Triton X-100 in PBS. Immediately before flow cytometer analysis, the cell suspensions were filtered using a 37- μ m nylon monofilament mesh filter. The fluorescein isothiocyanate-positive and -negative cells were sorted using a Beckman flow cytometer (Beckman Coulter, Brea, CA).^{35,36}

Immunofluorescence

The isolated hepatocytes were fixed with a methanol/acetic acid (3:1) mixture and were dropped onto poly(L-lysine)-coated slides for immunofluorescence staining. Antibodies against ac-H2AK5, ac-H2BK5, ac-H3K9, ac-H3K14, ac-H3K27, and ac-H3K9/14 were applied.

Immunoblot Analysis

Hepatocytic nuclear extract was prepared as previously described.³⁷ The nuclear proteins were analyzed by SDS-polyacrylamide gel electrophoresis with antibodies specific to ac-H2AK5, ac-H2BK5, ac-H3K9, ac-H3K14, ac-H3K27, and ac-H3K9/14. The amount of total H3 (sc-10809; Santa Cruz Biotechnology, Santa Cruz, CA) was used as the loading control. Protein bands were detected by an enhanced chemiluminescent reaction.

Measurement of HAT Activity

HAT activity in the nuclear extracts was measured using a HAT activity assay kit (Millipore) according to the manufacturer's protocol and as previously described.³⁸

ChIP-on-Chip and RT-PCR

To facilitate the antibody binding to nuclear antigens for flow cytometric sorting, cells were fixed with paraformaldehyde, and the cell membrane was perforated with Triton X-100. The mRNA purified from the sorted cells was severely degraded and would be no longer suitable for subsequent microarray analysis. Chromatin immunoprecipitation (ChIP)-on-chip analysis offers a rational strategy to reveal the global transcriptional activity because the specimen for the ChIP reaction is DNA. The binding of RNA polymerase II (Pol II) to the DNA sequence of target genes always indicates the priming and elongation of transcription.^{39–41} Using ChIP, the Pol II-bound genes are coprecipitated with anti-Pol II antibody. The precipitated genes could be further analyzed by microarray (chip), thus to obtain the global transcriptome profile of the cells.^{42,43} The sorted cells from three mice were mixed, and DNA was purified and immunoprecipitated with Pol II antibody (Millipore). ChIP assay was performed using an EZ-ChIP Kit (Millipore). The ChIP DNA was amplified and then hybridized to a Roche-NimbleGen MM8 RefSeq mouse promoter chip (C4222-00-01), and all of the hybridizations were repeated two times. The hybridized chips were scanned, and the results were analyzed with GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA); genes with a mean peak false discovery rate of ≤ 0.2 were regarded as being bound by Pol II.⁴⁴ We then performed reverse transcription-polymerase chain reaction (RT-PCR) to partially verify the ChIP-on-chip results. Seven genes important in metabolic process were selected for analysis (see Table 1). Total RNA was subjected to RT-PCR using a PrimeScript One Step RT-PCR Kit (Takara, Dalian, China); the PCR products were electrophoresed in agarose gel containing ethidium bromide for visualization.

Statistical Analysis

Data were expressed as the mean $\pm$ SD. Statistical significance between the groups was determined via one-way analysis of variance using the SPSS 13.0 software (Armonk, New York). The significance of differences in proportions was analyzed using the χ^2 test. A *P* value < 0.05 was considered statistically significant.

Results

The Proportion of the Acetyl-Histone-Positive Hepatocytes Gradually Increases in Developing Cirrhosis

We examined the *in situ* expression levels of acetyl-histones in healthy and cirrhotic human livers. In healthy livers, less than half ($43.8\% \pm 9.9\%$) of the hepatocytes were positive and irregularly distributed in the parenchyma, either at the portal area or around the central vein (Figure 1, A, B, C, and F). In early-stage cirrhosis, the proportion of positive cells was significantly increased to around three-quarters ($70.8\% \pm 13.4\%$) (Figure 1, D and

Table 1. The Selected Metabolism-Related Genes and the Primers Used in RT-PCR

Gene	Full name	Primers	Product size
<i>Acsm1</i> NM_054094	Acyl-CoA synthetase medium-chain family member	Sense 5'-CAGGATGGATTCTGGCTAC-3' Antisense 5'-CAAGGCACTGAGTGATGG-3'	141 bp
<i>Atp5l</i> NM_013795	ATP synthase, H ⁺ transporting, mitochondrial F0	Sense 5'-CCCTGCTGAAATCCCTAC-3' Antisense 5'-CAAACATCATAGCCAACAAT-3'	186 bp
<i>Serhl</i> NM_023475	Serine hydrolase protein	Sense 5'-TTACATGGCTATGGATTTCG-3' Antisense 5'-CACCTTCGGACCTCACT-3'	100 bp
<i>Hk2</i> NM_013820	Hexokinase 2	Sense 5'-GGGACAGTCTTGCGAATA-3' Antisense 5'-CGTCCACTACTGCTCCTC-3'	160 bp
<i>Sdhb</i> NM_025848	Succinate dehydrogenase complex, subunit D	Sense 5'-TGCTGGGCTTTGCTACTT-3' Antisense 5'-AGGTGAACGGCATGGTA-3'	134 bp
<i>Gykl1</i> NM_010293	Glycerol kinase-like 1	Sense 5'-GTCTATTATGCCCTGGAA-3' Antisense 5'-TGGGACGAAGTAACAAC-3'	147 bp
<i>Ptdss1</i> NM_008959	Phosphatidylserine synthase 1	Sense 5'-TACACGAGAAGCGGACAT-3' Antisense 5'-CCATAACTACGGATCAACAA-3'	141 bp
<i>GAPDH</i> BC_145810	Glyceraldehyde-3-phosphate dehydrogenase	Sense 5'-AAATGGTGAAGGTCGGTGTGAACG-3' Antisense 5'-CATTTGATGTTAGTGGGTCTCG-3'	257 bp

F), and in end-stage cirrhosis, nearly all of the nuclei in the regenerative nodules, markedly enlarged with loose chromatin, were strongly positively stained (Figure 1, E and F). Correspondingly, with the progression of cirrhosis, the proportion of the negative hepatocytes was gradually decreased, and these cells were completely exhausted in patients with a Child-Pugh score of C. All of the acetyl-histone markers had the same expression patterns (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

The Acetyl-Histone-Positive Hepatocytes Possess Much More Active Transcription

We isolated hepatocytes from the intact mouse liver and examined the expression of ac-H2AK5, ac-H2BK5, ac-H3K9, ac-H3K14, ac-H3K27, and ac-H3K9/14 using immunofluorescence. Similar to healthy human liver, only part of the hepatocytes were labeled by green fluorescence (Figure 2).

To directly clarify the different transcriptional activity between the acetyl-histone-positive and -negative hepatocytes, we sorted the cells based on their different expression of ac-H2AK5 (Figure 3A). The global transcriptome profiles by ChIP-on-chip analysis showed that in ac-H2AK5-expressing hepatocytes, 479 genes were

identified; however, 86 genes were identified in the negative hepatocytes (Figure 3B), and 31 genes overlapped these two groups (for a detailed gene list, see Supplemental Table S1 at <http://ajp.amjpathol.org>).

It was still needed to determine whether all of the active markers were expressed in the same cells. We examined the expression of each acetyl-histone in ac-H2AK5-positive and -negative cells. As shown in Figure 3C, all of the active markers were simultaneously and robustly expressed in the ac-H2AK5-positive cells.

Liver Function Is Completely Compensated after Partial Hepatectomy, Independent of Regeneration

To further confirm that a small part of the liver can compensate the entire function, we pretreated the mice with retrorsine before PH. Forty-eight hours after PH, mitosis was seldom seen in the livers, suggesting that liver regeneration was severely inhibited. On the contrary, many of the hepatocytes had entered mitosis in the mice that received PH alone (Figure 4, A and B). Histologically, no remarkable necrosis was seen in either group, although hepatocytic hypertrophy was prominent in PH + retrorsine mice at day 7 (Figure 4C). Despite the strong inhibition of DNA synthesis in retrorsine-pretreated mice (Fig-

Figure 1. Hepatocytic histone acetylation differs in healthy and cirrhotic human livers. Liver tissues of healthy humans (A), and patients suffering from early (D) or end-stage cirrhosis (E) were subjected to ac-H2AK5 immunohistochemistry (at a magnification of $\times 200$). B and C: The fields that are boxed in (A) show the distribution of the ac-H2AK5-positive cells in the central zone and portal zone, respectively. F: The proportion of positive hepatocytes was recorded based on 10 randomly selected fields at a magnification of $\times 200$. * $P < 0.01$. The other acetylation markers present a similar manner of expression; for details, see Supplemental Figure S1 at <http://ajp.amjpathol.org>.

Figure 2. Histone acetylation markers are positively expressed in part of the hepatocytes. The isolated hepatocytes from the intact mouse were subjected to immunofluorescence staining of ac-H2AK5, ac-H2BK5, ac-H3K9, ac-H3K14, ac-H3K27, and ac-H3K9/14. DAPI was used to stain the nuclei. Scale bar = 50 μ m.

ure 4D), their remnant livers were well reconstituted at day 7, and there was no significant difference in liver/body weight ratio between the PH and PH + retrorsine groups (Figure 4, E and F). Additionally, retrorsine administration alone did not lead to cell injury since the increased levels of enzymes seemed only correlated to surgery (Figure 4G). All of the animals in both groups survived the entire experimental period, suggesting that the remaining livers were competent with or without regeneration. We then performed the ICG-R15 test to manifest the retained liver function after operation. Indeed, the ICG retention was increased 48 hours after operation; however, there was no significant difference between the two groups. At day 7, the ICG retention of both groups was decreased to normal level (Figure 4H). These results indicate that the liver function was entirely compensated independent of liver regeneration.

The Expression of Acetyl-Histones Is Robustly Elevated after PH

If the acetyl-histone-negative hepatocytes act as a functional reservoir, they will be activated to compensate the initial parenchymal loss. Indeed, after PH, all of the remnant hepatocytes, with or without retrorsine pretreatment, were marked by acetyl-histones (Figure

Figure 3. The different transcriptional activity in hepatocytes. **A:** The ac-H2AK5⁺ and ac-H2AK5⁻ hepatocytes were sorted by flow cytometer. **B:** The flow cytometric-sorted hepatocytes were subjected to ChIP-on-chip assay, and the Pol II-bound genes were identified. **C:** Immunoblots of the histone acetylation markers in ac-H2AK5⁺ and ac-H2AK5⁻ hepatocytes are shown. The expression of total histone H3 was used as a loading control. FDR, false discovery rate; FS, forward scatter; SS, side scatter.

Figure 4. Retrorsine (Retro) inhibits hepatocyte proliferation but does not interfere with liver reconstitution or lead to direct hepatocyte damage. **A:** BrdU immunohistochemistry of the differently treated mice at day 2. **B:** The average BrdU-positive cells in 10 randomly chosen visual fields per section at a magnification of $\times 200$ at day 2. **C:** The histology of liver tissue at day 7. **D:** The genomic DNA content of the livers at day 7. **E:** The gross view of the reconstitution of the remnant liver at day 7. **F:** The ratios of liver to body weight at day 7. **G:** The serum ALT and AST levels at day 2. All of the error bars represent the SD from the mean of three mice assays of an individual experiment. * $P < 0.05$. **H:** ICG-R15 test was performed to manifest the liver function at days 2 and 7. * $P < 0.05$ versus control (Con).

5A, only the expression of ac-H2AK5 is presented here), and immunoblot analysis also showed that the expression of all of the active markers was robustly elevated (Figure 5B).

Different Genes Are Activated in Mice Receiving PH With or Without Retrorsine Pretreatment

DNA extracted from hepatectomized mice with or without retrorsine pretreatment was subjected to ChIP-on-chip assay with Pol II antibody. The global transcriptome profiles indicated that a great number of genes were primed in hepatocytes of both groups of mice: 802 genes in the PH-alone mice and 911 genes in the PH + retrorsine mice (Figure 6A).

Because it has been proved that histone acetylation plays a crucial role in the regulation of RNA synthesis and DNA replication in regenerating liver, we then hypothesized that different genes should be activated in retrorsine-pretreated liver, in which regeneration was severely deprived. We classified the identified genes into three subgroups, the metabolism-related genes, cell proliferation-related genes, and the uncategorized genes, using the online software of Gene Ontology (<http://www.geneontology.org> and Ref. 45). In mice that received PH alone, 28 (3.5%) and 235 (29.3%) genes belonged to the proliferation-related and metabolism-related gene categories, respectively. In mice that received PH + retrorsine, the number was 18 (2.0%, $P = 0.057$ compared to PH mice) and 334 (36.7%, $P = 0.001$ compared to PH mice), respectively (Figure 6A). We then performed semi-

quantitative RT-PCR to verify the ChIP-on-chip results. All of the selected genes that encode key enzymes involved in metabolism were notably up-regulated in both groups, especially in the PH + retrorsine mice (Figure 6B). For a detailed gene list, see Supplemental Table S2 at <http://ajp.amjpathol.org>.

Inhibition of Acetyl-Histone Expression Leads to Depressed Liver Function and Increased Animal Mortality

We have demonstrated that the elevation of histone acetylation seems closely correlated with functional compensation; therefore, inhibition of histone acetylation would be destined to produce a bad outcome. To clarify this hypothesis, we treated the mice with AA to inhibit acetyl-histone expression. As expected, in AA-treated mice, the HAT activity was tremendously inhibited (Figure 7A); as a result, the expression of acetyl-histones was remarkably decreased (PH + Retro + AA, Figure 5), and the metabolism-related genes were notably depressed (Figure 6B). We did not find any AA-related histological damage or increased serum enzyme levels in AA-treated mice (data not shown); however, AA administration did significantly increase the ICG retention (Figure 7B). AA administration alone did not lead to animal death; however, when AA was combined with retrorsine, PH, or both, the mortality was significantly increased (Figure 7C).

Figure 5. The expression of acetyl-histones is robustly elevated after PH, especially in retrorsine (Retro)-pretreated mice, but is robustly decreased in AA-treated mice. **A:** The immunofluorescence of ac-H2AK5 in different groups. Scale bar = 50 μ m. **B:** The immunoblots of acetyl-histones in different groups; the expression of total histone H3 was used as a loading control (Con).

Discussion

In this study, we proposed a novel concept of “functional heterogeneity” of the hepatocytes based on their different expression level of acetyl-histones and transcriptional activity. We demonstrated that normally, part of the hepatocytes are activated to satisfy the routine physiological requirement, and the inactive cells act as a functional reservoir for future activation, thus compensating for the initial massive loss of hepatocytes even without proliferation.

It is generally accepted that liver regeneration is the major compensatory mechanism after massive parenchymal loss.^{2,11} However, knowledge from clinical cases and animal experiments has confirmed that both human and rodents can survive on a small part of their liver even without regeneration.^{4–9} It is still unclear why the liver possesses such a powerful compensatory capability.

Histone acetylation is commonly considered to be a marker of active transcription,^{15,46} and the different expression of acetylated histones often indicates the transcriptional status of cells. By examining liver tissues, we found that only part of the hepatocytes in healthy human and mouse livers are marked by acetyl-histones. By examining the flow cytometric-sorted hepatocytes, we confirmed that all included acetyl-histone markers are simultaneously and robustly expressed in the ac-H2AK5-positive cells but are weakly expressed in the negative cells. The global transcriptome profiles directly demonstrated that the ac-H2AK5-positive hepatocytes possess active transcription, but to the negative cells, the tran-

scription is robustly depressed. Thus, we can divide the hepatocytes into two subgroups, the active and the inactive subgroups, based on their different expression of ac-H2AK5 or other acetyl-histone markers. Therefore, our findings offer a novel manifestation of the “functional heterogeneity” of hepatocytes, which is completely different from the established concept of the functional heterogeneity of the periportal and perivenous hepatocytes.⁴⁷ The active hepatocytes are competent for routine physiological requirement; the inactive cells, then, might act as a functional reservoir. In progressing cirrhosis, the residual cells are extensively activated, accompanied by the gradual exhaustion of the inactive cells. Once the reserve cells are completely exhausted, the liver becomes destined for failure. Therefore, a biopsy to evaluate the proportion of acetyl-histone-positive hepatocytes might offer a potential strategy to reveal hepatic functional reserves. Additionally, the irregular distribution of the active cells in the parenchyma suggests that the cells might be alternately activated, thus to ensure all of the cells obtain the chance to rest and repair the damaged or mutated DNA. The sustained histone acetylation might in turn lead to transcriptional deregulation, which would partly explain why cirrhosis often results in hepatoma.^{48,49}

To prove directly that a small part of the liver can function as an intact one, we established a 70% PH mouse model in which liver regeneration was severely deprived by retrorsine pretreatment. Retrorsine is a well-

Figure 6. Different genes are activated in mice receiving PH with or without retrorsine pretreatment. **A:** DNA extracted from hepatectomized mice with or without retrorsine (Retro) pretreatment was subjected to ChIP-on-chip assay with Pol II antibody. The identified genes were then categorized as metabolism-related genes, cell proliferation-related genes, or uncategorized genes. **B:** Seven selected genes that encode key enzymes involved in metabolism were analyzed using reverse transcription-PCR; *GAPDH* was used as a loading control (Con).

Figure 7. Administration of anacardic acid (AA) leads to HAT inhibition, function deterioration, and increased mortality. **A:** The relative HAT activity in different groups. **B:** The ICG retention rate at day 2 (2d; $n = 3$) and day 7 (7d; all of the animals survived at day 7). **C:** The survival curve of the different groups. * $P < 0.05$. Con, control; Retro, retrorsine.

established agent that inhibits hepatocyte proliferation through inhibition of DNA replication and cell cycle arrest.^{5,6,26–29} With PH and retrorsine treatment, hepatocyte hypertrophy, accompanied by an enlargement of the mitochondria, Golgi apparatus, and endoplasmic reticulum, indicates that energy metabolism and protein synthesis have been notably enhanced.⁴ A previous study in rat⁴ and our experiment in mouse both proved that 7 days after operation, the liver could be well reconstituted to its original weight through hypertrophy in the absence of regeneration. However, in the aspect of gene activation, little is understood regarding the functional compensation in the residual hepatocytes. Our data manifested that all of the residual hepatocytes are transcriptionally activated as indicated by acetyl-histones staining. The tran-

scriptome profile also indicated that a great number of genes are activated. Our results demonstrated that the extensive activation of the remnant hepatocytes is the major pathway to maintain and compensate the liver function. Previous studies revealed that liver regeneration after PH in retrorsine-exposed rats is accomplished through proliferation and differentiation of small hepatocyte-like progenitor cells²⁹; however, the immature hepatocytes only begin to appear at 3 days post-PH. Obviously these cells could not be responsible for functional execution, especially during the immediate days post-PH.⁵⁰ Indeed, we found that a few BrdU-positive cells emerged at day 3 after operation; however, these cells might be the progenitor cells rather than the regenerating hepatocytes since they were much smaller and showed colony-like proliferation.

In regenerating livers, the hepatocytes have to be responsible for their self-proliferation and functional compensation. It is well known that histone acetylation plays a critical role in regulation of RNA synthesis and DNA replication.^{22–24} To date, little has been clarified as to how the proliferating hepatocytes could maintain their function.^{11,14} Our data indicate that numerous mitosis-related genes as well as metabolism-related genes are activated, suggesting that the remnant hepatocytes can keep a good balance between self-proliferation and functional execution. When liver regeneration is deprived, however, the metabolism-related genes seem to be preferentially transcribed. These results suggest that the cells possess the ability to determine which gene cluster should be preferentially activated, thus to adapt different physiopathological processes.

To explore whether the elevation of acetyl-histones expression is necessary for functional compensation after PH, mice were treated with AA. AA is one of the most commonly used inhibitors of p300 and p300/CBP-associated factor,^{30,31} both of which are members of the HAT family.^{19,51,52} It has been demonstrated that some hepatotoxic chemicals, such as hydralazine derivatives, have an impact on acetylation inhibition.⁵³ In our experiment, the repression of the acetyl-histones expression, gene transcription, and functional deterioration was subsequent to AA injection. The proper HAT activity is critical to regulate cell cycle,²⁰ thus, the severe inhibition of HAT might disturb cell proliferation. Indeed, the regeneration of hepatectomized liver was moderately inhibited by AA administration (data not shown); however, it did not directly lead to animal death. We cannot rule out the effect of the systemic toxicity of AA, because all of the emerged data concerning this chemical have been based on *in vitro* experiments,^{54–56} and its global toxicity *in vivo* has yet to be fully explored. Our data indicate that no remarkable histological damage or increased serum enzyme levels were observed in AA-treated mice; it is likely that the deteriorated liver function and increased mortality should be ascribed to HAT inhibition and the subsequent inhibition of histone acetylation and gene activation.

In summary, we have raised, at least in part, a novel concept of functional heterogeneity of the hepatocytes to explain the powerful compensatory mechanism of the liver via gene transcription. Accordingly, under normal

conditions, the hepatocytes lacking acetylated histones are inactive in gene transcription and act as a functional reservoir. These cell are activated to transcription after massive parenchymal loss, thus to maintain liver function, even without the restoration of cell mass.

Acknowledgments

We are grateful to Xiangli Yin, Ruina Yang, Jun Wan, and Lijia Cheng for their assistance with this experiment.

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